Phosphorylation of myelin basic protein by glycogen phosphorylase kinase

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The ability of homogeneous glycogen phorphorylase kinase (Phk) from rabbit skeletal muscle to phosphorylate bovine brain myelin basic protein (MBP) was investigated. Phk could incorporate a maximum of 1.9 mol phosphate/mol MBP. The apparent K_m and V_{max} for Phk phosphorylation of MBP were 27 μM and 90 nmol/min per mg enzyme, respectively. Properties of MBP phosphorylation by Phk are similar to those of phosphorylase as a substrate. Only serine residues of MBP are phosphorylated by Phk. Phosphorylation sites of MBP by Phk are not identical to those by cAMP-dependent protein kinases.

Glycogen phosphorylase kinase *Myelin basic protein* *Rabbit skeletal muscle* *cAMP-dependent protein kinase*

1. INTRODUCTION

Muscle Phk (EC 2.7.1.38) phosphorylates glycogen phosphorylase and synthase [1,2]. The enzyme is also known to phosphorylate various proteins including troponins, H1 histone and myosin light chain [3–7]. These observations seem to indicate the broad specificity of muscle Phk, although the physiological role of this enzyme in processes other than glycogen breakdown has not yet been substantiated. Phosphorylation of MBPs has been reported in both in vivo and in vitro systems [8]. In fact various protein kinases, including protein kinases A [8], brain Ca^{2+}- and calmodulin-dependent protein kinase [9] and Ca^{2+}- and phospholipid-dependent protein kinase [10], have a capacity for phosphorylation of this protein. However, no physiological significance of this phosphorylation has been clarified. In an effort to identify substrates of Phk we examined the ability of Phk to phosphorylate MBP. We report here that Phk has the potential to phosphorylate seryl residues of MBP with considerable reaction rates and the sites of its phosphorylation are not identical to those by protein kinase A.

Abbreviations: Phk, glycogen phosphorylase kinase; MBP, myelin basic protein; protein kinase A, cAMP-dependent protein kinase; HPLC, high-performance liquid chromatography

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2. MATERIALS AND METHODS

Purified Phk [11], purified phosphorylase *b* [12], and catalytic subunit of protein kinase A [13] were prepared from rabbit skeletal muscle. MBP was purified from bovine brain as in [14] and further purified by Sephadex G-100 column chromatography. [γ-^{32}P]ATP was prepared as in [15]. Tosylphenylalanyl chloromethyl ketone-treated trypsin was obtained from Worthington. Other chemicals were obtained from commercial sources.

Phosphorylation of MBP and phosphorylase *b* was assayed by measuring the amount of ^{32}P incorporated into MBP and phosphorylase *b*. The standard reaction mixture (0.1 ml) contained 5 μmol Tris–HCl (pH 8.5), 1 μmol magnesium acetate, 2 nmol $CaCl_2$, 30 nmol [γ-^{32}P]ATP (5–20 cpm/

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pmol), 50 μg MBP or 500 μg phosphorylase *b* and 1–2 μg Phk. The incubations for MBP and phosphorylase *b* were usually carried out at 30°C for either 15 min or 3 min, respectively. The reaction was stopped by the addition of 10% trichloroacetic acid, and acid-precipitable materials were collected on a membrane filter (TM-2, Toyo Roshi). Phosphorylation of MBP by protein kinase A was as follows. The reaction mixture (0.1 ml) contained 3 μmol Tris–HCl (pH 7.0), 0.5 μmol magnesium acetate, 30 nmol [γ-^{32}P]ATP (20 cpm/pmol), 50 μg MBP and 3 μg protein kinase A. Other conditions were the same as described above. The radioactivity was determined with a liquid scintillation spectrometer. SDS-polyacrylamide gel electrophoresis was carried out as in [16] on 15% gels. For autoradiography an X-ray film (type IV, Fuji Photo Film) was exposed for about 20 h to the gels under test.

For analysis of phosphorylation sites, a radioactive preparation of fully phosphorylated MBP was digested for 4 h at 37°C with trypsin (MBP : enzyme = 50 : 1) in 0.2 M ammonium bicarbonate buffer (pH 8.0). After digestion, [^{32}P]phosphopeptides were separated by HPLC. Separations were carried out on a C_{18} reverse-phase column (SynChropack RP-P) with a Gilson HPLC system. The column was eluted for 30 min at a flow rate of 1 ml/min with a gradient of 0–40% acetonitrile containing 0.1% trifluoroacetic acid. Peptides were detected by absorbance at 210 nm. Each peptide peak was collected and analyzed for radioactivity by Cerenkov radiation. Acid hydrolysis (6 N HCl for 3 h) of radioactive MBP and determination of phosphoserine and phosphothreonine were carried out as in [17].

3. RESULTS AND DISCUSSION

Fig.1 shows the time course of phosphorylation of MBP by muscle Phk. Approx. 2 mol phosphates are incorporated into MBP. MBP could not be phosphorylated by itself. Under this condition autophosphorylation of Phk could hardly be seen.

To rule out the possibility that some other proteins, which could possibly be a contaminant in the Phk preparation, might be responsible for the reaction, the phosphorylated proteins were subjected to SDS-polyacrylamide slab gel electrophoresis followed by autoradiography (fig.2). Only MBP was phosphorylated and practically no

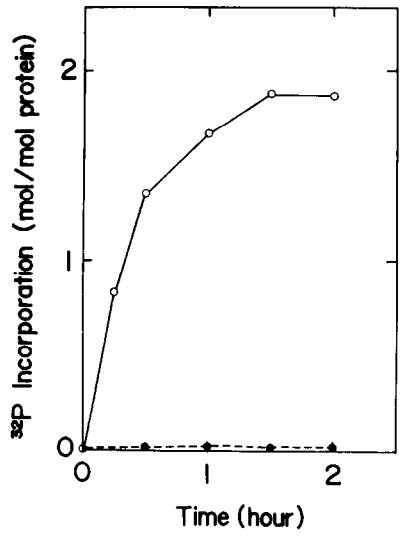

Fig.1. Time course of the phosphorylation of myelin basic proteins by phosphorylase kinase. Reaction conditions were as described in section 2 except that 10 μg MBP was employed. (○,●) Phosphorylation with and without MBP, respectively.

phosphorylation could be seen in α and β subunits of Phk.

Table 1 summarizes the properties of MBP phosphorylation by Phk in comparison with those of phosphorylase phosphorylation. The optimum pH is 8.5–9 and the activity ratio of pH 6.8/8.2 is about 0.35. The apparent K_m and V_{max} for MBP of Phk were determined to be 27 μM and 90 nmol/min per mg enzyme, respectively. The V_{max} is approx. 30-times lower than that with phosphorylase *b* as substrate. Although the phosphorylation of phosphorylase by Phk is absolutely dependent on Ca^{2+}, that of MBP is partially dependent on Ca^{2+}. Polylysine which is a strong inhibitor of phosphorylase phosphorylation [18], also completely inhibited MBP phosphorylation. These results suggested that properties of MBP phosphorylation by Phk were similar to those by phosphorylase. Acid hydrolysis of the radioactive MBP revealed that Phk phosphorylated exclusively serine residues, and phosphothreonine obtained was less than 1% of the phosphoserine (fig.3). On the other hand, protein kinase A phosphorylated both serine and threonine residues.

To distinguish the phosphorylated sites of MBP by Phk from those by protein kinase A, HPLC was

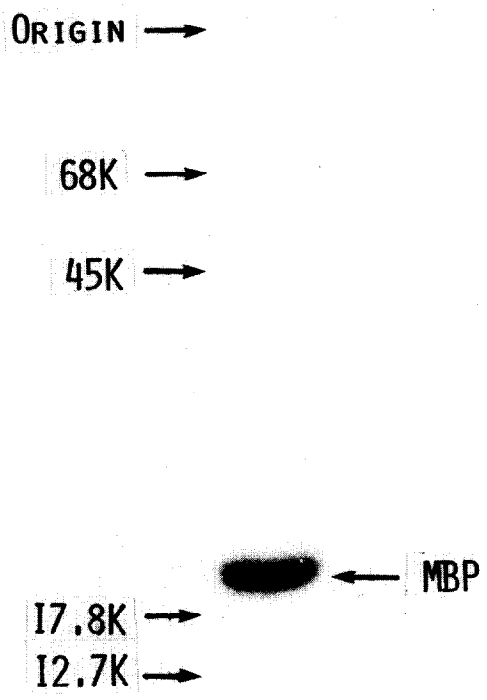

Fig.2. SDS-polyacrylamide gel electrophoresis of the phosphorylated MBP and autoradiography of the gel. MBP (50 μg) was incubated with 1 μg Phk under standard assay conditions. After termination of the reaction and heat treatment, aliquots (40 μl) were subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography as described in section 2. Standard protein used were bovine serum albumin (68 kDa), ovalbumin (45 kDa), myoglobin (17.8 kDa) and cytochrome *c* (12.7 kDa).

used to separate and quantitate tryptic [^{32}P]phosphopeptides. As shown in fig.4 tryptic phosphopeptides patterns by Phk are different from those by protein kinase A. About 16 radioactive peaks were obtained by the action of both Phk and protein kinase A, among which at least two peaks seemed to be specific for Phk phosphorylation. The results together with the data that protein kinase A phosphorylated preferably threonine residues rather than serine residues [8] suggested that phosphorylation sites of MBP by Phk were not identical to those by protein kinase A.

It has been reported that most of the serine and threonine residues of MBP are phosphorylated in vivo [8,14,19]. Various protein kinases have been identified as candidates for in vivo phosphorylation of MBP [8–10,19]. We have here demonstrated that like other kinases, Phk is also able to phosphorylate MBP. Although Phk is well known to be one of the key enzymes of glycogen metabolism, accumulating evidence shows that the kinase could also phosphorylate various proteins [3–7]. Phk seems to be one of the candidates of in vivo phosphorylation of MBP. However, no physiological significance can be assigned at present for the phosphorylation of MBP by Phk. In brain cytosol the presence of both MBP and Phk has been reported, in particular the specific activity of Phk is much higher than those in liver and heart [20]. In addition, partially purified rat brain Phk phosphorylated MBP (unpublished). In the light of those findings the possibility could arise that Phk is responsible for MBP phosphorylation in some

Table 1

Comparison of phosphorylation reactions of myelin basic protein and phosphorylase *b* by phosphorylase kinase

	Myelin basic protein	Phosphorylase *b*
Optimum pH	8.5–9	8.5–9
pH 6.8/8.2 ratio	0.35	<0.1
K_m for ATP (M)	1.25×10^{-4}	0.8×10^{-4}
V_{max} (μmol/mg per min)	0.09	2.7
K_m for substrate (μM)	27	22
Optimum Mg^{2+} (mM)	20	10
Inhibition (%) by EGTA (1 mM)	30–50	95
Inhibition by polylysine (100 μg/ml)	+	+
Mol phosphate incorporated	1.9	1
Phosphorylated amino acids	serine	serine

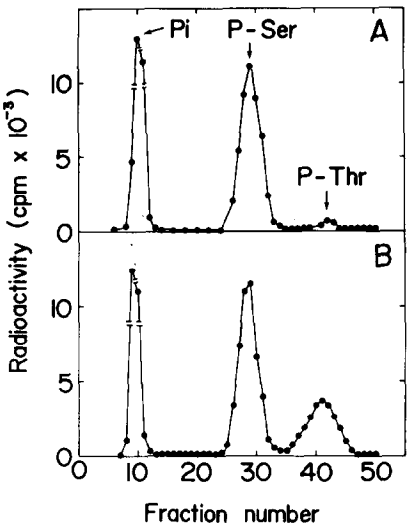

Fig.3. Acid hydrolysis of radioactive myelin basic proteins phosphorylated by Phk (A) and protein kinase A (B). Each hydrolysate was applied to an AG 50 W × 8 column (0.8 × 41 cm) which had been equilibrated with 50 mM HCl. Fractions of 1.5 ml were collected.

metabolic processes. It is well known that two adjacent basic amino acids near the phosphorylatable serine are spatially important for the recognition by protein kinase A [3]. While not stressing the physiological events, it is noteworthy that the phosphorylation of MBP by Phk may serve as a good tool for studying the recognition of sequence of amino acids by Phk. The role of Phk in controlling biological processes other than glycogen metabolism and the phosphorylation sites of MBP by Phk are currently under investigation.

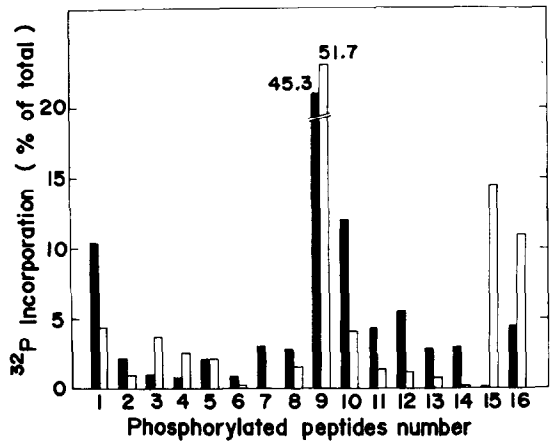

Fig.4. HPLC separation of tryptic [^{32}P]phosphopeptides. HPLC of MBP samples was as described in section 2. (■) Phosphorylated by Phk; (□) phosphorylated by protein kinase A. Phosphorylations were carried out for 2 h as described in section 2 except that 200 cpm/pmol [γ-^{32}P]ATP was employed. Total counts: 29 × 10^4 cpm by Phk and 28 × 10^4 cpm by protein kinase A, respectively.

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