Phosphorylation of Myelin Basic Protein*

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SUMMARY

Myelin membranes prepared from rat brain possess both the enzyme and substrates to incorporate \(^{32}\)P from \([\gamma-^{32}\text{P}]\text{ATP}\) into membrane protein constituents. Of the myelin polypeptides, only the two basic proteins were phosphorylated; and both components appeared to be equally good substrates for endogenous or added protein kinases. The \(^{32}\)P was transferred primarily to serine residues of the basic protein. With myelin membranes in vitro, the phosphorylation reaction was linear for less than 1 min at 24\(^\circ\), and was not stimulated by cyclic adenosine 3\:'5\:'-monophosphate. Myelin basic protein was also labeled following intracranial injection of \(^{32}\)P. Basic protein of myelin prepared from adult rats was more readily phosphorylated than the basic protein of myelin prepared from 14-day-old rats. However, when differences in myelin protein kinase activity were minimized by the addition of rabbit muscle protein kinase to heated or native myelin preparations, the basic protein of 14-day-old myelin was phosphorylated more readily and to a greater extent than adult myelin. These studies suggest that myelin basic protein is phosphorylated in vitro and in vivo and such phosphorylation may have potentially profound effects on myelin structure and function.

Phosphorylation of brain membrane polypeptides has been reported from several laboratories (1–8). In many of these brain membrane preparations, both the enzyme (protein kinase) and the polypeptide substrates have been localized to the same fraction. In all such investigations a considerable number of polypeptide constituents have been shown to be phosphorylated. Crude myelin preparations from brain have also been shown to possess both protein kinase activity and endogenous substrates (4). However no detailed examination has been undertaken of the phosphorylated substrates. A recent report has suggested that basic protein isolated from myelin is an extremely effective substrate for an exogenous protein kinase isolated from bovine muscle (9).

In the present study, we demonstrate that basic protein within a purified myelin fraction can be phosphorylated both under in vitro and in vivo conditions, while other protein components show no incorporation of \(^{32}\)P. This phosphorylation changes with development and with varied conditions of incubation.

MATERIALS AND METHODS

Wistar rats of various ages were used throughout. Rats were killed by decapitation, and the brains were removed quickly and placed in iced sucrose buffer (0.32 M sucrose and 0.1 M Tris-HCl, pH 7.4). Subcellular fractionation was prepared by a technique previously described (10). The method consists primarily of a mild homogenization with a Teflon glass homogenizer, removal of the crude nuclear fraction by centrifugation at 1000 \(\times g\) for 10 min, and isolation of the crude mitochondrial fraction by centrifugation at 14,500 \(\times g\) for 15 min. The crude mitochondrial pellet was used for the preparation of purified myelin according to the method of Banik and Davison (11).

After centrifugation at 53,000 \(\times g\) for 1 hour, a crude myelin fraction accumulates at the interface of 0.32 and 0.8 M sucrose. The myelin is then purified by osmotic shock and spun at 53,000 \(\times g\) for 1 hour on a discontinuous sucrose gradient where it is collected from the interface of 0.32 and 0.85 M sucrose. The myelin was washed in 30 mM Tris-HCl, pH 7.4, and stored at 20\(^\circ\). To prepare myelin from the brains of young rats in sufficient quantity up to 15 animals were used for each preparation. For adult rats two brains were sufficient for the preparation of adequate amounts of myelin. For in vitro experiments only preparations that were left frozen for the same period of time and thawed once were used.

Proteins were determined by the method of Lowry et al. using bovine albumin as standard (12).

Protein phosphorylation was measured in an incubation volume of 0.2 ml at 24\(^\circ\) for 20 s to 20 min. The standard assay unless otherwise indicated contained 80 to 100 \(\mu\)g of membrane protein, 10 mM Tris-HCl, pH 7.4, 5 mM MgCl\(_2\), 10 \(\mu\)M \([\gamma-^{32}\text{P}]\text{ATP} (1.5 to 2.5 \times 10^{5}\) counts per min). The reaction was stopped by addition of 0.1 ml of a solution of 2% sodium dodecyl sulfate, 8 M urea, and 2% \(N\text{-}\text{methyl}-\text{N\text{-}tetramethylenediamine, and 0.5% ammonium persulfate. The samples were run on gels,}\)

MATERIALS AND METHODS

The abbreviations used are: PPO, 2,5-diphenyloxazole; dimethyl POPOP, \(p\)-bi(3-(6-phenylxanoethyl)benzenec. 5416
per liter of dimethyl-POPOP as well as 30% Triton X-100 and counted in a Packard scintillation counter. After blinks were subtracted, basic protein phosphorylation was measured as the sum of the radioactivity of the two basic protein components and results expressed as picomoles of $^{32}$P per mg of basic protein.

Quantification of myelin basic protein on polyacrylamide gel was done by cutting and weighing sections of the stained profiles (15). The staining of individual peaks yielded a scan that was proportional to protein concentration. Determinations were performed for each preparation and agreed within ±5%.

Molecular weights were determined by calibration of the gels with polypeptides of known molecular weight (cytochrome c, α-chymotrypsin, ovalbumin, transferrin, and phosphorylase A).

Basic protein was extracted from myelin according to a technique adapted from Eylar et al. (18). Five to ten milligrams of myelin were defatted in 6 volumes of acetone precooled to -20°. The protein residue was then mixed with 20 volumes (w/v) of dilute HCI to adjust and keep the pH at 1.7 for the period of the acid extraction, i.e. about 17 to 24 hours. After the acid extraction, the suspension was spun at 16,000 × g for 20 min, the residue was discarded and the supernatant fluid was adjusted to pH 7.0 with 1 N NaOH. A slight precipitate formed and was removed by centrifugation. The supernatant fluid was lyophilized and then redissolved in water for electrophoresis.

The method of Panyim and Chalkley (19) was used to characterize the basic protein by polyacrylamide gel electrophoresis at acid pH. The gels were composed of 15% acrylamide, 0.1% methylene-bisacrylamide, 5.4% glacial acetic acid, 0.5% N,N',N',N'-tetramethylenediamine, 0.25 M urea, and 0.125% ammonium persulfate. The final pH after pre-electrophoresis was 3.2. Tray buffer was 0.9 N acetic acid. The samples were run on gels, 6 mm × 8 cm, at 2 ma per gel and 120 volts for 3 to 4 hours.

Fixation, staining, and solubilization were done as described above.

For in vivo experiments five 20-day-old rats were used for each preparation and killed 5 min or 2 hours following intracranial injection of $^{32}$P-labeled ATP. For in vitro experiments five to ten milligrams of myelin were incubated with $^{32}$P-labeled ATP in a volume of 1.0 ml under the standard assay conditions. The reaction was then stopped after 30 s by addition of acetic acid precooled at -20° and the basic protein extracted at an acid pH.

The acid-extracted material yielded two polypeptide bands corresponding to the basic peptides of rat myelin (Fig. 2). The estimated molecular weight of basic protein isolated by this technique was 18,000 for the slow migrating component and 15,000 for the fast migrating component which is a good agreement with values published by others (23). The two basic protein components migrated so close to one another that the two radioactive peaks in these experiments were not resolved.

Following acid extraction the basic protein was water-soluble, and could be characterized by conventional gel electrophoretic techniques employed for basic proteins. Fig. 3 shows the electrophoretic pattern of basic protein in 15% polyacrylamide gels at acid pH according to the technique of Panyim and Chalkley (19). This technique was found to give a better separation of the two basic protein components and confirms the observation of several investigators that gel electrophoresis of rat central nervous system myelin basic protein at acid pH reveals two major components (24, 25). This was true, however, only when basic protein was extracted from isolated myelin. If basic protein was isolated from whole central nervous system tissue treated with chloroform-methanol, additional protein bands appeared with electrophoretic mobilities similar to those of histones. Thus, the acid extract of whole central nervous system tissue contains basic proteins that are not myelin constituents (26).
The phosphorylation reaction had similar requirements to those described for other protein kinases, and there was an absolute requirement for magnesium (1, 4, 6). Under conditions of initial rate there was no significant stimulation of phosphorylation of the basic protein by the addition of cyclic adenosine 3':5'-monophosphate. Calf thymus histones were also a good substrate for the endogenous kinase.

When incubated at 24°C the phosphorylation of basic protein continued for about 12 min after which little increase in total incorporation was observed. The incorporation was proportional to the quantity of membrane protein present in the reaction mixture over a range of membrane concentrations from 20 to 250 μg of protein. The enzyme was completely inactivated by treatment of the membranes at 50°C for 5 min. However, heated membrane could be used as a substrate for exogenous enzyme; and the pattern of phosphorylated protein on dodecyl sulfate polyacrylamide gels was similar to that obtained with native membranes.

In order to obtain more direct evidence that phosphate was incorporated into protein and to identify the amino acid residues being phosphorylated the reaction was stopped by 10% trichloroacetic acid and the phosphorylated proteins were separated from free a2P by repetitive washing with trichloroacetic acid and hydroxylamine (16). The resulting precipitate was hydrolyzed for 3 hours in 6 N HCl and subjected to high voltage electrophoresis according to the method of Allerton and Perlmann (17). Phosphoserine and phosphothreonine were hydrolyzed under identical conditions and used as standards. Positions of the amino acids were determined after electrophoresis by spraying with ninhydrin. The electrophoresis paper was cut into 1-cm strips and counted by scintillation spectrometry. After correction for hydrolysis of phosphoserine and phosphothreonine (17) 60% of the total incorporated phosphate was associated with phosphoserine, 7% was associated with phosphothreonine with the remaining activity recovered as a2P.

In Vivo Phosphorylation of Basic Protein Following Intracranial Injection of a2P—There was no labeling of basic protein 5 min after the intracranial injection of a2P, but significant labeling 2 hours after the injection of the isotope. When basic protein was extracted from whole brain, many polypeptides were found to be phosphorylated, and it was extremely difficult to specify the myelin basic protein with certainty. To identify the phosphorylated substrate following in vivo labeling, myelin was first isolated. Then basic protein was isolated from myelin according to the same method used for in vitro studies (18). The electrophoretic pattern of basic protein on the Chalkley gel and the corresponding phosphorylation is shown in Fig. 4. The extracted phosphorylated polypeptide also migrated at an Rf identical with myelin basic protein on dodecyl sulfate gels. In Fig. 4, it appears that only one of the basic proteins is phosphorylated. The fact that both components could be phosphorylated in vitro suggests the possibility that only certain basic protein locales are available in vivo. However, both components were labeled on dodecyl sulfate gels (Fig. 5); and it is more likely that phosphorylation of the less concentrated component on Chalkley gels has been obscured by the low rate of incorporation and the gel-slicing technique employed.

The resolution of total myelin on dodecyl sulfate gels was found less satisfactory for in vivo studies because of the presence of the labeled membrane lipids appearing as a fast running band in front of the bromphenol blue tracking dye (Fig. 5). When myelin was extracted with acetone most of these lipids were extracted and the radioactivity in this region of the gel was diminished markedly. Incorporation of radioactivity in these membrane lipids was only observed when myelin was labeled with a2P phosphate in vivo or after long incubation in vitro of myelin with [γ-32P]ATP.

Having demonstrated a2P incorporation into basic protein following intracerebral incubation, it was important to show that phosphorylation did not take place during the subcellular fractionation. [γ-32P]Phosphate was added to a homogenate of control brains and subcellular fractionation carried out. No radioactivity was detectable in basic protein when a2P was added...
to the homogenate. A mixing experiment was also carried out in which a 40,000 × g for 20 min supernatant, following intracerebral ^32P injection was added to control brain homogenate. Following rehomogenization, myelin was separated and the basic protein was extracted. Under these conditions no radioactivity was recovered in basic protein.

Changes of in Vitro Basic Protein Phosphorylation during Development—Definite changes have been noted in the relative amounts of myelin proteins with increasing postnatal age (27). For this reason it was necessary to express our results based upon milligrams of basic protein rather than on a total myelin basis. Following electrophoresis, the basic protein peak represented 31% of total adult myelin protein, while it was only 21% of 14-day myelin. These results are similar to reports from other investigators (27, 28).

At Day 8 when only trace amounts of basic protein could be demonstrated, no significant endogenous or exogenous phosphorylation of basic protein was found. The rate of incorporation of radioactive phosphate into the basic protein of 14 days and adult myelin in vivo is shown in Fig. 6. After a 20-min incubation, the ^32P phosphate of adult basic myelin protein had a specific activity more than 3 times higher than in young rats.

To determine whether the greater specific activity was related to the amount of endogenous enzyme or the state of the basic protein, myelin membranes were heated at 50° for 5 min to inactivate the enzyme. Muscle protein kinase was then added to heated myelin of adult and 14-day rats. A significantly greater phosphate incorporation was observed in heated myelin preparations following addition of exogenous enzyme to myelin fractions obtained from 14-day-old rats as compared to adult (Fig. 7). Both the initial rate of phosphorylation and the final level of phosphorylation in basic protein were greater in myelin isolated from 14-day-old animals. Such results were not merely an artifact of the heating process since higher initial rates of incorporation of radioactive phosphate were also noted when native unheated myelin was used as a substrate in the presence of exogenous protein kinase activity and the endogenous activity subtracted (Fig. 7). Thus the lower rate of endogenous phosphorylation noted with exogenous enzyme and substrate in myelin from 14-day-old animals was not related to substrate availability but to lower enzyme activity in the younger animals.

An additional interesting finding was revealed with the addition of exogenous protein kinase to unheated but not to heated membranes. After 3 min of incubation of exogenous enzyme with 14-day native myelin, and after 6 min of incubation of exogenous enzyme with adult native myelin, a significant decrease in ^32P phosphate incorporation into basic protein was observed. These data would suggest that phosphate incorporated into the basic protein of unheated membranes may undergo dephosphorylation. This turnover appeared to be a property of the unheated myelin and not the exogenous protein kinase preparation because the latter was present in both incubations yet the turnover could not be demonstrated with heated myelin.

**DISCUSSION**

Phosphorylation of membrane protein represents a reaction with potentially profound effects on membrane structure and function (1–8). Although soluble substrates have been characterized, membrane substrates have not been solubilized and purified to help define the reaction. Myelin is a membrane which can be isolated with high purity, and its limited polypeptide profile provides an excellent model for the detailed analysis of protein phosphorylation. Of the myelin polypeptides, only the two basic proteins were
phosphorylated; and in vitro both components appeared to be equally good substrates for the endogenous or the exogenous protein kinases. Because both components migrated so closely on dodecyl sulfate gels, incorporation of radioactive phosphate into the basic protein was calculated on the basis of the total amount of [32P]phosphate transferred to the two basic protein components. Under conditions of maximal phosphorylation (incubation of myelin for 20 min in the presence of exogenous protein kinase), 1000 pmoles of [32P] were incorporated per mg of basic protein. Assuming an average molecular weight of 17,500 for the two components of the basic protein, less than 0.02 mole of phosphate was incorporated per mole of protein. The reason for this low level of phosphorylation is unknown although a phosphate was incorporated per mole of protein. The reason for this low level of phosphorylation is unknown although a higher rate would be anticipated with a more purified system. In fact, Carnegie et al. (9) recently demonstrated an in vitro incorporation of 0.37 mole of [32P] per mole of protein employing purified myelin basic protein and bovine muscle protein kinase.

The most significant question in the present investigation is whether phosphorylation of basic protein is merely a potential reaction or an actual in vivo event. Other laboratories (29) have reported that the isolated basic protein had no phosphate. By direct analysis, this would mean that less than 0.1 mole of phosphate per mole of protein could be detected, and our in vivo and in vitro phosphorylation would not have been noted. Our data clearly show that myelin basic protein may be phosphorylated in vivo. Yet the fact that we cannot demonstrate higher specific activity suggests that only certain basic protein locales may be subject to phosphorylation.

Fourteen-day-old myelin was found to be a better substrate for protein phosphorylation than adult myelin. This was true for both heated or native myelin and may be explained by better substrate accessibility to exogenous enzyme. In the young animal the myelin sheath consists of only a few lamellae. In such a structure, the basic protein might be more accessible to the exogenous enzyme than more mature animals where myelin is a compact multilamellar structure. An alternative explanation of the enhanced rate of phosphorylation of basic protein in myelin from 14-day-old rats might depend upon the structure of the myelin membrane and the local milieu of the basic protein at different ages. The lipid composition is known to change during development (30, 31) and such changes may influence myelin structure and the extent to which the basic protein was phosphorylated. Unfortunately the lack of a definitive membrane localization for basic protein prevents a more detailed explanation of our noted differences.

The present study concentrated primarily on myelin basic protein as a substrate for protein phosphorylation in vivo and in vitro. However we also noted endogenous protein kinase activity in all enriched preparations of myelin including a highly purified fraction prepared by multiple hypotonic treatments and density gradient centrifugation (kindly supplied by Dr. E. Day, Duke University). At present, it is not clear to what extent the endogenous protein kinase is associated with the myelin sheath or some closely related structure such as axolemma or the oligodendroglial cell membrane.

REFERENCES