The Contribution of Phosphorylation and Loss of COOH-terminal Arginine to the Microheterogeneity of Myelin Basic Protein

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Two guinea pig myelin basic protein preparations which differed markedly in their contents of high pH electrophoretic or chromatographic forms were studied in an attempt to elucidate the causes of their microheterogeneity. Both total preparations and components isolated therefrom were examined for their amino acid compositions, NH₂-terminal and COOH-terminal residues, total phosphorus contents, and contents of phosphoamino acids. The results showed that the five components differed sequentially by a single charge and that the microheterogeneity arose as a result of secondary modifications of a single species (Component 1) of basic protein. Two modifications were demonstrated: viz. phosphorylation of serine and threonine and loss of COOH-terminal arginine. These two modifications were insufficient to account completely for the observed microheterogeneity; an additional cause, deamidation, was postulated. From the relationship between the number of components present in the total basic protein, the phosphorus and phosphoamino acid contents of the components, and the changes in relative electrophoretic mobility of the components which accompanied their phosphorylation and dephosphorylation we conclude that in the native basic protein no more than two sites in any polypeptide chain are phosphorylated.

Myelin basic protein isolated from central nervous system tissue of vertebrates exists in several forms which differ in charge, as judged by their behavior during alkaline-pH gel electrophoresis (1, 2) or ion exchange chromatography (2–4). These different forms have been isolated and shown to have essentially the same amino acid compositions (2, 4) and encephalitogenic activities (4). All forms contained unsubstituted arginine, N²-monomethylarginine, and N⁶,N⁶-di-methylarginine at position 106, a finding which eliminated differential methylation of this residue as a cause of the microheterogeneity (2, 4).

A possible cause of at least some of the observed microheterogeneity has been suggested by Martenson et al. (3), who pointed out that since the COOH terminus of the protein is -Ala-Arg-Arg, partial loss of these arginines would yield three forms of the protein differing by a single charge.

Recently the basic protein has been found to exist in a partially phosphorylated state. Direct chemical analyses of the protein from bovine brain have demonstrated that the phosphorylation involves both serine and threonine residues (5, 6).

In vivo studies in the rat have shown the rapid uptake of radioactive orthophosphate into serine and threonine residues of the basic protein (5–7). Studies by Carnegie et al. (8, 9) have shown that endogenous and exogenous protein kinases phosphorylate specific serine and threonine residues in the myelin basic protein in vitro. As an explanation of the microheterogeneity of the basic protein, Miyamoto and Kakiuchi (6) have suggested that the different forms of the protein result from different extents of phosphorylation.

In the present study we describe experiments designed to examine these two possibilities (loss of COOH-terminal arginine and phosphorylation) for their role in producing the protein's microheterogeneity. The results show that both factors are involved and a third as well. In vivo the basic protein exists in three forms by virtue of phosphorylation: a major form which is not phosphorylated, a minor form which is diphosphorylated, and an intermediate form which is monophosphorylated. A brief report of part of this work has appeared previously (10).

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MATERIALS AND METHODS

Reagents—Escherichia coli alkaline phosphatase-BAPC, carboxypeptidase A, diisopropylphosphorofluoridate, and carboxypeptidase B-diisopropylphosphorofluoridate were obtained from Worthington Biochemical Corp., Freehold, N. J. All other materials were obtained from commercial sources. Portions (50 μl) of carboxypeptidase B (10 μg/μl in 0.1 M NaCl) were frozen and stored at -30°C until used. Carboxypeptidase A crystals were washed twice with H₂O and dissolved in 0.2 M NH₄HCO₃, 100 μl portions (10 μg/μl) were stored frozen (11, 12).

Myelin Basic Protein—Myelin basic protein was prepared from quick-frozen guinea pig brains as described by Deibler et al. (13) and purified further by gel filtration through Sephadex G-100. The protein was subsequently chromatographed at high pH on carboxymethyl cellulose, and the five chromatographic components were isolated as previously described (4). Components 3, 4, and 5 contained some contaminants and were purified further on Sephadex G-100. This protein preparation and components derived therefrom were compared with the corresponding material prepared and described earlier (4). The present preparation will henceforth be referred to as preparation A; the earlier, preparation B.

Determination of Protein—A known dry weight of salt-free, lyophilized preparation A and each of its components were dissolved in a known volume of known volumes of 0.1 M NaCl for various analyses and lyophilized. One portion was subjected to amino acid analysis, and from the yield of glutamic acid (10 mol/mole of protein of M₉ = 18,300 (14)) the actual protein content of each sample subjected to the various analyses was determined. From the glutamic acid content a dry weight of 1.00 mg was found to correspond to 0.81 to 0.94 mg of protein. For preparation B and its components 1.00 mg dry weight was assumed to correspond to 0.86 mg of protein.

Determination of Protein—A known dry weight of salt-free, lyophilized preparation A and each of its components were dissolved in 0.2 M NH₄HCO₃ at concentrations of approximately 5 mg/ml. After portions serving as zero time controls had been removed, carboxypeptidase A and carboxypeptidase B were added (in that order) such that the approximate enzyme to substrate (w/w) ratios were 1:35 (carboxypeptidase A) and 1:70 or 1:15 (carboxypeptidase B). The solutions were incubated at 25°C for appropriate time intervals (up to 2 hours) portions for subsequent electrophoretic and amino acid analyses were removed, added to 2 to 4 volumes of 0.2 M acetic acid, and lyophilized twice. As controls, mixtures of carboxypeptidase A and B and protein samples minus the enzymes were incubated at 37°C for 2 hours and treated as described above. Analyses of zero time and 2 hour controls showed no detectable free amino acids; no notable differences in the electrophoretic patterns of the basic proteins.

Determination of Phosphorus—Protein (2.0 mg dry weight) was dissolved in 1.0 ml of H₂O, then precipitated with trichloroacetic acid and washed with ethanol-ether as described by Adams et al. (15). The precipitate was dissolved in 200 μl of H₂O, and the solution was divided into two portions and analyzed for total phosphorus by the dry ash method of Ames (16).

Determination of Phosphoamino Acids—Samples were hydrolyzed in vacuo in constant boiling HCl at 105°C for periods varying from 3 to 8 hours. Quantitation of phosphoserine plus phosphothreonine in the hydrolysates was generally carried out with the use of a 20-μm column of DC 6A resin (Durrum Chemical Corp., Palo Alto, Calif.) equilibrated at 55°C with 0.2 M sodium citrate buffer, pH 3.25, and operated at a flow rate of 70 ml/hour. In one experiment a 50-μm column of Beckman AA-15 resin was used. The eluate was monitored at 254 nm. Separation of phosphoserine and phosphothreonine was accomplished by high voltage electrophoresis at pH 1.9 (11) on cellulose “chromagram” sheets (20 × 20 cm) (Eastman Kodak Co., Rochester, N. Y.) for 3 hours at 400 volts. The phosphoamino acids were located with ninhydrin spray (Sigma).

Phosphorylation Reactions—Samples of Component 1 (preparation A) were phosphorylated for periods of 1 to 24 hours at 30°C with bovine brain adenosine 3′5′-monophosphate-dependent protein kinase according to the procedure of Miyamoto and Kakinouchi (6). Each reaction mixture contained 1 mg of Component 1, 50 μmol of sodium acetate buffer (pH 6.0), 10 μmol of magnesium acetate, 1 nmol of cyclic AMP, 1 μmol of ATP, and 70 μg of protein kinase in a total volume of 1.0 ml. The reaction was terminated by heating for 30s in a boiling water bath.

The solutions were dialyzed overnight at 5°C against 0.1 M acetic acid and lyophilized.

Dephosphorylation Reactions—The total basic protein and its components were treated with Escherichia coli alkaline phosphatase as described by Bathorn et al. (18) except that 0.05 M NH₄HCO₃ pH 8.2, was used instead of Tris-HCl. Enzyme to substrate ratios were approximately 1:15 (w/w). The control proteins were incubated under identical conditions but without enzyme; the control contained the same amount of (NH₄)₂SO₄ as present in the enzyme suspension. Incubation was carried out for 24 hours at 25°C, after which the samples were lyophilized twice.

Other Analytical Procedures—Standard amino acid analyses were carried out as described previously (14). NH₂-terminal residues were determined by reaction of the basic protein with tryptic or chymotryptic enzyme (17) and by reaction of the protein with diisopropylphosphorofluoridate as described by Tamura et al. (19) and separation of the dansyl amino acids on polyamide sheets (7.5 × 7.5 cm) by the procedure of Hartley (20). Polyaacrylamide gel electrophoresis was carried out in 5% gels containing 8 M urea and either 0.01 M sodium glycinate, pH 10.6, or 1 M acetic acid, pH 2.4 (13). Proteins were stained with Amido black, and gels were scanned at 500 or 690 nm with a Beckman DU monochromator fitted with a Gilford model 2410 linear transport having a slit plate (0.05 × 2.36 mm). Areas under the peaks were measured with a planimeter.

In order to compare the different protein samples with regard to their specific component compositions, equal volumes of samples containing comparable amounts of protein were subjected to electrophoresis in parallel. Where possible, duplicate samples were included in the same run; otherwise the entire electrophoretic analysis was repeated. For some procedures it was found to yield extremely reproducible mobilities for any given electrophoretic component when the gels were run in parallel, thereby permitting direct comparison of the patterns by alignment of the gel origins. In addition, in every set of experiments in which proteins were treated with carboxypeptidases, the time course of the reaction was followed by electrophoresis in parallel of the samples obtained at the different time points (the first being at 5 min). This established unequivocally which of the components were undergoing change and the correctness of the component designation.

When maximal resolution of components for the purpose of quantitation was desired, the amount of protein applied to the gels was limited to 50 μg. Under these conditions repeated electrophoretic analyses of the same sample yielded relative area values agreeing to within 5% for well resolved components and 10% for less well resolved ones.

RESULTS

Treatment of Basic Protein with Carboxypeptidases A and B—It is known from studies with carboxypeptidases A and B (21, 22) that the COOH terminus of the intact guinea pig myelin basic protein is -Ala-Arg-Arg and that removal of the fourth residue, methionine, is inhibited (22). In the bovine protein this inhibition is due to the presence of an adjacent prolid residue (23), and it can be assumed that the same is true for the guinea pig protein as well.

In order to assess the importance of the intact COOH terminus in contributing to the over-all pattern of microheterogeneity, the basic protein preparations were treated with carboxypeptidases A and B (Fig. 1, left). Loss of COOH-terminal residues from preparation A resulted in a complete anodic shift such that the relative proportions of Components 3, 4, and 5 in the new electrophoretic pattern were essentially the same as those of Components 1, 2, and 3 in the pattern obtained prior to enzyme treatment. It appeared as if Components 1 to 5 differed sequentially by a single charge and that removal of the two COOH-terminal arginines had converted Components 1, 2, and 3 to Components 3, 4, and 5, respectively. Loss of COOH-terminal residues from preparation B, on the other hand, resulted in relatively little change in the pattern other than the loss of Components 1 and 2. From the behavior of the two preparations it was obvious that the
The resolution of preparation A (right) was inferior to that of the same retic mobilities at acid and alkaline pH. Components of slightly higher electrophoretic mobility. The corresponding fraction of polypeptide chains having the intact COOH terminus was much greater in preparation A than in B.

**Treatment of Basic Protein with Alkaline Phosphatase**—The role of phosphorylation in contributing to the microheterogeneity was examined in an analogous fashion (Fig. 1, right). Dephosphorylation of the basic protein resulted for both preparations in a partial shift to Components 1 and 2. The effect was shown most clearly with preparation A, where appreciable reduction in the relative amounts of Components 3 and 4, as well as 5, occurred. Chemical analyses of these preparations before and after enzyme treatment showed that the electrophoretic shift was accompanied by loss of 98 to 100% of the phosphorus originally present. These results indicated that Components 3, 4, and 5 existed in a partially phosphorylated form, whereas Components 1 and 2 were unphosphorylated.

**Isolation of Components**—Detailed studies on the relationship between microheterogeneity and modification of the basic protein were made possible by the isolation of the individual components by ion exchange chromatography. The partial characterization of components obtained from preparation B has been described previously (4). Those obtained from preparation A appeared to be essentially homogeneous by electrophoresis at alkaline pH (Fig. 2). Electrophoresis at acid pH, however, revealed that Components 3, 4, and 5 were still contaminated to the extent of approximately 10% with protein of slightly higher electrophoretic mobility. The corresponding components of preparations A and B had identical electrophoretic mobilities at acid and alkaline pH.

**Amino Acid Compositions of Components**—As shown in Table I there were no significant differences in amino acid composition among the components of preparation A. Amino acid analyses of preparation B components (4) have led to similar conclusions, although in the earlier study the precision of the data was not as high. No NH2-terminal residue was detected in Components 1, 3, and 5. The trace of NH2-terminal methionine found in Components 3, 4, and 5 undoubtedly arose from the contaminating protein which had been detected in these components upon their electrophoresis at acid pH. A trace of NH2-terminal methionine was also found in Components 1 and 2 of preparation B.

Relative Electrophoretic Mobility versus Charge—In order to establish the relationship between relative electrophoretic mobility and charge, each of the components of preparation A was incubated with carboxypeptidases A and B. If each of these components consists of some polypeptides having the intact COOH terminus -Ala-Arg-Arg, short term treatment with the exopeptidases should yield from each component three polypeptide chains differing from one another by a single charge. After 10 min of incubation the results shown in Fig. 3 were obtained, showing that Components 1 to 5 did, in fact, differ sequentially by a single charge and that each consisted of some polypeptide chains having the intact COOH terminus.

**COOH-terminal Analyses**—Incubation with carboxypeptidases A and B for 90 or 120 min resulted in 100% conversion of Component 1 of both preparations, via Component 2, to Component 3. Component 2 was converted completely to a mixture of Components 3 and 4. Components 3, 4, and 5, on the other hand, differed markedly in their ability to convert, depending upon their source. Those from preparation A underwent appreciable conversion to the less basic forms, whereas those from preparation B underwent minimal conversion (Table II). No conversion of any components occurred upon incubation for 90 or 120 min in the absence of the enzymes. A further difference between the two preparations lay in the nature of the final products of Components 3 and 5. In preparation A Components 3 and 4, respectively, were ultimately converted to Components 5 and 7 only; in preparation B the final spectrum included Components 4 and 6 as well (Table II).

Since the conversion of any component to the one of next
TABLE I

Amino acid compositions of guinea pig myelin basic protein and its components

| Total basic protein | Components | Theoretical |
|---------------------|------------|-------------|
|                     | 1  | 2  | 3  | 4  | 5  |     |
| Asp                 | 11.2| 10.6| 11.1| 10.8| 10.3| 11.0|
| Thr                 | 7.7 | 7.4 | 7.4 | 7.1 | 7.1 | 7.6 |
| Ser                 | 16.1| 16.6| 18.3| 17.6| 16.7| 16.8|
| Glu                 | 10.0| 10.0| 10.0| 10.0| 10.0| 10.0|
| Pro                 | 9.5 | 10.0| 10.6| 10.4| 10.6| 10.4|
| Gly                 | 22.1| 22.8| 24.2| 23.3| 22.7| 23.1|
| Ala                 | 13.5| 12.6| 13.3| 12.8| 12.4| 12.7|
| Val                 | 3.3 | 2.3 | 2.1 | 2.1 | 2.1 | 2.2 |
| Met                 | 1.7 | 1.6 | 1.7 | 1.5 | 1.5 | 1.5 |
| Leu                 | 3.6 | 3.5 | 3.4 | 3.4 | 3.4 | 3.3 |
| Tyr                 | 3.6 | 3.7 | 3.6 | 3.6 | 3.6 | 3.6 |
| Phe                 | 8.3 | 8.3 | 8.4 | 8.7 | 8.3 | 8.3 |
| NH₂ terminus        | Blocked | Blocked | Blocked | Trace | Trace | Blocked |

|                     | 1  | 2  | 3  | 4  | 5  | 6  | 7  |
|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| higher number involved the loss of one positive charge, the data in Table II were used to estimate the per cent of polypeptides in each initial component having 0, 1, or 2 arginines at the COOH terminus. For example, Component 3 of preparation A consisted of polypeptides 92% of which had the 2 COOH-terminal arginines and 8% of which lacked both. Component 3 of preparation B contained polypeptides 25% of which had the intact COOH terminus, 13% of which lacked 1 arginine, and 62% of which lacked both. These data were used to calculate the theoretical yield of COOH-terminal arginine for each component; e.g. for Component 3 of preparation A: 0.92(2) = 1.84 mol/mol; for Component 3 of preparation B: 0.25(2) + 0.13(1) = 0.63 mol/mol.

Portions of the samples analyzed electrophoretically were analyzed for free arginine and alanine on the amino acid analyzer. The actual yields of these amino acids obtained after 90- or 120-min treatment with carboxypeptidases A and B are presented in Table III, together with the theoretical yields of arginine calculated on the basis of the data in Table II. All components released approximately 1 mol of alanine/mol of protein but no additional amino acids other than arginine and a trace of methionine. The actual yields of arginine determined directly were in reasonably good agreement with the theoretical contents of COOH-terminal arginine estimated by electrophoretic analysis.

As a further comparison of the direct and indirect methods for determining the COOH terminus of each component, the time courses of amino acid release and component conversion were followed. Each component released arginine in parallel with its conversion to the next higher homologue that differed by two charges. For example, it can be seen (Fig. 4, left) that conversion of Components 3 and 5 to Components 5 and 7, respectively, proceeded via Intermediates 4 and 6 and that the extent of conversion of Components 3 and 5 was much greater for preparation A than for B. As shown in Fig. 4 (right) arginine was released faster than alanine from the A components, whereas the reverse was true for the B components. The results obtained with Component 4 paralleled those shown for Components 3 and 5.

Relative Electrophoretic Mobility versus Extent of Phosphorylation—Component 1, which is phosphorus-free (see below), was phosphorylated by brain protein kinase. In the course of 24 hours approximately 3.4 g atom of phosphorus/mol were incorporated. As shown in Fig. 5 (top) a whole series of

TABLE II

Conversion of basic protein components with carboxypeptidases A and B

| Initial component | Final components |
|-------------------|------------------|
| 1                 |                 |
| 2                 |                 |
| 3                 |                 |
| 4                 |                 |
| 5                 |                 |
| 6                 |                 |
| 7                 |                 |

| Preparation A | 3 | 4 | 5 | 6 | 7 |
|---------------|---|---|---|---|---|
| 1             | 1.00 | | | | |
| 2             | 0.17 | 0.83 | | | |
| 3             | 0.08 | | 0.92 | | |
| 4             | 0.15 | 0.20 | 0.65 | | |
| 5             | 0.21 | | | 0.79 | |

| Preparation B | 3 | 4 | 5 | 6 | 7 |
|---------------|---|---|---|---|---|
| 1             | 1.00 | | | | |
| 2             | 0.38 | 0.62 | | | |
| 3             | 0.62 | 0.13 | 0.25 | | |
| 4             | 0.66 | 0.16 | 0.18 | | |
| 5             | 0.71 | 0.14 | 0.15 | | |

*a Preparation A. The results are averages of duplicate analyses on the same hydrolysate. Tryptophan and amide contents were not determined.

*b Determined from the amino acid compositions of constituent peptic peptides of the protein (14).

*c Based upon 10 mol of glutamic acid/mol of protein.
TABLE III

COOH-terminal residues of myelin basic protein and its components

Preparation A and its components were treated with carboxypeptidases A and B for 120 min. Preparation B and its components were treated for 90 min. The amino acids released were quantitated on an amino acid analyzer. Values in parentheses are theoretical ones calculated from electrophoretic data in Table II (see text).

| Preparation | Total basic protein | Components |
|-------------|---------------------|------------|
|             |                     | 1         | 2         | 3         | 4         | 5         |
| Preparation A |                   | 1.19      | 0.98      | 1.15      | 1.00      | 0.99      | 1.11      |
|              | Ala                 | 1.82      | 1.83 (2.0)| 1.84 (1.8)| 1.68 (1.8)| 1.40 (1.5)| 1.82 (1.6)|
| Preparation B |                   | 0.86      | 0.83      | 0.81      | 0.83      | 1.02      | 0.81      |
|              | Ala                 | 0.56      | 1.74 (2.0)| 1.23 (1.6)| 0.38 (0.6)| 0.35 (0.5)| 0.33 (0.4)|

FIG. 4. Left, time-dependent conversion of Components 3 (top) and 5 (bottom) to higher numbered components upon incubation with carboxypeptidases A and B. Right, corresponding release of COOH-terminal amino acids from Components 3 (top) and 5 (bottom) upon incubation with the enzymes. —, preparation A; ——, preparation B.

Components differing from one another by two charges were generated. The earliest products resulting from phosphorylation of Component 1 were Components 3 and 5. These must contain one and two phosphate groups in their respective polypeptide chains. The other components must have arisen from the successive incorporation of additional phosphate groups. It is obvious that Components 7, 9, and 11 are not detectable in the total isolated basic protein. The importance of carrying out electrophoresis above the pK, of phosphoric acid and, more significantly, close to the isoelectric point of the basic protein is shown in Fig. 5 (bottom). Electrophoresis at acid pH failed to reveal any microheterogeneity, and only a slight progressive decrease in electrophoretic mobility accompanied the increase in phosphorylation of Component 1.

Phosphorus Analyses—The extent to which each component of the basic protein was phosphorylated was examined in a manner analogous to that used for COOH-terminal analyses. Incubation of Components 1 and 2 from either preparation with alkaline phosphatase for 24 hours had no effect on their electrophoretic mobilities. Components 3 and 4, however, underwent partial conversion to Components 1 and 2, respectively, while Component 5 underwent partial conversion to Components 1 and 3. These two-charge changes were precisely those expected from dephosphorylation. No changes in electrophoretic mobility occurred when incubation was carried out in the absence of the enzyme. The capacities of Components 3, 4, and 5 of preparation A to convert to Components of higher electrophoretic mobility (lacking two or four negative charges) were much greater than those of preparation B. As an example, Fig. 6 shows that more than one-half of Component 3 of preparation A underwent conversion to Component 1, whereas the conversion of Component 3 of preparation B to Component
FIG. 6. Electrophoresis in parallel of Component 3 (50 µg) before and after treatment with alkaline phosphatase. A, preparation A; B, preparation B. Control and enzyme-treated samples are designated C and E, respectively. Electrophoretic conditions were the same as in Fig. 1.

1 was barely significant. The results of treatment of Components 1 to 5 of both preparations with alkaline phosphatase are summarized in Table IV. They indicate the fraction of each initial component which contained either one or two phosphate groups; e.g. for preparation A Component 3 consisted of polypeptides 53% of which were monophosphorylated, while Component 5 consisted of polypeptides 44% of which were monophosphorylated and 29% of which were diphosphorylated. As described above for the electrophoretic data on the carboxypeptidases A and B treatment of components, the data in Table IV permitted the calculation of theoretical phosphorus contents: for preparation A Component 3 contained 0.53 g atom of phosphorus/mol, while Component 5 contained 0.29 (2) + 0.44(1) = 1.02 g atom of phosphorus/mol.

Table V presents the experimental data from chemical analyses of phosphorus in the components, together with the theoretical values calculated from the electrophoretic data in Table IV. The agreement between the two sets of data was excellent. Summation of the products (g atom of phosphorus/mol × fraction of total basic protein) for each component yielded a value of 0.16 or 0.18 g atom of phosphorus/mol of total preparation B, depending upon whether the actual or theoretical phosphorus values were used. Similarly, for total preparation A a single value of 0.23 g atom/mol was calculated. These values agreed well with those (0.16 and 0.20 g atom/mol, respectively) determined directly.

Analyses for Phosphoamino Acids—Examination of 8-hour hydrolysates of the total basic protein preparations and their Components 3, 4, and 5 on an amino acid analyzer revealed a single symmetrical peak of strongly acidic material eluting in the position of authentic phosphoserine and/or phosphothreonine. High voltage electrophoresis of these hydrolysates demonstrated in each case the presence of both phosphoserine and phosphothreonine. These two amino acids together accounted for 67 to 85% of the phosphorus present in the samples prior to hydrolysis. On occasion, hydrolysates of phosphorus-containing basic proteins also contained a highly acidic ninhydrin-positive substance which migrated ahead of phosphoserine. Its occurrence was not eliminated by trichloroacetic acid precipitation and ethanol-ether washing of the protein prior to hydrolysis. Acid hydrolysates (8 hour) of pepsin and ovalbumin contained the material as well. Since the phosphorylated residue in pepsin and ovalbumin is known to be O-phosphoserine (24), it would appear that the unknown compound might be a phosphorus-containing degradation or rearrangement product of this amino acid which forms during acid hydrolysis of certain phosphoproteins. An electropherogram illustrating the composition of 8-hour hydrolysates is depicted in Fig. 7.

DISCUSSION

In the present study we have examined two preparations of myelin basic protein with regard to the COOH termini and phosphoamino acid contents of their constituent components. The results are summarized in Table VI. The two preparations differed markedly in the relative proportion of their components: in one preparation (A) it was the most basic form of the protein (Component 1) and in the other (B) the intermediate...
form (Component 3) which predominated. These differences were found to be related largely to the extent to which the COOH-terminal arginines of the protein had been lost. In both preparations Component 1 consisted entirely of intact polypeptide chains. In the remaining components of preparation A the intact COOH terminus was present in 65 to 92% of the polypeptide chains. Loss of most of these COOH-terminal arginines would have resulted in a preparation the properties of which would be those of preparation B; viz., a preparation with more alanine than arginine at the COOH terminus and consisting mostly of the less basic polypeptides, Components 3, 4, and 5. These components would show a decreased concentration of phosphorus as a result of their dilution with nonphosphorylated forms.

From the results of phosphorus and COOH-terminal analyses it is evident that Components 3, 4, and 5 are mixtures of phosphorylated and nonphosphorylated polypeptides. Component 3 can be derived from Component 1 by monophosphorylation or loss of 2 arginines. Component 4 can be derived from Component 2 by monophosphorylation or from phosphorylated Component 3 by loss of 1 arginine. Component 5 can be derived from Component 1 by diphosphorylation or from nonphosphorylated (di-desarginyl) Component 3 by monophosphorylation. If phosphorylation and loss of COOH-terminal arginines were the sole causes of microheterogeneity, the per cent of polypeptides in Component 3 or 4 which contained phosphorus should have equaled the per cent having an intact COOH terminus. Similarly, the per cent of Component 5 polypeptides which were diphosphorylated should have equaled the per cent having an intact COOH terminus. Perusal of the data in Table VI shows, on the contrary, that the per cent of intact polypeptides always exceeded the per cent of mono- or diphosphorylated polypeptides. One instance (Component 5 of preparation A) by a factor of 2.7 (79/29). This observation indicates that some additional factor(s) must have been involved in the conversion of Component 1 to less basic polypeptides, a conclusion confirmed by the finding that

| Component | % of total basic protein | Composition (% of component)a |
|-----------|--------------------------|-------------------------------|
|           | COOH terminus            | Degree of phosphorylation     |
| Preparation A |
| 1          | 49                       | Ala-Arg-Arg(100)              | None                              |
| 2          | 14                       | Ala-Arg-Arg(83)               | None                              |
| 3          | 24                       | Ala-Arg(17)                   | Mono- (53)                        |
| 4          | 7                        | Ala-Arg-Arg(92)               | Mono- (55)                        |
| 5          | 6                        | Ala-Arg-Arg(65)               | Mono- (44); di- (29)              |
| Preparation B |
| 1          | 10                       | Ala-Arg-Arg(100)              | None                              |
| 2          | 8                        | Ala-Arg-Arg(62)               | None                              |
| 3          | 35                       | Ala-Arg-Arg(25)               | Mono- (10)                        |
| 4          | 19                       | Ala-Arg-Arg(18)               | Mono- (11)                        |
| 5          | 28                       | Ala-Arg-Arg(15)               | Mono- (33); di- (6)               |

*Values calculated from electrophoretic data in Tables II and IV.

in preparation A 83% of the polypeptides in Component 2 were intact, yet they differed from Component 1 by a single charge. Possibly, this additional mechanism of component conversion might have been deamination of a limited number of the 10 amidated dicarboxylic acid residues reported (23) to be present in the protein. This possibility is currently under investigation.

The preparations which have been described represent two extremes in a large number of basic protein preparations which have been studied in our laboratory. Most resemble preparation A in containing predominantly Component 1, with Component 3 next in quantitative significance. Recently, we have prepared preparations of basic protein obtained from freeze-blown guinea pig brain. In the freeze-blowing procedure the tissue is instantaneously removed from the conscious animal and frozen so as to virtually eliminate postmortem changes (25). The electrophoretic pattern of basic protein obtained from tissue obtained in this manner is identical with that of preparation A. It is obvious, therefore, that the basic protein of preparation B cannot be representative of the basic protein as it exists in vivo, inasmuch as it has undergone extensive alterations at the COOH terminus.

Recently Chou et al. (26) have described an analysis of the COOH termini of basic proteins corresponding to Components 1 and 3. These investigators treated the components with BrCN, isolated the fragment COOH-terminal to the methionine near the end of the polypeptide chain, and found the amino acid composition in each case to correspond to the sequence Ala-Arg-Arg. These data are in complete agreement with our results with preparation A.

Microheterogeneity of myelin basic protein becomes a very

* R. E. Martenson and A. J. Kramer, unpublished data.
significant phenomenon in light of the discovery that the protein undergoes phosphorylation \textit{in vitro} and \textit{in vivo}. Our studies have shown the relationship between the high pH electrophoretic mobility of a basic protein component and its degree of phosphorylation. Components 1 and 2 contain no phosphorylated polypeptides, whereas Components 3, 4, and 5 do. Phosphorylated polypeptides present in Components 3 and 4 are monophosphorylated and consist of a mixture of two polypeptides, one containing phosphoserine and the other phosphothreonine. Components 5 contain no phosphorylated polypeptides, whereas Components 3 and 4 do. Phosphorylated polypeptides present in Components 3 and 4 are monophosphorylated and consist of a mixture of two polypeptides, one containing phosphoserine and the other phosphothreonine. Components 5 contain no phosphorylated polypeptides, whereas Components 3 and 4 do. Phosphorylated polypeptides present in Components 3 and 4 are monophosphorylated and consist of a mixture of two polypeptides, one containing phosphoserine and the other phosphothreonine. Components 5 contain no phosphorylated polypeptides, whereas Components 3 and 4 do. Phosphorylated polypeptides present in Components 3 and 4 are monophosphorylated and consist of a mixture of two polypeptides, one containing phosphoserine and the other phosphothreonine. Components 5 contain no phosphorylated polypeptides, whereas Components 3 and 4 do. It is important to note that in the native basic protein no more than two sites in any polypeptide chain are phosphorylated. Our studies have shown that brain phosphokinase converts Component 1 \textit{in vitro} to Components 3 and 5, but not to Components 2 and 4, and that Components 1 to 5 differ sequentially by a single charge. Therefore, Component 5 must differ from Component 1 by no more than four charges; i.e. two phosphate groups. A phosphoamino acid present at three sites in the polypeptide chain would yield an electrophoretic species differing from Component 1 by six charges and would appear as Component 7. This species as well as Components 9 and 11 can, in fact, be generated from Component 1 by prolonged incubation with brain phosphokinase. However, no forms of basic protein corresponding to Component 7, 9, or 11 have been detected either in basic protein preparations or in crude pH 3.0 extracts obtained from quick-frozen or freeze-blown tissue.

The phosphorus content of a basic protein preparation is bound to vary depending upon which components are removed in the course of its purification. Since Components 1 and 2 contain no phosphorus, it is obvious that "highly purified" preparations consisting only of these two components would be phosphorus-free. Inclusion of Component 3 would yield a preparation containing significantly less than the 0.2 g atom of phosphorus/mol of protein found in our studies and in those of Miyamoto and Kakiuchi (6). This relationship between phosphorus content and component composition could explain why Eylar and Thompson (27) reported a phosphorus content of less than 0.1 g atom/mol for their basic protein preparation.

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