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# Large Peptides of Bovine and Guinea Pig Myelin Basic Proteins Produced by Limited Peptic Hydrolysis†

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ABSTRACT: Bovine and guinea pig myelin basic proteins were cleaved with pepsin at pH 3.0 or pH 6.0 (enzyme/substrate, 1:500, w/w), and the peptides were isolated and identified. At pH 3.0 cleavage of the bovine protein occurred principally at three sites: Phe-Phe (88-89), Phe-Phe (42-43), and Leu-Asp (36-37). Minor cleavages occurred at Leu-Ser (110-111), Phe-Ser (113-114), and Ile-Phe (152-153). A study of the time course of the hydrolysis showed that the reaction was biphasic; nearly all of the protein was cleaved at Phe-Phe (88-89) before significant cleavages at other sites occurred. At pH 6.0 cleavage of the bovine protein occurred almost exclusively at a single site, the Phe-Phe bond at position 88-89, resulting in bisection of the protein. Treatment of the guinea pig protein with pepsin under the same conditions resulted in the production of peptides which were identical with those of the bovine protein in chromatographic and electrophoretic properties and in N-terminal and C-terminal residues but which differed slightly in amino acid composition.

Myelin sheaths of the central nervous system of vertebrates contain a highly basic protein which accounts for about 30% of the total protein (Kies *et al.*, 1964; Autilio, 1966; Eng *et al.*, 1968). It induces an autoimmune disease, experimental allergic encephalomyelitis, when injected with Freund's complete adjuvant into a number of different species of animal, including guinea pig, rat, rabbit, and monkey (for a review, see Kies, 1973). Since the complete amino acid sequences of the bovine (Eylar *et al.*, 1971; Brostoff *et al.*, 1974), human (Carnegie, 1971), and the smaller of the two rat (Dunkley and Carnegie, 1974) myelin basic proteins have been determined, this protein provides an excellent model for the study of the relationship between primary structure and immunological activity.

Sequence studies by Eylar *et al.* (1971) and Carnegie (1971) have shown that the myelin basic protein can be extensively digested by a variety of proteolytic enzymes. At pH 3.0 and 37° with an enzyme/substrate ratio of 1:50 (w/w), pepsin cleaves the protein into at least 17 peptides, the largest of which consists of 46 residues (Eylar *et al.*, 1971). In order to obtain additional, relatively large fragments of the basic protein as part of a systematic exploration of immunologically active regions, we have cleaved the protein at a limited number of peptide bonds by decreasing the pepsin/substrate ratio to 1:500. The present report describes the preparation, purification, and characterization of the several relatively large peptides produced in high yield by limited peptic cleavage of the bovine and guinea pig myelin basic proteins.

## Materials and Methods

*Myelin Basic Proteins.* Bovine and guinea pig myelin basic proteins, isolated from quick-frozen brain by the procedure of Deibler *et al.* (1972), were further purified and

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separated into multiple forms by ion-exchange chromatography on carboxymethylcellulose (Whatman CM-23) at high pH (Deibler and Martenson, 1973a). Since we wished to see if any of the peptic peptides subsequently produced likewise existed in multiple forms, the material collected and used generally consisted of a mixture of components 1, 2, and 3. In one study pure component 1 of the guinea pig protein was used. Components 5 and 4 which are eluted from CM-23 prior to component 3 were not collected.

*Analytical Techniques.* Polyacrylamide gel electrophoresis was carried out in 10% gels containing 8 M urea and 1 M acetic acid (Martenson and Deibler, 1975). Proteins and peptides were stained with Amido Black; gels were scanned at 500 nm with a Beckman DU monochromator fitted with a Gilford Model 2410 linear transport having a 0.05 × 2.36 mm slit plate.

Amino acid analyses were carried out in duplicate with a Beckman 121 automatic amino acid analyzer equipped with a Beckman System AA computing integrator. Acid hydrolysis was carried out as described previously (Deibler and Martenson, 1973a). For analysis of tryptophan the peptides were hydrolyzed in the presence of 4% thioglycolic acid (Matsubara and Sasaki, 1969). Analysis of basic amino acids was generally carried out with an 8-cm column of Beckman PA-35 resin eluted with 0.35 N sodium citrate buffer (pH 5.28) and operated at 53.5° at a flow rate of 70 ml/hr. In this system, $N^G$,$N^G$- and $N^G$,$N'^G$-dimethylarginine (Deibler and Martenson, 1973b) were eluted at 42 and 45 min, respectively; $N^G$-monomethylarginine and arginine were not resolved. Quantitation of both mono- and dimethylarginines was carried out with a 20-cm column of Durrum DC-6A resin operated at 56° at a flow rate of 70 ml/hr. The starting buffer, 0.35 N sodium citrate (pH 5.28), was changed at 82 min to 0.35 N sodium citrate (pH 4.12). The base-line shift due to the buffer change occurred immediately after the ammonia peak. The following elution times were obtained: $N^G$,$N^G$-dimethylarginine, 137 min; $N^G$,$N'^G$-dimethylarginine, 147 min; $N^G$-monomethylarginine, 171 min; arginine, 180 min. Buffers were made from the Beckman standard pH 5.26 buffer by the addition of 50% NaOH or concentrated HCl.

Determination of N-terminal and C-terminal residues was carried out as described previously (Martenson *et al.*, 1975) by reaction with 5-dimethylaminonaphthalene-1-sulfonyl chloride and carboxypeptidases A and B, respectively. The quantity (nanomoles) of each peptide analyzed for C-terminal residues was determined from the yield of glutamic or aspartic acid obtained upon acid hydrolysis of a given weight of the peptide.

A preliminary estimate of the molecular size of peptides was obtained by gel filtration of 5–10 mg of peptide or peptide fraction through a Sephadex G-50 fine column (3.2 × 95 cm) equilibrated with 0.01 M HCl at 5° and monitored at 225 nm. The column was calibrated with acetic acid (to yield the parameter $V_t$), 5–10 mg of chemically derived fragments of the bovine myelin basic protein (residues 1–115, 20–115, 116–169, and 1–19 [Martenson *et al.*, 1975]), horse heart cytochrome *c*, and peptides derived from cytochrome *c* by partial cleavage with BrCN (residues 1–80, 1–65, 66–104, 81–104, and 66–80). The latter were isolated by repeated gel filtration as described above and identified by amino acid analysis.

*Peptic Cleavages of Myelin Basic Protein.* Myelin basic protein was dissolved at a concentration of 4 mg/ml either in 0.1 M acetic acid adjusted to pH 3.0 with 1 M ammonium acetate or in 0.1 M ammonium formate adjusted to pH 6.0 with 88% formic acid. Porcine pepsin (Worthington PM 2HA, 3000 units/mg) was dissolved in the above buffer at a concentration of 0.2 mg/ml and after 10 min was added to the basic protein solution such that the ratio of pepsin to basic protein was 1:500 (w/w). For preparative purposes involving relatively large amounts of material hydrolysis was allowed to proceed at 24° for 2 hr at pH 3.0 or for 1 hr at pH 6.0. Subsequently the solution was adjusted to pH 8 with 1 M $NH_4OH$ and allowed to stand for 1 hr to ensure pepsin denaturation. A small sample was removed for electrophoretic analyses, and the solutions were frozen and lyophilized twice. For studies on the time course of the hydrolysis aliquots were removed from the reaction mixture at appropriate intervals, added to 3 vol of 8 M urea–0.2 M $NH_4OH$ (2:1, v/v), and subjected to electrophoresis.

*Initial Fractionation of the Digests.* The lyophilized digests were dissolved in 4 M urea and applied to a Sephadex G-50 fine column (3.2 × 95 cm) equilibrated and operated as described above. Appropriate fractions were lyophilized directly.

*Ion-Exchange Chromatography.* Peptides were chromatographed at 5° on columns of Whatman CM-23 at pH 8.2 with elution monitored at 230 nm. Columns were prepared for use and regenerated by flux of two column volumes of 0.5 M ammonium bicarbonate (pH 8.2), followed by equilibration with the same buffer appropriately diluted. Peptides were applied to the column in several milliliters of deionized water made weakly acid (if necessary to effect complete solution of peptides) by the addition of several drops of 1 M acetic acid. Elution of peptides was carried out with a linear gradient of ammonium bicarbonate made with the use of two identical communicating bottles, each initially containing 2 l. of buffer. The gradient was determined from the concentration of buffer entering the column at the end of the run. This was calculated from the molarity of starting buffer initially present in the mixing vessel, the molarity of buffer in the reservoir, and the total volume of buffer remaining. Peptides were recovered by direct lyophilization (twice) of the column effluents.

## Results

*Purification of Peptides.* Gel filtration of the pH 3.0 digest (Figure 1a) resulted in the separation of peptides into two fractions with elution volumes corresponding to those of peptides consisting of approximately 80 and 45 residues. The F80 fraction was subjected to a second gel filtration, then chromatographed on Whatman CM-23 (Figure 1b), yielding three peaks, the relative proportions of which corresponded closely to those of components 1, 2, and 3 of the basic protein used as starting material. Subsequent gel filtration of each F80 component was carried out to remove any contaminants present. Ion-exchange chromatography of fraction F45 yielded three major components and two minor ones (Figure 1c). Component F45-3 was rechromatographed under the original conditions while F45-1 and F45-2 were rechromatographed with a gradient of 0.01–0.1 M buffer. Component F45-5 was freed of minor contaminants by gel filtration. Component F45-4 was separated into two subcomponents, A and B, by gel filtration (Figure 1d).

Gel filtration of the pH 6.0 digest yielded two major, nearly overlapping peaks, corresponding in elution volumes to 90- and 80-residue peptides, plus a relatively small amount of shorter fragments. Chromatography of the mix-

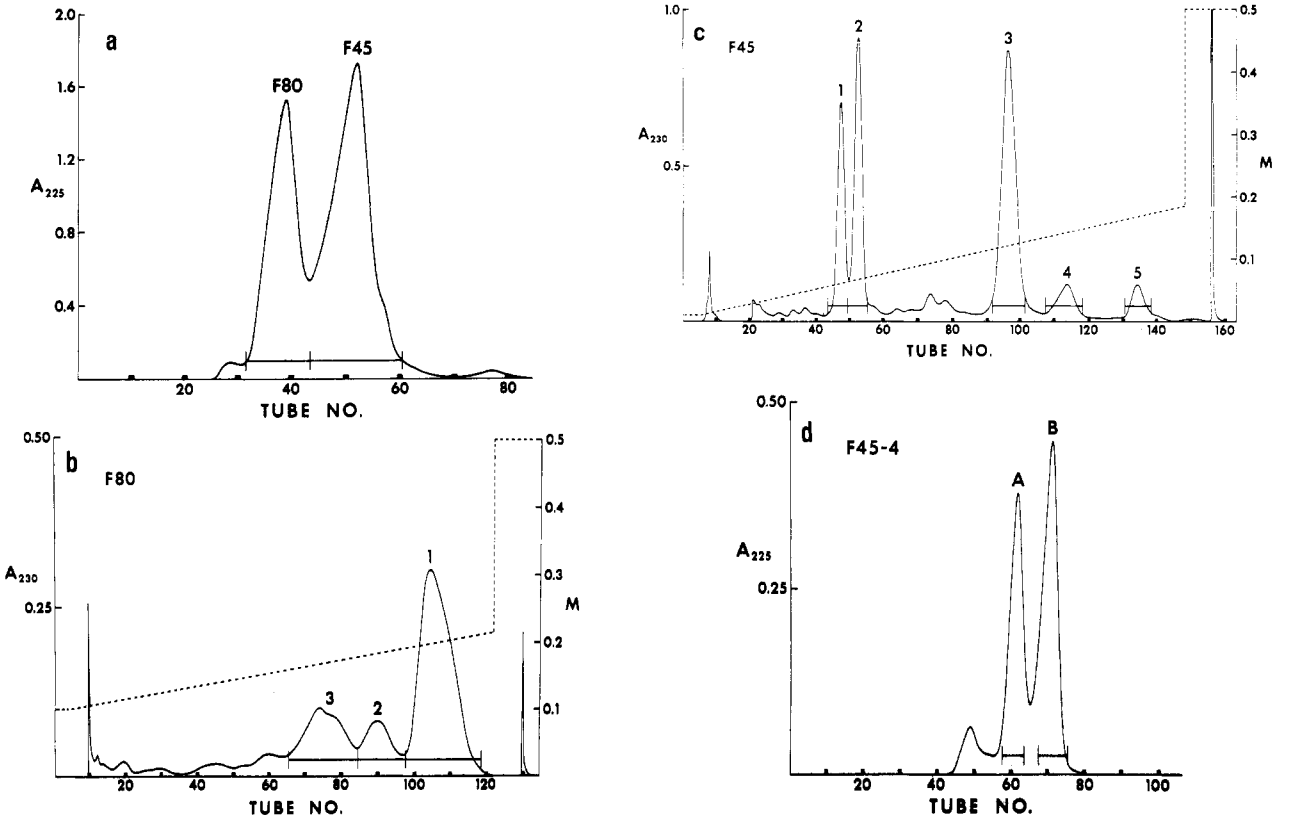


FIGURE 1: Purification of peptides produced by peptic cleavage at pH 3.0. Essentially identical results were obtained with bovine and guinea pig materials. Fractions were combined as shown. (a) Gel filtration of bovine pepsin digest (220 mg) through Sephadex G-50 fine. The flow rate was 33 ml/hr; fractions of 9.8 ml were collected. (b) Chromatography of guinea pig fraction F80 (164 mg) on a column (2.0 × 46 cm) of Whatman CM-23. The molarity of buffer entering the column is indicated. The flow rate was 90 ml/hr; fractions of 19.4 ml were collected. (c) Chromatography of guinea pig fraction F45 (465 mg). Except for the buffer gradient and flow rate the conditions were identical with those in b. The flow rate was 98 ml/hr; fractions of 21.0 ml were collected. (d) Gel filtration of guinea pig component F45-4 through Sephadex G-50 fine. The flow rate was 32 ml/hr; fractions of 6.3 ml were collected.

ture of F90 and F80 peptides on CM-23 yielded results shown in Figure 2. Components F80-1 and F80-2 were present in this digest (pH 6.0) in the same proportion as they were in the pH 3.0 digest. The small amount of F80-3 present was eluted in the tail of the F90 peak. Because of the shallow gradient used in the experiment depicted in Figure 2, F80-1 was resolved into two subcomponents (A and B). When the F90 + F80 mixture derived from pure basic protein component 1 was chromatographed under identical conditions, no material corresponding to F80-2 and F80-3 was found. The F90 component was further purified by rechromatography under the original conditions followed by gel filtration; F80-2 was purified further by gel filtration. From 800 mg of guinea pig and bovine proteins, respectively, the following yields of purified components were obtained from the pH 3.0 digest: F80-1, 66 and 71 mg; F80-2, 17 and 27 mg; F80-3, 31 and 25 mg; F45-1, 53 and 61 mg; F45-2, 74 and 61 mg; F45-3, 129 and 117 mg. From 300 mg of basic protein treated with pepsin at pH 6.0 approximately 60 mg of purified F90 component was obtained.

Figure 3 illustrates the results of peptic digestion of basic protein and the purity of the isolated components as judged by polyacrylamide gel electrophoresis. Cleavage of the basic protein appeared to be complete. With the exception of F45-3 and F45-4B all of the F45 components appeared to be highly pure (Figure 3a). Component F45-3 consisted of two peptides. Those comprising bovine F45-3 were separated and isolated by recycling gel filtration; they were obtained in a homogeneous state by five cycles of the fraction

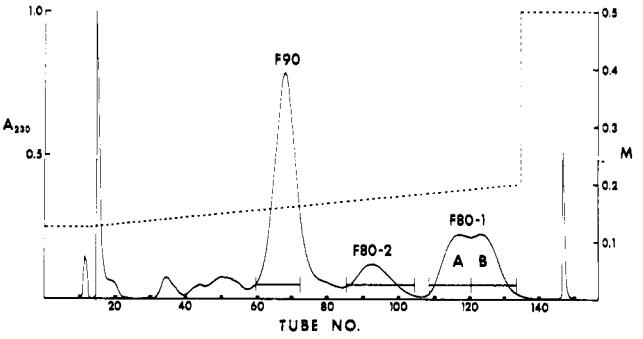


FIGURE 2: Chromatography of bovine fraction F90 + F80 (227 mg) produced by peptic cleavage at pH 6.0 on a column (2.5 × 49 cm) of Whatman CM-23. The molarity of buffer entering the column is indicated. The flow rate was 82 ml/hr; fractions of 19.7 ml were collected and combined as shown.

and collection of the first and last one-fourths of the resultant broad peak. Component F45-4B consisted predominantly of a major peptide with the same mobility as F45-5 plus a trace of a faster running contaminant. The three F80 peptides obtained by cleavage at pH 3.0 (Figure 3b) were similar, but not identical, in electrophoretic mobilities; peptide F80-3 showed slight contamination. As shown in Figure 3c the slower migrating peptide, F90, and the faster migrating F80-1A and F80-1B resulting from cleavage at pH 6.0 appeared to be highly pure; peptide F80-2 was slightly contaminated. Peptides F45-4A, F45-4B, F45-5, and F80-1, 2, and 3 each contained a trace of a component of lower

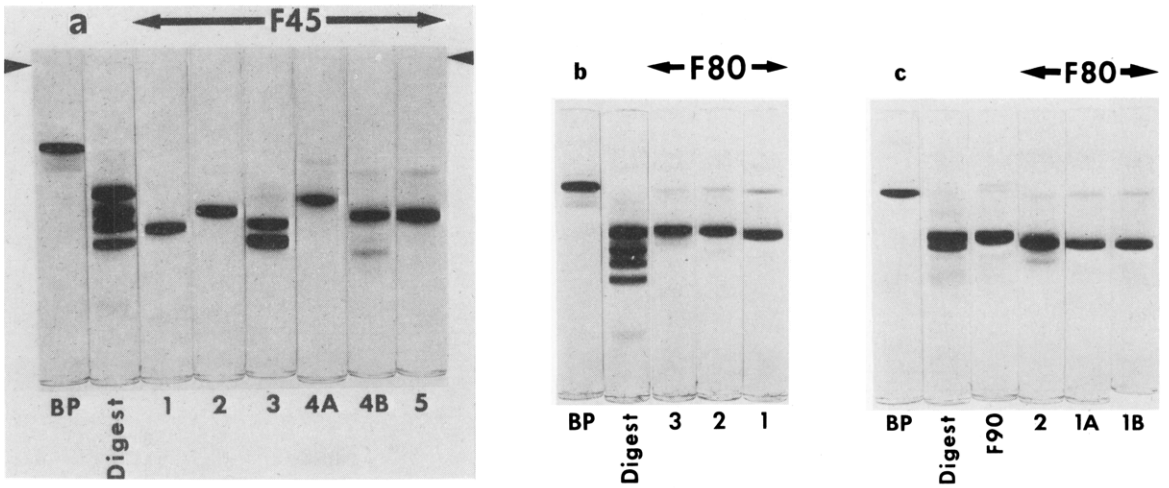


FIGURE 3: Electrophoresis in parallel of basic protein, pepsin digest, and purified chromatographic components. Samples are labeled as in Figures 1 and 2. Electrophoresis was carried out toward the cathode (bottom) for 3.5 hr at 1.5 mA/gel. Results with guinea pig and bovine materials were essentially identical. (a) Guinea pig basic protein (25 $\mu$g), pH 3.0 digest (100 $\mu$g), and F45 components (F45-3, 50 $\mu$g; all others, 25 $\mu$g). The arrows indicate the origin. (b) Guinea pig basic protein (25 $\mu$g), pH 3.0 digest (100 $\mu$g), and F80 components (25 $\mu$g). (c) Bovine basic protein (25 $\mu$g), pH 6.0 digest (50 $\mu$g), and isolated components (25 $\mu$g).

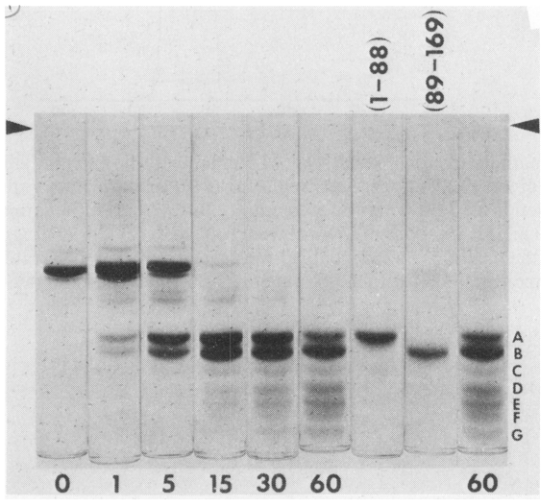


FIGURE 4: Electrophoresis in parallel of bovine basic protein (25 $\mu$g), its cleavage products (100 $\mu$g) obtained after varying periods (shown in minutes) of incubation with pepsin at pH 3.0, 24°, and purified peptides (1–88) and (89–169) (25 $\mu$g). Electrophoresis was carried out toward the cathode (bottom) for 4.0 hr at 1.5 mA/gel. The arrows indicate the origin. Components were identified as follows: (A) peptide (1–88); (B) peptide (89–169); (C) peptide (89–152); (D) mixture of peptides (37–88), (111–169), and (114–169); (E) peptide (1–42); (F) peptide (43–88); (G) peptide (1–36). Identical results were obtained with the guinea pig basic protein.

mobility, probably a dimer which formed during lyophilization. Dimerization of the basic protein (Martenson *et al.*, 1972) and its chemically derived C-terminal 54-residue fragment (Martenson *et al.*, 1975) have been found to occur upon similar treatment.

*Characterization of Peptides.* The identities of the peptides were established from their amino acid compositions and N-terminal and C-terminal residues. The corresponding guinea pig and bovine peptides yielded identical terminal residues but differed slightly in amino acid compositions. The results are summarized in Table I, where the complete data for the guinea pig peptides are given. Data for the bovine peptides which differ from data for the guinea pig peptides are indicated. Of all peptides analyzed chemically only F45-4B showed contamination (0.10 and 0.14 residue of Val/mol of bovine and guinea pig peptides, respectively). The larger, electrophoretically slower peptide of bovine F45-3 had the amino acid composition and C-terminal residues (Arg,Phe) of peptide (1–42); the smaller, electrophoretically faster peptide had the composition and C-terminal residues (Ile,Leu) of peptide (1–36). Densitometric scanning of polyacrylamide gels of bovine F45-3 indicated the molar ratio, peptide (1–36)/(1–42), of 1.2. For guinea pig F45-3 the ratio was 1.9. These proportions were reflected in the amino acid compositions and yields of C-terminal residues of the peptide mixtures. Bovine and guinea pig peptides F80-2 and F80-3 differed from F80-1 only in their contents of arginine at the C-terminus. Whereas F80-1 yielded 1.9–2.0 mol of C-terminal arginine per mole of peptide, the respective yields from F80-2 and F80-3 were 1.7 and 1.2–1.4. These results are comparable to those which have been obtained with components 1, 2, and 3 of the basic protein (G. E. Deibler, unpublished observation). The amino acid compositions of the F80-1 and F80-2 peptides obtained by pH 6.0 digestion were identical with those obtained by digestion at pH 3.0. Bovine F80-1A and F80-1B (residues 89–169) were identical in amino acid compositions, as were the guinea pig counterparts, except for their contents of methylarginine. Bovine and guinea pig peptide (89–169) molecules in fraction A contained, respectively, 93 and 100% of the $N^G$,$N'^G$-dimethylarginine and 72 and 81% of the $N^G$-monomethylarginine of F80-1.

*Relative Rates of Cleavage of Phe–Phe (42–43) and Phe–Phe (88–89) at pH 3.0.* Bovine and guinea pig proteins were incubated with pepsin (enzyme/substrate, 1:500) at pH 3.0 (24°) for varying periods of time, and the digests were examined by electrophoresis. The results (Figure 4) showed that the bond most susceptible to cleavage at pH 3.0 was Phe–Phe (88–89) and that a biphasic breakdown of the protein occurred. After 5 min of incubation 57% of the basic protein had undergone cleavage, yielding relatively large quantities of peptides (1–88) and (89–169) but only traces of the smaller peptides. Two very minor components with mobilities intermediate between those of peptide (1–88) and the basic protein were observed. These are probably peptides (43–169) and (37–169), resulting from cleavage at Phe–Phe (42–43) and Leu–Asp (36–37), respectively. After

Table I: Amino Acid Compositions and End Groups of Guinea Pig Peptic Peptides.[a]

| | Residues/mole of Peptide[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | F90 | F45-1 | F45-2 | F45-3 | F45-4A | F45-4B | F45-5 | F80-1 |
| Trp | | | | | 0.9 (1) | 0.7 (1) | 0.7 (1) | 0.9 (1) |
| Lys | 5.0 (5) | 3.1 (3) | 3.3 (3) | 2.2 (2) | 7.3 (7) | 5.9 (6) | 6.2 (6) | 7.7 (8) |
| His | 8.2 (8) | 4.0 (4); 4.9 (5) | 4.2 (4); 5.2 (5) | 4.0 (4); 3.0 (3) | 1.0 (1); 2.0 (2) | 1.5 (1); 2.1 (2) | 1.1 (1); 2.0 (2) | 1.0 (1); 1.9 (2) |
| $N^G$, $N'^G$-$(Me)_2$Arg | 0.00 (0) | 0.00 (0) | 0.00 (0) | 0.00 (0) | Present | 0.00 (0) | 0.00 (0) | 0.16 |
| Arg (total) | 10.1 (10) | 4.0 (4) | 4.8 (5) | 5.2 (5.4) | 3.7 (4) | 4.2 (5) | 6.6 (6) | 7.5 (8) |
| Asp | 6.9 (7) | 3.9 (4) | 4.8 (5) | 2.1 (2.4) | 3.0 (3) | 3.0 (3) | 3.0 (3) | 4.0 (4) |
| Thr | 4.7 (5); 3.7 (4) | 1.9 (2) | 1.8 (2) | 2.7 (3); 1.8 (2) | 2.7 (3) | 1.2 (1) | 1.0 (1) | 2.7 (3) |
| Ser | 9.8 (11); 7.2 (8) | 5.1 (6); 2.5 (3) | 6.1 (7); 3.2 (4) | 3.7 (4.4) | 5.0 (6); 6.0 (7) | 5.1 (6); 5.7 (7) | 6.0 (7); 6.8 (8) | 7.7 (9); 8.7 (10) |
| Glu | 6.0 (6) | 4.0 (4) | 4.0 (4) | 2.0 (2) | 3.9 (4) | 2.7 (3) | 3.0 (3) | 4.0 (4) |
| Pro | 4.9 (5); 6.5 (6) | 3.1 (3); 3.6 (4) | 3.2 (3); 4.1 (4) | 1.9 (2) | 5.6 (5) | 2.1 (2) | 2.1 (2) | 5.7 (6) |
| Gly | 8.1 (8); 11.1 (10) | 3.9 (4); 6.9 (7) | 5.1 (5); 8.3 (8) | 3.4 (3.4); 2.6 (2.5) | 12.6 (12) | 11.4 (12) | 12.9 (12) | 15.0 (15) |
| Ala | 8.3 (8); 9.5 (9) | 3.9 (4) | 4.2 (4) | 4.0 (4); 5.0 (5) | 3.8 (4) | 4.9 (5) | 5.1 (5) | 4.7 (5) |
| Val | 1.4 (2) | 1.4 (2) | 1.4 (2) | 0.00 (0) | 0.9 (1) | 0.14 (0) | 0.00 (0) | 0.7 (1) |
| Met | 0.8 (1) | 0.00 (0) | 0.00 (0) | 0.8 (1) | 0.00 (0) | 0.8 (1) | 0.8 (1) | 0.9 (1) |
| Ile | 1.8 (2); 0.9 (1) | 0.00 (0) | 1.0 (1); 0.00 (0) | 1.2 (1.4); 0.9 (1) | 1.5 (2) | 0.9 (1) | 0.9 (1) | 1.7 (2) |
| Leu | 3.9 (4); 5.0 (5) | 1.1 (1) | 1.0 (1); 2.1 (2) | 3.0 (3); 3.5 (3.5) | 3.0 (3); 4.0 (4) | 2.2 (2); 3.1 (3) | 2.2 (2); 3.4 (3) | 4.3 (4); 5.0 (5) |
| Tyr | 2.0 (2) | 0.7 (1) | 0.8 (1) | 0.8 (1) | 2.1 (2) | 1.9 (2) | 2.0 (2) | 2.0 (2) |
| Phe | 4.5 (4) | 1.7 (2) | 3.0 (3) | 1.2 (1.4) | 4.2 (4); 3.1 (3) | 3.1 (3); 1.9 (2) | 4.0 (4); 3.0 (3) | 5.1 (5); 3.8 (4) |
| Total | 88 | 44; 46 | 50; 52 | 38 + 44; 36 + 42 | 62; 64 | 54; 56 | 57; 59 | 79; 81 |
| N-Terminus[c] | Blocked | Phe | Asx | Blocked | Phe | Ser | Ser | Phe |
| C-Term residues[d] | 0.8 0.7 0.7 Val, His, Phe | 0.9 0.8 0.8 Val, His, Phe[e] | 1.0 0.9 1.1 Val, His, Phe[e] | 0.6 0.7 0.1 0.3 Ile, Leu, Arg, Phe | 0.2 0.2 0.8 0.8 Leu, Ser, Lys, Ile[e] | 0.8 1.7 Ala, Arg[e] | 0.8 2.1 Ala, Arg[e] | 0.6 2.0 Ala, Arg[e] |
| Presumed sequence | Res. "1–88" | Res. "43–88" | Res. "37–88" | Res. "1–36" + "1–42" | Res. "89–152" | Res. "114–169" | Res. "111–169" | Res. "89–169" |

[a] The *left* column under each heading gives the data for the guinea pig peptide. Data obtained for the corresponding bovine peptide which differed from those of the guinea pig are given in the *right* column. Guinea pig values in parentheses are the most probable numbers of residues; for F45-1 (residues "43–88") they are the theoretical numbers (Shapira *et al.*, 1971). Bovine values in parentheses are the theoretical numbers (Eylar *et al.*, 1971; Brostoff *et al.*, 1974). Fractional values in parentheses (F45-3) are those that would result from a mixture of peptides in the ratio (1-36)/(1-42) of 1.9 (guinea pig) or 1.2 (bovine). [b] Based upon the integral values for Glu (F90, F45-1, F45-2, F45-3, F80-1) or Asp (F45-4A, F45-4B, F45-5) shown. [c] Determined by reaction with 5-dimethylaminonaphthalene-1-sulfonyl chloride. [d] Mole/mole of peptide released after 10-min (F90), 20-min (F45-1, F45-2, F45-3), 60-min (F45-4A, F45-4B, F45-5), or 120-min (F80-1) incubation with carboxypeptidases A and B. [e] First amino acid released.

15 min practically no basic protein was left, yet a relatively large quantity of peptide (1–88) was present. With time the latter underwent progressive cleavage at Phe–Phe (42–43) and Leu–Asp (36–37) to yield increasingly larger amounts of the smaller derivative peptides.

## Discussion

The present studies have shown that it is possible to control the peptic cleavage of myelin basic protein so that only a limited number of fragments result. A study of the time course of hydrolysis of the protein at pH 3.0 (enzyme/substrate ratio, 1:500, w/w) demonstrated a preferential cleavage of Phe–Phe (88–89): cleavage at this bond was nearly complete before appreciable cleavages of Phe–Phe (42–43) and Leu–Asp (36–37) occurred. The high sensitivity of Phe–Phe (88–89) to cleavage by pepsin was shown dramatically by the results obtained at pH 6.0: after 1 hr complete and nearly exclusive hydrolysis of this bond had occurred. This difference in susceptibility to peptic cleavage of the two Phe–Phe bonds illustrates the importance of secondary interactions between pepsin and amino acid side chains flanking the sensitive bond (Hollands *et al.*, 1969; Medzihradszky *et al.*, 1970; Sachdev and Fruton, 1969, 1970).

The relative susceptibility to cleavage of the two Phe–Phe bonds of the basic protein by pepsin is markedly different from their relative susceptibility to cleavage by liver cathepsin D. Brostoff *et al.* (1974) have shown that incubation of the bovine basic protein with the latter enzyme at pH 3.5 and 25° for up to 3 hr resulted in nearly exclusive cleavage at Phe–Phe (42–43) with the sparing of Phe–Phe (88–89). A marked difference in the specificity of bovine spleen cathepsin D as compared with pepsin in the cleavage of certain synthetic peptides containing a Phe–Phe linkage has also been observed (Ferguson *et al.*, 1973).

Both Phe–Phe linkages of the basic protein have been shown by other investigators to be particularly prone to cleavage by pepsin-like enzymes from brain (Bergstrand, 1971; Benuck *et al.*, 1974). In those studies no cleavage at Leu–Asp (36–37) was noted. In the present studies with pepsin this Leu–Asp bond was at least as susceptible to cleavage as Phe–Phe (42–43). Cleavage at Leu–Asp (36–37) by pepsin has been shown by others to occur when the reaction is carried out at low pH with relatively high pepsin/substrate ratios (Carnegie, 1971; Eylar *et al.*, 1971).

In addition to the major cleavages at the Phe–Phe and Leu–Asp bonds which took place in the present studies, minor cleavages were found to occur elsewhere within the protein: at Leu–Ser (110–111), Phe–Ser (113–114), and Ile–Phe (152–153). These cleavages have been shown previously to occur at relatively high pepsin/substrate ratios (Carnegie, 1971; Eylar *et al.*, 1971; Bergstrand, 1972).

Cleavage of Ile–Phe (152–153) at a relatively low pepsin/substrate ratio (1:500, w/w) was surprising. Synthetic substrates containing this bond have been found to be completely resistant to cleavage by pepsin (Trout and Fruton, 1969). A survey of bonds in several proteins cleaved by pepsin failed to reveal any in which Ile contributed the carboxyl peptide bond, although a number of susceptible bonds were found in which cleavage occurred carboxy-terminal to Val (Tang, 1963; Hirs, 1970). It is perhaps noteworthy that in the present study, where only the most susceptible bonds were cleaved, five of the six bonds which underwent significant cleavage were located between residues flanked on one or both sides by a cationic residue. The one exception was Leu–Asp (36–37).

Peptic cleavage of myelin basic protein preparations which consisted of three chromatographic forms (components 1, 2, and 3) resulted in the production of three F80 peptides. One of these, F80-1, was identified as peptide (89–169). The other two, F80-2 and F80-3, differed from F80-1 in having slightly less arginine at the C-terminus. Cleavage of homogeneous basic protein (component 1) yielded only F80-1. Results of detailed studies on these peptides and the contribution they make to the microheterogeneity of the basic protein will be described in detail elsewhere (R. E. Martenson, A. J. Kramer, and G. E. Deibler, manuscript in preparation).

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# Histone–Histone Associations within Chromatin. Cross-Linking Studies Using Tetranitromethane†

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ABSTRACT: Treatment of chromatin with the protein cross-linker tetranitromethane (TNM) results in a product identified as an F2a1–F2b dimer. The same product appears after treatment with TNM of HeLa cells growing in culture. Furthermore acid-extracted histones which have been fractionated into the five separate species can be recombined and mixed with DNA to produce a nucleohistone preparation which is also cross-linked by TNM to give the F2a1–F2b dimer. F1 and F3 can be excluded from the reconstitution mixture without effect on the dimer production. In contrast, the presence of F2a2 is essential to the proper reconstitution of F2a1 and F2b with DNA. The specificity of TNM and the characteristics of the reaction suggest that F2a1 and F2b are cross-linked at their specific binding sites. These results provide evidence that F2a1, F2a2, and F2b interact specifically in chromatin.

Recent developments strongly suggest that the structure of chromatin is based on a linear array of subunits (Olins and Olins, 1974; Hewish and Burgoyne, 1973; Axel et al., 1974; Weintraub and Van Lente, 1974; Sahasrabuddhe and Van Holde, 1974; Noll, 1974). X-Ray data which had previously been thought to reflect a supercoil have now been reinterpreted in the context of this new subunit model (Kornberg, 1974). Although some workers still prefer the supercoil concept (Pooley et al., 1974) recent data of Weintraub and Van Lente (1974) argue strongly in favor of histone clusters as a fundamental structural feature of chromatin.

There are no direct data bearing on the stoichiometry of the histones within the putative subunits. However, a number of considerations suggest that for the most part all of the histones, with the possible exception of F1, are represented in each subunit. First of all, in solution, heterologous histone–histone interactions are much stronger than the homologous ones. Moreover the interactions are very specific and can be fit most easily into a scheme which proposes the clustering of the four smaller histones (i.e., F2a1, F2a2, F2b, and F3) into an octamer containing two of each type (D'Anna and Isenberg, 1974b; Kornberg and Thomas, 1974). These considerations are consistent with electron micrographs of chromatin which reveal subunits of a size compatible with a hypothetical histone octamer and about 200 base pairs of DNA (Olins and Olins, 1974). Finally, Kornberg and Thomas (1974) report that the X-ray diffraction pattern characteristic of native chromatin can be obtained from reconstituted chromatin only when all four of the smaller histones are present. Although Richards and Pardon (1970), using a different reconstitution procedure, have regenerated the X-ray pattern with fewer histones, it seems likely that many or most subunits in chromatin contain all four of the smaller histones and probably two of each.

We have studied the arrangement of the histones in chromatin by means of chemical cross-linking and report here the results obtained with the cross-linking agent tetranitromethane (TNM). The mechanism by which this reagent cross-links proteins is not completely clear. Nevertheless the primary site of TNM action is tyrosine and, in those cases which have been studied, dityrosine is recovered from the cross-linked protein (Williams and Lowe, 1971). Probably TNM produces a tyrosine free radical which inserts itself directly onto an appropriately situated and precisely adjacent neighbor. The TNM does not itself become incorporated as a bridge and thus is effectively a zero length cross-linker. The probability that the hydrophobic portions of the histones, where the tyrosine residues are found, are involved in specific and very tight histone–histone interactions in chromatin (Hayashi and Iwai, 1971; Bradbury and Rattle, 1972; Bradbury et al., 1973; Shih and Fasman, 1971; Weintraub and Van Lente, 1974) suggests that TNM, which can penetrate hydrophobic clusters (Myers and Glazer, 1971), may serve as a cross-linking probe specific for complexed rather than merely adjacent histones.

Using TNM we have found the rapid formation of a single cross-linked histone product with the molecular weight of a dimer. This cross-linked product is formed by treatment with TNM of either growing cells in culture, isolated chromatin, or reconstituted nucleohistone. On the basis of reconstitution of DNA with separated radioactive histones the cross-linked product has been identified as an F2b–F2a1 dimer.

## Experimental Section

*Materials.* Tetranitromethane (TNM) was purchased from Sigma. For reconstitution of calf thymus nucleohis-

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