Immunochemistry of Sperm Whale Myoglobin. II. Modification of the Two Tryptophan Residues and Their Role in the Conformation and Antigen–Antibody Reaction*

M. Z. Atassi† and D. R. Caruso

ABSTRACT: Apomyoglobin (ApoMb) was treated with 2-hydroxy-5-nitrobenzyl bromide under two different conditions. A derivative modified at tryptophan-7 and another modified at tryptophans-7 and -14 were obtained and characterized chemically and physicochemically. Modification at tryptophan-7 did not alter the electrophoretic behavior of the protein and only slightly altered the spectral behavior. Also, upon modification of this tryptophan residue, the Stoke's radius and the molar frictional coefficient of the molecule remained unchanged. Modification of both tryptophans resulted in drastic changes in all these physical properties and especially, there was an almost twofold increase in the values of the Stoke's radius and the molar frictional coefficient. The antigenic reactivity decreased drastically upon blockage of the two tryptophans, as a result, most likely, of definite unfolding (or marked deviation from the spherical shape) of the molecule. ApoMb modified at tryptophan-7 and HNB-Mb was identical immunochemically with apomyoglobin pretreated with 8 M urea–5% methanol at pH 3.0 (U-ApoMb) and with metmyoglobin prepared by complex formation between ferriheme and U-ApoMb, respectively. It was therefore concluded that tryptophan-7 is not an essential part of an antigenic site in metmyoglobin.

The chemical modification approach for probing the nature of antigenic sites in proteins is extremely valuable, especially if the specificity of the reagent is well established and the derivative is purified and characterized and any resultant conformational alterations determined. When conclusions are based solely on the activity of certain peptide fragments, the approach is often criticized on the basis that the isolated peptide will not exist appreciably with (and might never be able to assume) the configuration it had in the parent protein. The critcism, valid as it is, will not render the technique useless, especially where positive results of activity with peptides are obtained. However, if studying the activity of peptide fragments is coupled with the chemical modification approach, a more comprehensive picture concerning the antigenic structure of a protein will be obtained. In a recent report from this laboratory (Atassi, 1967a), it was demonstrated that the two methionine residues at positions 55 and 131 were not essential parts of the antigenic sites of Mb.[1] This was based on the preparation of a metmyoglobin derivative, specifically modified at the methionine sites. We wish now to report on the role of the two tryptophan residues in Mb.

It has been shown (Koshland *et al.*, 1964) that 2-hydroxy-5-nitrobenzyl bromide reacts specifically with tryptophan residues in proteins, especially when the latter do not contain any free SH groups (as is the case in Mb). This paper therefore describes the preparation and chemical and physiochemical characterization of two myoglobin derivatives modified specifically at one and both tryptophan residues respectively. The immunochemical reactivity of each derivative is also reported.

Materials and Methods

Myoglobin and Apomyoglobin. Myoglobin used in these studies was the major component, 10 (MbX), obtained by CM-cellulose chromatography (Atassi, 1964). The present work was done on the met form. The apoprotein was prepared by a method similar to that described by Theorell and Åkenson (1955).

Reaction of Apomyoglobin with 2-Hydroxy-5-nitro-

* From the Departments of Biochemistry and Oral Biology, State University of New York, Buffalo, New York 14214. *Received August 10, 1967.* Part of this work was presented on Oct 19, 1966, in a lecture on the "Chemistry of Myoglobin" at the American University of Beirut, Beirut, Lebanon. The work was done during the tenure (to M. Z. A.) of an Established Investigatorship of the American Heart Association, the Investigatorship being supported in part by the Heart Association of Erie County, N. Y. This investigation was supported by Grant (AM-08804) from the National Institutes of Health, and by the Office of Naval Research, Biochemistry Branch, Contract No. Nonr-4564(00).

† Established Investigator of the American Heart Association.

[1] Abbreviations used: Mb, metmyoglobin; ApoMb, apomyoglobin; MbX, the major component 10 obtained by CM-cellulose chromatography (Atassi, 1964); HNB, 2-hydroxy-5-nitrobenzyl bromide; HNB-Mb, metmyoglobin containing 1 mole of HNB; HNB_2-Mb, metmyoglobin containing 2 moles of HNB; U-ApoMb, apomyoglobin pretreated with 8 M urea–5% methanol at pH 3.0; U-Mb, metmyoglobin prepared by complex formation between ferriheme and U-ApoMb.

TABLE I: Amino Acid Compositions of Apomyoglobin and Its HNB Derivatives.[a]

Amino Acid	Amino Acid Composition (residues/mol)					
	ApoMb		HNB-ApoMb		HNB_2-ApoMb	
	Acid Hydrolyses	Alkaline Hydrolyses	Acid Hydrolyses	Alkaline Hydrolyses	Acid Hydrolyses	Alkaline Hydrolyses
Aps	7.83		8.16		7.99	
Thr	4.98		4.83		4.76	
Ser	5.75		5.79		5.82	
Glu	18.9		18.8		19.1	
Pro	3.86	3.95	4.03	4.21	4.07	3.88
Gly	11.0		11.1		11.08	
Ala	17.0		16.9		16.84	
Val	7.65		7.49		7.68	
Met	1.94	2.02	1.82	2.02	1.88	1.96
Ile	8.52		8.42		8.60	
Leu	18.0		17.9		17.8	
Tyr	2.86	3.01	2.79	3.09	2.87	2.79
Phe	6.1	5.98	5.83	5.90	5.97	6.02
Trp		1.93		1.06		0.08
Lys	18.8		19.0		18.8	
His	12.1		12.2		12.2	
Arg	3.99		4.08		4.07	

[a] The results represent the average of four acid hydrolyses (two at 22 and two at 72 hr) and two alkaline hydrolyses for each sample. Values for serine and threonine were obtained by extrapolation to zero hydrolysis time.

benzyl Bromide. Reaction of ApoMb with HNB was carried out under two different conditions. (a) Apomyoglobin (4 μmol) was dissolved in 10 ml of 8 M–urea–5% methanol solution at pH 3.0. To the protein solution, 50 μmol of solid HNB (Calbiochem) was added and the mixture was allowed to stir magnetically for 1.5 hr. The mixture was then centrifuged (0°, 0.5 hr, 5600 rpm) and the supernatant was filtered in two portions on a column (2.2 × 15 cm) of Sephadex G-25 in order to remove excess reagent. The column was preequilibrated and eluted with 8 M urea at pH 3.0. The tubes containing the protein fraction were combined, dialyzed extensively against distilled water, and then freeze dried. (b) In this experiment, ApoMb (4 μmol) was treated with 200 M excess of solid HNB, using the same solvent employed in a above. The reaction was allowed to continue for 4 hr at room temperature, after which the reaction mixture was treated as described under a. Since ApoMb might suffer some denaturation, under the urea–acid conditions, which might affect its antigenic reactivity, two control preparations were carried out in which ApoMb was subjected to the conditions mentioned under a or b except that in this case HNB was not added.

Reconstitution of Metmyoglobin. For reconstitution with ApoMb crystalline hemin chloride (Eastman Organic Chemicals) was dissolved in 0.1 M–Na_2HPO_4 and added drop by drop to cold (0°), magnetically stirred ApoMb solution in water. The ferriheme was in about 2 M excess (and one-fifth the volume) of the ApoMb solution and the operation was carried out away from direct light. When the addition of ferriheme to ApoMb was complete, stirring was continued at 0° for 2 more hr. The mixture was then dialyzed against three changes of ten times its volume of 0.1 M Na_2HPO_4. This step resulted in the removal of most of the excess heme. To remove the last traces of heme, the solution was dialyzed against 0.01 M NaH_2PO_4 containing 0.01% KCN (pH 6.2), centrifuged, and applied on columns (1.5 × 10 cm) of CM-cellulose preequilibrated with the same phosphate–KCN solvent at pH 6.2. The column was eluted with this solvent when heme came out completely unabsorbed. The cyanmetmyoglobin solution remained absorbed on the upper (1 cm) segment of the column and was only eluted when the latter was washed with 0.01 M phosphate buffer containing 0.01% KCN at pH 7.6. This combination procedure resulted in the precipitation of only a very small amount of the protein.

Analytical Methods. These were similar to those described in the previous communication (Atassi and Saplin, 1968). In addition, electrophoresis was on starch gel, at room temperature in the discontinuous buffer system of Poulik (1957); the buffers contained KCN (0.05%) and the gels were stained with Amido Black (Smithies, 1959). Continuous spectra were done in a Cary Model 14 spectrophotometer. Sedimentation experiments were carried out in a Spinco

Model E analytical centrifuge, using a red filter and infrared film in 0.01 M phosphate–0.01% KCN (pH 7.2).

Digestion with Chymotrypsin. For chymotryptic hydrolysis of ApoMb and its HNB derivatives, the protein (8–9 mg) was dissolved in 1.5 ml of water, adjusted to pH 8.8 with 0.1 N NaOH, followed by the addition of 10 μl of a 1% solution of chymotrypsin (three-times crystallized, Worthington Biochemical Corp.) in 0.001 N HCl. The pH was maintained at 8.8 by the addition of 0.1 N NaOH on the pH-Stat and hydrolysis was continued at room temperature until alkali consumption ceased (18–20 hr). The reaction was terminated by lowering the pH to 4.0 with 2 N acetic acid. The slight cloudiness obtained at this stage was removed by centrifugation. Chromatography and electrophoresis of the soluble chymotryptic peptides was by the procedure described in the preceding paper (Atassi and Saplin, 1968).

Antisera. These were prepared by the procedure previously described (Atassi, 1967a). Antisera from individual animals were kept separate and stored in 8–10-ml portions at −40°. Rabbit antisera 11 and 100 and goat antiserum G1 were used in the present studies.

Gel Filtration. Mb- or HNB-modified myoglobin (1–2 mg) was dissolved in 0.5 ml of cold (0°), 0.01 M phosphate buffer, containing 0.01% KCN at pH 7.01 and applied onto a column (2.2 × 51 cm) of Sephadex G-75. Elution was carried out with the same phosphate buffer at 0°. The column was calibrated with horse heart cytochrome C, ovalbumin, human serum albumin, and bovine ribonuclease. The effluent was monitored with a Canalco Model DA double-beam flow analyzer equipped with an automatic scale expansion and cuvets with 10-mm light path.

Results

Determination of the Modified Amino Acid Residues. Amino acid analyses of acid and alkaline hydrolysates of ApoMb and the two derivatives obtained from its reaction with HNB are shown in Table I. The results show that on reaction of ApoMb with 13 M excess of HNB for 1.5 hr at room temperature, the tryptophan content decreased by one residue. No other amino acids suffered modification. Results of the amino acid analysis agreed with calculations based on the new absorption peak at 320 mμ (Figure 1). The number of HNB groups incorporated was 0.784 mol/mol of ApoMb. In order to locate the modified tryptophan residue, the derivative was subjected to digestion with chymotrypsin. Digestion with trypsin will not be helpful in this case since the segment containing the two tryptophan residues of myoglobin is in the insoluble portion of the tryptic hydrolysate (Edmundson, 1963). The soluble chymotryptic peptides were subjected to peptide mapping. The yellow spot, which was also revealed by staining with a weak ninhydrin solution (0.05% in ethanol), was eluted with 10% pyridine, flash evaporated on a rotary evaporator, and subjected to hydrolysis and amino acid analysis. The amino

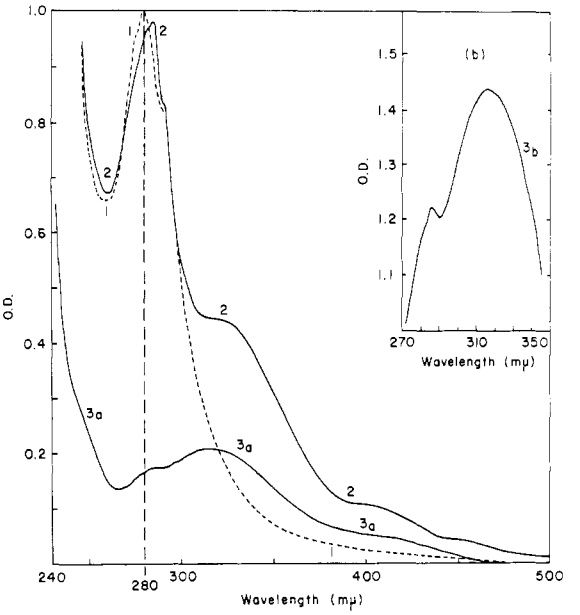

FIGURE 1: Absorption spectra of (1) ApoMb, 151.4 μg of N/ml; (2) ApoMb containing 1 mol of HNB/mol, 176.0 μg of N/ml; (3a) ApoMb containing 2 mol of HNB/mol, 22.6 μg of N/ml, and (3b) the same derivative as in 3a but the solution was more concentrated, 183.9 μg of N/ml. Solutions were in distilled water.

acid composition obtained at this stage suggested the spot comprised more than one chymotryptic peptide. To overcome this, the soluble chymotryptic peptides were subjected to chromatography (14 hr) and the yellow spot obtained was cut out, eluted, and dried. It was then redissolved and subjected to rechromatography (22 hr) followed by electrophoresis (75 min). Three spots were obtained which, after elution and hydrolysis, were analyzed; peptide 1: Asp, 0.91; Glu, 1.0; Gly, 1.89; Ala, 2.30; and Met, 0.72; peptide 2: (yellow in color) Ser, 0.88; Glu, 1.45; and Gly, 1.00; while composition of peptide 3 did not correspond to any of the chymotryptic peptides of Mb as indicated by the positions of chymotryptic attack reported by Edmundson (1965). Peptide 3 was therefore most likely still a mixture. The most likely locations for these peptides, based on their composition, the published sequence of Mb and the locations of chymotryptic attack (Edmundson, 1965) are peptide 1, sequence 124–131; and peptide 2, sequence 3–7. It can therefore be concluded that HNB was attached to tryptophan-7.

When ApoMb was treated with 200 M excess of HNB for 4 hr at room temperature, both tryptophan residues suffered modification (Table I). The contents of the other amino acids remained unchanged. Calculations from spectral data showed that 2.14 mol of HNB was incorporated/mol of Mb. The two tryptophan residues in Mb are located at positions 7 and 14 (Edmundson, 1965).

Properties of the Modified Derivatives. The absorption spectra of ApoMb and its two derivatives modified

TABLE II: Absorption Maxima and Ratios of Optical Densities at These Maxima of Metmyoglobin and Its Various Derivatives.[a]

	λ_{max} (mμ)				Ratio of Optical Densities at Absorption Maxima		
	A	B	C	D	A/D	B/D	C/D
Mb	279	360	424	542	3.21	2.85	10.05
U-Mb	270	360	423	542	4.15	2.85	10.15
HNB-Mb	270	360	423.5	542	4.04	3.33	9.51
HNB_2-Mb		360	408	540		5.82	6.55

[a] All solutions were in 0.01 M phosphate buffer (pH 7.2) containing KCN (0.01%).

at one and both tryptophans are shown in Figure 1. When 1 mol of HNB is incorporated into the protein, a new peak appears in the visible region of the spectrum in the form of a large shoulder between 309 and 325 mμ. On the other hand, the absorption in the ultraviolet region was still present but suffered a slight shift (from 278 mμ in ApoMb) to 284 mμ and, in addition, the extinction coefficient at the new absorption maximum decreased by 15.7% relative to ApoMb. When both tryptophan residues were blocked with HNB, the absorption maximum at 278 mμ disappeared completely and the derivative showed maxima at 287, 315, and 410 mμ (Figure 1).

Myoglobins were then formed by combination of ferriheme with these HNB apomyoglobin derivatives. The spectrum of HNB-Mb showed maxima at 270, 360, 423, and 542 mμ. There was a slight increase in the extinction at 270 mμ (relative to MbX) in U-Mb and HNB-Mb. In addition, the latter showed an increase in the extinction at 360 mμ, probably due to the contribution of HNB. On the other hand, the changes in the spectral behavior of HNB_2-Mb were appreciable (Figure 2). The peak at 278 mμ was completely absent. The peak at 360–370 mμ was a shoulder in the curve and showed a twofold increase in magnitude (relative to MbX). The Soret peak appeared at 408 mμ and in magnitude was only 0.60 that of Mb (at 423 mμ). Table II summarizes the absorption maxima and the ratios of the extinctions at these maxima for cyanmetmyoglobin and its various derivatives.

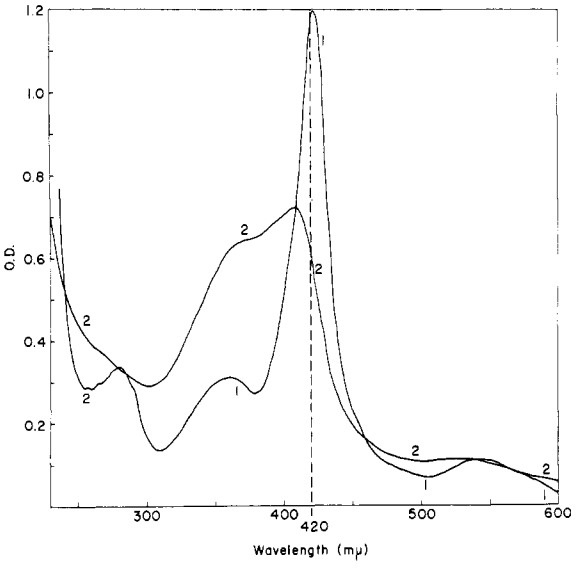

FIGURE 2: Spectra of U-Mb (1) and of HNB_2-Mb (2) in their cyanmet forms. Solutions were in 0.01 M phosphate buffer (pH 7.2) containing KCN (0.01%) and adjusted so that they had identical optical densities at 540 mμ.

On starch gel electrophoresis, HNB-Mb behaved in a similar manner to native MbX. HNB_2-Mb however showed four bands on electrophoresis all of which moved faster than MbX with mobilities (relative to MbX = 1) of 1.83, 2.68, 3.36, and 3.85 (trace). The slowest band was separated from the rest by column chromatography on CM-cellulose and the two groups had identical amino acid composition and quantitative immunochemical behavior. This splitting might be related to the existence of Mb in various molecular species with identical chemical and immunochemical properties but differing only in charge (Atassi, 1964, 1967b). It can be seen from the amino acid composition that the apparent increase in the negative charge of the protein is not due to modification of any basic amino acid residues.

Determination of Conformational Alterations. In the ultracentrifuge, Mb, HNB-Mb, and HNB_2-Mb sedimented at the same rate when the solutions had the same concentration. However, the $s^0_{20,w}$ values were not determined. Our spectral and electrophoretic data on HNB_2-Mb suggested, however, that certain changes might have taken place in the conformational organization of the molecule. There is evidence from results on succinylated bovine serum albumin (Habeeb, 1967) that $s^0_{20,w}$ may be insensitive for the determination of conformational changes. It has been well shown (Porath, 1963; Ackers, 1964; Laurent and Killander, 1964; Siegel and Monty, 1965, 1966) that gel filtration

TABLE III: Gel Filtration Data and Molecular Parameters of Standard Proteins, Myoglobin, and Modified Myoglobins.[a]

Protein	Mol Wt	Elution Vol (ml)	K_D	a (Å)	f/f_0
		Standard Proteins[b]			
Cytochrome C	12,400	137.6	0.5858	17	1.09
Ribonuclease	13,400	133.2	0.5492	16.5	1.04
Ovalbumin	46,000	90.0	0.1908	27.7	1.16
Human serum albumin	69,000	75.0	0.0664	37.0	1.36
		Mb and Derivatives[c]			
Mb	17,816	125.50	0.4854	18.5	1.06
U-Mb	17,816	125.40	0.4850	18.5	1.06
HNB-Mb	17,967	125.41	0.4850	18.5	1.06
HNB_2-Mb	18,118	78.43	0.0948	34.0	1.95

[a] Each gel filtration value represents the average of three determinations. Range was $< \pm 2\%$. [b] Values for cytochrome C were obtained from Margoliash and Lustgarten (1962). Values of f/f_0 for the other proteins were calculated from $s^0_{20,w}$ and M was obtained from Altman and Dittmer (1964). The values of a were then calculated from eq 1. [c] Molecular parameters were calculated from gel filtration data. For explanation, see text.

on calibrated Sephadex columns can be employed for the calculation of the Stoke's radii and the molar frictional coefficients (f/f_0) of proteins. Habeeb (1966) used this technique successfully to monitor conformational changes associated with various modifications of bovine serum albumin.

In gel filtration on a Sephadex column, the distribution coefficient K_D is given by $K_D = (V_e - V_0)/V_i$, where V_e is the effluent volume at the apex of a protein peak, V_0 is the void volume of the column, and V_i is the volume of unbound solvent within the gel phase (this can be computed from (total volume of column $- V_0$) $\times$ 0.95; Rogers *et al.*, 1965). Equation 9 and Table III of Ackers (1964) were used to obtain a value for a/r from the experimental K_D, where a is the Stoke's radius and r is the effective pore radius within the gel. The known values of a for the standard proteins (Table III) were used for the calculation of r. The deduced value of r was then used to calculate the Stoke's radii of the modified myoglobins. Alternatively, a could be obtained from the plot of a *vs.* $(K_D)^{1/3}$ for a set of standard proteins (Figure 3) and is known to be linear (Siegel and Monty, 1966). Finally, f/f_0 was calculated from eq 1. $\bar{V}$ is the partial

$$f/f_0 = a \Big/ \left(\frac{3\bar{V}M}{4\pi N}\right)^{1/3} \qquad (1)$$

specific volume (0.7398 ml/g for cyan-Mb; Atassi, 1964), M is the molecular weight, and N is Avogadro's number (6.02×10^{23}). Table III gives the elution volumes and molecular parameters for Mb, U-Mb (used as a control to investigate any permanent effect of urea on the shape of the molecule), and the two HNB derivatives. Each of these protein species eluted as a single, symmetrical peak. The results show that the Stoke's radius and f/f_0 for HNB-Mb and U-Mb had identical values with those of the unmodified protein. On the other hand, in HNB_2-Mb there was an almost twofold augmentation in these molecular parameters caused by an increase in the swelling or asymmetry of the molecule. It is noteworthy, that treatment of apomyoglobin with urea does not induce (after removal of urea) a permanent asymmetry in the Mb molecule formed from it. Sephadex G-75 was employed here since the equilibrium molecular exclusion effect was shown (Ackers, 1964) to be more sensitive to molecular size in the G-75 and G-100 grades than in the G-200.

Antigenic Reactivity of the Modified Derivatives.

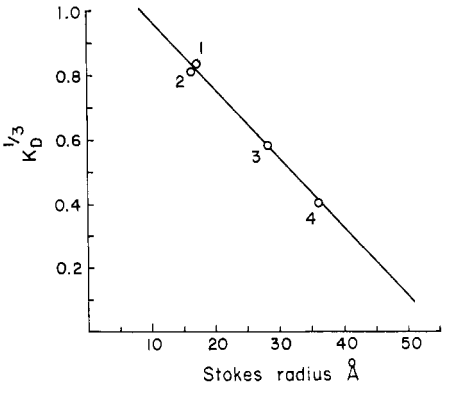

FIGURE 3: Correlation of K_D with Stoke's radius for four standard proteins according to the correlation of Porath (1963). The proteins were cytochrome *c* (1), bovine ribonuclease A (2), hen ovalbumin (3), and human serum albumin (4). The calculated Stoke's radii for these proteins shown are in Table III.

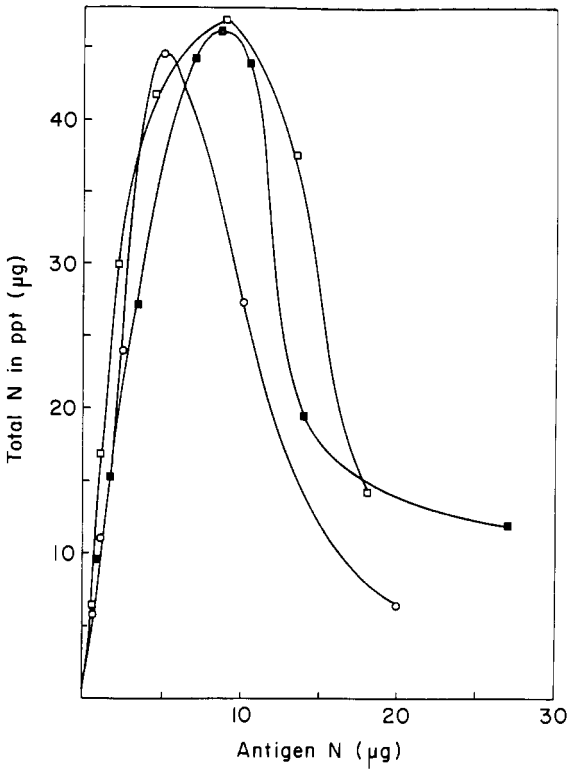

FIGURE 4: Precipitin analyses with rabbit antiserum 100 and ApoMb (0), pH 3.0 urea-pretreated ApoMb (□) and ApoMb modified with HNB at tryptophan-7 (■). For reaction of these proteins with other sera, see Table IV.

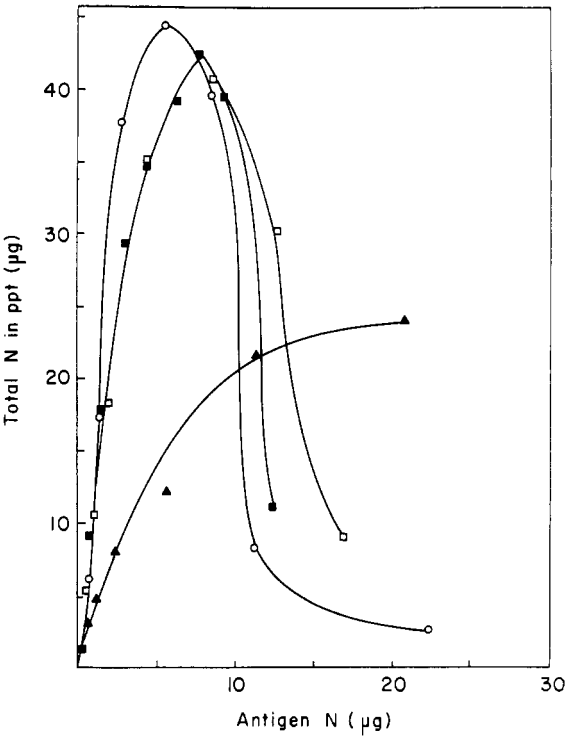

FIGURE 5: Precipitin analyses with rabbit antiserum 100 and HNB-modified myoglobins. ○, Mb obtained by recombination of ApoMb with ferriheme; □, U-Mb; ■ HNB-Mb; ▲, HNB_2-Mb. For reaction of HNB-Mb with other sera, see Table IV.

The Mb derivative which was modified at tryptophan-7 gave, in agar double-diffusion experiments, a single precipitin line with rabbit and goat antisera to MbX. No spurs or intersections were formed with the line due to MbX or that of U-Mb. Figure 4 shows the results of precipitin reactions of ApoMb, U-ApoMb, and the apoprotein modified at tryptophan-7 with rabbit antiserum 100. The interactions of the corresponding myoglobins (*i.e.*, recombined Mb, U-Mb, and HNB-Mb) are shown in Figure 5. Table IV summarizes the results of all the precipitin reaction experiments with antisera 11, 100, and G1. It can be seen that U-Mb and HNB-Mb were immunochemically identical, but precipitated maximum antibody nitrogen at an antigen concentration slightly higher than that required by the homologous antigen. It should be pointed out here that MbX and Mb obtained from recombination of ApoMb with ferriheme have already been shown to be immunochemically and physicochemically identical (Atassi, 1967a,b).

Myoglobin modified at the two tryptophans gave, in agar double diffusion, one precipitin line which was weaker than the line of U-Mb or MbX, but no spurs or intersections were formed. The precipitin reaction of HNB_2-Mb with antiserum 100 is shown in Figure 5. HNB_2-Mb reacted poorly with all the antisera to MbX precipitating between 55 and 61% of antibody nitrogen relative to U-Mb. Immunochemical studies could not be performed on apomyoglobin modified at both tryptophans since this derivative was almost completely insoluble in 0.15 M NaCl.

Discussion

The preparation of two apoprotein derivatives modified at one and both tryptophan residues indicates that these residues differ in their reactivity toward HNB. The difference in reactivities is obtained under conditions where the protein is expected to be completely unfolded. A similar observation was reported for α-chymotrypsin where, of the five reactive tryptophan residues, one reacted more easily with HNB than the other four (Koshland *et al.*, 1964). In a previous communication from this laboratory (Atassi, 1967c), it was shown that tryptophan-7 undergoes oxidation more easily than tryptophan-14 when ApoMb is subjected to periodate oxidation. The reactivity of HNB with ferriheme has never been investigated. Therefore, it was decided, in order to avoid the possibility of such a reaction, to react ApoMb with HNB first then subject the derivatives to reconstitution with unmodified ferriheme. This precaution will overcome any complications which might arise from

TABLE IV: Relative Amounts of Precipitation Formed by Apomyoglobin and Metmyoglobin Derivatives Modified at One Tryptophan.[a]

	Modified ApoMb			HNB-Mb		
	% Ppt Rel to Reaction of Antiserum with U-ApoMb[b]			% Ppt Rel to Reaction of Antiserum with U-Mb[b]	
Antiserum	Expt	Av	Range	Expt	Av	Range
11	2	98.6	97.4–99.8			
100	6	95.6	93.7–98.9	5	104.5	100.4–106.3
G1	3	101.4	100– 103.2	2	98.8	97.6– 99.9

[a] The precipitation reaction was carried out with two rabbit antisera and a goat antiserum against Mb X. The percentage of precipitation relative to U-Mb was based on the total nitrogen values in the precipitates at the point of maximum precipitation. [b] It is considered that U-ApoMb and U-Mb (rather than ApoMb or Mb X) constitute the appropriate controls here since the effect of urea, low-pH treatment on the antigenic reactivity (see Figures 4 and 5) must be taken into account.

changes in antigenic reactivities upon modification of the heme group (Atassi, 1967b).

Since HNB-Mb and U-Mb were immunochemically identical, this suggested that tryptophan-7 is not an essential part of an antigenic site in Mb. It is noteworthy that the reactivity of these two preparations was slightly different from that of native Mb. It is significant that no major conformational alterations were observed, since the Stoke's radii and molar frictional ratios were unchanged. This does not exclude the possibility that immunochemical methods can reveal small changes in folding, which are undetectable by gel filtration. The sensitivity of the antigenic reactivity of Mb to conformational alterations has already been reported (Atassi, 1967b). Our results suggest therefore that urea, low-pH treatment leads to some slight irreversible changes detectable only by immunochemical methods. The present conclusion that tryptophan-7 is not located in a reactive region agrees with the finding in the preceding paper (Atassi and Saplin, 1968) and those of Crumpton and Wilkinson (1965) where it was shown that peptide 1–7 did not inhibit the interaction of Mb with its antibodies. Also, it was shown by Atassi and Saplin (1968) that peptide 8–153, obtained by cleavage with periodate, was antigenically as efficient as periodate oxidized (but not cleaved at position 7) ApoMb.

Modification at the two tryptophan residues provided a Mb derivative which was antigenically much less reactive than Mb. The modification however was associated with a great degree of swelling or unfolding. It is not possible at this stage to determine whether the decrease in reactivity is caused by the conformational change or indeed happens as a direct result of modifying the two tryptophans. It should be pointed out here that although blockage of tryptophan-7 results in no detectable conformational changes and that blockage of the two tryptophans leads to a great degree of unfolding, this does not necessarily imply that the native three-dimensional structure of the protein in solution is sensitive only to the modification of tryptophan-14. The possibility of a cooperative effect upon blockage of the two tryptophans must be considered.

References

Ackers, G. K. (1964), *Biochemistry 3*, 723.

Altman, P. L., and Dittmer, D. S. (1964), Biology Data Handbook, Federation of the American Society of Experimental Biology, Washington, D. C., p 388.

Atassi, M. Z. (1964), *Nature 202*, 496.

Atassi, M. Z. (1967a), *Biochem. J. 102*, 478.

Atassi, M. Z. (1967b), *Biochem. J. 103*, 29.

Atassi, M. Z. (1967c), *Arch. Biochem. Biophys. 120*, 56.

Atassi, M. Z., and Saplin, B. J. (1968), *Biochemistry 7*, 688 (this issue; preceding paper).

Crumpton, M. J., and Wilkinson, J. M. (1965), *Biochem. J. 94*, 545.

Edmundson, A. B. (1963), *Nature 198*, 354.

Edmundson, A. B. (1965), *Nature 205*, 883.

Habeeb, A. F. S. A. (1966), *Biochim. Biophys. Acta 121*, 21.

Habeeb, A. F. S. A. (1967), *Arch. Biochem. Biophys. 121*, 652.

Koshland, D. E., Karkhanis, Y. D., and Latham, H. G. (1964), *J. Amer. Chem. Soc. 86*, 1448.

Laurent, T. C., and Killander, S. (1964), *J. Chromatog. 14*, 317.

Margoliash, E., and Lustgarten, J. (1962), *J. Biol. Chem. 237*, 337.

Porath, J. (1963), *Pure Appl. Chem. 6*, 233.

Poulik, M. D. (1957), *Nature 180*, 1477.

Rogers, K. S., Hellerman, L., and Thompson, T. E. (1965), *J. Biol. Chem. 240*, 198.

Siegel, L. M., and Monty, K. J. (1965), *Biochem. Biophys. Res. Commun. 19*, 494.

Siegel, L. M., and Monty, K. J. (1966), *Biochim. Biophys.*

Acta 112, 346.
Smithies, O. (1959), *Advan. Protein Chem. 14*, 141.
Theorell, H., and Åkeson, Å. (1955), *Ann. Acad. Sci. Fennicae 60*, 303.

Immunochemical Studies on the Poly-γ-D-glutamyl Capsule of *Bacillus anthracis*. III. The Activity with Rabbit Antisera of Peptides Derived from the Homologous Polypeptide*

Joel W. Goodman, Danute E. Nitecki, and Inge M. Stoltenberg

ABSTRACT: The poly-γ-D-glutamic acid capsular polypeptide from a strain of *Bacillus anthracis* was partially hydrolyzed with 3 N HCl at room temperature for 6 days. Peptides from the tripeptide to the hexapeptide were resolved by high-voltage electrophoresis of the hydrolysate on sheets of DEAE-cellulose paper at pH 3.5. The peptides were recovered by elution from the papers and characterized for molecular size and optical activity. The peptides were assayed for reactivity with three rabbit antisera specific for the parent polypeptide, prepared by immunization with either intact bacilli or purified polypeptide–methylated albumin complexes, by inhibition of the quantitative precipitin reaction. With two of the sera, increments in inhibiting efficiency were obtained up to the pentamer, which was equal in inhibiting capacity to the hexamer. The third antiserum could distinguish between the pentapeptide and the hexapeptide. These results indicate a maximum size for the region on the polypeptide which combines with the antibody site of six residues of D-glutamic acid, and also demonstrate heterogeneity of the combining sites of the antibodies in the sera tested. However, an unresolved mixture of larger peptides, with an average polymer size of nine amino acids, gave almost fourfold better inhibition than the hexapeptide on a molar basis with all three sera.

The capsular polypeptide of *Bacillus anthracis*, a polymer composed exclusively of residues of D-glutamic acid (Hanby and Rydon, 1946; Goodman and Nitecki, 1966) linked by γ-peptide bonds (Bruckner and Kovács, 1957), and its homologous antibody comprise an ideal system for investigating the extent of the region on a protein antigen which combines with the antibody molecule. In the initial stages of this study, the eight possible dipeptides and four branched tripeptides of glutamic acid were synthesized (Nitecki and Goodman, 1966) and used to inhibit the precipitin reaction of rabbit antiserum and the homologous anthrax polypeptide (Goodman and Nitecki, 1966). The best inhibitor of these proved to be a branched tripeptide consisting of a residue of L-glutamic acid substituted at both carboxyl groups with residues of D-glutamic acid.

In the present communication, the study has been extended using a series of peptides of increasing chain length up to the hexapeptide, obtained from a partial acid hydrolysate of the native capsular polypeptide of *B. anthracis*, to delineate the extent of the combining region on the polypeptide with three different rabbit antisera.

Materials and Methods

Polypeptide and Antisera. The preparation of purified polypeptide from strain M36 of *B. anthracis* and the pool of rabbit antisera prepared by immunization with heated suspensions of the organism have been previously described (Goodman and Nitecki, 1966). Rabbit antisera from individual animals prepared by immunization with purified polypeptide–methylated bovine serum albumin electrostatic complexes have also been detailed (Goodman and Nitecki, 1967). These are designated antisera 7 and 10. The former had a precipitating antipolypeptide antibody content of 400 μg of protein/ml, while the content of the latter was about 165 μg of protein/ml.

Degradation of Peptides of Glutamic Acid by Serum Enzymes. It has been shown that serum enzymes degrade peptides of L-amino acids and influence their apparent interaction with antibody (Schechter *et al.*, 1966). Accordingly, the eight synthetic dipeptides of glutamic acid (Nitecki and Goodman, 1966) were assayed for susceptibility to proteolysis by serum. A 1% solution of each peptide was mixed with an

* From the Department of Microbiology, University of California School of Medicine, San Francisco, California 94122. *Received October 18, 1967.* Supported by grants from the National Institutes of Health (AI 05664, ITI AI 299, and AM-08527) and the Committee on Research of the University of California.