Reaction of Peptide 89–169 of Bovine Myelin Basic Protein with 2-(2-Nitrophenylsulfenyl)-3-methyl-3′-bromoindolenine†

Russell E. Martenson,* Gladys E. Deibler, and Allan J. Kramer‡

ABSTRACT: The C-terminal half of the bovine myelin basic protein, peptide 89–169, was treated with BNPS-skatole [2-(2-nitrophenylsulfenyl)-3-methyl-3′-bromoindolenine], and the products were isolated by repeated gel filtration through Sephadex G-50. They consisted of uncleaved peptide 89–169 in which approximately 30% of the tyrosine had been monobrominated and the tryptophan converted to oxindolealanine, peptide 116–169 modified by partial bromination (30%) of the tyrosine, and two chromatographic forms of peptide 89–115. The major form contained the lactone of dioxindolealanine at the C terminus; the minor form contained the uncyclized oxidation product. Each form of peptide 89–115 was resolved into several components by electrophoresis in polyacrylamide gels (10%, w/w) containing 1 M acetic acid and 8 M urea. The presence of three of these components could be explained by partial deamidation of Asn-91 and Gln-102. Studies on the oxidation of tryptophan-containing model peptides by BNPS-skatole indicated that the reaction can also include partial bromination of the dioxindole and its lactone and partial cleavage at the amino peptide bond of the tryptophan.

The oxidative brominating agent, 2-(2-nitrophenylsulfenyl)-3-methyl-3′-bromoindolenine (BNPS-skatole),[1] was recently introduced (Omenn et al., 1970) to cleave proteins specifically at the carboxyl peptide bond of tryptophanyl residues (for review, see Fontana, 1972). Use of this reagent has helped investigators to localize immunologically active regions in the highly basic encephalitogenic protein of central nervous system myelin (Burnett and Eylar, 1971; Bergstrand, 1971, 1972; Bergstrand and Källén, 1972; Martenson et al., 1975a; Whitaker et al., 1975) and to prepare fragments of this protein for use in studies of protein–lipid interactions (Mateu et al., 1973; London et al., 1973).

We have recently used BNPS-skatole to locate the position of an encephalitogenic region in the C-terminal half (residues 89–169) of the bovine myelin basic protein (Martenson et al., 1976a). The studies reported herein show that, under the conditions we employed to cleave peptide 89–169, 77% of the Trp-115 bonds were cleaved, yielding chromatographically separable dioxindole and dioxindole lactone derivatives of peptide 89–115. In addition, 30% of the tyrosyl residues in peptide 116–169 and in the oxidized but uncleaved peptide 89–169 were monobrominated, and it appeared that some deamidation and loss of C-terminal dioxindolealanine occurred as well. Some of these reactions, in addition to creating potential antigenic determinants, also introduce a heterogeneity into the products which may, in some instances, complicate their isolation and identification.

Materials and Methods

Reagents. The sources of most reagents used have been given previously (Martenson et al., 1975a). Others were as follows: trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone, Worthington Biochemical Corp., Freehold, N.J.; 3,5-dibromo-L-tyrosine, L-lysyl-L-tyrosine monohydrate, and glycyl-L-tryptophylglycine monohydrate, Cyclo Chemical, Los Angeles, Calif.; glycyl-L-tryptophan and ninhydrin spray, Sigma Chemical Co., St. Louis, Mo.; *N*-bromosuccinimide (NBS) and pyridine (Sequanal grade), Pierce Chemical Co., Rockford, Ill.; cellulose "chromagram" sheets (20 × 20 cm), Eastman Kodak Co., Rochester, N.Y.

Peptide 89–169. Peptide 89–169 was prepared from highly purified bovine myelin basic protein by limited peptic hydrolysis as described previously (Martenson et al., 1975b). The peptide, previously designated F80-1, was electrophoretically homogeneous at both acid and alkaline pH and contained no phosphorus (Martenson et al., 1976b).

Treatment of Peptides with BNPS-Skatole. Peptide 89–169, glycyltryptophan, and glycyltryptophylglycine were treated with BNPS-skatole under the conditions used previously (Martenson et al., 1975a). The peptide (1.7 μmol/ml) and BNPS-skatole (17 μmol/ml) in 75% acetic acid were incubated at 37 °C for 24 h. Subsequently, excess reagent and its by-products were removed from the reaction mixture by repeated extraction with ethyl acetate (Fontana et al., 1973), and the aqueous solution was lyophilized.

Treatment of Glycyltryptophan and Glycyltryptophylglycine with NBS. To prepare oxindole, dioxindole (lactone), and bromodioxindole (lactone) derivatives, samples of the model peptides (2.0 μmol/ml in 50% acetic acid) were reacted with 0.5, 2.0, or 4.0 equiv, respectively, of NBS (100 μmol/ml in 50% acetic acid). After incubation for 1 h at 25 °C excess NBS was destroyed by the addition of 10 volumes of 0.01 M HCOOH (Barakat et al., 1955), and the samples were lyophilized.

Analysis of Oxidation Products of Glycyltryptophan and Glycyltryptophylglycine. Derivatives of the model peptides formed by their oxidation with BNPS-skatole or NBS were separated by chromatography on a column (2.5 × 99 cm) of Sephadex G-10 equilibrated at 5 °C with 0.2 M acetic acid. Elution was carried out in succession with 0.2 and 2.0 M acetic acid and monitored at 260 nm. An initial ultraviolet absorption spectrum was recorded for the contents of each "peak" tube. After appropriate pooling of fractions, each compound was lyophilized, subjected to amino acid analysis, rechromato-

† From the Section on Myelin Chemistry, Laboratory of Cerebral Metabolism, National Institute of Mental Health, Bethesda, Maryland 20014. *Received June 24, 1976.*

‡ Supported by Grant No. 828-A-4 from the National Multiple Sclerosis Society.

[1] Abbreviations used: BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3′-bromoindolenine; NBS, *N*-bromosuccinimide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

graphed, and analyzed again in the same manner. After lyophilization some of the compounds were partially converted to products having much lower Sephadex elution volumes. In general, these new compounds had the same elution times on the amino acid analyzer as the originals. They were present in relatively small amounts in the initial mixtures of oxidized peptides.

Identification of Tryptophan Oxidation Products. Tryptophan oxidation products were identified by comparison of their spectral characteristics with those of 3-substituted oxindoles published by Hinman and Bauman (1964), specifically, oxindole-3-propionic acid, dioxindole-3-propionic acid, dioxindole-3-propionic acid lactone, 3-methyldioxindole, and 5-bromo-3-methyldioxindole. To test for the presence of bromine, a drop of a concentrated solution of the material (as the acetate salt) dissolved in 2 M acetic acid was applied to a copper wire and volatilized in a flame; a green color indicated the presence of a volatile copper halide.

Amino Acid Analyses. Amino acid analysis of peptide 89–169 and its cleavage products was carried out as described previously (Martenson et al., 1975b) with analysis of basic amino acids done on either an 8-cm column of Beckman PA-35 resin or a 21-cm column of Durrum DC-6A resin. The latter column was also used for analysis of bromotyrosines and products of the reaction between the tryptophan-containing model peptides and BNPS-skatole or NBS. For these analyses, however, the starting buffer was 0.2 N sodium citrate, pH 4.25; this was changed at 24 min to 0.35 N sodium citrate, pH 5.28. The column was operated at 56 °C at a flow rate of 70 ml/h. The elution times of tyrosine, 3-bromotyrosine, 3,5-dibromotyrosine, and lysine were 36, 61, 83, and 93 min, respectively. 3,5-Dibromotyrosine was found to have a ninhydrin color factor 107% that of tyrosine.

Tryptic Peptide Mapping. Peptide 89–115, 0.3 to 0.4 mg, was reacted with trypsin (enzyme–substrate, 1:50 or 1:250 by weight) in 100 μl of 0.1 M ammonium bicarbonate, pH 8.2, for 4 h at 37 °C. After lyophilization the sample was dissolved in 2 μl of H_2O, applied to a 20 × 20 cm cellulose "chromagram" sheet, and subjected to electrophoresis at pH 4.7 (pyridine–acetic acid–H_2O, 1:1:48 by volume) for 100 min at 400 V, followed by chromatography (1-butanol–pyridine–acetic acid–H_2O, 122:189:38:151 by volume) for 150 min. The sheet was sprayed for ninhydrin- or Sakaguchi-positive peptides (Easley, 1965). Spots from three maps lightly sprayed with ninhydrin were scraped from the plastic backing, and the peptides were extracted from the cellulose powder with two 1-ml portions of 0.1 N HCl. The extracts were lyophilized, hydrolyzed for 22 h at 110 °C in constant-boiling HCl in vacuo, and analyzed for amino acids.

Additional Procedures. End-group analyses were carried out as described previously (Martenson et al., 1975a). Ultraviolet absorption measurements were made with a Beckman DU monochromator updated with Gilford accessories; spectra were determined from measurements made at 1- or 2-nm intervals. Polyacrylamide gel electrophoresis was carried out in 10% (w/w) gels (6 × 70 mm) containing 1 M acetic acid–8 M urea, pH 3.2 (Martenson and Deibler, 1975). Peptides were stained with Amido Black, and gels were scanned as previously described (Martenson et al., 1975b).

Results

Isolation of Cleavage Products of Peptide 89–169. Sephadex G-50 *fine* chromatography of the peptide mixture resulting from treatment of peptide 89–169 with BNPS-skatole yielded four peptide peaks (Figure 1). Recovery of material

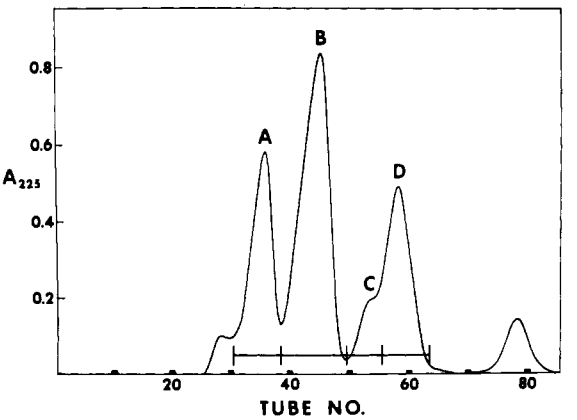

FIGURE 1: Initial fractionation of 95 mg of BNPS-skatole-treated bovine peptide 89–169 on a column (3.2 × 95 cm) of Sephadex G-50 fine equilibrated and eluted with 0.01 N HCl at 5 °C. The flow rate was 33 ml/h; fractions of 10.1 ml were collected, combined as shown, and lyophilized. The recovery (dry weight) of material eluted in tubes 26 to 63 was 92 mg.

(lyophilized dry weight) eluted between tubes 25 and 64 was 97% of that applied to the column. The material eluted at tubes 73–84 yielded a brownish oil upon lyophilization, apparently a by-product of BNPS-skatole. Lyophilization of peaks A and B yielded 20.6 and 44.7 mg of peptide, respectively. Based on the identification of these peaks (see below), the extent of cleavage was 77%. Peaks A and B were incubated with thioglycolic acid to regenerate methionine from the sulfoxide (Omenn et al., 1970), rechromatographed, and recovered as chromatographically homogeneous peptides in yields of 15.8 and 37.9 mg, respectively. Peaks C and D without prior thioglycolic acid treatment were rechromatographed twice and yielded respectively 4.6 and 13.3 mg of chromatographically homogeneous peptide.

Amino Acid and Amino Terminal Analysis. As shown in Table I, the amino acid compositions of peaks A and B were those expected for pure peptides 89–169 and 116–169, respectively. Comparison with the unreacted peptide 89–169 showed that some destruction (approximately 30%) of tyrosine had resulted from BNPS-skatole treatment and that not all of the methionine had been regenerated. The loss of tyrosine could be completely accounted for by monobromination. The amino acid composition of peak D was that expected for pure peptide 89–115 with no evidence of contamination. The composition of peak C indicated that it too was peptide 89–115, but slightly contaminated as shown by the presence of small amounts of histidine, alanine, tyrosine, and 3-bromotyrosine. Reaction of the peptides with dansyl chloride showed that, like the parent peptide 89–169, peaks A, C, and D also contained phenylalanine (residue 89) and a trace of lysine (residue 90) present at the N terminus. Peak C contained, in addition, a contaminant with N-terminal glycine. As expected, the N terminus of peak B was glycine (residue 116).

Tryptic Peptide Maps of Peaks C and D. Peaks C and D yielded identical tryptic peptide maps, save for some very faint spots found exclusively in peak C, which undoubtedly arose from the contaminants known to be present. The peptide map (Figure 2) identifies the peptides according to the designation of Eylar et al. (1971); amino acid analysis established the identities of the peptides. In all cases the amino acid recovered in reduced yield, other than lysine, corresponded to the theoretical N-terminal residue, as expected from its reaction with ninhydrin. Curiously, peptide T16A was Sakaguchi-negative,

TABLE I: Amino Acid Compositions of Peptide 89–169 and Its BNPS-Skatole Reaction Products.[a]

Residue	Mol/mol of Peptide[b]				
	Peptide 89–169	Peak A	Peak B	Peak C	Peak D
Trp[c]	Present (1)	Absent	Absent (0)	Absent	Absent
3-Bromo Tyr[d]	(0.00 (0)	0.51	0.48	0.03 (0)	0.00 (0)
Lys	7.9 (8)	8.2 (8)	6.3 (6)	1.9 (2)	2.0 (2)
His	2.0 (2)	2.1 (2)	2.0 (2)	0.12 (0)	0.00 (0)
N^G,N'^GMe$_2$Arg	(0.19	0.20	0.00 (0)	0.24	0.15
N^G-MeArg	0.21	0.24	0.00 (0)	0.22	0.26
Arg (total)	7.9 (8)	8.1 (8)	5.0 (5)	2.6 (3)	3.0 (3)
Asp	3.8 (4)	3.8 (4)	3.0 (3)	0.88 (1)	0.92 (1)
Thr	2.6 (3)	2.6 (3)	0.96 (1)	1.4 (2)	1.7 (2)
Ser	8.6 (10)	8.4 (10)	5.1 (6)	2.8 (4)	3.3 (4)
Glu	4.0 (4)	4.0 (4)	3.0 (3)	1.0 (1)	1.0 (1)
Pro	5.9 (6)	5.5 (6)	2.1 (2)	3.0 (4)	3.9 (4)
Gly	16.6 (15)	16.0 (15)	13.0 (12)	3.2 (3)	3.0 (3)
Ala	5.4 (5)	5.2 (5)	5.1 (5)	0.35 (0)	0.00 (0)
Val	0.69 (1)	0.66 (1)	0.04 (0)	0.48 (1)	0.63 (1)
Met	0.79 (1)	0.53 (1)	0.53 (1)	0.00 (0)	0.00 (0)
Ile	1.4 (2)	1.4 (2)	0.92 (1)	0.53 (1)	0.61 (1)
(Leu	4.8 (5)	4.9 (5)	3.1 (3)	1.7 (2)	1.9 (2)
(Tyr	1.8 (2)	1.3 (2)	1.3 (2)	0.05 (0)	0.00 (0)
Phe	3.9 (4)	3.6 (4)	1.9 (2)	1.5 (2)	1.8 (2)
Presumed sequence		89–169	116–169	89–115	89–115

[a] Amino acid analyses were done on two separate hydrolysates and the results averaged. [b] Calculated on the basis of the integral values of glutamic acid shown. Values in parentheses are the theoretical ones based upon the sequence (Eylar et al., 1971). [c] Determined from the ultraviolet absorption spectra of peptides. [d] For quantitation of 3-bromotyrosine, the ninhydrin color factor of 3,5-dibromotyrosine was used.

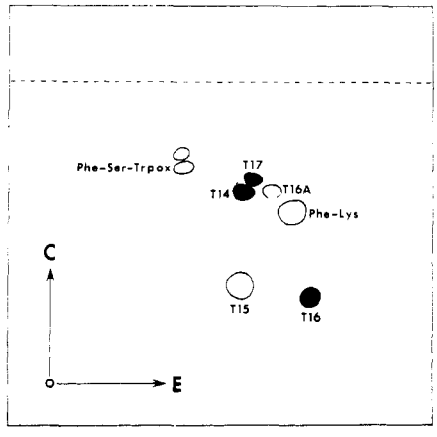

FIGURE 2: Two-dimensional electrophoresis (E)–chromatography (C) of tryptic peptides from peptide 89–115 carried out as described under Materials and Methods. The chromatographic solvent front is indicated; Sakaguchi-positive peptides are depicted in black. T14, Asn-Ile-Val-Thr-Pro-Arg; T15, Thr-Pro-Pro-Pro-Ser-Gln-Gly-Lys; T16, Gly-Arg; T16A, Gly-Arg(Me)$_{1,2}$-Gly-Leu-Ser-Leu-Ser-Arg; T17, Gly-Leu-Ser-Leu-Ser-Arg.

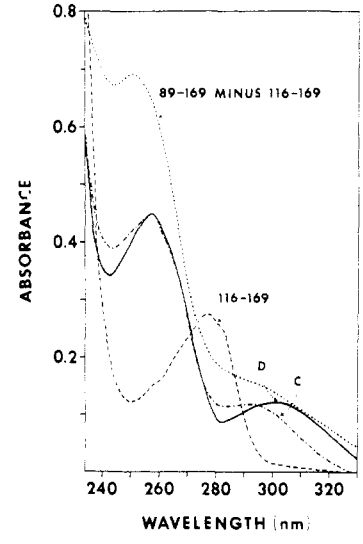

FIGURE 3: Ultraviolet absorption spectra of products formed from the reaction of peptide 89–169 with BNPS-skatole. Samples were dissolved in deionized water and read against water in 1-cm cells. Except for peak C peptide 89–115 (- - - -), their concentration was 0.1 mM. The concentration of dioxindole in peak C peptide 89–115 could not be precisely determined because the peptide was not sufficiently pure; its extinction coefficient at 258 nm was therefore assumed to be the same as that of peak D peptide 89–115 (—) for comparative purposes. Minor corrections were made for the small amounts of tyrosine and 3-bromotyrosine present in peak C peptide 89–115. The theoretical absorption spectrum (. . . .) of the oxidized tryptophan in the uncleaved peptide 89–169 was obtained by subtraction of the peptide 116–169 spectrum (- - - -) from the spectrum of the oxidized peptide.

although it contained arginine. An unidentified spot was present with electrophoretic mobility identical with, but with chromatographic mobility slightly greater than, that of Phe-Ser-Trpox.

Ultraviolet Absorption Spectra. The ultraviolet absorption spectra of the products resulting from BNPS-skatole treatment of peptide 89–169 were compared (Figure 3). Only phenolic absorption was shown by peptide 116–169 as expected. Subtraction of the peptide 116–169 spectrum from that of the oxidized peptide 89–169 yielded a characteristic oxindole spectrum with λ_{max} ~252 nm and a shoulder at ~295–300 nm. The spectrum of the peak D peptide showed a prominent absorption peak at λ ~302 nm, characteristic of a dioxindole lactone. In the peak C peptide, the long wavelength peak was less prominent and occurred at ~292 nm, typical of an uncyclized dioxindole derivative. Both of these peptides had their

major absorption peak at 258 nm. Incubation of peak D at 25 °C for 18 h in 0.05 M Tris-HCl buffer, pH 8.1, converted its absorption spectrum (in this buffer) to one that was essentially identical with that of peak C (in deionized H_2O), indicating lactone ring opening. Identical treatment of peak C had no significant effect on its absorption spectrum. In order to determine whether or not the dioxindole derivative was brominated, peak D (which was not contaminated by bromotyrosine-containing material) was passed through Sephadex G-10 equilibrated with 2 M acetic acid and lyophilized. Flame tests showed the peptide to be halogen negative.

Carboxy Terminal Analysis of Peaks C and D. Previous studies (Martenson et al., 1975a) have indicated that peptides with dioxindolealanine at the C terminus are resistant to the action of carboxypeptidase A. This resistance was exploited to check if both peaks C and D did, in fact, result from a specific cleavage at tryptophan-115. Treatment of a bovine basic protein control with a mixture of carboxypeptidases A and B for 30 min resulted in quantitative release of C-terminal arginine and alanine (1.8 and 1.0 mol, respectively, per mol of protein). Treatment of peaks C and D with the enzymes for 60 min, on the other hand, yielded only minor amounts of amino acids. Those released in greatest amounts (leucine, serine, arginine, and phenylalanine; 0.2 to 0.3 mol per mol of peptide) appeared to have arisen from the sequence Leu-Ser-Leu-Ser-Arg-Phe-Ser, residues 108–114, since these amino acids are not located together elsewhere in peptide 89–169. Their limited release could be explained either by a limited ability of carboxypeptidase A to remove C-terminal dioxindolealanine or by a prior limited removal of the tryptophan from peptide 89–115 in a side reaction accompanying or subsequent to its oxidation by BNPS-skatole.

In order to determine if oxidation of a tryptophanyl residue can result in amino peptide bond scission, a sample of glycyltryptophan was treated with BNPS-skatole as described under Materials and Methods; a control sample was incubated in 75% acetic acid alone. Each sample was subsequently treated with ethyl acetate, and portions of the aqueous phase were analyzed for glycine on the amino acid analyzer before and after acid hydrolysis. BNPS-skatole treatment of the dipeptide was found to result in 20% cleavage; in the absence of BNPS-skatole no cleavage occurred. These results show that, under the conditions used for cleavage of peptide 89–169 and isolation of the products, some cleavage at the amino peptide bond of tryptophan can occur.

Electrophoretic Analysis of Peaks C and D. Polyacrylamide gel electrophoresis showed that both peaks C and D were heterogeneous (Figure 4). The two peaks differed in the quantitative distribution and mobilities of their constituent components (the latter was verified by electrophoresis of a C plus D mixture). The components of peak D moved slightly faster than the corresponding components of peak C. The electrophoretic heterogeneity observed in each peak could not be explained by resolution of peptide 89–115 into components containing monomethyl-, dimethyl-, and unsubstituted arginine at position 106 since the relative proportions of the three major components within each peak bore no resemblance to the relative proportions of these three amino acids. When the analysis was repeated after the peptides had been stored desiccated at 5 °C for 22 months, a significant change in electrophoretic patterns was noted. A single component, which appeared to have been the slowest migrating major component in the original material, now accounted for a much larger fraction of each preparation. The change was not accompanied by any significant change in ultraviolet absorption spectra from those originally observed, indicating that changes other than those involving the dioxindolealanine were involved.

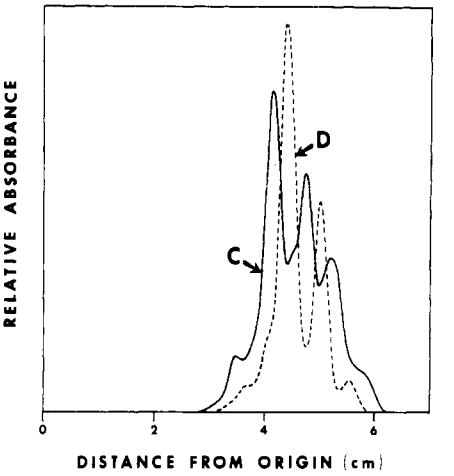

FIGURE 4: Composite electrophoretogram of chromatographic peaks C (—) and D (- - - -). The samples (100 μg) were subjected to electrophoresis toward the cathode in 10% polyacrylamide gels containing 8 M urea and 1 M acetic acid (pH 3.2) for 3.5 h at 1.25 mA/gel. After the peptide had been stained with Amido Black, the gels were scanned at 580 nm; 100% of the ordinate corresponds to 0.75 A for C and 1.5 A for D.

Oxidation of Glycyltryptophan and Glycyltryptophylglycine with BNPS-Skatole. The tryptophan-containing model peptides were treated with 10 equiv of BNPS-skatole and the reaction products separated on a 2.5 × 99 cm Sephadex G-10 column. The major products were characterized by their ultraviolet absorption spectra, presence or absence of halogen, and chromatographic properties (Table II). Elution with 0.2 M acetic acid at 5 °C yielded peaks of the corresponding oxindole derivatives and peaks identified as glycyldioxindolealanine (peak 1) and its lactone (peak 2). Subsequent elution with 2.0 M acetic acid at 5 °C yielded two additional peaks identified as the 5-bromo derivatives of glycyldioxindolealanine (peak 3) and its lactone (peak 4). A number of unidentified minor products were also formed in the reactions; these were detected on the amino acid analyzer by the appearance of small additional peaks with unique elution times. In addition, a significant amount of free glycine was generated from glycyltryptophan and was eluted from the Sephadex column prior to elution of the oxindole derivative.

Discussion

As shown in this study, BNPS-skatole can be a highly efficient, as well as specific, reagent for cleavage of proteins and peptides at tryptophanyl residues. This reagent, however, also oxidizes methionine (Omenn et al., 1970) and partially destroys tyrosine (Bergstrand, 1972; Fontana et al., 1973). The methionine sulfoxide formed can be converted back to methionine by treatment with a reducing agent, while destruction of tyrosine can be avoided by conducting the cleavage reaction in the presence of added phenol (Fontana et al., 1973). In the present study, where a scavenger for excess Br^+ was not used, approximately 30% of the tyrosine was brominated at the 3 position of the ring.

From the reaction mixture of peptide 89–169 and BNPS-skatole, two chromatographically distinct forms of peptide 89–115 were isolated, each of which appeared to be lacking about 20% of its C-terminal oxidized tryptophan. The ultraviolet absorption spectra in deionized H_2O and at pH 8.1 after overnight incubation at this pH indicated that the major form (peak D) contained the lactone of dioxindolealanine, while the

TABLE II: Chromatographic and Spectral Properties of Tryptophan-Containing Model Peptides and Their Major Oxidized Derivatives.

Compound	Sephadex G-10 Elution Volume (ml)[a]	Durrum DC-6A Elution Time (min)[b]	λ_{max}[c] I	λ_{max}[c] II	A_{II}/A_{I}[c]
Glycyltryptophan	1400	91			
Glycyltryptophylglycine	860	104			
Glycyloxindolealanine	510	71	250	280 (sh)[e]	0.19
Glycyloxindolealanylglycine	450	76	252	280 (sh)[e]	0.21
Peak 1 (glycyldioxindolealanine)	930	36	255	286	0.26
Peak 2 (lactone of peak 1)	1120	41	255	299	0.39
Peak 3 (5-bromo derivative of peak 1)	*d*	61	261	302	0.17
Peak 4 (lactone of peak 3)	*d*	67	261	310	0.21

[a] Except where indicated otherwise, the column (2.5 × 99 cm) was equilibrated and eluted with 0.2 M acetic acid; elution volume of acetic acid, 370 ml. [b] The column (0.9 × 21 cm) was eluted as described in Materials and Methods. [c] In the eluting solvent (0.2 or 2.0 M acetic acid). [d] Peaks 3 and 4 were eluted in succession with 2.0 M acetic acid. [e] sh, shoulder.

other (peak C) contained the dioxindole in the uncyclized form. Each form of peptide 89–115 was resolved into a unique set of several components by electrophoresis in polyacrylamide gel.

Since in the native basic protein the two acidic residues located in the sequence 89–115 are amidated (Asn-91 and Gln-102) (Eylar et al., 1971), their partial deamidation under the rather extreme conditions employed for cleavage could readily account for the three major components observed in each of the two peaks. This explanation was supported by the finding that prolonged storage of each peak resulted in an apparent conversion of the two cathodically faster migrating components to the slower migrating one without significant alteration of the material's ultraviolet absorption spectrum. The observation that the components of peak D moved slightly faster than the corresponding ones of peak C is understandable since in peak D, where the dioxindolealanine was lactonized, a free carboxy terminus would be lacking.

BNPS-skatole treatment of the model peptides glycyltryptophan and glycyltryptophylglycine yielded four major products in common. These had to have been formed by oxidation of the tryptophan to dioxindolealanine and loss of C-terminal glycine from the tripeptide since common products could not have arisen otherwise. On the basis of ultraviolet absorption spectra and halogen content, the products were identified as glycyldioxindolealanine and glycyldioxindolealanine lactone and the corresponding derivatives brominated at the 5 position of the dioxindole ring. Treatment of glycyltryptophan with BNPS-skatole resulted in amino peptide bond cleavage with release of 20% of the glycine originally present. Except for bromination of the oxidized tryptophan, these results parallel those obtained with peptide 89–169.

The formation of four common dioxindole derivatives from the model peptides with different affinities for Sephadex G-10 follows from the nature of oxidative bromination reactions (Lawson et al., 1960; Patchornik et al., 1960) and the known selectivity of binding of aromatic compounds to tightly cross-linked dextran (Eaker and Porath, 1967; le Cam, 1973) or polyacrylamide (Thornhill, 1972; Welinder, 1972) gels. Many studies have shown that aromatic compounds have binding affinities that appear to be related to their electron polarizabilities. These in turn can be assessed from the energies and oscillator strengths of electronic transitions. Thus, in an homologous series of compounds, such as bromotyrosine (Welinder, 1972), the affinity of binding increases with increasing ϵ and λ_{max} in the order tyrosine < 3-bromotyrosine < 3,5-dibromotyrosine. The same relationship appears to hold for the oxidized tryptophans (oxindole < dioxindole < dioxindole lactone < 5-bromodioxindole < 5-bromodioxindole lactone) and can be conveniently utilized for their separation and analysis. It is noteworthy that the differences in binding affinities of the dioxindole and dioxindole lactone derivatives are apparently sufficient to permit their separation even when they are present in a 27-residue peptide chromatographed on the relatively loosely cross-linked Sephadex G-50 gel.

It is obvious that considerable microheterogeneity can be introduced into peptides as a result of BNPS-skatole cleavage reactions. This could greatly interfere with their isolation, purification, and identification, particularly if the peptides involved are of similar size so that separation by gel filtration is not feasible. Microheterogeneity resulting from a mixture of dioxindole and dioxindole lactone derivatives of the peptide could probably be eliminated by incubation and gel filtration of the peptides at alkaline pH. Partial monobromination of tyrosyl residues, if allowed to occur, would create some phenolic hydroxyl groups titrating in the region of pH 8 and would potentially complicate ion-exchange chromatography if it were carried out at alkaline pH. An even greater potential problem is deamidation, for the resulting microheterogeneity could affect ion-exchange chromatography of the peptide over virtually the entire operational pH range. In a protein such as myelin basic protein, where 10 of 21 acidic residues are amidated (Eylar et al., 1971), the problem could be particularly acute. The potential for inadvertent creation of new antigenic sites must also be kept in mind when BNPS-skatole is used to prepare peptides for immunological studies (Bergstrand and Källén, 1972; Martenson et al., 1975a,c).

Supplementary Material Available

Supplementary material includes preparation of lysylbromotyrosine standards, spectrophotometric determination of 3-bromotyrosine in the presence of tyrosine, identification of *O*-dansylbromotyrosines, and absorption spectra of oxidized tryptophan-containing model peptides. (12 pages). Ordering information is given on any current masthead page.

References

Barakat, M. Z., El-Wahab, M. F. A., and El-Sadr, M. M. (1955), *J. Am. Chem. Soc. 77,* 1670–1672.

Bergstrand, H. (1971), *Eur. J. Biochem. 21,* 116–124.

Bergstrand, H. (1972), *Eur. J. Biochem. 27,* 126–135.

Bergstrand, H., and Källén, B. (1972), *Cell. Immunol. 3,* 660–671.

Burnett, P. R., and Eylar, E. H. (1971), *J. Biol. Chem. 246,*

3425-3430.
Eaker, D., and Porath, J. (1967), *Sep. Sci. 2,* 507-550.
Easley, C. W. (1965), *Biochim. Biophys. Acta 107,* 386-388.
Eylar, E. H., Brostoff, S., Hashim, G., Caccam, J., and Burnett, P. (1971), *J. Biol. Chem. 246,* 5770-5784.
Fontana, A. (1972), *Methods Enzymol. 25 B,* 419-423.
Fontana, A., Vita, C., and Toniolo, C. (1973), *FEBS Lett. 32,* 139-142.
Hinman, R. L., and Bauman, C. P. (1964), *J. Org. Chem. 29,* 2431-2437.
Lawson, W. B., Patchornik, A., and Witkop, B. (1960), *J. Am. Chem. Soc. 82,* 5918-5923.
le Cam, A. (1973), *J. Chromatogr. 77,* 450-454.
London, Y., Demel, R. A., Geurts van Kessel, W. S. M., Vossenberg, F. G. A., and van Deenen, L. L. M. (1973), *Biochim. Biophys Acta 331,* 520-530.
Martenson, R. E., and Deibler, G. E. (1975), *J. Neurochem. 24,* 79-88.
Martenson, R. E., Deibler, G. E., Kramer, A. J., and Levine, S. (1975a), *J. Neurochem. 24,* 173-182.
Martenson, R. E., Deibler, G. E., Kramer, A. J., and Levine, S. (1976a), *Trans. Am. Soc. Neurochem. 7,* 182.
Martenson, R. E., Kramer, A. J., and Deibler, G. E. (1975b), *Biochemistry 14,* 1067-1073.
Martenson, R. E., Kramer, A. J., and Deibler, G. E. (1976b), *J. Neurochem. 26,* 733-736.
Martenson, R. E., Levine, S., and Sowinski, R. (1975c), *J. Immunol. 114,* 592-596.
Mateu, L., Luzzati, V., London, Y., Gould, R. M., Vossenberg, F. G. A., and Olive, J. (1973), *J. Mol. Biol. 75,* 697-709.
Omenn, G. S., Fontana, A., and Anfinsen, C. B. (1970), *J. Biol. Chem. 245,* 1895-1902.
Patchornik, A., Lawson, W. B., Gross, E., and Witkop, B. (1960), *J. Am. Chem. Soc. 82,* 5923-5927.
Thornhill, D. P. (1972), *Biochim. Biophys. Acta 279,* 1-7.
Welinder, B. S. (1972), *Biochim, Biophys. Acta 279,* 491-497.
Whitaker, J. N., Chou, C.-H. J., Chou, F. C.-H., and Kibler, R. F. (1975), *J. Biol. Chem. 250,* 9106-9111.

Magnetic Resonance Studies of the Binding of ^{13}C-Labeled Carbon Monoxide to Myoglobins and Hemoglobins Containing Modified Hemes†

Richard B. Moon, Kilian Dill, and John H. Richards*

ABSTRACT: The effects of changes in the groups attached to the periphery of the porphyrin ring of the heme of various hemoglobins and myoglobins on the environment experienced by the ligand, carbon monoxide, have been studied by observation of the chemical shift of the bound ^{13}CO. The results indicate that the major interaction between bound ligand and substituents around the porphyrin is that transmitted electronically from substituent to ligand. The nature of the protein environment around the ligand and the interaction between the proximal histidine (F8) and the ligand (through the iron atom) impose differences between subunits of hemoglobin and between myoglobins and hemoglobins which are largely, but not entirely, independent of these substituent effects. To assess the influence of protein structure on the chemical shifts of bound ligand, the shifts of ^{13}CO bound to myoglobin and hemoglobins from a wide range of species have also been measured.

Considerable work has been devoted to gaining an understanding of the various molecular interactions between the heme and the protein which modulate the kinetic and thermodynamic aspects of heme-ligand binding in hemoglobins and myoglobins. An unquestionably crucial role in the ligand affinity of hemoglobin arises from the conformational changes which occur on ligation of this tetrasubunited protein and which account in large measure for the positive allosteric cooperativity shown for hemoglobin ligation by such ligands as oxygen and carbon monoxide. Thus steric constraints operate to exclude ligands from the heme pocket when the protein is in one conformation; in the other conformation there is essentially unobstructed approach to the heme available to ligand (Perutz, 1970). Moreover, the ease of moving the iron atom into the plane of the porphyrin ring (for ligand binding) from a position out of that plane on the proximal face of the porphyrin probably also contributes importantly; the ease of this movement depends on steric factors which are ultimately transferred to the iron through the proximal histidine (F8) (Perutz, 1970).

To what degree do the electronic characteristics of the hemes influence the process of ligation and the nature of the bound ligand? Considerable work has been devoted to this question by studies of reconstituted myoglobins and hemoglobins containing hemes modified at the 2 and 4 positions of the porphyrin ring from the vinyl groups normally present (protoheme). In principle these studies can be of three general types: (i) observations of the effects of heme modification on the kinetic or thermodynamic aspects of ligation, including the effect of heme modification on the allosteric behavior of hemoglobins; (ii) observations on the nature of the modified heme within the globin protein; and (iii) the effect of heme modification on the

† Contribution No. 5315 from the California Institute of Technology, Division of Chemistry and Chemical Engineering, the Chemical Laboratories, Pasadena, California 91109. *Received April 19, 1976.* Supported by grants from the United States Public Health Service (NIHL 15196 and NIHL 15162).