Immunochemistry of Sperm Whale Myoglobin. I. The Specific Interaction of Some Tryptic Peptides and of Peptides Containing All the Reactive Regions of the Antigen*

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ABSTRACT: Sperm whale apomyoglobin was digested with trypsin and the soluble peptides were fractionated by chromatography. Five peptides, which occupied mostly corners in the three-dimensional structure of Kendrew *et al.* (Kendrew, J. C., Watson, H. C., Strandberg, B. E., Dickerson, R. E., Phillips, D. C., and Shore, V. C. (1961), *Nature 190*, 666) were purified and their immunochemical reactivities were studied. These peptides occupied in the parent protein the corners A–B (*i.e.*, the bend between helices A and B in the three-dimensional model), E–F, F–G, and G–H and also most of helix B, all of helices F and C and the C-terminal hexapeptide. None of the peptides precipitated antibody and only inhibitory activities were detected. The greatest inhibitory activity resided in the segment A–B. This was followed, in degree of reactivity by segment E–F–G (*i.e.*, corner between helices E and F, all of helix F and corner between helices F and G).

The C-terminal hexapeptide was also reactive as was the segment comprising the bend G–H. The peptide corresponding to helix C showed no or very little inhibitory activity. It was therefore concluded that the foregoing corners on the surface of the molecule are present in antigenically active regions. Apomyoglobin was also subjected to cleavage at tryptophan-7 and/or at the two methionine sites. The shortened apopoprotein was immunochemically intact (relative to oxidized, uncleaved control). This, in addition to the finding that fragment 1–7 was inactive, suggested that the latter fragment is not part of an antigenic site in the intact molecule. There are indications, however, that sequence 1–7 might be important for the proper orientation of the reactive region(s) on fragment 1–55. Fragments 1–55 and 56–131 gave immune precipitates with antibodies to the whole protein. Fragment 132–153 showed only inhibitory activity with all the five sera tested. With a given antiserum, a greater portion of the reactivity appeared in the core fragment (*i.e.*, 56–131). However, the three fragments obtained by cleavage at the methionines accounted for almost all the immunochemical reactivity of the intact antigen. The significance of these findings is discussed.

Specific chemical modification procedures are employed for investigating the correlation of protein structure with its biological function. An additional advantage presents itself in the study of antigens in that if a reactive region is removed from the intact molecule, by chemical, enzymic, or physical cleavage procedures, it might still interact with antibodies to the whole molecule. Landsteiner (1942) gave the first such demonstration in which he showed that peptides obtained from silk fibroin inhibited the interaction of the latter with its antibody. Fragments from several proteins (including sperm whale myoglobin; Crumpton and Wilkinson, 1965) have since been shown to interact with the antibody to the intact parent protein.

A recent report from this laboratory (Atassi, 1967b) showed that the two methionine residues at positions 55 and 131 were not essential parts of the antigenic sites of Mb.[1] Similarly, tryptophan at position 7 is not an essential part of a reactive site (Atassi and Caruso, 1968). It was therefore concluded that cleavage of the protein at the two methionine residues and tryptophan-7 may not physically rupture a reactive *region*[2] of the molecule as might happen when the enzymic procedure is employed. Cleavage at methionine can be accomplished with cyanogen bromide (Gross and Witkop, 1962) and the procedure was useful in the sequence

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[1] Abbreviations used: ApoMb, apomyoglobin; Mb, metmyoglobin; Mb X, major chromatographic component 10 obtained by CM-cellulose chromatography (Atassi, 1964).

[2] In the present terminology, a reactive *region* is distinct from a reactive *site* and might represent any portion of (or a whole) reactive site. A *site* could incorporate various adjacent folds (reactive *regions*) which are close in the three-dimensional structure but might be distant in sequence.

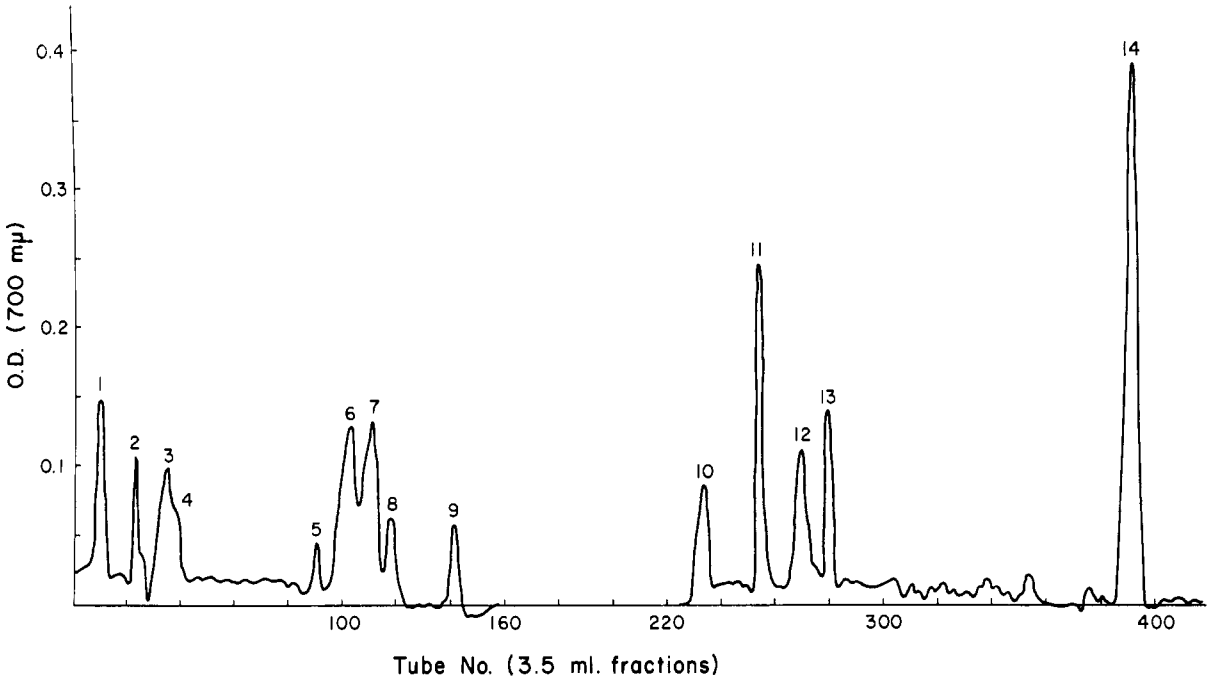

FIGURE 1: Chromatogram of the soluble tryptic peptide of ApoMb on Dowex 1-X2. The soluble tryptic digest was applied onto the column which had been preequilbrated with pyridine–collidine–AcOH buffer (pH 8.2). The column was developed at 35° by the polygradient elution system (Table I of Funatsu, 1964) at the rate of 40 ml/hr. 0.1-ml portions of each fraction were analyzed by the Folin–Lowry method (Lowry *et al.*, 1951). The correspondence of some of these peptides with the amino acid sequence of the protein was determined and was peptide 1 = sequence 99–102; peptide 6 = sequence 80–96; peptide 7 = sequence 79–96; peptide 9 = sequence 141–145; peptide 10 = sequence 35–42; peptide 11 = sequence 119–133; peptide 12 = sequence 17–31; peptide 13 = sequence 51–56; and peptide 14 = sequence 148–153.

studies on Mb (Edmundson, 1963). Atassi (1967a) has shown that cleavage of tryptophyl peptide bonds can be effected with periodate. The present paper describes the immunochemical reactivities of peptides obtained by cleavage of ApoMb at these amino acids. In addition, the interaction of five peptides, isolated from tryptic digests of sperm whale apomyoglobin, is reported.

Materials and Methods

Preparation of Metmyoglobin and Apomyoglobin. Sperm whale Mb used in these studies was the major chromatographic component 10 (Mb X), obtained by CM-cellulose chromatography (Atassi, 1964). The apoprotein was prepared from Mb X by the procedure of Theorell and Åkeson (1955).

Preparation of Tryptic Peptides. Apomyoglobin (200 mg) was dissolved in water (20 ml), the temperature was brought to 40°, and the pH was adjusted to 8.0 with 0.2 N NaOH. Hydrolysis was started by the addition of 0.5% trypsin in 0.001 N HCl (200 μl) and the pH was maintained at 8.0 by the addition of 0.1 N NaOH on the pH-Stat. Three more aliquots of trypsin solution (200 μl each) were added over a period of 1 hr and digestion was allowed to continue until alkali consumption ceased. The total digestion time was 2 hr. The pH of the digestion mixture was then adjusted to 7.0 with 0.1 N HCl and the precipitate obtained was removed by centrifugation. The peptides in the supernatant from the tryptic digest were fractionated by chromatography on columns (1.0 × 153 cm) of Dowex 1-X2 by a procedure similar to that described by Funatsu (1964). The peptides were purified by rechromatography on the same column.

Cleavage at the Methionine and Tryptophan Sites and Preparation of the Peptides. Reaction with cyanogen bromide was carried out on the apoprotein and the resulting fragments were separated by gel filtration on appropriate grades of Sephadex (Edmundson, 1963). Preparation and characterization of the peptides obtained by cleavage at the tryptophan sites have already been described (Atassi, 1967a). The purity of the peptides was confirmed by peptide mapping.

Antisera. Rabbit and goat antisera were prepared against MbX and against ApoMb. The preparation of antisera of MbX has been described in detail by Atassi (1967b). Antisera to ApoMb were prepared by a similar procedure. Only those sera which gave a single line by agar double diffusion were used. Antisera from individual animals were kept separate and stored in 8–10-ml portions at −40°. Rabbit antisera

TABLE I: Amino Acid Compositions of ApoMb-Tryptic Peptides and Their Calculated Molecular Weights.[a]

Amino Acid	Peptide 7	Expected for Sequence 79–96	Peptide 12	Expected for Sequence 17–31	Peptide 11	Expected for Sequence 119–133	Peptide 10	Expected for Sequence 35–42	Peptide 14	Expected for Sequence 148–153
					Peptide					
Asp	0.010		1.88	2	2.83	3			0.075	
Thr	0.899	1					0.993	1		
Ser	1.02	1					0.816	1	0.024	
Glu	2.85	3	1.92	2	0.940	1	1.93	2	2.18	2
Pro	0.937	1			1.02	1	0.972	1		
Gly	1.08	1	1.98	2	3.00	3	0.035		2.02	2
Ala	2.80	3	1.75	2	3.11	3	0.034		0.038	
Val			1.84	2						
Met					1.04	1				
Ile			1.65	2						
Leu	1.83	2	1.00	1			1.03	1	0.957	1
Tyr	0.03								1.05	1
Phe	0.05				1.13	1				
Lys	2.96	3	1.00	1	0.982	1	0.948	1		
His	2.60	3			0.860	1	1.06	1		
Arg			0.96	1						
Total number of residues	18		15		15		8		6	
Molecular weight	1982		1594		1515		940		666	

[a] Values are expressed in moles of amino acid per mole of peptide.

TABLE II: Amino Acid Sequence and Location of Tryptic Peptides in the Primary Structure of Mb as Reported by Edmundson (1965).[a]

Peptide	Position	Sequence
7	79–96	Lys-Gly-His-His-Glu-Ala-Glu-Leu-Lys-Pro-Leu-Ala-Gln-Ser-His-Ala-Thr-Lys 79 96
12	17–31	Val-Glu-Ala-Asp-Val-Ala-Gly-His-Gly-Gln-Asp-Ile-Leu-Ile-Arg 17 31
11	119–133	His-Pro-Gly-Asn-Phe-Gly-Ala-Asp-Ala-Gln-Gly-Ala-Met-Asn-Lys 119 133
10	35–42	Ser-His-Pro-Glu-Thr-Leu-Glu-Lys 35 42
14	148–153	Glu-Leu-Gly-Tyr-Gln-Gly 148 153

[a] Peptides are given in the order of size.

11, 80, and 100 and goat antisera G1 and G4 were against Mb. Rabbit antiserum 93 was against ApoMb. All these antisera were employed in the present studies.

Analytical Methods. Optical density was measured with a Zeiss PMQII spectrophotometer. The purity of each peptide was confirmed by peptide mapping on Whatman No. 3MM papers (63 × 25 cm). Ascending chromatography in butanol–acetic acid–water (4:1:5, v/v) was followed by electrophoresis at pH 3.75 in a Savant electrophoresis apparatus with pyridine–acetic acid–water buffer (1:10:289, v/v) and a potential gradient of 24.5 V/cm for 60 min. The peptide spots were revealed at room temperature with ninhydrin (0.2% solution in ethanol). Nitrogen determinations were done in a micro-Kjeldahl apparatus similar to that described by Markham (1942). The concentrations of protein and peptide solutions were determined from their nitrogen contents.

Double diffusion in 1% agar was by the method of Ouchterlony (1949). Precipitin experiments were done according to the procedure of Heidelberger and Kendall (1935), with 0.2 ml of antiserum and various amounts of antigen in 0.2 ml of 0.15 M NaCl. For inhibition studies, aliquots (0.2 ml) containing various amounts of peptide in 0.15 M NaCl were mixed each with 0.2 ml of antiserum, in conical glass centrifuge tubes, at 0°. When addition of peptides to antiserum aliquots was complete, the tubes were removed from the ice bath and allowed to stand at room temperature for 2 hr. Weakly inhibiting peptides were, in addition, allowed to stand overnight at 0° with no apparent changes in inhibitory activity. Aliquots (0.2 ml) containing an amount of antigen, necessary to saturate the antibody present at equivalence, were added. The mixture was left at 37° for 1 hr and then at 0° for 24 hr with occasional mixing. The precipitates were centrifuged, washed, dissolved, and subjected to nitrogen determination in the usual manner (Atassi, 1967c). For determination of small amounts of immune precipitates (1–10 μg of N) the Folin–Lowry method (Lowry *et al.*, 1951) was employed after dissolving these precipitates. Results were expressed in per cent inhibition, *i.e.*, 100 × (total precipitate with antigen − total precipitate in presence of peptide)/total precipitate with antigen. Amino acid analyses of acid hydrolysates (110°, 22 hr, constant-boiling HCl, in nitrogen-flushed, evacuated, sealed tubes) were done on Spinco Model 120 C amino acid analyzer.

Results

Preparation of Tryptic Peptides. Chromatography on Dowex 1-X 2 resolved the soluble portion of the tryptic digest of ApoMb into 13–14 peptides (Figure 1). Nine of these peptides were characterized, after purification by rechromatography, with regard to their position in the primary structure of Mb. Table I summarizes the amino acid compositions of the five

TABLE III: Molecular Weights and Nitrogen Contents of Peptides.[a]

Protein or Peptide	Mol Wt	Nitrogen Content (%)
Mb	17,816	17.36
ApoMb	17,200	17.66
8–153	16,398	17.83
56–131	8,266	18.29
1–55	6,414	17.03
8–55	5,596	17.51
132–153	2,556	16.98
1–7	819	13.68

[a] Values for each peptide were calculated from its amino acid composition. Peptides are referred to by their location in the primary structure of Mb (Edmundson, 1965).

TABLE IV: Maximum Inhibition of the Precipitin Reactions of Mb and ApoMb by Some ApoMb–Tryptic Peptides.[a]

Peptide	Serum: Antigen:	Anti-Mb Sera G1 Mb[b]	Anti-Mb Sera 80 Mb[c]	Anti-Mb Sera 80 ApoMb[d]	Anti-ApoMb Serum 93 ApoMb[e]
79–96			11 (80)	11 (85)	15.5 (41)
17–31		6 (12)	6 (12)	11 (11)	19.1 (15)
119–133			5 (45)		13 (35)
35–42		0	0	0	8 (100)
148–153			6 (120)	11 (100)	17.1 (48)

[a] Results are expressed in per cent inhibition of the precipitation reaction. Values in parentheses represent peptide/antigen molar ratio at 50% maximum inhibition. Values represent the average of three determinations which ranged ±1.5% or better. The variations in analyses of uninhibited controls were: [b] ±1.3% from four analyses, [c] ±1.0% from three analyses, [d] ±1.4% from three analyses, and [e] ±1.1% from three analyses.

peptides used in the present studies. The location of the peptides in the primary structure of the protein together with their individual sequences are shown in Table II.

Preparation of the Peptides Obtained by Cleavage at the Methionine and Tryptophan Sites. Procedure for cleavage of ApoMb with periodate at the tryptophan sites and isolation and purification of the resultant peptides has already been described (Atassi, 1967a). It was shown that with this procedure 92–94% cleavage was obtained at tryptophan-7 (fragments IA and III). Fragments obtained by cleavage at tryptophan-7 were isolated in high purity as determined from their amino acid compositions and quantitative N-terminal analyses (Atassi, 1967a). Peptides obtained by cleavage at the methionine sites were identical in amino acid compositions and N-terminal analyses with those reported by Edmundson (1963). Table III lists all the peptides obtained by chemical cleavage and used in the present work with their molecular weights and nitrogen contents together with the corresponding data for Mb and ApoMb. To avoid ambiguity these fragments will not be referred to by numbers or letters but will be designated by their location in the primary structure of ApoMb as reported by Edmundson (1965). To economize on space, the sequences of the peptides used here will not be given and the paper of Edmundson (1965) may be referred to.

Immunochemical Reactivities of the Peptides. A. TRYPTIC PEPTIDES. The soluble fraction of the tryptic digest was investigated with regard to its ability to inhibit the precipitation of antibodies in sera G1 and 80 with both MbX and ApoMb. With G1 its maximum inhibitory activity was 25% (with MbX) and 30% (for ApoMb). With rabbit antiserum 80 and ApoMb its maximum inhibitory activity was 36%. These results suggested that an investigation of the activity of isolated individual tryptic peptides should prove valuable. The inhibitory activity of some of these tryptic peptides is shown in Table IV. Peptide 79–96 inhibited the precipitation of antibodies to Mb with the homologous antigen to the extent of 10%. Its inhibitory activity of the reaction anti-ApoMb with ApoMb was even greater (15.5%). Peptide 119–133 showed only a slight inhibitory activity of the Mb–antiMb system but a greater inhibitory activity was obtained with the ApoMb–anti-ApoMb system. It can also be seen in Table IV that peptide 17–31 had an appreciable inhibitory activity with the anti-Mb–ApoMb system but exhibited a larger activity with the anti-ApoMb–ApoMb system. On the other hand, peptide 35–42 did not inhibit the precipitation of Mb with anti-Mb and only slightly inhibited the precipitation of ApoMb with anti-ApoMb. Finally, peptide 148–153 was able to inhibit the reaction of MbX and ApoMb with antiserum 80 to MbX, and more so the reaction of ApoMb with antiserum 93 to ApoMb.

B. PEPTIDES FROM CLEAVAGE AT TRYPTOPHAN AND METHIONINE SITES. Peptide 1–7. This peptide did not inhibit the reaction of Mb or ApoMb with sera 11, 100, and G1 even when a very large molar excess of peptide to antigen was employed (Table V). It may be pointed out that a similar peptide, obtained from a chymotryptic digest of ApoMb, was reported to be noninhibitory with two pools of antisera from various numbers of rabbits (Crumpton and Wilkinson, 1965). It appears that this peptide has a similar behavior with goat antiserum.

Fragment 8–153. This short ApoMb gave a precipitate with antisera to Mb. Figure 2 shows typical precipitin curves of this peptide with rabbit antisera 11 and 100. The precipitin curves of the homologous reactions for these two sera are also shown for comparison. The precipitation of antibodies by peptide 8–153 was subjected to an exhaustive investigation in numerous experiments with appropriate controls. This was done in order to rule out any precipitation due to a decrease of solubility as a result of shortening the apoprotein molecule. Thus reaction of this peptide with preimmune sera and sera quantitatively absorbed with Mb showed a very small amount of precipitation

TABLE V: Maximum Activity of the Inhibitory Fragments from ApoMb Obtained by Cleavage at Methionine and/or Tryptophan.[a]

Anti-serum	Peptide: Antigen:	1–7		132–153		8–55	
		Mb	ApoMb	Mb	ApoMb	Mb	ApoMb
G1		0 (200)	0 (185)	21.0 (4.1)	18.5 (5.0)	Precipitate[b]	
G4				13.3 (13.4)	14.0 (9.1)	10.4 (13)	
11		0 (400)	0 (400)				
80				10.4 (4.1)			
100		0 (300)	0 (280)	10.9 (9.4)	20.8 (6.1)		
93[c]					50.7 (2.4)	Precipitate[b]	

[a] Results are expressed in maximum per cent inhibition by the peptide of the precipitin reactions of Mb and ApoMb with various antisera. Values in parentheses represent peptide/antigen molar ratio at 50% of maximum inhibition, except for peptide 1–7 where they represent the maximum molar excess of peptide used in the inhibition experiment. Inhibition values represent the average of three analyses which ranged ±1.2% or better. [b] Peptide 8–55 gave an immune precipitate with these sera (see Table VII). [c] Rabbit antiserum to ApoMb.

(about 5% relative to reaction with anti-Mb sera). However, this compared well with the small amounts of precipitation given by ApoMb and by periodate oxidized (but not cleaved at tryptophan-7) ApoMb[3] with preimmune or absorbed sera. Additional evidence, that the precipitation with peptide 8–153 and Mb antisera was due to specific reaction with antibodies and did not result from possible decrease in solubility of the peptide, was obtained from absorption experiments with this peptide. Antisera 11, 100, and G1 were absorbed with the minimum amount of peptide necessary to bring about maximum precipitation and the supernatants were tested for their reactivities with Mb both by gel diffusion and by quantitative precipitin analysis. None of the three sera absorbed with peptide 8–153 showed any reaction with the homologous antigen indicating in each case complete removal of the antibodies to Mb by the peptide. In a similar absorption experiment with an antiserum to serum albumin, little or no precipitate was obtained with the peptide and, absorbed serum retained its full reactivity with albumin. Finally with all the antisera tested the precipitin curves given by the peptide superimposed well with the curves given by periodate-oxidized (but not cleaved at tryptophan-7) ApoMb. Table VI summarizes the reactivity of this peptide with various antisera.

Peptides 1–55 and 8–55. Peptide 1–55 was reacted with two rabbit and two goat antisera to MbX and one rabbit antiserum to ApoMb. In each case a precipitation reaction was obtained. The precipitin reaction of this peptide with rabbit antiserum 100 is shown in Figure 3. Table VII summarizes the results of the reaction of peptide 1–55 with various antisera. The peptide precipitated between 14 and 28% (reaction with G4 was less) of antibody nitrogen relative to the homologous reaction, and between 16 and 31% relative to the reaction of ApoMb with these sera. That the precipitate obtained was in fact a specific immune precipitate was confirmed by absorption experiments with this peptide. Rabbit antisera 11 and 100 and goat antisera G1 and G4 were absorbed with the minimum amount of peptide required to bring about maximum precipitation. The absorbed sera, after removal of the precipitates, showed a decrease in reactivity with Mb. The loss in reactivity compared well, in each case, with the relative values of precipitation (Table VII) given by the peptide with the unabsorbed antiserum. Controls with the corresponding preimmune sera showed no reactivity for the peptide with each of these sera. On the other hand, the reactivity of the absorbed sera with

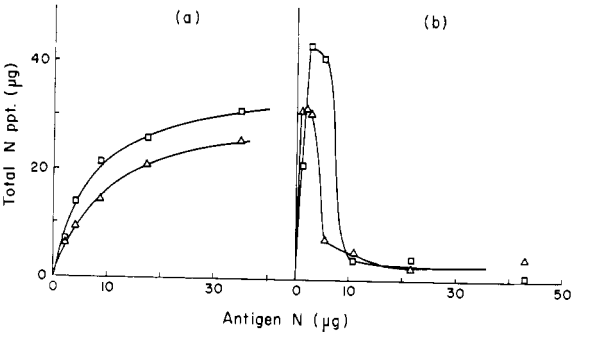

FIGURE 2: Precipitin analyses with rabbit antisera 11 (△) and 100 (□). (a) Reactions of peptide 8–153 with the two sera and (b) reactions of Mb X with the same sera. Antiserum 11 was diluted with 0.15 M NaCl (1:1) before use.

[3] Since cleavage at tryptophan is done in two steps (Atassi, 1967a), *i.e.*, (1) oxidation with periodate and (2) mild acid (0.1 N HCl) hydrolysis at room temperature, it is easy to prepare ApoMb which is oxidized but not cleaved.

TABLE VI: Relative Amounts of Precipitation Formed by Peptide 8–153 and Periodate-Oxidized ApoMb with Various Sera.[a]

Antiserum	% Ppt Rel to Reaction of Antiserum with Mb		% Ppt Rel to Reaction of Antiserum with ApoMb	
	Peptide 8–153	Oxidized ApoMb	Peptide 8–153	Oxidized ApoMb[b]
G1	105 (5.15)	102 (4.99)	96.4 (28.1)	98.8 (23.9)
100	79.0 (14.2)	81.1 (14.0)	82.7 (7.66)	85.1 (8.23)
11	80.6 (16.1)	83.2 (15.6)	76.1 (15.3)	80.0 (14.7)

[a] The percentage of precipitation relative to Mb X or ApoMb were based on the total nitrogen values in the precipitates at maximum precipitation. The molar ratios of peptide/antigen at their precipitin maxima are shown in parentheses. [b] Oxidized ApoMb is not as efficient in precipitating antibody as ApoMb. This is not surprising since the two tryptophan residues, the two methionine residues and one tyrosine were modified (Atassi, 1967b). In addition the cooperative effect of these modifications on the conformation of the protein has to be taken into account.

the core peptide 56–131 (see later) remained unchanged. Cleavage of peptide 1–55 at tryptophan-7 yielded the shorter fragment 8–55. This peptide precipitated less antibody nitrogen than peptide 1–55 when the reactions with the same serum were compared (Table VII). Also peptide 8–55 required a much larger amount of peptide nitrogen to reach maximum precipitation. With antiserum G4, the shorter peptide no longer precipitated antibody but instead exhibited an inhibitory activity of the precipitin reaction of Mb with this serum. Figure 4 shows the precipitin reaction of peptide 8–55 with antiserum 93. Therefore, it appears that although the N-terminal heptapeptide 1–7 is not a reactive region of the molecule (as shown by its lack of inhibitory activity and by the finding that peptide 8–153 and oxidized, uncleaved ApoMb had identical reactivities) it may nevertheless play a role in the interaction of peptide 1–55 with antibodies to Mb. This will be dealt with in the Discussion section.

Peptide 56–131. This was also a precipitating peptide. It invariably showed a higher precipitating efficiency than peptide 1–55 when reactions with the same serum were considered (Table VII). An example of the precipitin curve obtained with this peptide and antiserum 93 is shown in Figure 4. Its reaction with antisera 100 and G1 are shown in Figures 3 and 5b. The peptide precipitated in three sera between 40 and 80% of antibody nitrogen relative to the homologous reaction. Table VII summarizes the quantitative data for the precipitin reactions of peptide 56–131 and four different sera. Antisera absorbed with this peptide showed in each case a decrease in reactivity with Mb. The decrease in reactivity compared very well with the amount of antibody nitrogen which had been removed by the peptide from the antiserum. Figure 5 demonstrates an example of such an experiment with antiserum G1. It can be seen that peptide 56–131 brings down 18.5 μg of N at maximum (Figure 5b). The amount of antibody nitrogen precipitated by MbX decreased from 30.2 μg of N for the antiserum before absorption to 11.3 μg of N with the absorbed serum (*i.e.*, 98% of the decrease in reactivity of the absorbed serum is accounted for by the immune precipitate of the peptide). In addition when a serum

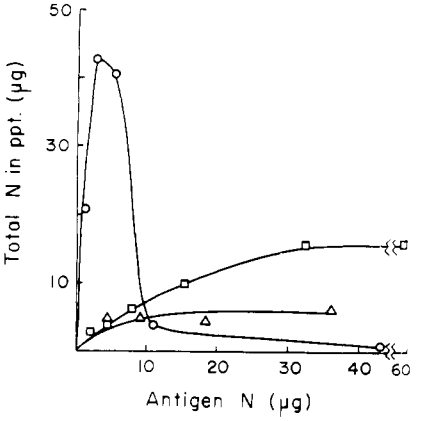

FIGURE 3: Precipitin studies with rabbit antiserum 100 and (○) Mb X, (△) peptide (1–55), and (□) peptide 56–131.

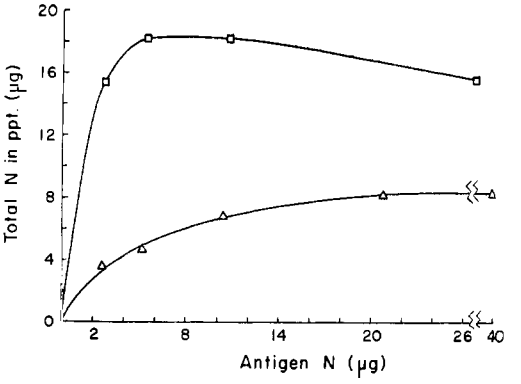

FIGURE 4: Precipitin reactions of rabbit antiserum 93 with peptides 8–55 (△) and 56–131 (□).

TABLE VII: Relative Amounts of Precipitation Formed by Peptides 1–55, 8–55, and 56–131 with Various Sera.[a]

Anti-serum	Peptide:	% Ppt Rel to Reaction of Antiserum with Mb 56–131	1–55	8–55	% Ppt Rel to Reaction of Antiserum with ApoMb 56–131	1–55	8–55
G1		62.6 (12.0)	27.6 (5.21)	13.11 (31.2)	56.9 (11.7)	30.9 (2.7)	12.3 (13.1)
G4		80.5 (1.14)	8.20 (9.62)	Inhibits[b]	81.9 (1.11)	7.95 (5.10)	Inhibits[b]
11			20.83 (17.1)			16.76 (15.4)	
100		40.0 (19.0)	14.12 (8.57)		45.9 (13.7)	16.2 (7.50)	
93					41.0 (3.43)	23.1 (16.6)	18.2 (24.6)

[a] Values are expressed as in Table VI. [b] Peptide 8–55 exhibits an inhibitory activity with G4 (see Table V).

was absorbed with this peptide its precipitating efficiency with fragment 1–55 or 8–55 was unaltered (Figure 5 shows its reaction with fragment 8–55).

Peptide 132–153. In all the five sera tested this peptide had an inhibitory activity which varied according to the serum and the antigen used in the precipitin reaction. This finding agrees with the recent report of Crumpton (1967) which appeared while this paper was being prepared for publication. The inhibitory activity of the peptide was usually greater with the precipitin reaction of ApoMb than with Mb. Table V summarizes the inhibitory activity of this peptide with rabbit antisera 80, 100, and 93 and goat antisera G1 and G4. The greatest amount of maximum per cent inhibition was obtained with antiserum 93 (50.7%). The value of peptide to antigen molar ratio at 50% of maximum inhibition for serum 93 was about 2 which was lower than the corresponding ratio with any of the other sera. The maximum per cent inhibition for the other sera ranged between 11 and 21% with Mb and between 14 and 21% with ApoMb. The molar ratios of peptide/antigen at 50% of maximum inhibition for those sera ranged between 4 and 13.

Absorption of Antisera with Fragments 1–55 and 56–131. Since both the N-terminal segment 1–55 and the central segment 56–131 give specific immune precipitates with all the antisera tested, a simple procedure for preparing sera with specificity towards the C-terminal region became available. Rabbit antisera 11, 80, 93, and 100 and goat antisera G1 and G4 were absorbed quantitatively with the minimum amount of each of peptides 1–55 and 56–131 necessary to bring down maximum antibody precipitation for each peptide. Each of the six absorbed sera was tested with respect to its reactivities with the above two peptides and with the homologous antigen. In no case was there a reaction obtained with either of these two peptides. However upon reaction of the absorbed sera with Mb X (or ApoMb for serum 93) a different result was obtained. Three sera (11, 93, and G4) gave no reaction at all with the homologous antigen over a wide range of antigen concentrations. On the other hand absorbed antisera 80 and 100 gave, in agar double diffusion, faint but definite lines (Figure 6) with MbX, while G1 gave an extremely faint reaction only with concentrated Mb solutions (70.8 μg of N/ml.). It is very significant that, although peptide 132–153 does not precipitate antibody nitrogen and is definitely an inhibitory peptide with these six sera, nevertheless antibodies directed against it can (although only in two cases out of six) form a specific immune precipitate

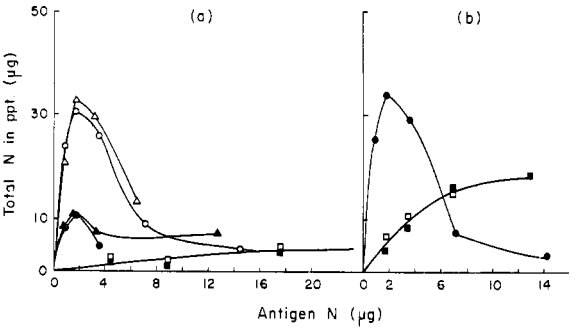

FIGURE 5: Absorption experiments with goat antiserum G1. Part a shows the reactivities of serum, preabsorbed with peptide 56–131, toward Mb X (●), ApoMb (▲), and peptide 8–55 (■); the reactions of unabsorbed serum (which had been diluted with a volume of 0.15 M NaCl equivalent to the volume of the peptide solution used in the absorption experiment) is also shown for comparison (open symbols). Maximum amount of precipitate obtained with peptide 8–55 and the diluted serum accounted, in duplicate analyses, only for 89 and 92% of the expected amount of precipitate from the results with the undiluted serum. Part b gives the reaction of serum which had been absorbed with peptide 8–55 toward peptide 56–131 (■) and ApoMb (●); the reactions of appropriately diluted but unabsorbed serum with peptide 56–131 (□) are also shown. Notice that the amount of antibody nitrogen precipitated by peptide 56–131 in b is equivalent to the loss in reactivity of the serum with Mb X shown in a upon absorption with this peptide while the reactivity against peptide 8–55 remained intact. Also absorption of the serum with peptide 8–55 did not remove any of the serum's reactivity toward peptide 56–131.

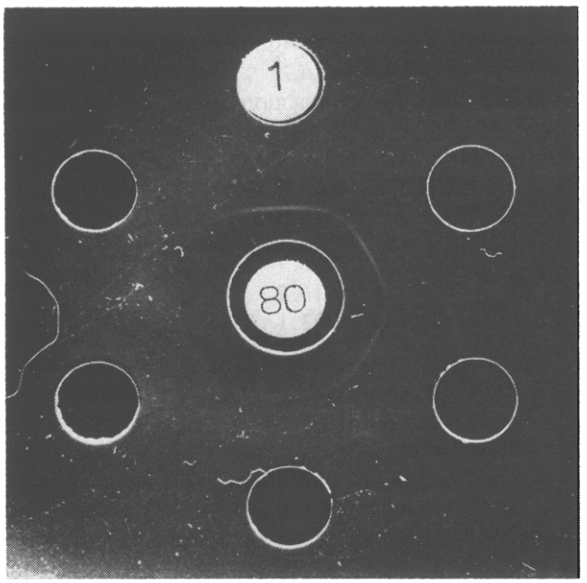

FIGURE 6: Agar double diffusion with antiserum to fragment 132–153 obtained from serum 80. An aliquot of serum 80 was absorbed with fragments 1–55 and 56–131 and the supernatant's reaction with Mb X is shown. Mb solutions had the following concentrations in micrograms of N per milliliter (from 1 clockwise): 70.8, 35.4, 17.7, 8.85, 4.43, and 2.21.

with the homologous antigen. This again points to the importance of looking at several sera when studying antigenic determinants and that these sera should be studied separately so that interesting individual specificities will not be diluted and lost upon pooling. A detailed study on the isolated specific antibodies to peptides 1–55, 56–131, and 132–153 will be reported separately.

Discussion

With most peptides, large amounts of the peptide are required to achieve maximum precipitation or inhibition. The possibility of contamination by small amounts of undegraded antigen was considered but was ruled out since: (a) all the peptides examined were absolutely homogeneous by peptide mapping even when the chromatograms were heavily overloaded; (b) the larger fragments were obtained by passage at least five times through Sephadex G-75 (2.2 × 150 cm) until at least in two consecutive runs single symmetrical peaks were obtained; (c) amino acid analysis confirmed the purity of these peptides; and (d) all the precipitating peptides gave a true plateau in the region of large antigen excess. This would not be possible had the precipitate been the result of contamination with undegraded antigen. Therefore the large amounts of peptide required to obtain maximum inhibition or precipitation can be rationalized only on conformational basis. It is likely that only a fraction of the isolated peptide achieves a configuration which approaches that it has in the native protein and which will give it a favorable fit with the antibody combining sites. The probability of finding such a configuration will improve with increase in the concentration of the peptide.

In the three-dimensional model (Kendrew *et al.*, 1961), peptide 79–96 includes the bend between helices E and F, all of helix F, and a portion of the bend between helices F and G. Having this location which occupies two corners on the surface of the molecule, it is not therefore surprising that this peptide should carry antigenically reactive region (or regions). Crumpton and Wilkinson (1965) found that the chymotryptic peptide 78–89 inhibited the precipitation of anti-Mb with ApoMb to the extent of 9% with one pool of sera but did not inhibit the reaction with another pool. Peptide 119–133 includes all of the corner between helices G and H and only a portion of helix H. In the parent protein, therefore, seven residues of this peptide assume a nonhelical conformation. It is likely that this segment is reactive in native Mb but due to unfavorable conformation in the isolated peptide, its inhibitory ability is limited. Since ApoMb has been shown to be more unfolded than Mb (Breslow *et al.*, 1965; Atassi and Cacciotti, 1966), the probability is greater that the free peptide can assume a configuration similar to that it has in ApoMb than in Mb. It is relevant to point out here that methionine at position 131 is not an essential part of a reactive site of Mb (Atassi, 1967b). Peptide 17–31 occupies all the corner between helices A and B and 75% of helix B. This is a region which is situated well on the surface of the molecule. It exhibited some inhibitory activity with all the sera tested, although to varying degrees. Peptide 35–42 corresponds to the small (five residues) helix C in the Kendrew model. In the free form, such a short peptide of which only a small portion is helical, might not be able to attain, to any appreciable degree, a configuration similar to that it has in the native protein.

Peptide 148–153 represents the C-terminal piece of the protein. The high inhibitory activity of this peptide with anti-ApoMb serum 93 represents only a fraction of the activity of the larger peptide 132–153 (50%, see Table V) with the same serum. This might suggest that the C-terminal hexapeptide represents only a portion of the reactive region(s) of the 22-amino acid terminal piece. In a recent report from this laboratory (Atassi, 1966), it was shown that the C-terminal dipeptide in Mb was not an essential part of a reactive site in the native protein. However, it has been reported (Crumpton, 1967) that when the dipeptide -Gln-Gly is removed from peptide 147–153, the inhibitory activity decreased drastically in the remaining pentapeptide. The apparent conflict may arise from the possibility that the C-terminal dipeptide, although not part of the reactive region, is nevertheless essential for the appropriate orientation of the isolated terminal heptapeptide for a more favorable fit onto the antibody site. This orientating influence of the dipeptide might not be required in the native molecule where other regions might exert a directive effect. Also, it is difficult

to correlate our data with those of Crumpton *et al.* due to possible differences in sera specificities. Our sera were obtained from a single bleeding of individual animals and studied separately, while the above authors worked with pools of sera from a number of bleedings and a number of rabbits.

The active tryptic peptides, therefore, usually occupy corners on the surface of the myoglobin molecule. The inhibitory activity is usually higher with apomyoglobin than with Mb especially when the serum is directed against ApoMb. This might be due to the fact that ApoMb is more unfolded than Mb and therefore new reactive regions might become uncovered. Also the free peptides in solution will be able to approximate more frequently the configuration they have in ApoMb than that they possess in Mb.

The absence of any inhibitory activity by peptide 1–7 suggested that this segment is not present in a reactive region of the molecule. However cleavage at tryptophan with periodate leads to the conversion of this amino acid into a spirolactone (Atassi, 1967a). It would be conceivable that the spirolactone structure at the C-terminal end of the peptide might cause the inactivation of an otherwise reactive peptide. There is a great deal of evidence which indicates that the N-terminal heptapeptide of Mb is in fact not part of a reactive region. Atassi and Caruso (1968) have shown that modification of tryptophan-7 with 2-hydroxy-5-nitrobenzyl bromide did not alter at all the antigenic reactivity of Mb with its antisera. Crumpton and Wilkinson (1965) also showed that the N-terminal heptapeptide isolated from a chymotryptic digest of ApoMb was noninhibitory. Further evidence is obtained from the finding that periodate-oxidized (but not cleaved at position 7) ApoMb had identical precipitin curves with those given by the shortened apoprotein (*i.e.*, fragment 8–153). However, the results obtained from the work on fragments 1–55 and 8–55 are very significant in this connection. It was shown that both fragments are precipitated by rabbit and goat antisera to Mb X. However, the precipitation obtained with fragment 8–55 was, for a given serum, considerably less than that from the reaction of fragment 1–55. In fact with one serum (G4), the reactivity changes from precipitation to inhibition as the N-terminal heptapeptide is removed from fragment 1–55. The finding throws an interesting light on the picture in that although the N-terminal heptapeptide is not an essential part for the reactivity of ApoMb with antibodies, it seems, however, to be important for the reactivity of the shorter segment 1–55. This is most likely dependent on the ability of the fragments in question to attain a favorable orientation for reactivity with the antibody combining site. It is very likely that in segment 1–55, the presence of the N-terminal heptapeptide is helpful in order for a reactive region(s) present in that segment (but not in peptide 1–7) to achieve the proper orientation. Such an example has already been cited with regard to the role of the C-terminal dipeptide in the antigenic reactivity of Mb. Therefore, in the process of delineating a reactive region in a peptide caution is necessary when conclusions are drawn from shortening the active peptide. This phenomenon with natural protein antigens is probably much less pronounced in polysaccharide antigens.

TABLE VIII: Total of Activities of Fragments 1–55, 56–131, 132–153.[a]

	Activities Rel to Mb			
Peptide	G1	G4	100	93
1–55	27.6	8.20	14.1	
56–131	62.6	80.5	40.0	
132–153	21.0	13.3	10.90	
Total	111.2	102.0	65.0	
	Activities Rel to ApoMb			
1–55	30.9	7.95	16.2	24.1
56–131	56.9	81.90	45.9	41.0
132–153	18.5	14.0	20.8	50.7
Total	106.3	103.9	82.7	115.8

[a] Values of activities represent either per cent precipitation relative to homologous reaction or per cent inhibition (see Tables V and VII).

Fragments 1–55 and 56–131 contain relatively large segments of predominantly hydrophobic regions which could cause a decrease in solubility. It is significant that all the insoluble core of the tryptic digest of Mb is derived from these two segments (Edmundson, 1963). The likelihood of poor solubility was indeed the reason why exhaustive controls were carried out. The reactive region with the antibody might be mostly hydrophilic and polar in nature (*e.g.*, active tryptic peptides 17–31 and 79–96), but the presence of hydrophobic centers associated with the reactive regions contributes to the formation of an immune precipitate. In addition, peptides 1–55 and 56–131 are sufficiently large, with molecular weights of 6414 and 8266, respectively, to contain more than one active region which would facilitate the formation of insoluble antigen–antibody clusters.

The C-terminal fragment 132–153 is probably too small to form an immune precipitate. This peptide includes almost all of helix H in the Kendrew model (Kendrew *et al.*, 1961) and the nonhelical C-terminal pentapeptide. This segment is very rich in hydrophilic and polar amino acids and a great portion of it therefore is exposed to the surface of the molecule.

It is significant that antibodies to peptide 132–153 can occasionally form immune precipitates with Mb. By holding the protein through its tail end, the antibody probably induces unfolding of the myoglobin molecule which helps in the formation of an immune precipitate, especially when this segment has more than one reactive region with different antibodies directed against these regions.

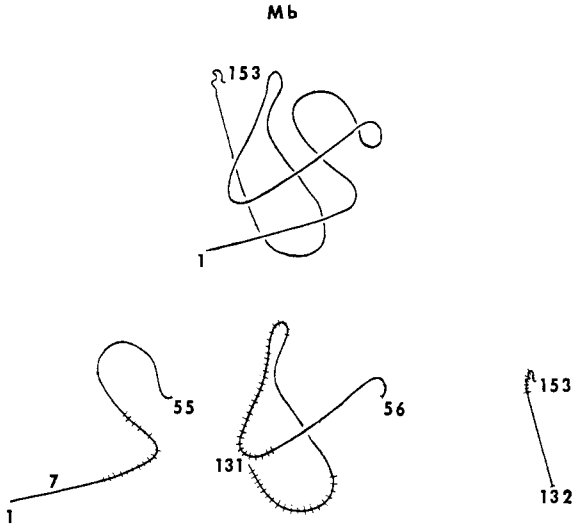

FIGURE 7: A very schematic line drawing of the mode of folding the sperm whale metmyoglobin polypeptide chain (adapted from Dickerson, 1964). The positions of cleavage at methionines-55 and -131 are indicated and the peptides obtained are dissected apart for clarity. The position of tryptophan-7 is also indicated on segment 1–55. The approximate locations of the reactive tryptic peptides are shown by the marking on the main line. It is not implied that the reactive peptides indicated on each segment represent the only reactive regions on that segment. Similarly the reactive regions may not necessarily include the whole length of the reactive tryptic peptides.

It is noteworthy that when the values of the relative reactivities of the three fragments 1–55, 56–131, and 132–153 are summed up, the total accounts (in three out of four sera where this has been done) to all the antigenic reactivity of Mb and ApoMb with each of these sera (Table VIII). The values add up to slightly more than 100% in G1 and in 93. This is probably due to the unmasking of new reactive regions upon breaking up the molecule. With antiserum 100 some loss of reactivity is observed especially with respect to Mb. The total reactivities relative to ApoMb are slightly better (83%). This suggests that upon fragmentation of the protein at methionines-55 and -131, the reactive regions remain essentially intact. At this stage the observations do not exclude that an antigenic site might comprise two or more reactive regions present on different segments of the molecule but adjacent in the three-dimensional structure. When these segments are separated, it is conceivable that each of the reactive regions on them might contribute its share of the reactivity.

The variation in extent of reactivity of each of the fragments with the various sera suggests a variation in specificities of these sera, each of which might contain antibodies against different regions of each segment. That a fragment such as 8–55 can be inhibitory in one case but give immune precipitates in the others suggests, in addition, that antibodies may vary in their affinities (Kitagawa *et al.*, 1965). In all the anti-Mb X sera, fragment 56–131 seems to be the immunodominant region. However, the extent of its immunodominance varies with the serum (see Table VII).

In conclusion, the findings show that the three fragments obtained by cleavage at the methionines are all immunochemically active and between them account for almost all the immunochemical reactivity of the antigen. Also, the short apoprotein obtained by cleavage at tryptophan-7 is immunochemically intact. It is not suggested that each of these fragments is a whole reactive site but rather each carries within it reactive region(s).

The work with tryptic peptides shows that reactive regions reside in segments 17–31 (corner A–B), 79–96 (E–F–G), 119–133 (corner G–H), and the C-terminal hexapeptide. The results are summed up very schematically in Figure 7.

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