Immunochemistry of Sperm Whale Myoglobin. VIII. Specific Interaction of Peptides Obtained by Cleavage at Proline Peptide Bonds*

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ABSTRACT: Cleavage of apomyoglobin at prolines-37, -87, and -120 has been effected by reaction with sodium in liquid ammonia. Five overlapping peptides have been isolated and characterized. These peptides corresponded, in the parent protein, to the following locations: 1–36, 37–87, 1–87, 37–119, and 120–153. The immunochemistry of these peptides was studied and compared with the previously reported immunochemical behavior of various overlapping peptides obtained by other chemical cleavage procedures. This approach yielded valuable information concerning the regions of overlap. Peptide 1–36 gave an immune precipitate with antisera to the native protein and, with a given serum, had a reactivity equal to that of peptide 1–55 suggesting that the sequence 37–55 does not carry a reactive region. This confirmed our previous findings derived from the modification of arginines-31 and -45 and methionine-55 and from the lack of activity of peptide 35–42. Peptide 37–87 showed inhibitory activity and, with a given serum, the sum of its activity and that of peptide 1–36 corresponded to the observed reactivity of peptide 1–87. The latter peptide, as well as peptide 37–119, gave immune precipitates with antisera to the whole protein. Finally, fragment 120–153 showed only inhibitory activity with all the sera tested. The activity of this peptide with each antiserum compared very well with that of the shorter peptide 132–153 indicating that region 120–132 is antigenically nonreactive. This is in agreement with our previous results concerning this region of metmyoglobin (Mb). With a given antiserum, a greater portion of the reactivity appeared in fragment 37–119. However, the three fragments 1–36, 37–119, and 120–153 accounted for almost all the immunochemical reactivity of the intact protein. These findings together with our previously reported results suggest that in Mb: (a) in the sequence 1–55 one reactive region exists within the portion 7–30 on the corner between helices A and B; (b) between residues 56 and 119 two reactive regions most likely exist, separated by a silent region around tyrosine-103; and (c) the C-terminal portion 120–153 carries one reactive region which is located C terminal to arginine-139 and is most likely centered around tyrosines-146 and -151.

Although information concerning the antigenic structure of sperm whale Mb[1] represents the most advanced such knowledge for a globular protein, nevertheless a great deal of work is still necessary for further accurate delineation of the antigenic reactive regions in the protein. Studies so far have utilized determination of the antigenic reactivities of peptides obtained both by enzymic and chemical cleavage procedures. Also specific chemical modification of selected amino acids in the protein and in immunochemically active peptides have aided greatly in the delineation of reactive regions. Further delineation of reactive regions by shortening the active peptide might lead to erroneous conclusions due to the inherent limitations of the technique (Atassi, 1968). In previous work from this laboratory (Atassi and Saplin, 1968) it has been shown that the reactivity of a peptide can be impaired upon shortening, even though the portion removed may not be part of the reactive region in the peptide. It has been suggested (Atassi, 1968) that the configuration of a peptide may be affected upon shortening so that it will no longer be able to attain a favorable orientation for reaction with the antibody combining site. Similarly for the same considerations of configuration, a nonreactive peptide may conceivably still comprise part of a reactive region in the intact protein. Specific chemical modification of selected amino acids in a reactive peptide avoids this problem (Atassi, 1968; Atassi and Thomas, 1969). Obviously, a limitation on this approach is imposed by the number of modification reactions that achieve a high degree of specificity. However, further information may be obtained from the isolation by various procedures, if possible, of long and overlapping peptides from various regions in the protein.

It has been shown that prolyl bonds in peptides and proteins may be cleaved by reaction with lithium in methylamine (Patchornik *et al.*, 1964), with lithium aluminum hydride (Ruttenberg *et al.*, 1964), or better with sodium in liquid ammonia (Benisek and Cole, 1965; Wilchek *et al.*, 1965; Benisek *et al.*, 1967). This paper details the application of this reaction to ApoMb. Five overlapping peptides have been obtained by cleavage of ApoMb at proline peptide bonds.

* From the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received April 2, 1970.* This work was supported by a grant (AM-13389) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service, and by a contract (N00014-69-C-0137) from the Biochemistry Branch, Office of Naval Research.

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[1] Abbreviations that are not listed in *Biochemistry 5*, 1445 (1966), are: Mb, metmyoglobin; ApoMb, apomyoglobin; MbX, the major chromatographic component 10 obtained by CM-cellulose chromatography (Atassi, 1964).

The purification and characterization of these peptides are described together with their immunochemical reactivities.

Materials and Methods

Materials. Sperm whale myoglobin used in these studies was the major chromatographic component 10 (MbX) obtained by CM-cellulose chromatography (Atassi, 1964). ApoMb was prepared from MbX by a procedure similar to that used for the preparation of apohemoglobin (Atassi and Skalski, 1969). Sodium metal was from Mallinckrodt Chemical Works (St. Louis, Mo.). Liquid ammonia was obtained from Matheson Co. (East Rutherford, N. J.).

Cleavage at the Proline Sites and Separation of the Peptides. Apomyoglobin (about 50 mg for test reactions and 500–600 mg for large-scale preparations) was placed into a two-neck round-bottom flask (250 ml for test reactions and 2 l. for large scale preparations). One outlet of the flask was fitted with a cold-finger condenser attached to a drying tube containing sodium hydroxide pellets. The other outlet of the flask was stoppered. All joints used were ground glass. The flask was submerged in a bath either at −20° or at −78°. First, air in the reaction vessel was replaced with anhydrous ammonia and then liquid ammonia (15 ml for test reactions and 125–150 ml for large-scale preparations) was allowed to accumulate at the bottom of the flask. The protein was suspended in the liquid ammonia by vigorous magnetic stirring, using a glass-covered magnetic bar. Appropriate amounts (see results) of small bits of sodium metal were introduced into the reaction vessel through the side arm, which is then stoppered quickly. The reaction time was carefully noted, taking the appearance of the blue color in the liquid ammonia after the addition of sodium metal as the start of the reaction. Reactions were carried out for various durations (see Results), and were terminated by oxidizing excess sodium with the rapid addition of ammonium acetate crystals. The liquid ammonia was then allowed to evaporate out through a drying tube, leaving a white crusty residue of peptides, uncleaved protein, and sodium and ammonium acetates. It was difficult to remove the salts without loss of peptides. Several techniques were employed for this purpose in the following order. (1) Residue was vacuum desiccated for 24 hr over sodium hydroxide pellets and concentrated sulfuric acid at 20° (Benisek and Cole, 1965); (2) sublimation for 48 hr at 20°; (3) the residue was then dissolved in water and the solution filtered at 0° in a Diaflo ultrafiltration cell (Amicon Corp., Cambridge, Mass.) using N_2 (80 psi) and a Diaflo membrane with a molecular weight cut-off of 500 (Diaflo, UM-05 membrane); the dissolved salts were extruded and the concentrated solution containing peptides and unreacted protein was again diluted with water and concentrated by ultrafiltration; the process of dilution and membrane filtration was repeated three-four times; finally (4) the concentrated solution was passed through a Sephadex G-10 column (40 × 3 cm) to remove the last traces of salt. The column was eluted with 0.1 N acetic acid and the fractions (4 ml) containing protein and peptides (detected by reading optical density at 280 mμ) were combined and freeze-dried.

For separation of the peptides, gel filtration on Sephadex G-75 was used. Two columns, connected in series, were employed and each was 5.0 × 85 cm. A solution of the reaction product (containing 150–200 mg of reaction product) in 0.1 N acetic acid (20 ml) was introduced onto the bottom of one column and the two columns were eluted in the ascending direction with 0.1 N acetic acid. Fractions (11–15 ml) were collected at 0° at the rate of 130 ml/hr. The effluents, emerging from the second column were monitored directly with a Canalco Model DA, double-beam flow analyzer equipped with automatic scale expansion and cuvets with 10-mm light path. In addition, fractions were systematically read at 230 mμ in a Zeiss PMQII spectrophotometer in order to reveal peptides which do not absorb at 258 mμ.

Antisera. Preparation of antisera against Mb X was carried out in goats and in rabbits by the procedure already described in detail (Atassi, 1967). Antisera from individual animals were kept and studied separately and were stored in 8 ml portions at −40°. Goat antisera G3 and G4 and rabbit antiserum 77 were used in the present studies.

Analytical Methods. Peptide mapping was carried out as described elsewhere (Atassi and Saplin, 1968). Optical densities were determined with a Zeiss PMQII spectrophotometer and continuous spectra were done in a Perkin-Elmer Model 120 spectrophotometer. Acid hydrolysis was performed at 110° for 22 or 72 hr in constant-boiling HCl (double distilled) in N_2-flushed evacuated sealed tubes. N-Terminal determination was carried out by the subtractive Edman degradation procedure described by Konigsberg and Hill (1962). Procedures for quantitative precipitin and inhibition experiments have already been described in detail (Atassi and Saplin, 1968). Results of inhibition experiments were expressed in per cent inhibition, *i.e.*, 100 × (total precipitate with MbX − total precipitate in presence of peptide)/total precipitate with MbX. Concentrations of protein and peptide solutions were based on their nitrogen contents which were calculated from their amino acid compositions (see Table I). Nitrogen contents of solutions were determined by a micro-Kjeldahl procedure (Markham, 1942) and by using Nessler's reagent standardized with ammonium sulfate. Three replicate analyses were done on each solution and they varied ±0.3%.

Results

Cleavage of Myoglobin at Proline Peptide Bonds. Reaction conditions for cleavage at proline peptide bonds in ApoMb were exhaustively studied at various temperatures, time intervals, and sodium metal concentrations. This was carried out in order to determine the most appropriate set of conditions which would give maximum cleavage at proline residues with little or no side reactions at other amino acid residues. The extent of the cleavage reaction in each case was monitored by peptide mapping of the desalted reaction product, and in some cases, by actual separation of the peptides on Sephadex columns followed by amino acid analysis of each fraction and electrochromatography.

The reaction was studied at two different temperatures, *i.e.*, −78 and −33°, each with 600 atoms of sodium metal excess/mole of protein, and for 180 sec. At the lower temperature, −78°, little or practically no cleavage was obtained while at −33° most of the four proline peptide bonds were cleaved. No side reactions were observed at −33°. The extent of cleavage was determined at three different time intervals, 60, 160, and 210 sec each, at −33° with 600 sodium atoms excess/mole of protein. The reaction for 60 sec was

TABLE I: Amino Acid Composition of Various Peptides from ApoMb Obtained by Cleavage at Proline.[a]

	Peptide 1–36		Peptide 1–87		Peptide 37–87		Peptide 37–119		Peptide 120–153	
Amino Acids	Found	Calcd	Found	Calcd	Found	Calcd	Found	Calcd	Found	Calcd
Asp	1.90	2	4.23	4	2.10	2	2.08	2	3.87	4
Thr	0.08	0	3.97	4	3.90	4	4.96	5	0.11	0
Ser	1.74	2	2.90	3	1.01	1	4.10	4	0.18	0
Glu	5.00	5	11.86	12	6.78	7	9.78	10	4.29	4
Pro	0.12	0	1.10	1	1.03	1	2.90	3	0.95	1
Gly	2.92	3	6.23	6	3.20	3	3.24	3	4.90	5
Ala	3.23	3	8.15	8	5.16	5	8.12	8	6.20	6
Val	4.78	5	6.84	7	2.10	2	3.10	3	0.13	0
Met	0.04	0	0.93	1	0.88	1	0.84	1	0.87	1
Ile	2.13	2	3.19	3	1.14	1	5.95	6	1.20	1
Leu	4.85	5	11.81	12	6.97	7	10.13	10	3.08	3
Tyr	0.18	0	0.02	0	0.10	0	1.20	1	1.82	2
Phe	1.12	1	3.00	3	1.98	2	3.18	3	1.96	2
Lys	2.08	2	10.75	12	8.60	10	12.86	13	3.85	4
His	2.60	3	6.95	7	3.95	4	7.82	9	0.09	0
Arg	0.95	1	2.14	2	1.21	1	2.13	2	1.12	1
Trp[b]	*b*	2	*b*	2		0		0		0
Total number of residues		36		87		51		83		34
N terminal[c]	Val (0.91)	Val	Val (0.93)	Val	Pro (0.88)	Pro	Pro (0.85)	Pro	Pro (0.89)	Pro
Calculated molecular weight	4082		9848		5803		9481		3673	
Nitrogen contents (%)	17.50		17.49		17.83		17.87		17.16	

[a] Values are expressed in moles of amino acid per mole of peptide and represent the average of four analyses (two 22-hr and two 72-hr hydrolyses) for each peptide. Values of serine and threonine have been obtained by extrapolation to zero hydrolysis time. [b] Values for tryptophan were not determined. [c] Values in parentheses represent yield of N-terminal residue.

found to be too brief a reaction period resulting in negligible cleavage that gave a few long peptide chains and mostly uncleaved protein. By increasing the reaction period to 160 sec, extent of cleavage of protein at proline sites was considerably improved; however it was still a partial cleavage and gave only a poor yield of peptides. The reaction time period of 210 sec was found most satisfactory; cleavage at all the four proline peptide bonds was obtained without loss of any amino acids except the one immediately preceding proline residues in the sequence. Finally, effect of sodium metal concentration was investigated by employing an excess of 200, 600, or 800 sodium atoms per mole of apomyoglobin. In each case, the reaction was carried out at $-33°$ for 210 sec. Very little or no cleavage of the protein was obtained with 200 sodium atoms excess. In the peptide maps, big streaks at the point of application were observed. On the other hand, a sodium excess of 800 atoms/mole of protein was found to be too high a concentration as evidenced by numerous spots on the peptide maps. However, a 600 sodium atom excess gave best results, yielding only discreet major (and minor) spots in the peptide maps of the reaction product (Figure 1). Also no side reactions were noticed as determined by the amino acid analysis of the purified peptides.

As expected from the mechanism of the cleavage reaction, the amino acid immediately preceding a proline residue in the sequence where cleavage occurs is reduced to a certain extent under these conditions (see the values of histidine in peptides 1–36 and 37–119, and lysine in peptides 1–87 and 37–87 as reported in Table I). Therefore, for preparative purposes in the present work, the cleavage reactions were carried out on 570 mg of ApoMb (0.033 mmole) in 125 ml of liquid ammonium with 460 mg (20 mg-atoms) of sodium metal, that is, 604 atoms of sodium/mole of ApoMb. The reactions were performed at $-33°$ for 210 sec.

Separation and Characterization of the Peptides. Isolation and purification of the peptides in the reaction product of ApoMb with Na in liquid ammonia were accomplished by gel filtration on Sephadex G-75. Figure 2 shows an example of the gel filtration pattern of the cleavage product of ApoMb. Five components were apparent and fractions belonging to one peak were combined and freeze-dried. Purification of each fragment was accomplished by passage at least three times through the same two Sephadex G-75 columns, in series, until single symmetrical peaks were obtained. The purity of each fragment was confirmed by peptide mapping on heavily loaded chromatograms. Finally each

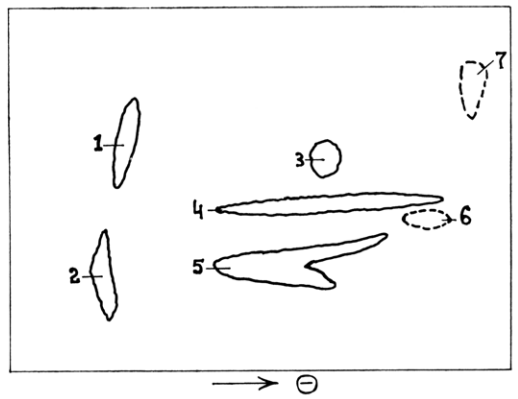

FIGURE 1: A tracing of the peptide map of the product of cleavage of ApoMb with Na in liquid ammonia. Reaction was carried out at −33° for 210 sec in the presence of 600 atoms of Na excess/mole of ApoMb. For experimental details, see text. By isolation and purification of the fragments from the cleavage reaction and their examination by peptide mapping, the spots shown here corresponded to the following sequences: spot 1, sequence 120–153; spot 2, sequence 1–36; spot 3, sequence 37–87; spot 4, sequence 37–119; spot 5, sequence 1–87. Spots 6 and 7 were present only in traces and were not isolated.

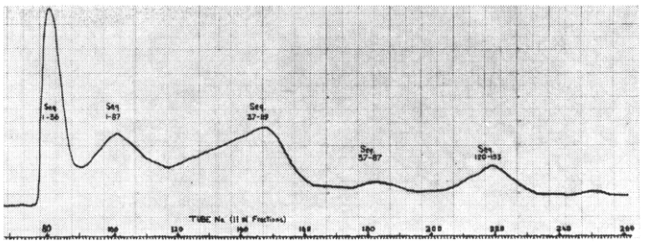

FIGURE 2: Gel filtration pattern of the cleavage product (170 mg) of ApoMb with sodium in liquid ammonia. The reaction product was the same as that used in Figure 1. Two Sephadex G-75 columns (each 5.0 × 85 cm) were employed, connected in series, and eluted in the ascending direction with 0.1 N acetic acid. The effluents were monitored directly at 258 mμ with a Canalco double-beam flow analyzer. The diagram shows an example of a direct scan from the flow analyzer. After extensive purification each peak was found to correspond to the sequences indicated.

peptide was characterized and its purity confirmed by amino acid and N-terminal analysis. Table I shows the amino acid composition and N-terminal residue of each of the present peptides, together with their calculated molecular weights and their nitrogen contents. From the amino acid composition data and the known sequence of Mb (Edmundson, 1965), the most likely locations of these peptides were as follows (in the order of appearance from the Sephadex columns): sequence 1–36, sequence 1–87, sequence 37–119, sequence 37–87, and sequence 120–153. 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).

It is relevant to point out here that the elution position of peptide 1–36 is not that expected from its size and molecular weight indicated in Table I. Also, on the basis of size alone, resolution of peptides 1–87 and 37–119 would not be expected or, at best, should take place with considerable overlap. The fact that these two peptides were well resolved suggested considerable aggregation. On calibrated Sephadex G-200 columns (Atassi and Caruso, 1968), peptide 1–36 eluted in the void volume indicative of a molecular weight that exceeded 250×10^3. The elution position of peptide 1–87 suggested an effective molecular weight around 80,000. These results are discussed later (see Discussion).

Immunochemical Reactivities of the Peptides. PEPTIDE 1–36. This peptide gave a precipitate with all the three sera tested. However, due to the high degree of aggregation of this peptide and its low solubility it was necessary to confirm that the precipitate obtained with the antisera was in fact an immune precipitate. Control experiments were therefore carried out on the peptide under the same conditions as those used in the precipition reaction, except that in this case preimmune sera (*i.e.*, sera that were obtained from the animal before immunization) from goats G3 and G4 and from rabbit 77 were used. With each preimmune serum, peptide 1–36 gave a precipitate that accounted to 30–35% (depending on the serum) of the precipitate with the antiserum proper. An example of the precipitin curve, after correction for the contribution of the nonimmune precipitate (as determined by reaction with the preimmune serum), obtained with this peptide and antiserum G3 is shown in Figure 3. Further confirmation that the precipitate obtained with this peptide and each antiserum was a true immune precipitate was derived from absorption experiments. Upon absorption of each antiserum with an amount of peptide necessary to achieve maximum precipitation, the reactivity remaining in the supernatant toward MbX was lower than the homologous

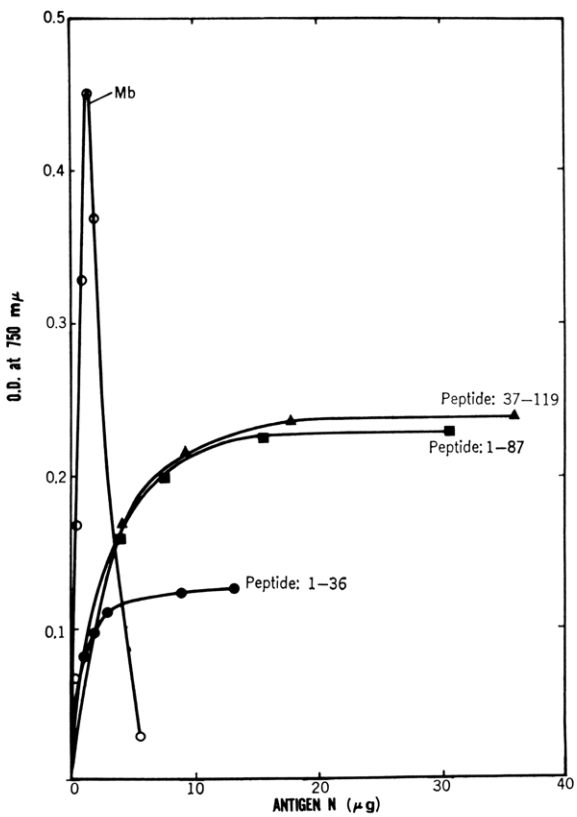

FIGURE 3: Precipitin analyses with goat antiserum G3 and MbX (○), peptide 1–36 (●), peptide 1–87 (■), and peptide 37–119 (▲). For experimental details, see text.

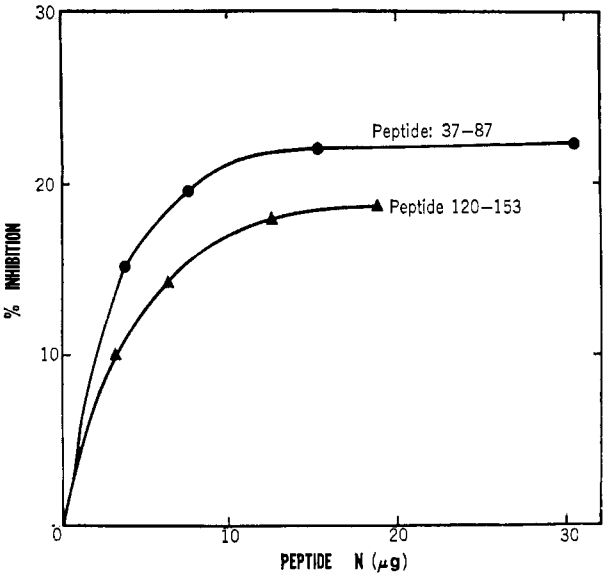

FIGURE 4: Inhibition experiments with antiserum G3 and peptides 120–153 (▲) and 37–87 (●).

reaction. The decrease was quantitatively equivalent to the amount of corrected immune precipitate brought down by the peptide at maximum. The reactivities of this peptide with antisera G3, G4, and 77 are summarized in Table II.

PEPTIDE 37–87. This peptide showed inhibitory activity with all the three antisera tested. Table III summarizes the inhibitory activity of this peptide toward MbX precipitin reaction with antisera G3, G4, and 77. The maximum per cent inhibition ranged from 12 (with rabbit antiserum 77) to 35% (with goat antiserum G4). The values of peptide to antigen molar ratio at 50% of maximum inhibition ranged only between 5 and 6. Figure 4 shows an example of the inhibitory activity of this peptide with antiserum G3.

PEPTIDE 1–87. This is an overlapping peptide that comprises the two previous peptides 1–36 and 37–87. Peptide 1–87 showed precipitating activity with all the antisera tested. A small amount of nonspecific precipitate (10%) was obtained with this peptide and each of the three preimmune sera. This nonimmune precipitation was subtracted from the total amount of immune precipitate with each antiserum to give the true amount of specific immune reactivity. The results shown in Table II have already been subjected to such correction. Figure 3 shows an example of a corrected precipitin curve for this peptide and antiserum G3. Also, the presence and extent of immune precipitate by this peptide was confirmed by absorption experiments. The amount of antibody nitrogen precipitated by MbX with each antiserum decreased by an amount quantitatively equivalent to the amount removed by the peptide (after correction for nonspecific precipitation as outlined above). Thus the reactivity of this peptide was quite high and ranged between 40 and 51% relative to reaction of antiserum with MbX.

PEPTIDE 37–119. This was also a precipitating peptide. It consistently showed the highest reactivity with each antiserum compared to the other two precipitating peptides 1–36 and 1–87. In this case control reactions with preimmune sera showed little nonspecific precipitation (5%). Quantitative absorption experiments also confirmed that the precipitate obtained with peptide and each antiserum was in fact a true immune precipitate. Figure 3 shows an example of the precipitin reaction of this peptide with antiserum G3. The curve has been corrected for the small amount (4.8%) of nonspecific precipitation as determined by reaction with preimmune serum from the same goat. The reactivity of this peptide with antisera G3, G4, and 77 is summarized in Table II. The reactivity of this peptide ranged between 49 (with antiserum 77) and 82% (with antiserum G4) relative to precipitation with MbX at equivalence.

PEPTIDE 120–153. With all the three sera tested this peptide showed appreciable inhibitory activity. The maximum per cent inhibition ranged between 14% (with G4) and 23% (with 77). The molar ratios of peptide to antigen at 50% of maximum inhibition ranged between 10 and 13. An example of an inhibition curve with antiserum G3 is shown in Figure 4 and Table III summarizes the activity of this peptide in per cent inhibition of the reaction of MbX with each of antisera G3, G4, and 77.

TABLE III: Maximum Inhibitory Activities of Peptides 37–87 and 120–153.[a]

Antiserum	Peptide 37–87	Peptide 120–153
G3	22.2 ± 1.2 (4.94)	18.8 ± 1.2 (9.90)
G4	34.7 ± 1.4 (5.03)	13.9 ± 0.9 (10.90)
77	11.8 ± 0.6 (5.88)	23.4 ± 1.3 (13.27)

[a] Results are expressed in maximum per cent inhibition by the peptide of the precipitin reaction of MbX with each antiserum. Values in parentheses represent peptide/antigen molar ratio at 50% maximum inhibition. Data were obtained from three independent determinations.

TABLE II: Relative Amounts of Precipitation Formed by Peptides 1–36, 1–87, 37–119.[a]

Antiserum[b]	% Ppt Rel to Reaction of Antiserum with MbX		
	Peptide 1–36	Peptide 1–87	Peptide 37–119
G3	28.2 ± 1.2	50.8 ± 1.4	52.8 ± 1.4
G4	5.0 ± 0.5	39.7 ± 1.3	82.3 ± 1.6
77	25.6 ± 1.1	39.4 ± 1.3	49.1 ± 1.4

[a] The percentage of precipitation relative to MbX was derived from maximum precipitation by peptide and precipitation at equivalence by MbX. Values were obtained from three independent determinations. [b] G3 and G4 are goat antisera and 77 is a rabbit antiserum, each to MbX.

Discussion

The specificity of cleavage and the presence of any other side reactions that will effect modification of amino acids within the cleavage products should be considered. Studies

of Benisek *et al.* (1967) have shown that specificity of cleavage at proline peptide bonds and the presence of side reactions depend on the amount of sodium used in the reaction and on the temperature and duration of the reaction. Reduction of insulin B chain (1000 molar excess of sodium, −78°, 180 sec) resulted in about 55% destruction of phenylalanine (in addition to cleavage at the proline bond). However, the authors did not observe this destruction of phenylalanine with apoferrodoxin or with insulin B chain under mild conditions (about 110 molar excess of sodium, −78°, 40 sec). Some small amount of nonspecific random cleavage of peptide bonds occurred when oxidized apoferrodoxin was treated with sodium in liquid ammonia under vigorous conditions (about 1400 molar excess of sodium, −33°, 15 min). On the other hand, nonspecific cleavage was not detected on reduction of insulin A chain under mild conditions (about 215 molar excess of sodium, −78°, 40 sec). While on reduction under vigorous conditions (682 molar excess of sodium, −33°, 15 min) resulted in appreciable cleavage at the peptide bond formed by the N-terminal amino acid. Under the conditions adopted in the present work for large scale preparation (600 molar excess, −33°, 210 sec) no destruction of phenylalanine was observed in any of the resultant fragments. Also no nonspecific cleavage penultimate to the N-terminal valine in Mb was detected. Homogeneity of the peptide fragments was confirmed by amino acid analysis, by peptide mapping on heavily loaded chromatograms, and finally by obtaining a true plateau with all the precipitating peptides in the region of antigen excess. The latter result would not have been possible had the immune precipitate been the result of contamination with undergraded antigen (Atassi and Saplin, 1968).

The N-terminal peptide 1–36 occupies two intact helices, A and B, and the bend between them in the three-dimensional model (Kendrew *et al.*, 1961). This peptide contains large segments of predominantly hydrophobic amino acids. In fact one of the insoluble core peptides obtained on tryptic digestion of Mb is derived from sequence 1–16 (Edmundson, 1963). The insolubility of region 1–16 will explain the aggregation of the larger peptide 1–36. It has already been shown (Atassi and Caruso, 1968) that tryptophan-7 is not located in an antigenic region in Mb. Also the N-terminal heptapeptide obtained by cleavage at tryptophan-7 either by periodate (Atassi, 1967) or by digestion with chymotrypsin (Crumpton and Wilkinson, 1965) is noninhibitory. Also fragment 8–153 was identical in reactivity with periodate-oxidized (but not cleaved at tryptophan-7) ApoMb (Atassi and Saplin, 1968). Therefore the reaction of peptide 1–36 is not due to the N-terminal heptapeptide. In addition, it has already been shown that arginines-31 and -45 are not present in a reactive region in Mb (Atassi and Thomas, 1969) and also peptide 35–42 is noninhibitory in Mb (Atassi and Saplin, 1968). The reactive region on peptide 1–36 can therefore be narrowed down to the region 7–30. In fact an active tryptic peptide comprising sequence 17–31 has already been reported (Atassi and Saplin, 1968). It is significant to mention here that the present data will give information concerning region 45–55 which has not yet been studied. It has already been shown that arginine-45 and methionine-55 (Atassi, 1967, 1969) are not part of a reactive region in Mb. However, since these two amino acids are separated by nine residues, it is conceivable that an antigenic reactive region might be located within that interval. This possibility is ruled out completely from the present data on the reactivity of peptide 1–36. Table IV summarizes the quantitative data for the precipitin reactions of peptides 1–36 and 1–55, the latter being obtained by cleavage at methionine-55, and has already been studied in detail (Atassi and Saplin, 1968). It can be seen that the two peptides possess identical reactivities with the three sera tested here. This result leads to the firm conclusion that the whole segment spanning sequence 37–55 constitutes an antigenically nonreactive region. Therefore, it can now be concluded that peptide 1–36 (and in fact peptide 1–55) carries one reactive region located around, but not necessarily including all of, sequence 17–30. This sequence occupies the bend AB and a small part of the two helices. Conclusions derived from the results on peptides 1–36 and 1–55 demonstrate the advantage of studying long and overlapping peptides. Further demonstration of the utility of this powerful approach was shown in the results from peptides 37–87, 1–87, and 37–119.

TABLE IV: Relative Amounts of Precipitation Formed by Peptides 1–36 and 1–55.

Antiserum	% Ppt Rel to Reaction of Antiserum with MbX[a]	
	Peptide 1–36[b]	Peptide 1–55
G3	28.2	29.1[c]
G4	5.0	8.2[d]
77	25.6	25.1[d]

[a] The percentage of precipitation relative to MbX was derived from maximum precipitation by peptide and precipitation at equivalence by MbX. [b] Values obtained from the present work (see Table II). [c] Value determined in the present work and representing the average of three analyses which ranged ±1.1%. [d] Values previously determined for these sera (Atassi and Thomas, 1969).

Peptide 37–87 carries the complete helices C, D, and E, and the bends between them, together with bend EF. This peptide showed appreciable inhibitory activity to reaction of each of the present antisera with MbX suggesting the presence on the peptide of region(s) with substantial antigenic reactivity. It has already been shown in the foregoing discussion that segment 37–55 is not in an antigenic reactive region. Therefore, the reactive region in peptide 37–87 must reside within sequence 56–87. An overlapping tryptic peptide from sequence 79–96 has been shown to possess inhibitory activity to the homologous reaction with one antiserum to MbX and one antiserum to ApoMb (Atassi and Saplin, 1968). A reactive region, therefore, will most likely be located toward the C-terminal end of peptide 37–87.

The reactive regions on peptide 1–87 should be those present in peptide 1–36 and peptide 37–87. Table V lists the reactions of peptides 1–36 and 37–87 with antisera G3, G4, and 77. For a given serum, the sum of the reactivities of peptides 1–36 and 37–87 agreed extremely well with the reactivity found experimentally for peptide 1–87. The data clearly confirm that peptide 1–87 carries no new reactive

TABLE V: Comparison of Relative Amounts of Activities of Peptides 1–36, 37–87, and 1–87.[a]

	% Act. Rel to Reaction of Antiserum with MbX			
Antiserum	A Peptide 1–36	B Peptide 37–87	A + B	Peptide 1–87
G3	28.2	22.2	50.4	50.8
G4	5.0	34.7	39.7	39.7
77	25.6	11.8	37.4	39.4

[a] Values of activities represent either per cent precipitation relative to homologous reaction (peptides 1–36 and 1–87, see Table II) or per cent inhibition (peptide 37–87, see Table III).

TABLE VII: Total of Activities of Fragments 1–36, 37–119, and 120–153.[a]

	Act. Rel to Reaction of Antiserum with MbX		
Peptide	Antiserum G3	Antiserum G4	Antiserum 77
1–36	28.2	5.0	25.6
37–119	52.8	82.3	49.1
120–153	18.8	13.9	23.4
Total	99.8	101.2	98.1

[a] Values of activities represent either per cent precipitation (for peptides 1–36 and 37–119, see Table II) relative to the homologous reaction, or per cent inhibition (for peptide 120–153, see Table III).

regions created by the joining of the two subfragments 1–36 and 37–87.

Since the reactivity of peptide 37–119 was appreciably greater than peptide 37–87 (see Tables II and III), it may be concluded that another reactive region is present within the sequence 88–119. This appears to possess substantial reactivity ranging from 30% (for G3) to 48% (for G4) relative to reaction of MbX with these sera. As previously mentioned, peptide 79–96 has been shown (Atassi and Saplin, 1968) to be inhibitory. The active region on this peptide, however, terminates before tyrosine-103 which is not located in an antigenic reactive region (Atassi, 1968). Therefore, another reactive region must be present after tyrosine-103. In fact arginine-118 has recently been shown to be part of a reactive region in Mb (Atassi and Thomas, 1969). Peptide 37–119 comprises the complete helices C, D, E, F, and G and the bends between them. Taking into account the above findings, it may be concluded that a reactive region is located on the bend EF. The second reactive region, C terminal to tyrosine-103 and which is situated around arginine-118, requires more work for further accurate delineation.

It appears from the high inhibitory activity of peptide 120–153 that a reactive region(s) exists within that peptide. It is relevant to point out here that, for a given serum, the inhibitory activity of this peptide is identical with that of the shorter peptide 132–153. Table VI gives a comparison of the inhibitory activities of the two peptides with antisera G3, G4, and 77. These results suggest that sequence 120–131 possesses no significant activity. Previously, it has been shown (Atassi and Saplin, 1968) that peptide 119–133, obtained by tryptic digestion of Mb, shows only some slight inhibitory activity of the precipitin reaction of Mb with one antiserum to Mb. In addition, it has also been shown that methionine-131 (Atassi, 1967, 1969) and arginine-139 (Atassi and Thomas, 1969) are not part of a reactive region in Mb. All these independent data point to the conclusion that sequence 120–139 does not carry a reactive region and activity of peptides 120–153 and 132–153 must reside within the sequence 140–153. However, the C-terminal dipeptide is not part of a reactive region (Atassi, 1966) but the C-terminal hexapeptide has some inhibitory activity (Crumpton, 1967; Atassi and Saplin, 1968) and nitration of tyrosines-146 and -151 in peptide 132–153 abolishes the inhibitory activity of this peptide completely (Atassi, 1968). It is therefore clear that in the sequence 120–153 only one reactive region exists and is located around tyrosine-146 and -151. It terminates at tyrosine-151 but is not clear whether it extends part of (or all) the way to lysine-140. This reactive region is located at the end of helix H and the beginning of the randomly folded C-terminal pentapeptide.

With each peptide, relative to MbX, a large amount of the peptide is required to achieve maximum reactivity (precipitation or inhibition). In the absence of contamination by small amounts of undegraded antigen, these observations can be explained only on a conformational basis. In solution, only a fraction of the free peptide may achieve a configuration that approaches that which it has in the native protein. Such a native configuration will be necessary in order that the peptide may gain appropriate fit onto the antibody combining site. Recent results from this laboratory (Singhal and Atassi, 1970) on the optical rotatory dispersion and circular dichroism behavior of these peptides have confirmed that the helical content of each of these free peptides in solution was only a fraction of the value the peptide possessed

TABLE VI: Comparison of the Inhibitory Activities of Peptides 120–153 and 132–153.

	Max. % Inhibn of Homologous Reaction	
Antiserum	Peptide 120–153[a]	Peptide 132–153[b]
G3	18.8	18.3
G4	13.9	13.5
77	23.4	20.5

[a] Values are obtained from the present work (see Table III). [b] Inhibitory activity of peptide 132–153 with these three sera has previously been determined (Atassi and Thomas, 1969).

in the native protein. Similar conclusions were also derived from the immunochemistry of the peptides obtained by cleavage at the methioninine sites (Atassi and Saplin, 1968) and these were confirmed by the conformational studies of Epand and Scheraga (1968).

When the total activities of peptides 1–36, 37–119, and 120–153 are summed up, disregarding the high molar excesses (relative to MbX) required to achieve maximum activity, the total accounts for the total reactivity of MbX. Table VII shows the total of the relative reactivities of these peptides with antisera G3, G4, and 77. This suggests that on cleavage of Mb at prolines-37 and -120, the reactive regions remained essentially intact. It may be pointed out here that the results do not yet exclude that two or more reactive regions from different segments might come together in the three-dimensional structure of the native molecule to be incorporated into a reactive site.[2]

In conclusion, by reaction with sodium in liquid ammonia, cleavage of ApoMb has been effected at prolines-37, -87, and -120. Overlapping peptides have been isolated and characterized. The immunochemistry of these peptides has been studied, yielding valuable information, especially concerning the regions of overlap. The results have been discussed in the light of previous findings from this laboratory on the antigenic structure of Mb.

Acknowledgments

The authors wish to thank Mrs. Q. Taqi and Mr. M. Loren for their technical assistance.

[2] The distinction between antigenic reactive *regions* and antigenic reactive *sites* is made here according to the definitions previously given (Atassi and Saplin, 1968).

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