In Vitro and in Vivo Phosphorylation of Myelin Basic Protein by Exogenous and Endogenous Adenosine 3':5'-Monophosphate-dependent Protein Kinases in Brain

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RISIHI MIYAMOTO AND SHIRO KAKIUCHI

From the Research Division and Clinical Laboratory, Nakamiya Mental Hospital, Hirakata, Osaka, Japan

SUMMARY

Myelin basic protein, an encephalitogenic protein, was phosphorylated by adenosine 3':5'-monophosphate (cyclic AMP)-dependent protein kinase from bovine brain, and its phosphorylation was stimulated by cyclic AMP. The ability of the protein to serve as substrate for the protein kinase was comparable to that of histone fractions and better than that of casein, protamine, or phosphorylase. The apparent K_m of the enzyme for the protein was 2 X 10^{-3} M. The protein phosphorylated by the protein kinase showed a different migration on disc gel electrophoresis from that of the native protein, suggesting that the modification of the protein molecule by phosphorylation resulted in an alteration of physicochemical properties of the protein. The protein caused the activation of the protein kinase, and preincubation of the enzyme with the protein resulted in a substantial increase of cyclic AMP-independent activity. Protein kinase modulator inhibited the enzyme activity in the presence or absence of cyclic AMP when myelin basic protein was used as substrate.

Amino acid residues of myelin basic protein which were phosphorylated by bovine brain protein kinase were seryl and threonyl. The maximum amount of phosphate incorporated into the protein was 3.80 moles per mole of protein, which was 3.6 times higher than that incorporated into histone on the basis of weight. Native myelin basic protein contained 0.20 mole of phosphorus per mole of protein, which was 3.6 times higher than that incorporated into the protein. Incorporated phosphate was released in the course of time. The results indicate that the phosphorylation and dephosphorylation of myelin basic protein may be in a dynamic state and that the turnover rate of phosphate in the protein may be relatively rapid.

Adenosine 3':5'-monophosphate-dependent protein kinase has been initially found in rabbit skeletal muscle (1) on the basis of the findings of phosphorylase kinase activation by cyclic AMP (2). A class of the enzymes has been shown to be widespread in the animal kingdom, and it was proposed that the diverse actions of cyclic AMP are mediated through regulation of protein kinases (3, 4). According to the hypothesis, the actions of cyclic AMP are brought about by the protein kinase in a given tissue and by the phosphorylation of the substrates of the protein kinases in that tissue. Subsequent to the discovery of cyclic AMP-dependent protein kinase in mammalian brain (5, 6), it was demonstrated that the protein kinase is associated with particulate subcellular organelles in a highly chromate (7). The studies of endogenous substrates in rat cerebrum have shown that all particulate subfractions are capable of becoming phosphorylated and that the highest substrate activities are found in synaptic plasma membrane, microsomal, and synaptic fractions (8). Furthermore, work from the same laboratory has demonstrated that the endogenous phosphorylation of a specific protein component of synaptic membrane fractions showed a marked dependence upon the presence of added cyclic AMP (9). Weller and Rod-}

\[ \text{The abbreviation used is: cyclic AMP, adenosine 3':5'-monophosphate.} \]
Myelin of brain and spinal cord contains a basic protein, which constitutes about 30% of the total protein in myelin (15, 16). The protein is unique in inducing experimental allergic encephalomyelitis in animals when injected with adjuvant. The protein has been purified to homogeneity from human and bovine brain and bovine spinal cord as shown by disc gel electrophoresis and ultracentrifugation (17-19). Recently, Carnegie (20) and Eylar et al. (21) have presented the complete amino acid sequences of the proteins from human brain and bovine spinal cord. The partial methylation of the arginyl residue at position 107 has been noted (22-24). Deibler and Martenson have shown that myelin basic protein prepared from a wide variety of species contains N,N'-dimethylarginine and N-monomethylarginine (25).

The myelin basic protein is considered to be a membrane protein. In view of this, it would be interesting to know whether it is phosphorylated. The present investigation describes the in vitro and in vivo phosphorylation of myelin basic protein which was catalyzed by exogenous and endogenous cyclic AMP-dependent protein kinases. The native protein is partially phosphorylated in its seryl and thrcnyl residues. A preliminary communication has been reported.3

EXPERIMENTAL PROCEDURE

Materials—Frozen bovine brains were obtained from a local slaughterhouse and stored at −20°C until use. Histone mixture (calf thymus) obtained from Schwarz-Mann was the histone preparation used throughout this study, unless otherwise specified. Lysine-rich histone, protamine, phosvitin, O-phospho-L-serine and N-terminal O-phosphothreonine were purchased from Sigma; slightly basic histone and arginine-rich histone were obtained from Schwarz-Mann; casein was purchased from Wako Chemicals. Cyclic AMP was obtained from Schwarz-Mann; casein was purchased from Wako Chemicals. Cyclic AMP was purchased from Japan Radioisotope Association. [32P]Orthophosphate (carrier-free) was purchased from Japan Radioisotope Association.

Preparation of Protein Kinase and Assay for Substrate Phosphorylation—Cyclic AMP-dependent protein kinase was purified through the DEAE-cellulose column chromatography step from bovine brain according to the method of Miyamoto et al. (26). The specific activity of the protein kinase was 13.6 mmoles of phosphate transferred per 5 min per mg of protein at 30°C. The phosphorylation of substrate was measured in an assay mixture consisting of 10 mmoles of sodium acetate buffer, pH 6.0, 1.0 m mole of [γ32P]ATP (2 to 5 × 106 cpm), 0.06 m mole of ethylene glycol bis(b-aminomethyl)ether)-N,N′-tetracetic acid, 2.0 mmoles of magnesium acetate, the amount and kind of substrate indicated, and 4.9 m g of bovine brain cyclic AMP-dependent protein kinase in the presence or absence of 0.2 m mule of cyclic AMP in a total volume of 0.2 m l. Assays were carried out for 5 min at 30°C. The reaction was stopped by addition of 4.0 m l of 6% trichloroacetic acid containing 0.25% sodium tungstate and 0.06 m eeluric acid. The procedure for washing with alkalii and acid and for counting the precipitate was as described earlier (26).

Preparation of Myelin Basic Protein—Myelin basic protein was prepared from whole bovine brain essentially according to the method described by Oshiro and Eylar (18) using Cellex-P column chromatography as a final step. The protein preparation, which showed one major single band on analytical disc gel electrophoresis described below, was used in this study.

Preparation of Myelin Fraction—Myelin fraction was prepared from rat brain by the procedure of Uyemura et al. (27) with some modifications. Animals were killed by decapitation, and the method described by Oshiro and Eylar (18) using Cellex-P column chromatography was followed. All sucrose solutions used in the succeeding steps of the fractionation contained 10 m m Tris-Cl buffer, pH 7.5, and blotted with filter paper. All sucrose solutions used in the succeeding steps of the fractionation contained 10 m m Tris-Cl buffer, pH 7.5. After mincing with scissors, the tissue was homogenized in 10 volumes of ice-cold 0.8 m osm solution with 12 up-and-down strokes at 900 rpm in a glass homogenizer with Teflon pestle of 0.28-mm clearance. This fraction was referred to as the crude homogenate. The crude homogenate in 0.8 m osm solution was centrifuged 65,000 × g for 60 min. The supernatant layer was carefully removed, resuspended in 0.8 m osm solution with 0.2 m mole of MgCl2 and centrifuged at 65,000 × g for 60 min. After the centrifugation, the interphase layer was suspended in 0.15 m osm solution and centrifuged at 27,000 × g for 15 min. The resulting pellet was suspended in 0.15 m osm solution and centrifuged at 27,000 × g for 15 min. The supernatant solution was centrifuged at 27,000 × g for 30 min, the supernatant solution decanted to another centrifuge tube, and the pH of the solution measured.

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High Voltage Paper Electrophoresis—High voltage paper electrophoresis was carried out at 3 kV for 1 hour in formic-acetic acid buffer, pH 1.9, according to the method of Allerton and Perlmann (28).

Analytical Disc Gel Electrophoresis—Analytical disc gel electrophoresis was performed at pH 4.3 in 15% acrylamide in accordance with the procedure of Uyemura et al. (27) without stacking gel. Gels were stained with aniline blue and destained electrophoretically. Radioactivity in the gel was located by two methods. In one method, the gel was stained and destained, and then cut into 2-mm slices. Each slice of the gel was crushed with a spatula and protein extracted from the gel with 0.1 m l of 0.5 N NaOH. Radioactivity of each sample was determined by a liquid scintillation spectrometer of Packard model 3510. In the other method, the gel, after being stained and destained, was placed on a wet filter paper and wrapped in Saran wrap. Radioactivity was located by autoradiography with Fuji KX medical x-ray films.

Acid Hydrolysis of 32P-labeled Myelin Basic Protein and Histone—After 100 μg of myelin basic protein or histone were labeled for 6 min at 30°C in the standard assay system for substrate phosphorylation. In order to radioactively label or 6 m l of HCl and 32P-labeled histone, the usual procedure by repeating washing and precipitating except for the absence of added bovine serum albumin as carrier protein. The final pellet was resuspended in 0.5 m l of 2 N or 6 N HCl and hydrolyzed for 3 hours at 120°C, as indicated, at 100°C in ampoules sealed under nitrogen. After drying in a vacuum desicator, the residues were dissolved in small volumes of water and an aliquot of the solution applied to Toyo Roshi No. 51 paper. The components were separated by high voltage paper electrophoresis as described above, with authentic [γ32P]orthophosphate, phosphoserine, and phosphothreonine as markers. Radioactivity was located by two methods. In one method, the paper was cut into 1-cm strips following electrophoresis, and the radioactivity of each strip was counted. In the other method, autoradiography was carried out with Fuji KX medical x-ray films.

Isolation, Identification, and Determination of Phosphoserine and Phosphothreonine in Myelin Basic Protein—Phosphoserine and phosphothreonine can be separated rapidly from other amino acids and peptides through an automatic amino acid analyzer on an Aminex resin column as described below, but are eluted close to each other. In contrast, these phosphoamino acids migrate clearly in different positions on high voltage paper electrophoresis. Therefore, phosphoserine and phosphothreonine were first separated by high voltage paper electrophoresis and then determined by the automatic amino acid analyzer. Myelin basic protein was partially hydrolyzed with 6 N hydrochloric acid at 100°C for 4 hours in ampoules sealed under nitrogen. After drying in a vacuum desicator, the dried residues were dissolved in a small volume of water. Inorganic phosphorus was determined.
on an aliquot of the solution by the method of Fiske and Subba-Roy (30). Another aliquot of the solution corresponding to 20 mg of myelin basic protein was subjected to preparative high voltage paper electrophoresis at 5 kV for 1 hour. Phosphoserine and phosphothreonine bands were located by staining guide strips along the sides of the paper with ninhydrin. From the portion of the paper which corresponded to each compound, phosphoamino acids were separately extracted by water. The extracted solution was dried in a vacuum desiccator over sodium hydroxide and sulfuric acid. The dried residues were dissolved in a small volume of water. Aliquots of the solution were subjected to the determination of phosphoserine and phosphothreonine, and of organic phosphorus (31, 32) in phosphoserine and phosphothreonine. Phosphoserine and phosphothreonine were determined by an automatic amino acid analyzer, Yanagimoto type LC-5, on a column.

In Vivo Phosphorylation of Myelin Basic Protein-Male Sprague-Dawley rats (8 weeks old and weighing 200 to 250 g) were given injections in the right ventricle with a neutralized solution of 6.1 μCi of [32P]orthophosphate (carrier-free) in a total volume of 10 μl by means of a permanent cannula implanted 1 week before the experiment, as described by Hayden et al. (34). Animals were killed by decapitation at 30, 60, or 120 min after injection of the radioactive phosphate, and the cerebrum of each animal was quickly removed. Crude homogenate and myelin fraction were obtained as described above. Protein fraction from crude homogenate or from myelin fraction was obtained as follows. The aliquot of crude homogenate or of myelin fraction was subjected to washing with 8% trichloroacetic acid containing 0.25% sodium tungstate plus 0.06 N H2SO4 three times, washing with ethyl alcohol twice, washing with acetone twice, washing with ethyl alcohol once, washing with chloroform-methanol (2:1) twice, and being incubated at 80°C for 30 min with 1 N perchloric acid twice in this order. At each step, the precipitate was obtained by centrifuging at 100 x g for 5 min and suspended in the appropriate solution. The final precipitate was dissolved in 0.1 ml of 0.5 N NaOH and radioactivity counted by scintillation spectrometry. A hydrochloric acid extract of the myelin fraction was obtained as described above. The radioactivity of the hydrochloric acid extract of the myelin fraction was measured for [32P] after being precipitated by addition of 4.0 ml of 5% trichloroacetic acid containing 0.25% sodium tungstate and 0.06 N sulfuric acid and washed as indicated for the assay of substrate phosphorylation.

Preparation of Protein Kinase Modulator-Protein kinase modulator was prepared from bovine brain through the step of trichloroacetic acid precipitation essentially as described by Donnelly et al. (35). Other Methods—[γ-32P]ATP was prepared by the method of Post and Son (36). Protein was measured by the method of Lowry et al. (37), with bovine serum albumin as the protein standard.

RESULTS

Cyclic AMP-dependent Phosphorylation of Myelin Basic Protein-The effect of myelin basic protein concentration on the activity of the protein kinase is shown in Fig. 1. The amount of the phosphate incorporated into myelin basic protein increased markedly in the presence of 1 μM cyclic AMP and almost reached a plateau at 200 μg per 0.2 ml of reaction mixture. The concentration of myelin basic protein required to reach half-maximal velocity was 70 μg per 0.2 ml of reaction mixture, determined in the presence of 1 μM cyclic AMP. This corresponded to 2 x 10^-4 M, assuming that the molecular weight of bovine brain myelin basic protein is 18,000 (19), although a double reciprocal plot of the enzyme activity, obtained in the presence of cyclic AMP, was not linear with respect to myelin basic protein concentration (Fig. 1). The ability of myelin basic protein to serve as substrate was compared to that of other proteins including several fractions of histone, casein, protamine, phosvitin, and bovine serum albumin (Table I). Histone mixture was the best substrate for the enzyme under the conditions used, and the phosphorylation of myelin basic protein was found to be less than that of lysine-rich histone and slightly lysine-rich histone but higher than that of arginine-rich histone, casein, protamine, and phosvitin.

In order to study the extent of phosphate incorporation into myelin basic protein and histone, each protein was incubated in the presence of a high concentration of ATP for longer times in the presence or absence of 1 μM cyclic AMP (Fig. 2). Interestingly, the incorporation of phosphate into the myelin basic protein increased almost linearly for 6 hours and reached a plateau at 24 hours of incubation. During the 24-hour incubation, 3.8 moles of phosphate per mole of myelin basic protein...
dependent protein kinase in the presence (---) or absence (-----) of 1 mM cyclic AMP. Other experimental conditions were as described in the text. Values of activities have been corrected for those obtained in the absence of added substrate at each time indicated.

After analytical disc gel electrophoresis, the gels were cut into 2-mm slices, and the location of the radioactivity was examined. Radioactivity was found only in the slices which corresponded to the myelin basic protein band, and the phosphorylation was stimulated by cyclic AMP (Fig. 3). The autoradiogram also showed the location of the radioactivity to be in accordance with the myelin basic protein band (Fig. 4). Cyclic AMP-dependent phosphorylation was observed on the autoradiogram, and the radioactivity was not found in the gels of the tubes in which the reaction mixture was incubated in the absence of myelin basic protein and the enzyme, myelin basic protein, or the enzyme (Fig. 4). The positions of the phosphorylated and native proteins were compared on analytical disc gel electrophoresis. The phosphorylated protein migrated more slowly than the native one (Fig. 5). Rf values of the phosphorylated and native proteins with cytochrome c as the marker protein were 0.52 and 0.58, respectively. This may be explained by the fact that myelin basic protein acquired a more negative charge upon phosphorylation.

In order to obtain evidence that phosphate incorporation was into the protein and to identify the amino acid residues, phosphorylated, myelin basic protein was hydrolyzed at 100°C for 4 hours in 6 N hydrochloric acid after being phosphorylated, precipitated, and washed as described above. The hydrolysate was subjected to high voltage paper electrophoresis. A typical result is shown in Fig. 6. After correction for hydrolysis of phosphoserine and phosphothreonine, 35% of the total phosphate incorporated was found to be associated with phosphoserine, 45% associated with phosphothreonine, and 10% associated with other amino acids or polypeptides. The remaining phosphate was recovered as inorganic phosphate. Under the milder conditions of acid hydrolysis (2 N hydrochloric acid, 3 hours), there was less radioactivity in phosphoserine and phosphothreonine with more radioactivity in polypeptides, whereas, under the more drastic conditions of acid hydrolysis (6 N hydrochloric acid, 12 hours), radioactivity decreased in the position of polypeptides and increased in the position corresponding to inorganic phosphate. Thus, 80% of total radioactive phosphate was accounted for in association with these two amino acid residues under the conditions of acid hydrolysis examined. In contrast to myelin basic protein, histone showed only phosphorylation of seryl residues under the conditions of mild or drastic acid hydrolysis studied (Fig. 6). This finding is consistent with those obtained by Langan (38) and Maeno and Greengard (39). The results suggest that the kind of phosphorylated amino acid residues is not determined by the source of the enzyme, but by the substrate itself.

Native Phosphoamino Acids in Myelin Basic Protein—Table Ii shows the content of phosphoamino acids and phosphorus in native myelin basic protein obtained from bovine brain. Total phosphorus in myelin basic protein was 0.203 mole per mole of the protein. Phosphoserine and phosphothreonine were determined to be 0.072 and 0.091 mole, respectively, per mole of myelin basic protein. The amount of organic phosphorus contained in phosphoserine and phosphothreonine was found to be 0.073 and 0.095 mole per mole of the protein, in fairly good agreement with the values for the corresponding phosphoamino acid. Thus, about 82% of the total phosphorus included in myelin basic protein was found to be associated with phosphoserine and phosphothreonine.

Activation of Cyclic AMP-dependent Protein Kinase by Myelin Basic Protein—The protein kinase was preincubated with bovine...
Phosphorylation of Myelin Basic Protein by Endogenous Myelin-

serum albumin, myelin basic protein, or histone, followed by assay of the enzyme activity in the absence of cyclic AMP (Table III). Preincubation of the protein kinase in the presence of myelin basic protein or histone caused a substantial increase in the activity of the enzyme in the absence of cyclic AMP, whereas myelin basic protein were incubated for zero (A) or 24 hours (B) with 1 μm cyclic AMP in the presence of 1 mM ATP and 14 μg of bovine brain protein kinase. Other experimental conditions were as described in the text. After incubation the aliquot of each solution or the combination of both (C), which corresponded to 10 μg of each protein, was subjected to analytical disc gel electrophoresis, which was carried out at 4 ma per tube for 2 hours and 60 min as described in the text. The protein migrated from top to bottom.

Fig. 4 (left). Analytical disc gel electrophoresis of myelin basic protein and autoradiography of its gels. One hundred μg of myelin basic protein were incubated at 30° for 5 min under the following conditions. A, the enzyme and myelin basic protein were removed from the complete system of the reaction mixture; B, the enzyme was removed from the complete system; C, myelin basic protein was removed from the complete system; D, cyclic AMP was removed from the complete system; and E, the complete system. Other experimental conditions were as described in the text. After incubation the tubes were placed at 0°, and an aliquot of the reaction mixture, which corresponded to 20 μg of the protein, was subjected to analytical disc gel electrophoresis which was carried out at 4 ma per tube for 2½ hours. Protein migrated from top to bottom. Left, protein staining pattern. Right, autoradiogram, performed as described in the text. The gels were placed in close contact with film for 3 days and the film was developed.

Fig. 5 (center). Analytical disc gel electrophoresis of the phosphorylated and native myelin basic protein. One hundred μg of bovine serum albumin was ineffective in activating the enzyme. Histone showed more increase of the activity than myelin basic protein on the basis of weight, suggesting that the activation of the enzyme is correlated well with substrate specificity under the conditions used. Thus, myelin basic protein not only serves as a good substrate for the protein kinase but also causes the activation of the enzyme. The results confirm our earlier studies (26, 40) in which substrate proteins for the enzyme also caused the dissociation and activation of the protein kinase.

Effect of Protein Kinase Modulator on Phosphorylation of Myelin Basic Protein—The protein kinase modulator from bovine brain decreased the activity of bovine brain cyclic AMP-dependent protein kinase both in the presence and absence of cyclic AMP when myelin basic protein was used as substrate. Thus, 4 μg of the modulator per 0.2 ml of the incubation mixture inhibited 32 and 21% of the enzyme activity for myelin basic protein (200 μg) and histone (40 μg) respectively, in the presence of cyclic AMP, and increasing the amount of the modulator to 6 μg caused 41 and 61% inhibition of the enzyme activity for each protein, respectively. Higher amounts of the modulator inhibited the activity for histone more than for myelin basic protein. In contrast to the results obtained with protamine (35, 41), myelin basic protein reacted to the modulator in a manner similar to that of histone.

### Table II

<table>
<thead>
<tr>
<th>Compound</th>
<th>Phosphoamino acid</th>
<th>Phosphorus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mole/mole protein</td>
<td></td>
</tr>
<tr>
<td>Phosphoserine</td>
<td>0.072</td>
<td>0.073</td>
</tr>
<tr>
<td>Phosphothreonine</td>
<td>0.091</td>
<td>0.095</td>
</tr>
<tr>
<td>Inorganic phosphor</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>0.203</td>
<td></td>
</tr>
</tbody>
</table>

Determination of phosphoamino acids and phosphorus was conducted at least four times, including the experiments in which different conditions of partial acid hydrolysis were used. The distribution of values of phosphoamino acids and phosphorus were in good agreement from experiment to experiment. One set of results is shown in the table. Values for phosphoserine, phosphothreonine, and organic phosphorus derived from them were corrected for 82.5% recovery for the extraction procedure from the paper on high voltage paper electrophoresis.
TABLE III

<table>
<thead>
<tr>
<th>Test protein</th>
<th>Preincubation</th>
<th>No preincubation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>- Test protein</td>
<td>+ Test protein</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Myelin basic protein</td>
<td>1.9</td>
<td>3.4</td>
</tr>
<tr>
<td>20 µg per tube</td>
<td>2.6</td>
<td>4.7</td>
</tr>
<tr>
<td>60 µg per tube</td>
<td>3.5</td>
<td>6.2</td>
</tr>
<tr>
<td>100 µg per tube</td>
<td>2.9</td>
<td>5.1</td>
</tr>
<tr>
<td>40 µg per tube</td>
<td>8.3</td>
<td>18.8</td>
</tr>
</tbody>
</table>

**Phosphorylation of myelin fraction in the presence or absence of added histone or protein kinase**

Protein kinase activity was assayed as described in the text, except for addition of myelin fraction (82 µg of protein), of 200 µg of histone, or of 4 µg of cyclic AMP-dependent protein kinase from bovine brain to the samples indicated.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Protein kinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- Cyclic AMP</td>
</tr>
<tr>
<td>Myelin fraction</td>
<td>16.0</td>
</tr>
<tr>
<td>Myelin fraction plus histone</td>
<td>19.0</td>
</tr>
<tr>
<td>Myelin fraction plus protein kinase</td>
<td>23.4</td>
</tr>
</tbody>
</table>

**In Vivo Phosphorylation of Myelin Basic Protein**—Values for in vivo phosphorylation of protein fractions are presented in Table V. Radioactivity was highest at 30 min after injection of orthophosphate in the three protein fractions examined, and decreased with later times. On a per mg of protein basis, the hydrochloric acid extract of the myelin fraction showed very little stimulation by cyclic AMP. An autoradiogram of the gel obtained from disc gel electrophoresis also showed that radioactivity corresponded only to the two major protein bands of myelin basic protein (Fig. 8).
FIG. 8 (left). Analytical disc gel electrophoresis of hydrochloric acid extract of rat myelin fraction and autoradiography of the gels. Myelin fraction (820 µg of protein) obtained from rat cerebrum was incubated at 30°C for 5 min in the absence (A) or presence (B) of 1 µM cyclic AMP, precipitated, and washed as described in the text, except for the absence of added bovine serum albumin as carrier protein. The hydrochloric acid extract (50 µg of protein) of myelin fraction was subjected to analytical disc gel electrophoresis which was carried out at 4 mA per tube for 2 hours and 40 min, followed by autoradiography of the gels. The gels were placed in close contact with film for 7 days, and the film was developed. A photograph of the protein staining pattern of the gels is shown on the left. The resulting autoradiogram is shown on the right.

FIG. 9 (right). Autoradiography of in vivo phosphorylated myelin basic protein. Experimental conditions of the in vivo studies were as described in the text. Hydrochloric acid extract (60 µg of protein) of myelin fraction from rat cerebrum was subjected to analytical disc gel electrophoresis which was carried out at 4 mA per tube for 2 hours and 40 min, followed by autoradiography of the gels. The gels were placed in close contact with film for 28 days, and the film was developed. The samples were obtained from animals sacrificed at 30 min (A) and 60 min (B), respectively, after injection of [32P]orthophosphate into brain.

Table V

In vivo phosphorylation of protein fractions after injection of [32P]orthophosphate

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactivity (cpm/mg protein)</th>
<th>30 min after injection</th>
<th>60 min after injection</th>
<th>120 min after injection</th>
</tr>
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<tbody>
<tr>
<td>Protein fraction from crude homogenate</td>
<td>93.8</td>
<td>37.9</td>
<td>14.5</td>
<td></td>
</tr>
<tr>
<td>Protein fraction from myelin fraction</td>
<td>145.6</td>
<td>64.5</td>
<td>48.6</td>
<td></td>
</tr>
<tr>
<td>Hydrochloric acid extract of myelin fraction</td>
<td>491.2</td>
<td>152.8</td>
<td>69.7</td>
<td></td>
</tr>
</tbody>
</table>

TABLE V

Radioactivity (cpm/mg protein) for 30 min, 60 min, and 120 min after injection of [32P]orthophosphate.

Discussion

It is important to document that the protein phosphorylated in the in vitro experiment was myelin basic protein and not a contaminating protein. This is considered to be the case for the following reasons. (a) Myelin basic protein was purified by an established method (18), and the preparation of the protein showed homogeneity on analytical disc gel electrophoresis under the acidic conditions used. (b) Radioactivity in the gel after analytical disc gel electrophoresis, which was measured directly by counting or by autoradiography, coincided well with the band of the myelin basic protein. (c) Both seryl and threonyl residues of the myelin basic protein were phosphorylated. This is different from histone, which was phosphorylated exclusively at seryl residues. The capacity of the myelin basic protein for phosphate incorporation was much larger than that of histone. (d) The ratio of phosphoserine and phosphothreonine in the native protein was in good agreement with the ratio of labeling of both amino acids. (e) Myelin basic protein obtained from the isolated myelin fraction of rat brain was also phosphorylated in vitro and in vivo. Thus, the results obtained in this study indicate that myelin basic protein, an encephalitogenic protein, is phosphorylated by bovine brain cyclic AMP-dependent protein kinase in the presence of ATP and Mg++, and its phosphorylation is stimulated by cyclic AMP. Maeno and Greengard (19) have independently observed that myelin basic protein, obtained from E. H. Eylar, is comparable to various histone fractions in its effectiveness as a substrate for cyclic AMP-dependent protein kinase prepared from bovine brain. The maximum amount of phosphate incorporated into the protein was 3.8 moles per mole, and, therefore, the total amount of phosphate incorporated into the protein was 4.0 moles per mole including 3.8 moles of exogenous phosphate catalyzed by protein kinase and 0.2 mole of endogenous phosphate. On the other hand, Maeno and Greengard have observed that myelin basic protein, obtained from E. H. Eylar, is comparable to various histone fractions in its effectiveness as a substrate for cyclic AMP-dependent protein kinase prepared from bovine brain.

8 H. Maeno and P. Greengard, personal communication.
basis of weight, myelin basic protein showed about a 4 times greater capacity for incorporation of phosphate than histone. Myelin basic protein was also found to be phosphorylated by endogenous protein kinase in well washed myelin fraction and in vivo after injection of orthophosphate. The phosphorylation of purified myelin basic protein catalyzed by exogenous protein kinase was cyclic AMP-dependent, whereas the endogenous phosphorylation of myelin basic protein catalyzed by myelin-associated protein kinase showed almost no stimulation by cyclic AMP. When histone was added to the endogenous protein kinase system as exogenous substrate, there was slight stimulation of phosphorylation by cyclic AMP. Further studies are required to clarify the properties of myelin-associated protein kinase system as exogenous substrate, there was slight stimulation of phosphorylation by cyclic AMP. When histone was added to the endogenous protein kinase system as exogenous substrate, there was slight stimulation of phosphorylation by cyclic AMP. Further studies are required to clarify the properties of myelin-associated protein kinase. Efforts are now in progress in our laboratory to determine whether or not the in vivo phosphorylation of the protein is cyclic AMP-dependent.

As far as we are aware, this is the first report which describes the natural occurrence of phosphoserine and phosphothreonine in myelin basic protein. The fact that these phosphoamino acids have not been detected may be due to the fact that amino acid analysis of the protein was performed by drastic acid hydrolysis which resulted in complete destruction of these phosphoamino acids. In fact, acid hydrolysis of phosphoserine and phosphothreonine, which was carried out at 100°C in 6 M HCl for 6 hours, resulted in 48 and 23% release of total phosphate, respectively, and 24-hour acid hydrolysis under these conditions brought about almost 100% release of phosphate from the phosphoamino acids. These findings were similar to those obtained by Allerton and Perlmann (28).

The amount of phosphorus in myelin basic protein, which was determined to be about 0.20 mole per mole of the protein, corresponded to a molecular weight of 19,600. Since the molecular weight of the protein is 18,000 (19), the amount of native phosphorus included in the protein would have had little influence on the value of the molecular weight presented in previous reports. About 80% of total phosphorus in native myelin basic protein could be accounted for in association with phosphoserine and phosphothreonine. In view of the difficulty of recovering these phosphoamino acids by partial acid hydrolysis without loss, it seemed likely that all phosphorus included in myelin basic protein could be associated with the phosphoamino acids and that only seryl and threonyl residues might be phosphorylated. It is of interest that the ratio of phosphoserine to phosphothreonine in native myelin basic protein was 0.77 and that the threonyl residue is more easily phosphorylated than the seryl residue by bovine brain cyclic AMP-dependent protein kinase, since the ratio of the content of seryl to threonine in myelin basic protein is 2:14 (19). The results suggest that myelin basic protein is not homogeneous, but heterogeneous with respect to the state of phosphorylation of its constituent amino acids. In this connection, it should be noted that heterogeneity of myelin basic protein was shown in terms of methylarginine content by Carnegie (22), Drostoff and Eylar (24), and Deibler and Martenson (25), who found that the amount of N\textsuperscript{a}-N\textsuperscript{a}-dimethylarginine and N\textsuperscript{a}-monomethylarginine in myelin basic protein from bovine brain was 0.20 and 0.30 mole per mole of the protein, respectively (29).

The protein which was phosphorylated by 5-min incubation with lower concentration of ATP migrated in the same position as the native protein on analytical disc gel electrophoresis and still showed a single band at pH 4.3. The native protein or the protein phosphorylated after a brief incubation may contain fewer molecules of phosphate per molecule of protein, since when 4 molecules of phosphate were incorporated, the protein migrated more slowly.

Deibler and Martenson have presented evidence of microheterogeneity of myelin basic protein (4). They have demonstrated the electrophoretic pattern of the myelin basic protein shows multiple forms. When the protein was examined on analytical disc gel electrophoresis at pH 10.6, it was clearly separated into five bands. This is not explained by methylation at its arginyl residues, since it seems likely that the changes in either the pK values of the protein or the molecular weight are enough to produce the difference on analytical disc gel electrophoresis. Kies\textsuperscript{4} suggested a possible removal of arginyl residue at the COOH-terminal by carboxypeptidase. However, the heterogeneity may be more easily explained by the different extent of phosphorylation of the protein. Each phosphoryl group at a seryl or threonyl residue contributes two negative charges at this pH, assuming that the three pK values of phosphate groups of phosphoserine and phosphothreonine are the same as those of phosphoserine and phosphothreonine. This could cause a significant decrease in the net positive charge of the myelin basic protein.

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REFERENCES


\textsuperscript{4} M. W. Kies, personal communication.
In Vitro and in Vivo Phosphorylation of Myelin Basic Protein by Exogenous and Endogenous Adenosine 3' : 5'-Monophosphate-dependent Protein Kinases in Brain

Eishichi Miyamoto and Shiro Kakiuchi


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