

MOLECULAR INTERNALIZATION OF A REGION OF MYELIN BASIC PROTEIN*

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Myelin encephalitogenic or basic protein (BP)¹ accounts for 30% of the protein in central nervous system (CNS) myelin (1), where BP is postulated to serve a structural role. BP is unique to myelin, and a similar, if not identical, protein designated P1 (2) is present in peripheral nervous system (PNS) myelin (2-4). BP has a monomeric mol wt of 18,500 daltons and is comprised of 169 amino acids. The amino acid sequence has been determined for BP from human (5) and bovine (6) sources and the small BP of the rat (7). In addition to its extensively investigated property of inducing experimental allergic encephalomyelitis (EAE), BP may also sensitize lymphocytes and stimulate antibody formation. Lymphocyte sensitization and antibody formation to BP may occur independent of each other and of EAE (8-10). The molecular portion of BP responsible for inducing EAE is different for the guinea pig (11), rabbit (12, 13), and Lewis rat (14), and there appear to be differences in the antigenic determinants recognized by guinea pig (15) and rabbit (16) antibodies to BP.

The conformation of BP is likely to be important in the stability of the myelin sheath and in influencing the immune response after injection. The conformation of BP in the CNS myelin membrane and in solution is unresolved (17). While some studies have indicated that BP has only random-coil structure (18-23) other investigations have provided findings suggestive of a prolate ellipsoid conformation (24, 25). In a previous study of the antigenic determinants of bovine BP (16), we reported that peptide 43-88 had limited participation as an antigenic determinant recognized by complement (C)-fixing antibodies in two rabbit antisera to BP. This finding prompted speculation that peptide 43-88

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¹ *Abbreviations used in this paper:* BP, basic protein; CM-cellulose, carboxymethyl-cellulose; CNS, central nervous system; DA-RIA, double-antibody radioimmunoassay; EAE, experimental allergic encephalomyelitis; HRP, horseradish peroxidase; MBSA, methylated bovine serum albumin; PNS, peripheral nervous system; QMCT, quantitative microcomplement fixation test; RSA, rabbit serum albumin; TA-MBSA, Tris-acetate buffer containing methylated bovine serum albumin.

might have an internal position in the BP molecule. The present study provides data strongly supportive of this possibility as well as suggesting that there are differences in the region of the BP molecule involving residues 43-88 and the comparable region of P1 in PNS myelin.

Materials and Methods

Materials. Carboxymethyl (CM)-cellulose (CM52) was obtained from Reeve Angel, Clifton, N. J., carrier-free ^{125}I from Amersham/Searle Corp., Arlington Heights, Ill., methylated bovine serum albumin (MBSA) from Sigma Chemical Co., St. Louis, Mo., α -chymotrypsin, pepsin, and trypsin (treated with L-[tosylamido 2-phenyl] ethyl chloromethyl ketone) from Worthington Biochemical Corp., Freehold, N. J., lactoperoxidase from Calbiochem, La Jolla, Calif., citraconic anhydride from Aldrich Chemical Co., Inc., Milwaukee, Wis., Bio-Gel P-2 from Bio-Rad Laboratories, Richmond, Calif., Sephadex and AH Sepharose 4B from Pharmacia Fine Chemicals, Inc., Piscataway, N. J., guinea pig brain tissue from Pel-Freez Bio-Animals, Inc., Rogers, Ark., and plastic tubes (no. 2053) from Falcon Plastics, Div. of BioQuest, Oxnard, Calif. Guinea pigs were obtained from Camm Research Institute, Inc., Wayne, N. J. Bovine brain tissue was obtained from a local slaughterhouse.

Purification of BP. Bovine BP and guinea pig BP were purified by extraction and cation-exchange chromatography (16). The last major peak (16, 26), designated as component 1,² to elute from the CM-cellulose was used as the starting material for fragmentation. Components 1-4 from bovine BP were mixed and used for preparation of the BP solid-phase immunoabsorbent. Electrophoresis of the individual BP components on polyacrylamide gels in acetic acid-urea (27) and at pH 4.3 (28) showed essentially one band. Combining the major components of bovine BP for use in solid-phase immunoabsorbence seemed justified since previous studies (26) have shown that these components probably do not differ in their amino acid sequence but differ because of modification by phosphorylation and/or deamidation reasonably restricted to specific sites in the polypeptide chain.

Fragmentation of BP (Component 1). Peptides 1-88,³ 1-115, 20-166, and 43-88 were prepared and isolated from BP component 1 (1-169) as previously described (16). Peptides 43-88 and 1-88 were obtained from limited peptic digests of BP, peptide 1-115 was derived from BP reacted with BNPS-skatole, and peptide 20-166 was generated from BP by cyanogen bromide fragmentation.

Peptide 43-88 served as the starting material for the preparation for smaller peptides. Bovine peptide 43-88 was subjected to limited chymotrypsin digestion (protein to enzyme ratio, 1,000:1) to produce peptides 43-67 (C2) and 68-88 (C1) which were separated on CM-cellulose and further purified on Sephadex G50 (14). Peptide 79-88 was prepared by tryptic digestion of the guinea pig peptide 43-88 pretreated with citraconic anhydride followed by regeneration in dilute acid. The peptide was isolated on CM-cellulose and further purified on Sephadex G25 (14). The guinea pig peptide 43-88 was used instead of the corresponding bovine peptide since it is not possible to obtain peptide 79-88 from the bovine peptide 43-88 because of a resistant Arg-Pro peptide bond at position 78-79. The guinea pig peptide 79-88 differs from the corresponding bovine peptide by a single serine for proline substitution at position 79 (29). Peptide 64-73 was prepared by treating guinea pig peptide 43-88 with trypsin at an enzyme:substrate ratio of 1:50 at pH 8, 37°C for 4 h. The peptide was eluted from CM-cellulose at pH 4 with a NaCl gradient and was further purified on Sephadex G25 (14). Peptide 64-73 has the same amino acid sequence in bovine and guinea pig BP (29).

Characterization of BP Fragments. The BP fragments were characterized by amino acid analysis and by analysis of tryptic peptides isolated from two-dimensional paper chromatography-high voltage electrophoresis peptide maps as described previously (16).

Preparation of Antisera. 3 mg of bovine BP peptide 43-88 were dissolved in 1.5 ml of distilled H₂O. After 3.2 mg of rabbit serum albumin (RSA) and 3.8 mg of 1-ethyl-3-(dimethylaminopropyl)-

² Component 1 designates the last of the six components to elute from the column. This numbering system is that proposed by Deibler and Martenson (39) rather than that used by the present authors in a previous publication (16).

³ The numbering of residues of BP is based on the revised sequence data of 169 amino acid residues (16, 37) in which the serine previously assigned to position 2 is absent.

carbodiimide HCl were added, the solution was stirred at 25°C for 20 h and dialyzed at 4°C for 4 days against distilled H₂O. For the primary immunization the peptide-RSA mixture was diluted with 0.01 M phosphate-buffered 0.15 M NaCl, pH 7.2, and emulsified with complete Freund's adjuvant. Two 2-kg female New Zealand white rabbits (R95 and R96) were injected with an inoculum of 2 ml containing 200 µg of peptide 43-88. Secondary immunization was performed 4 wk later with an identical amount of peptide in incomplete Freund's adjuvant. Boosting immunizations of the peptide-RSA immunogen containing 250 µg of peptide in 0.15 M NaCl were injected intravenously at monthly intervals. Blood was obtained after the second and later immunizations. For the detailed immunochemical studies described below, blood on R95 was obtained after the second intravenous boost and on R96 after the fourth intravenous boost. Goat anti-rabbit IgG was prepared as previously described (4).

Absorption of Anti-Peptide 43-88 with BP. In order to remove antibodies to intact BP, a BP-immunoadsorbent was prepared. 1 g of AH Sepharose 4B was washed with 300 ml 0.5 M NaCl and 100 ml distilled H₂O after which it was suspended in 10 ml distilled H₂O. 15 mg each of bovine BP microheterogeneous components 1-4 (total of 60 mg) were added, and the pH adjusted to 4.5 with 0.1 N HCl. With the pH maintained at 4.5, 5 ml of distilled H₂O containing 60 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl was added. This mixture was gently agitated at 25°C for 18 h, washed successively with 200 ml distilled H₂O, 100 ml 0.5% acetic acid, and 200 ml borate-saline buffer (30, 31), and stored in the borate-saline buffer.

The BP-AH Sepharose was placed in a 1.5 × 5 cm column at 25°C and washed thoroughly with the borate-saline buffer. 2 ml of antiserum was passed through the column with the effluent monitored at 280 nm. After emergence of the unretarded serum material, the attached substances were eluted with 0.1 M glycine-HCl, pH 2.8, in 0.1 M NaCl. Both the flow-through and eluate fractions were dialyzed against phosphate-buffered saline and concentrated by vacuum dialysis to 2 ml. The BP-immunoadsorbent was regenerated by washing with the borate-saline buffer.

Radioiodination of BP and Peptide 43-88. BP and peptide 43-88 were radioiodinated with carrier-free ¹²⁵I by the lactoperoxidase method (32, 33). To a 12 × 75 mm polypropylene tube were added in sequence 40 µl 0.3 M acetate buffer, pH 5.5, 10 µg of BP or BP peptide 43-88 in 5 µl of the acetate buffer, 2.5 µg of lactoperoxidase in 10 µl of the acetate buffer, 10 µl of 0.5 M NaH₂PO₄, and 10 µl of ¹²⁵I. 25 µl of 0.4 M H₂O₂ were added in five aliquots of 5 µl each at 60-s intervals. 6 min after the initial addition of H₂O₂, 10 µl of 0.022 M KI was added. Radiolabeled antigen was separated from free iodine on a 0.7 × 13 cm Bio-Gel P-2 column equilibrated with 0.01 M HCl containing 1 g of bovine serum albumin/100 ml (33). A single tube (0.25 ml) in the initial peak (approximately 25% of the gel bed volume) containing the maximum number of counts per minute was used. The specific activity was calculated to be 20 µCi/µg for BP and 40 µCi/µg for peptide 43-88. This indicated that 900 pg of ¹²⁵BP or 300 pg of ¹²⁵I-BP-peptide 43-88 was added to each assay tube in the double-antibody radioimmunoassay (DA-RIA). 5% TCA precipitated 95-98% of the counts per minute added with ¹²⁵I-BP. At a concentration of 5% TCA, 70-80% of the counts per minute associated with radioiodinated peptide 43-88 were precipitable. This low percentage of precipitability may be due in part to the size of the polypeptide. Since only 90% of the ¹²⁵I-peptide 43-88 could be precipitated with low dilutions of certain anti-BP peptide 43-88 antisera, radioiodination-induced changes of peptide 43-88 must also be considered. Radiolabeled antigens were used no longer than 2 wk after radioiodination.

Radioimmunoassays. The DA-RIA was performed in a total vol of 0.95 ml in 12 × 75 mm polypropylene plastic tubes. The assay mixture consisted of 0.2 M Tris-acetate buffer, pH 7.2, containing 0.2 g MBSA (TA-MBSA)/100 ml, 0.2 ml of rabbit antiserum appropriately diluted in the Tris-acetate buffer containing a 1:200 dilution of normal rabbit serum, known amounts of BP or BP fragment in 50 µl of TA-MBSA, and 17,000-20,000 cpm of ¹²⁵I-BP peptide 43-88 or ¹²⁵I-BP in 50 µl of the Tris-acetate buffer.

After the initial mixture had stood at 4°C for 20 h, 0.2 ml of goat anti-rabbit IgG was added and the mixture allowed to stand for 24 h at 4°C. The tubes were centrifuged, the pellet washed with 2 ml of the Tris-acetate buffer, and the radioactivity of the pellet determined on an autogamma counter. In each DA-RIA all determinations of inhibition by peptides were done in triplicate. For those determinations of reactivity of antiserum or fractions thereof or of the optimal dilution of antiserum for studying inhibition by peptides in the DA-RIA, the assay was performed in a similar manner except that the unlabeled BP or BP fragments were omitted.

The BP and BP fragments tested for inhibition were stored at -20°C at a concentration of 10 µg/

ml in the working buffer used for quantitative C fixation (4, 34) and diluted to the desired extent in TA-MBSA before the DA-RIA. For calculation of molar concentrations, peptides were assigned a molecular weight based on 100 daltons per amino acid residue.

Other Methods. The quantitative micro-complement fixation test (QMCT) (34) was performed as previously described (4). Apparent association constants were calculated based on Scatchard analysis (35, 36). Indirect immunocytochemistry was performed (4) using cryostat sections of guinea pig cerebellum and sciatic nerve. Tissue was processed and horseradish peroxidase (HRP)-Fab prepared as previously described (4) except that fixation was in methanol (98.3 parts)-H₂O₂ (1.7 parts of 30% solution) at 25°C for 20 min. Guinea pig cerebellum and sciatic nerve were also obtained from animals perfused *in situ* with 4% paraformaldehyde in Millonig's buffer (38). Cryostat sections of perfused tissue were further fixed and processed as the cryostat sections of fresh frozen material.

Results

Fragmentation of BP Peptides. Bovine BP peptides 43-88, 1-88, 1-115, and 20-166 were the same preparations as those previously described (16). Peptide 43-88 contained approximately 40% of peptide 37-88. Peptide 1-88 and 1-115 were of high purity, but peptide 20-166 was partially contaminated with intact BP.

Limited chymotrypsin digestion cleaved peptide 43-88 at Tyr-Gly (residues 67-68) to produce peptides 43-67 and 68-88. Chymotrypsin also cleaved the contaminating peptide 37-88 at Phe-Phe (residues 42-43) to generate the small peptide 37-42 which was removed by subsequent purification procedures (14). Amino acid and tryptic peptide map analysis showed these two major peptides, 43-67, 68-88, to be of high purity (14). Approximately 40% of the preparation of peptide 64-73 of guinea pig BP was contaminated with another peptide (Table I). Analysis of the two-dimensional peptide map prepared from this preparation showed that the contaminating peptide was peptide 52-56. Peptide 79-88 was of high purity as shown in Table I. As noted above, this peptide differs from the corresponding bovine peptide in that there is a serine at position 79 rather than proline (29).

Reactivity of Anti-Peptide 43-88 with BP. Serum obtained before immunization of both R95 and R96 had no antibody activity to bovine BP or peptide 43-88. After immunization, antibody to peptide 43-88 was detected after the second immunization and showed a gradual increase in titer with repeated boosting (Figs. 1 and 2). The amount of radioiodinated peptide 43-88 precipitated never exceeded 90% even with antiserum dilutions as low as 1:100.

In contrast to their reactivities with peptide 43-88, antisera from both R95 and R96 throughout the course of immunization did not contain appreciable amounts of antibody to intact BP (Figs. 1 and 2). R95 had slightly more antibody activity to BP than did R96. When these antisera were absorbed with the BP-immunoadsorbent, antibody activity to peptide 43-88 was unchanged (Table II). The small amount of anti-BP activity was removed and recovered in the eluate.

Studies on the Antigenic Site of Peptide 43-88. In order to identify the region of peptide reactive with these antisera and by inference "buried" in the BP molecule, inhibition of the DA-RIA reaction between antisera and ¹²⁵I-peptide 43-88 by various BP peptides was investigated. It was clearly shown that peptide 68-88 contained all of the reactant site(s) for R96 with none present in peptide 43-67 or peptide 64-73 (Fig. 3). On further fragmentation of peptide 43-88 it could be shown that all of the activity to peptide 68-88 in R96 was actually against 79-88.

TABLE I
Amino Acid Composition of Peptides Derived from BP Region 43-88

Amino acid	43-67	68-88	64-73	79-88
Lysine	1.9 (2)	1.0 (1)	1.6*(1)	
Histidine	2.5 (3)	2.1 (2)	1.0 (1)	0.8 (1)
Arginine	3.0 (3)	0.8 (1)	0.6*(0)	
Aspartic acid	2.0 (2)	2.0 (2)		2.1 (2)
Threonine	1.8 (2)		2.0 (2)	
Serine	1.8 (2)	0.9 (1)	1.8*(1)	0.8 (1)
Glutamic acid		4.0 (4)	1.0 (1)	2.0 (2)
Proline	0.9 (1)	2.9 (3)	1.0 (1)	1.0 (1)
Glycine	4.8 (5)	1.8 (2)	2.2*(1)	
Valine		1.0‡(2)		0.7 (2)
Leucine		1.0 (1)	0.8 (1)	
Tyrosine	0.9 (1)		0.8 (1)	
Phenylalanine	0.7 (1)	1.0 (1)		0.9 (1)

1 mg of each peptide was hydrolyzed in 6 N HCl at 110°C for 48 h and the nanomoles per residue are based on the content of glutamic acid.

* This peptide is contaminated with approximately 40% of peptide 52-56 (Arg-Gly-Ser-Gly-Lys).

‡ Low value of valine is due to a Val-Val sequence.

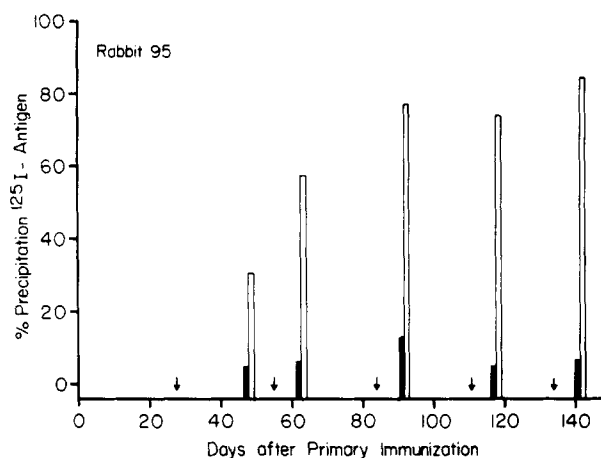


FIG. 1. Precipitation of radioiodinated BP (dark bars) and peptide 43-88 (light bars) by a 1:500 dilution of R95 sera obtained at varying intervals after primary immunization. Arrows (\downarrow) indicate times of immunization. Preimmune serum, diluted 1:100, precipitated neither antigen.

Comparable to the findings using R96 serum, R95 serum failed to react with peptides 43-67 and 64-73 (Fig. 4). However, the differences among peptides 43-88, 68-88, and 79-88 were less evident with these three peptides having nearly identical molar inhibitory activities (Fig. 4).

Since both antisera reacted well with peptide 43-88 and to a very limited extent with intact BP, investigations were undertaken to determine what fragments of BP containing peptide 43-88 would react with antibody to peptide 43-88. Intact BP and peptide 1-115 had little, if any, reactivity with either antiserum, previously absorbed with BP, at 100-fold higher molar concentrations (Figs. 5 and 6). Peptide 20-166 demonstrated some reactivity at high

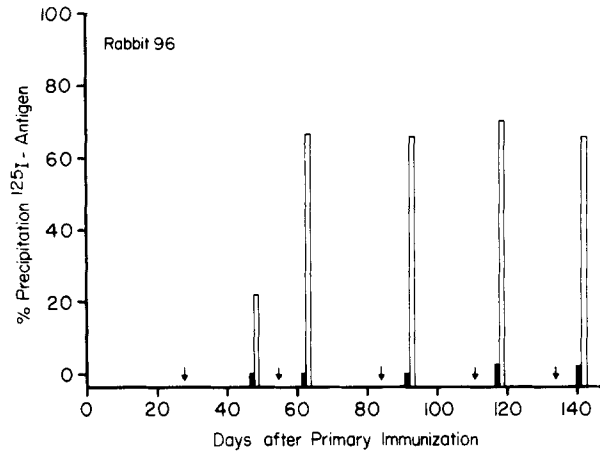


FIG. 2. Precipitation of radiiodinated BP (dark bars) and peptide 43-88 (light bars) by a 1:500 dilution of R96 sera obtained at varying intervals after primary immunization. Arrows (↓) indicate times of immunization. Preimmune serum, diluted 1:100, precipitated neither antigen.

TABLE II
Antibody Activity of Antisera Raised against Bovine BP Peptide 43-88

Rabbit serum or serum fraction*	% Precipitation of ^{125}I -antigen		Immunocytochemical reaction with:	
	BP	Peptide 43-88	CNS myelin	PNS myelin
R95 serum	13.0	43.7	+	+
R95 serum BP-absorbed	4.6	45.1	+	0
R95 serum BP-eluate	12.4	5.7	+	+
R96 serum	4.3	44.7	+	+
R96 serum BP-absorbed	2.8	40.8	+	0
R96 serum BP-eluate	1.5	7.7	+	+

* Diluted 1:500 for DA-RIA and 1:25 for immunocytochemistry.

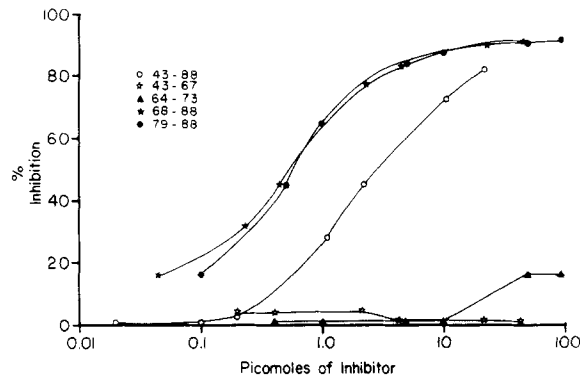


FIG. 3. Inhibition of the reaction between R96, absorbed with bovine BP and diluted 1:750, and radiiodinated bovine peptide 43-88 by varying amounts of peptides 43-88 (○), 43-67 (☆), 68-88 (★), 64-73 (▲), and 79-88 (●).

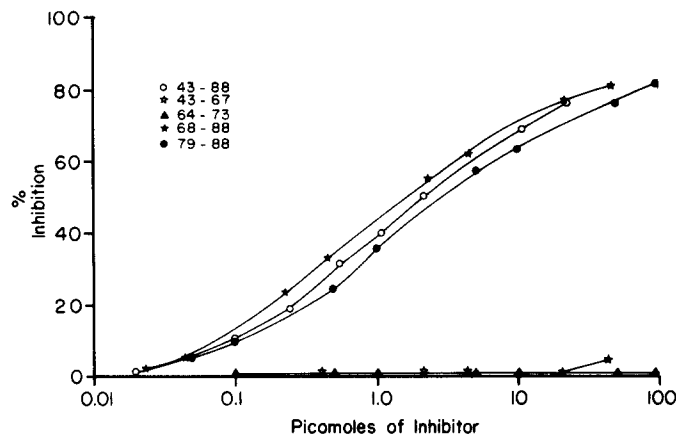


FIG. 4. Inhibition of the reaction between R95, absorbed with bovine BP and diluted 1:450, and radioiodinated bovine peptide 43-88 by varying amounts of peptides 43-88 (○), 43-67 (☆), 68-88 (★), 64-73 (▲), and 79-88 (●).

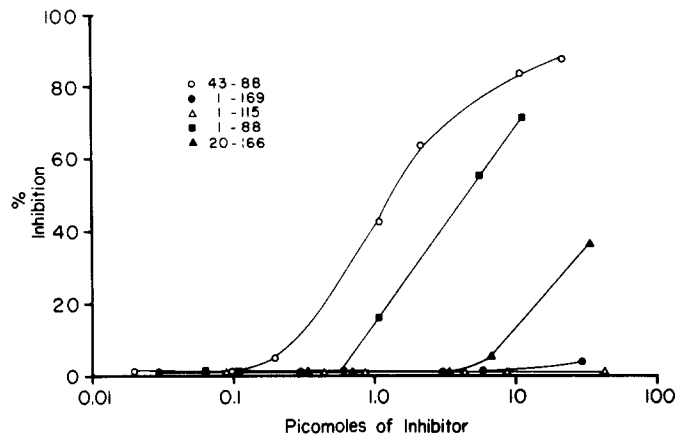


FIG. 5. Inhibition of the reaction between R96, absorbed with bovine BP and diluted 1:750, and radioiodinated bovine peptide 43-88 by BP (●) and peptides 43-88 (○), 1-88 (■), 1-115 (△), and 20-166 (▲).

concentrations. Peptide 1-88 had much greater reactivity and gave a displacement curve parallel to peptide 43-88.

Scatchard plots of the bound/free ratio of ^{125}I -peptide 43-88 as a function of the binding of peptides 1-88, 43-88, 68-88, and 79-88 were used to determine the apparent association constant (K_{assoc}) and reflected the reactions of R95 (Table III) and R96 (Fig. 7, Table III). R95 serum had a lower K_{assoc} , $8.8 \times 10^7 \text{ M}^{-1}$, for peptide 43-88 than did R96 serum which had a K_{assoc} of $1 \times 10^9 \text{ M}^{-1}$. The K_{assoc} of R96 for peptides 68-88 and 79-88 was higher than for peptide 43-88. The association constants shown by R95 serum for these peptides were lower but more nearly equal in magnitude (Table III).

Results of studies using QMCT also suggest a difference in BP or peptide 43-88 recognized by R95 serum but not by R96. At a 1:100 dilution R95, unabsorbed

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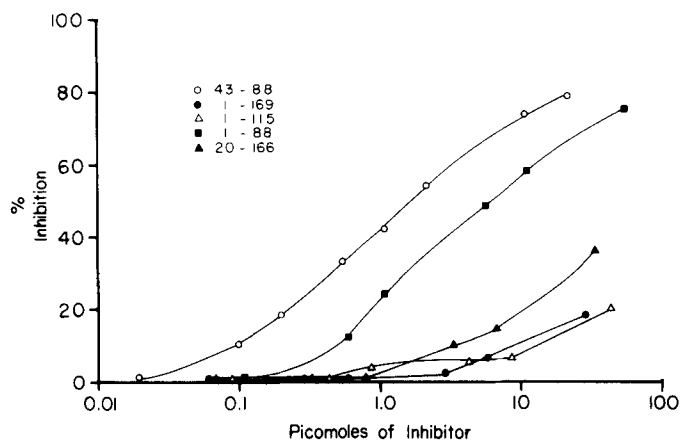


FIG. 6. Inhibition of the reaction between R95, absorbed with bovine BP and diluted 1:450, and radioiodinated bovine peptide 43-88 by BP (●) and peptides 43-88 (○), 1-88 (■), 1-115 (△), and 20-166 (▲).

TABLE III
Comparison of association constants

Peptide	R95 serum	R96 serum
43-88	1*	1
68-88	1.2	4.1
79-88	0.6	5.1
1-88	0.4	0.02

* Represents ratio of apparent K_{assoc} for given BP peptide to apparent K_{assoc} for BP peptide 43-88. K_{assoc} of R95 serum for peptide 43-88 was $8.8 \times 10^7 \text{ M}^{-1}$. K_{assoc} of R96 serum for peptide 43-88 was $1 \times 10^9 \text{ M}^{-1}$.

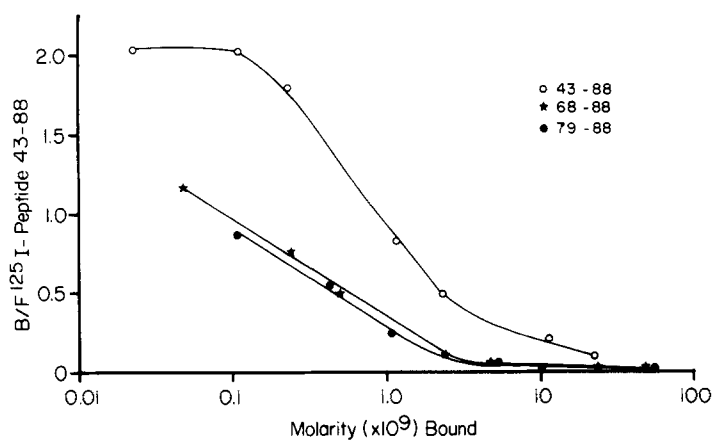


FIG. 7. Binding of peptides 43-88, 67-88, and 79-88 by R96 previously absorbed with BP. Bound/free ratio of radioiodinated peptide 43-88 plotted as a function of peptides 43-88 (○), 68-88 (★), and 79-88 (●).

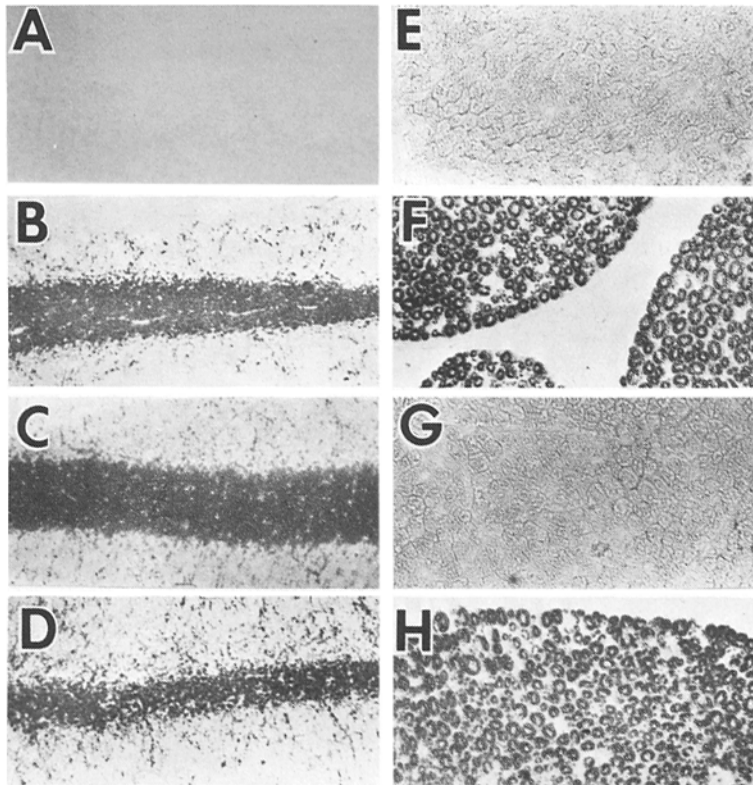


FIG. 8. Indirect HRP-immunocytochemical studies of R95 serum or fractions thereof reacting with cryostat sections of cerebellum (A-D) and peripheral nerve (E-H) removed from perfused guinea pigs. Preimmune serum did not react with either CNS (A) or PNS (E) myelin. R95 serum reacted with both CNS (B) and PNS (F) myelin. After exposure to a BP-immunoadsorbent, R95 serum continued to react weakly with CNS myelin (C) but showed no reactivity to PNS myelin (G). The antibodies eluted from the BP-immunoadsorbent reacted with both CNS (D) and PNS (H) myelin. All antiserum or antiserum fractions were studied at a 1:25 dilution. A-D, $\times 56$; E-H, $\times 140$.

with BP, fixed low levels of C in the reaction with intact BP but not peptide 43-88. The extent of reaction, large concentrations of BP required, and low dilution of antiserum used precluded hapten inhibition experiments with peptide 43-88 and fragments of peptide 43-88. At the same dilution R96 reacted with neither BP nor peptide 43-88 in the QMCT.

Immunocytochemical Studies. Because the two antisera reacted with peptide 43-88 but not the intact BP molecule, studies were performed to determine the reactivity of these antisera with fixed CNS and PNS tissue. Both R95 and R96 sera reacted with guinea pig CNS myelin at a 1:25 dilution before and after absorption with BP (Fig. 8, Table II). However, differences were encountered with the reaction to PNS myelin assessed at dilutions of 1:10 and 1:25. R95 and R96 sera reacted with PNS myelin but, after absorption with BP, this reaction was no longer detected. The eluate recovered from the BP-immunoadsorbent continued to react with PNS myelin. The binding to PNS myelin of R96 serum

and its fraction eluted from the BP immunoadsorbent was less than that of R95. This paralleled the binding to BP in the DA-RIA (Table II) and may be a result of differences in antigenic sites recognized by these two antisera (Table III).

Discussion

The present findings indicate that the conformation of BP peptide 43-88, used for immunization and immunochemical procedures, is different from the conformation of the same region as it exists in the BP molecule. Furthermore, the unreactivity of BP with antibodies to peptide 43-88 implies that some or all of this region has an internal position in the BP molecule.

The evidence may be summarized as follows: Antibodies produced in two rabbits by immunization with peptide 43-88 linked to RSA reacted well with radioiodinated peptide 43-88 but had little or no reaction with radioiodinated BP. In both instances the assumption is made that iodination did not alter immunoreactivity. Absorption of these antisera with a BP-immunoadsorbent eliminated the small reactivity to BP in R95 but did not remove the antibody activity to peptide 43-88 in either. These findings indicate that in intact BP, both free in solution in radioiodinated form and attached to an immunoadsorbent, the portions 43-88 reacting with these antisera are inaccessible in the intact molecule. The lack of inhibition by BP of the reaction between the two antisera and peptide 43-88 provides proof that intact BP in solution and not previously exposed to the conditions of radioiodination or linked to Sepharose is also incapable of furnishing an accessible antigenic site for attachment of the antibodies raised to peptide 43-88. The failure of the BP-immunoadsorbent, prepared with a mixture of microheterogeneous BP components 1-4 (26, 39), to remove antibodies to peptide 43-88 suggests that this inaccessibility is likely to be present in each of the microheterogeneous fractions as they are attached to Sepharose.

Efforts to further localize the portion of 43-88 bearing the antigenic determinant indicated that such was located in peptide 68-88. Since most protein antigenic determinants delineated possess approximately six amino acid residues (40, 41), this peptide might still have more than one determinant. No appreciable activity against peptide 64-73 existed, but peptide 79-88 was potent as an inhibitor, especially with R96 serum. This finding would indicate that the antigenic region of 43-88 reacting with R96 serum is in the region containing residues 79-88. The equal reactivity of guinea pig peptide 79-88 with bovine peptide 68-88 suggests that the proline residue at position 79, which is substituted with a serine in guinea pig BP (29), is probably not critical for the antigenic determinant in peptide 79-88 recognized by this rabbit antiserum. The antigenic region recognized by R95 also resided within region 68-88 but was not so restricted to peptide 79-88. This could indicate that a different or second determinant exists in peptide 68-88 that does not lie within peptide 79-88. The findings with R96 permit one to conclude that the antigenic determinant in region 79-88 is "buried" in the BP molecule. Whether some or all of the region of residues 43-78 are also buried cannot be determined with these two antisera; however, the findings with R95 are consistent with some of 43-88 outside 79-88 also being internalized in the BP molecule.

The point in the fragmentation of BP at which the antigenic determinants are exposed was explored by using large BP peptides of 1-88, 1-115, and 20-166. It should also be recognized that the contamination of BP or of the preparations of peptides 1-88, 1-115, or 20-166 by smaller fragments containing peptide 79-88 could alter the apparent reactivities of these three large peptides. Although this cannot be totally discounted, the gel filtration steps utilized and the results of amino acid analysis make such a contamination unlikely. The much greater reactivity of peptide 1-88 as compared to peptide 1-115 suggests that at least a portion of the region of BP encompassing residues 89-115 participates in the conformational alignment of BP which restricts access to region 79-88. A sharp bend in the BP molecule around the tri-proline residues of 98-100 has previously been postulated (42). High resolution nuclear magnetic resonance spectroscopy of bovine BP also indicates molecular folding in the region of residues 84-115 (43). Although the reactivity of peptide 20-166 was evident only at high concentrations, its greater reactivity than either intact BP or peptide 1-115 could indicate that the removal of residues 1-19 may effect a slight conformational alteration that increases exposure of region 68-88.

The immunocytochemical studies provide proof that peptide 43-88 does not have a shielded position in CNS myelin fixed by the described procedures. Both antisera, even after absorption with BP, continued to react with CNS myelin. In contrast, reactivity with PNS myelin was abolished by absorption with BP but recovered in the eluate from the BP-immunoadsorbent. This suggests that BP and its cross-reactant, presumably P1 (2), in PNS myelin have compositional or conformational differences involving peptide 43-88 in BP and the comparable region of P1. Such differences might account for selective involvement of CNS myelin by immune processes directed at BP. A more detailed immunological comparison of BP and P1 will require purified P1 protein.

The inaccessibility of molecular regions and potential antigenic sites has been demonstrated for fragment A of diphtheria toxin (44), fragment A in cholera enterotoxin (45), a portion of tetanus toxin (46), and the pepsin moiety in pepsinogen (47). Other proteins such as hemocyanin (48), human serum albumin (49), bovine serum albumin (50), RSA (51), human immunoglobulin (52), and rabbit immunoglobulin (53) contain determinants that are sequestered in the molecule. Some of the differences between the immunochemical reactivities of a protein fragment and its parent molecule may be due to the immunochemical method used and a requirement for multivalent antigens. For example, antibodies to peptide 99-126 of staphylococcal nuclease do not precipitate peptide 99-149 or intact nuclease (54). However, these antibodies bind to radiolabeled peptide 99-149 (54, 55), and this binding can be inhibited by intact nuclease (55). In the present study the reduced or absent reactivity between antibodies to BP peptide 43-88 and intact BP was demonstrated by methods which are affected by a single binding site.

These immunochemical findings may reflect a special role for peptide 43-88 or adjacent region of BP in the host immune response to BP, the metabolism of BP, and the alignment of BP in the myelin membrane. In the molecular region of residues 64-121 there occur the major encephalitogenic sites for the rabbit (13), Lewis rat (14), and guinea pig (11), a substrate for *in vivo* phosphorylation (26)

and glycosylation (56) at threonine-97, and a methylated arginine at residue 106 (57). The relationship of T-lymphocyte responsiveness to peptide 43-88 to the induction of EAE in rats has also been documented (33). Results of the present investigation have more general immunological implications regarding BP and other antigens in demonstrating that apparently distinct immunochemical reactions with tissue components may arise, not on a basis of different identities, but from the exposure of shielded antigenic regions. To the contrary, immune responses to a "buried" determinant may go undetected and cross-reactions missed if only the parent protein is used as antigen in immunological studies.

Summary

The conformation of myelin encephalitogenic or basic protein (BP) was investigated with a double-antibody radioimmunoassay by studying the reaction of BP or its fragments with antibodies produced in two rabbits against peptide 43-88 linked to rabbit serum albumin. Both antisera reacted well with peptide 43-88 but showed little or no reaction with BP. Absorption of these antisera with a BP-immunoabsorbent did not remove the antibody activity against peptide 43-88. Within the region of peptide 43-88 it was shown that peptides 68-88 and 79-88 gave an equivalent or better reaction than peptide 43-88, whereas peptides 43-67 and 64-73 had very little reactivity. In the BP fragments containing region 43-88, peptide 1-88 showed the best reactivity, peptide 20-166 showed minimal reactivity, while peptide 1-115 showed none.

These data document the internal position of at least a portion of peptide 43-88 and all of residues 79-88 in the BP molecule. The much greater reactivity of peptide 1-88 as compared to peptide 1-115 suggests that the region or a portion of the region of BP containing residues 89-115 participates in the conformational alignment of BP restricting access to peptide 79-88. After absorption with BP, neither of the antisera prepared to peptide 43-88 reacted with PNS myelin in fixed tissue sections but continued to react with CNS myelin in similarly treated sections. The present findings demonstrate the need to consider the role of shielded antigenic determinants in the investigation of antigens or of immune responses.

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