PHOSPHORYLATION ON BASIC AMINO ACIDS IN MYELIN BASIC PROTEIN

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SUMMARY

Isolated rat brain myelin when incubated with $\gamma^{32}P$ labelled ATP yields proteins bearing acid labile, base stable phosphoryl groups. Phosphorylated myelin basic protein can be isolated and degraded with trypsin and pronase to yield principally phosphoarginine and phosphohistidine. Only a very small amount of phosphoserine survives the base treatment used in the isolation procedure.

INTRODUCTION

Phosphorylation on serine and threonine residues in myeline basic protein has been studied as a posttranslational process in a number of laboratories (2, 6, 7) and similarly, phosphorylation of histones has been widely studied (5). Recently we have shown that, in addition to the hydroxy amino acids, several basic amino acids in histones I and IV are also phosphorylated (3). Most of the methodology developed to date to examine the phosphorylation of proteins involves either precipitation or electrophoresis under acidic conditions, a treatment which rapidly and completely destroys the extremely acid labile nitrogen-phosphate linkages.

We have examined the phosphorylation of myelin basic protein under neutral conditions and wish to report here the occurrence of base stable, acid labile phosphoryl bonds in myelin, specifically phosphohistidine and phosphoarginine.

METHODS

Isolation of myelin.

Myelin was prepared from fresh, unfrozen rat brains by centrifugation fractionation essentially as described by Whittaker (10). Brains were homogenized lightly in a Potter Elvehjam homogenizer in ice cold 0.32M sucrose and then centrifuged at 1000 g for 10 minutes to remove the crude nuclear pellet. The supernatant was then centrifuged at 10,000xg for 20 minutes to produce a crude mitochondrial pellet which was washed once and then resuspended in 0.32M sucrose and layered on 0.8M sucrose. After centrifugation at 56,000xg for 2 hours, the myelin remained at the top of the 0.8M sucrose while both mitochondria and synaptosomes pelleted to the bottom. The myelin was removed, diluted in cold water and again centrifuged at 100,000xg for 1 hr., after which the final pellet was resuspended in 5 ml of distilled water.

Phenol assay for acid labile phosphates in myelin.

Using the method essentially as described by Boyer and Bieber (1), 0.2 ml of myelin was incubated in 5 mM $MgCl_2$ and 30 μM γ^{32}P-ATP(2.2×10^7 cpm) for 15 minutes at 28°C. The reaction was stopped with 2 ml of 88% phenol (adjusted to pH 8.0 with 0.01 M sodium pyrophosphate, pH 8.1), and centrifuged at 2,500 rpm for five minutes. The upper aqueous layer was removed by careful aspiration. Washing was repeated 7 times with decreasing amounts of buffer until about 2.5 ml of phenol solution remained. 12.5 ml of cold acetone was added, the solution mixed, and stored overnight at -20°C. The precipitate was collected by centrifugation and lipids removed by successive extraction with 5 ml of a solution of chloroform and methanol (1:1), followed by 5 ml of chloroform methanol-water (20:10:1), and finally with 5 ml of methanol. The protein residue was then treated with 3 ml of 0.3 N trichloroacetic acid containing 1 mM sodium phosphate for 3 minutes in a boiling water bath in order to hydrolyze any acid labile phosphoryl bonds. The precipitated protein was then removed by centrifugation and 0.5 ml of the supernatant solution was assayed for radioactivity in aquasol.

Polyacrylamide gel electrophoresis of myelin.

0.1 ml of fresh myelin, prepared as before, was incubated in 10 mM $MgCl_2$ and 39.6 μM γ^{32}P-ATP(8.33×10^6 cpm) for 6 minutes at 30°C. The reactions were stopped with either 0.1 ml of 2% sodium dodecylsulfate (SDS) containing 8M urea and 2% β-mercaptoethanol or the same solution made up in 0.1 M sodium hydroxide. In order to insure a pH high enough to cleave the serine phosphates in the base treated mixture, 20 μl of 2N NaOH was added and the reaction mixture was heated to 60°C for 15 minutes. 50 μl of each reaction mixture was run on 7.5% acrylamide SDS gels made in accordance with Steck and Appel (7) and Weber and Osborn (9). The samples were run at 7 milliamps per gel, and were completed in 5 hours. Gels were removed, stained with 0.25% coomassie blue in 25% isopropanol overnight, and destained in 10% isopropanol. The distribution of protein throughout the gels was measured using a Gilford scanning spectrophotometer at 550 nm. Radioactivity was measured by the method of Tishler and Epstein (8). The gels were cut into 5 mm slices, allowed to dry, placed into test tubes with 0.2 ml of 30% hydrogen peroxide and heated at 55°C overnight in order to solubilize the gels. 1 ml of NCS tissue solubilizer was added, and the sample was counted in 10 ml of toluene base scintillation fluid.

Isolation and degradation of myelin basic protein.

A myelin pellet was prepared as before except that it was resuspended in

1 ml of 5 mM Tris·HCl buffer (pH 7.5) containing 5 mM $MgCl_2$ and 0.18 mM γ^{32}P-ATP (5×10^8 cpm) and incubated at 30°C for 10 min. The reaction was stopped with 1 ml of a solution containing 2% SDS, 2% β-mercaptoethanol, and 8 M urea, and then frozen.

50 µl of this opaque solution was run on each of nine 7.5% acrylamide SDS gels and stained as described above. The resultant bands of proteins were faint, but their location was the same as that reported by Steck and Appel (7) and a control rabbit myelin basic protein gratefully supplied by Dr. M. Kies. A typical SDS gel polypeptide profile is shown in figure 1A. These myelin basic protein bands were cut out, allowed to dry and placed into new gels that were freshly poured into tubes containing 1 cm of clay at the bottom. The clay was removed after the gel sat overnight and the resulting reservoir was covered with dialysis tubing. The myelin basic protein was electrophoretically run out of the gel into the reservoir buffer and held there by the dialysis tubing. The solution was removed and dialyzed against 400 ml of 1 mM Tris·HCl buffer, pH 7.5 at 5°C with two changes. The pH of the solution was adjusted to 8.2 and 40 µg of (10 µl) trypsin was added and allowed to incubate 1 hour at 37°C followed by 25 µg of pronase (5 µl) for 24 hours. The proteolyzed solution was applied to whatman 3 mm paper and chromatographed together with appropriate standard phosphoamino acids employing ethanol, water, triethylamine (30:30:39:1) as solvent (11). Alternatively, several samples were applied to a 20 x 20 cm thin layer silica gel plate and run in two dimensions using first EtOH, isopropanol, H_2O, triethylamine (30:30:39:1 v/v), then H_2O, 2-methoxyethanol, conc. NH_4OH, Methanol (70:10:10:10). The phosphoamino acids were located with ninhydrin and the appropriate radioactivity was determined in sequential rectangles (1 x 3 cm) of the paper or appropriate areas of the silica gel.

In a second experiment, a higher concentration of ATP was used (0.79 mM γ^{32}P-ATP) and the reaction was stopped by rapid freezing in solid CO_2. After lyophilization, lipids were extracted three times with 5 ml of methanol chloroform (1:1, v/v) to 200 mg of myelin. The residue was dried in a vacuum dessicator, after which it was dissolved in 0.2 M sodium phosphate buffer (pH 7.2) containing 1.1% SDS, 4.5 M urea, 5% glycerol, and 1% β-mercaptoethanol. 50 µl of the clear solution was run on each of eleven SDS gels producing clearer, darker myelin basic protein bands. The myelin basic protein was run off the gel, dialyzed, enzymatically hydrolyzed, and chromatographed as before.

RESULTS

By applying the phenol extraction assay of Boyer and Bieber (1) to rat brain myelin incubated with γ^{32}P-ATP, several experiments revealed that a significant portion of the ^{32}P remained as acid labile phosphoryl groups associated with myelin protein. Polyacrylamide gel electrophoresis was then used to determine which proteins in myelin were phosphorylated, and which of these were base stable. Figure 1B is the electrophoretic profile of phosphorylated myelin carried out at neutral pH throughout the experiment. Figure 1A demonstrates the effect of heating the phosphorylated myelin under basic

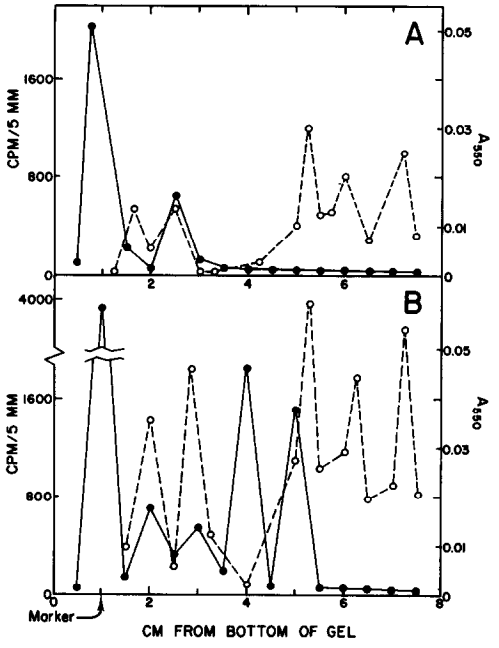

Figure 1. Polypeptide and radioactive profiles on sodium dodecyl sulfate acryamide gels of myelin incubated with γ^{32}P-ATP. —o— , absorbance at 550 mn along gel stained with coomasie blue. —o— , radioactivity present in gel slices assayed as described in the text.

A. Incubation mixture heated to 60°C for 15 min in presence of 0.25 M NaOH.
B. Incubation mixture of myelin heated to 60°C for 15 min at neutral pH.

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conditions prior to electrophoresis, thus eliminating serine phosphates. By using a lysozyme control it is estimated that about 4 µg total of myeline basic protein is present in these bands. The polypeptide profile on the gels is reproducible, with myelin basic protein the most constant component, although the strong base treatment appears to make these bands slightly more diffuse and less intense. The radioactivity associated with the slow moving myelin basic protein band, however, remains about equivalent to that seen without base treatment. A large unidentified peak of radioactivity moved just ahead of the fast migrating myelin basic protein band obscuring the base stable radioactivity normally seen with that band (figure 1A). In figure 1B there are peaks of radioactivity, presumably phospholipid or lipoprotein, that follow the

TABLE I

The incorporation of ^{32}P phosphoryl group from $\gamma^{32}P$-ATP to myelin basic protein with time

Incubation time minutes	Total Protein µg	Treatment prior to electrophoresis	Total phosphoryl incorporated cpm
0	2.0	neutral	0
0	2.0	basic	0
5	2.3	neutral	990
5	2.7	basic	1010
10	2.4	neutral	2130
10	2.8	basic	1410
15	2.3	neutral	750
15	2.7	basic	1040

0.1 ml myelin was incubated with $\gamma^{32}P$-ATP (51 M, 1.1 x 10^7 cpm) and 10 mM $MgCl_2$ for various times shown. The reactions were stopped and heated to 60°C either under neutral or basic conditions as outline in text. After SDS gel electrophoresis and staining, the absorbance at 550 nm and radioactivity were determined in the myelin basic protein bands. Approximate protein content was calculated from absorbance.

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myelin basic protein bands and disappear upon base treatment (figure 1A).

For the most part, the ^{32}P transferred to myelin basic protein from $\gamma^{32}P$-ATP, as shown in table 1, is base stable, a property not associated with phosphorylated hydroxy amino acids. It is evident from this time study that phosphoryl transfer to myelin basic protein is a dynamic process, but the kinetic details and the regulating components of this process are not now understood.

In order to determine which amino acids were phosphorylated, the phosphorylated myelin basic protein was isolated and hydrolyzed with trypsin and pronase, and the hydrolysate chromatographed with appropriate standards, figures 2A and 2B. In both chromatograms an approximately constant amount of phosphorylated hydroxy amino acid is seen. More striking is the appearance

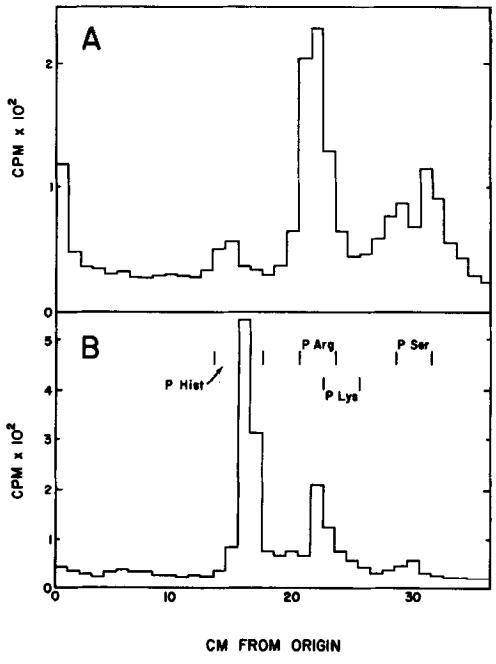

Figure 2. Distribution of radioactivity on chromatograms of proteolyzed phosphorylated myelin basic protein. Regions of standard phosphoamino acids located on chromatograph by ninhydrin spray are shown above the histogram of radioactivity.

A. Myelin incubated with 0.18 mM γ^{32}P-ATP and no lipid extraction.
B. Myelin incubated with 0.79 mM γ^{32}P-ATP and extracted after lyopholization with methanol:chloroform (1:1 v/v) prior to isolation of phosphorylated myelin basic protein.

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of phosphoarginine and a variable amount of phosphohistidine. Although there was not enough material to further identify phosphoarginine, the radioactivity was eluted from the paper and was converted to inorganic phosphate by heating in 0.1 M HCl for 10 min. at 60°C. The difference in ATP concentration in the incubation mixtures and the work-up procedure may account for the large difference in phosphohistidine seen in the two experiments reported here. In a completely separate experiment, an aliquot of the ^{32}P protein hydrolysate was applied to a 20 x 20 cm silica gel plate and developed in two dimensions at 90° to one another. The results revealed again that both phosphohistidine and phosphoarginine accounted for the majority of the radioactivity. None

was found in the phospholysine region of the plate. Some inorganic phosphate and some relatively immobile, presumably incompletely digested phosphopeptides, were also seen.

Apparently the kinase capable of phosphorylating myelin basic protein on histidine and arginine residues is a component of the myelin membrane, since no exogenous kinases were added to our preparations. Of the two experiments shown here, it is clear that the phosphorylation of myelin basic protein is a very dynamic process, since the level of phosphohistidine seen varies considerably. At present we know very little of the route or quantitative details of the turnover of the base stable phosphorylation, nor do we know its physiological function. The microheterogeneity of phosphorylated myelin basic protein seen by Deiber, Martenson, Kramer and Kies (4) may possibly be attributed to basic amino acid phosphorylation.

ACKNOWLEDGMENT

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