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

R. P. Singhal† and M. Z. Atassi‡

ABSTRACT: Derivatives have been prepared by reaction of myoglobin with diketene or with tetrafluorosuccinic, maleic, or citraconic anhydrides. The reversibility of the masking groups in each of these derivatives was examined by testing the electrophoretic homogeneity of the reaction product, by determining the number of free amino groups, by extent of reversion to the native conformation, and by immunochemical methods. Of the reversible blocking reagents tested here, only derivatives obtained by reaction with citraconic anhydride gave satisfactory results. These derivatives gave on deblocking homogeneous preparations with complete recovery of free amino groups, immunochemical properties, and native conformation. Specific cleavage at arginine residues was accomplished by tryptic digestion of citraconylmyoglobin (CT-Mb). Three peptides comprising the sequences 1–31, 46–118, and 119–131 were isolated and characterized. The immunochemical reactivity of these peptides with antisera to MbX was also determined. Peptides 1–31 and 46–118 gave immune precipitates with these sera while peptide 119–131 showed some slight inhibitory activity. By comparison of the present results with our previously reported data on the antigenic structure of Mb, it was concluded that sequence 1–55 carries one antigenic reactive region located in (but not necessarily including all of) the segment 8–30. In the region 56–118, two reactive regions most likely exist and more work is in progress for their further more accurate delineation. The remainder of the molecule, *i.e.*, sequence 119–153, carries only one reactive region located around tyrosines-146 and -151.

In previous communications from this laboratory the specific immunochemical interactions of peptides, derived from Mb[1] by various procedures, have been investigated. Thus studies were carried out on peptides that were obtained by tryptic digestion of the protein, and by chemical cleavage at tryptophan or methionine residues (Atassi and Saplin, 1968) or at proline residues (Atassi and Singhal, 1970a). As a result of the chemical and enzymic cleavage procedures employed, the peptides studied comprised considerable regions of overlap and therefore yielded valuable information concerning the immunochemical reactivity of the regions of overlap. Specific chemical modification of selected amino acids in a reactive peptide (Atassi, 1968; Atassi and Thomas, 1969) has been employed for further delineation of reactive regions[2] on these peptides. This avoided problems often encountered (Atassi, 1968; Atassi and Saplin, 1968) when using the alternative approach of shortening the active peptide.

Reversible blocking of amino groups is a useful method for rendering hydrolysis with trypsin specific for cleavage at arginine residues. Several reversible blocking reagents for amino groups have been reported. However, a careful investigation of their applicability and comparison of their specificity, ease of removal, homogeneity of the blocked and deblocked derivatives, and changes in their conformation and their biological activities have not been carried out. In the present work, the reactions of Mb with diketene (Marzotto *et al.*, 1967, 1968), maleic anhydride (Butler *et al.*, 1967), tetrafluorosuccinic anhydride (Braunitzer *et al.*, 1968), or citraconic anhydride (Dixon and Perham, 1968) have been investigated. The specificity and reversibility of the reactions and the homogeneity of the reaction products were examined. The results showed that of the reagents studied here, citraconic anhydride was the most satisfactory, giving upon deblocking a homogeneous protein that was identical with native Mb, both in its immunochemistry and its conformational and hydrodynamic parameters. Specific cleavage at arginine residues was accomplished by tryptic digestion of CT-Mb. The isolation and purification of the resultant peptides are described together with their immunochemical properties.

Experimental Section

Materials. Diketene was from Aldrich Chemical Co.; maleic anhydride from Fischer Scientific Co.; tetrafluorosuccinic anhydride from K & K Laboratories, Inc.; and citraconic anhydride from Eastman Organic Chemicals. Trypsin which had been treated with tosylphenylalanine chloromethyl ketone to eliminate any chymotryptic activity (trypsin L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone, twice crystallized) was obtained from Worthington Biochemical Corp. Sperm whale myoglobin was the major chromatographic component 10 (MbX) obtained by CM-cellulose chromatography (Atassi, 1964). ApoMb was obtained from MbX by a procedure similar to that used for the preparation of apohemoglobin (Atassi and Skalski, 1969).

* From the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received December 11, 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.

† Present address: Biology Division, Oak Ridge National Laboratory, Post Office Box Y, Oak Ridge, Tenn. 37830.

‡ Established Investigator of the American Heart Association; to whom to address correspondence.

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

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

Reaction of Mb with Diketene and Removal of Blocking Groups. Conditions were similar to those described by Marzotto *et al.* (1968). In the present work, Mb (40 mg) was dissolved in water (5 ml) and the solution adjusted to pH 8.50. Aliquots (25 μl each) of diketene (freshly distilled twice under reduced pressure) were added at 15-min intervals over a period of 4 hr. The pH was maintained at 8.5–8.6 on the pH-Stat by the addition of 3 N NaOH. When the addition of diketene was complete (400 μl), reaction was allowed to continue for 2 more hr at pH 8.60 at room temperature, until alkali consumption ceased. Solution was then dialyzed extensively against distilled water and freeze-dried.

Removal of the acetoacetyl blocking groups was carried out at 25° in 2% hydroxylamine hydrochloride at pH 7.0.

Reaction with Tetrafluorosuccinic Anhydride and Removal of the Blocking Groups. Portions (30 mg) of MbX were reacted with tetrafluorosuccinic anhydride and the blocking groups were removed according to the procedure described by Braunitzer *et al.* (1968).

Reaction with Maleic Anhydride or with Citraconic Anhydride. A solution (50 ml) of MbX (1.0 g) in water was adjusted to pH 8.2 on the pH-Stat. To the magnetically stirred Mb solution, six aliquots (100 μl each) of citraconic anhydride were added at 20-min intervals and pH was maintained at 8.2–8.4 on the pH-Stat by the addition of 5 N NaOH. When the addition of citraconic anhydride was complete, the mixture was allowed to stir at room temperature for 2 hr. It was then dialyzed extensively against water, which had been adjusted to pH 8.5–8.8 with NH_4OH and thereafter freeze-dried. Freeze-dried samples were kept at −10° and did not show any removal of blocking groups after 1 year storage. Reaction with maleic anhydride was by a procedure described elsewhere for lysozyme (Habeeb and Atassi, 1970).

Tryptic Digestion of Citraconyl-Myoglobin and Separation of the Peptides. CT-Mb (1.00 g) was dissolved in water (200 ml) which had been preadjusted to pH 8.2. Hydrolysis with trypsin was carried out at 35°, under an atmosphere of nitrogen, and the pH was kept constant at 8.2 by the addition of 0.5 N NaOH on the pH-Stat. Digestion was started by the addition of 1 ml of 0.5% trypsin solution in 10^{-3} N HCl. A second 1-ml aliquot of enzyme solution was added 3.5 hr after the first addition. Hydrolysis was allowed to continue for 16 hr, after which the temperature of the digestion mixture was lowered to 20° and pH was brought down to 3.5 with 4 N HCl. The solution was left stirring at pH 3.5 and 20° for 6 hr and then freeze-dried.

Separation of the peptides resulting from the tryptic digestion of CT-Mb was carried out by gel filtration on Sephadex G-25, followed by filtration of the appropriate peaks on Sephadex G-75. Two columns (5.0 × 85 cm), connected in series, were used in each case and were eluted in the ascending direction with 0.1 N acetic acid. A portion (about 200 mg) of the tryptic digest of CT-Mb was dissolved in 0.1 N acetic acid (20 ml) and centrifuged and the clear supernatant applied into the base of one column. Elution was at 2–3°, at the rate of 130 ml/hr, and 13–14-ml fractions were collected. 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.

Immunochemical Methods. The preparation of antisera against MbX in goats and rabbits has already been described in detail (Atassi, 1967a). Antisera used in the present studies were goat antisera G3 and G4 and rabbit antiserum 77 and these were kept and studied separately and stored in 8-ml portions at −40°. Procedures for quantitative precipitin and inhibition experiments have already been described elsewhere (Atassi and Saplin, 1968). Concentrations of protein and peptide solutions were based on their nitrogen contents (see Table III).

TABLE I: Antigenic Reactivity of Mb Derivatives with Blocked or Deblocked Amino Groups.

Protein	% Reaction with Antiserum[a]		
	G3	G4	77
MbX	100	100	100
TFSu-Mb	0	2	0
ML-Mb	0	0	0
CT-Mb	0	0	0
Deblocked TFSu-Mb	60.2	61.0	61.3
Deblocked ML-Mb	92.8	93	91.6
Deblocked CT-Mb	100	99.5	101.0

[a] Values represent per cent precipitation at equivalence relative to reaction of MbX with these sera. G3 and G4 are two goat antisera and 77 is a rabbit antiserum, each to MbX.

Analytical Methods. Peptide mapping, spectral determinations, starch gel electrophoresis, and amino acid analysis of acid hydrolysates of peptides and proteins were done as described elsewhere in detail (Atassi and Saplin, 1968). Nitrogen determinations were carried out by a micro-Kjeldahl procedure and by using Nessler's reagent standardized with ammonium sulfate. Three replicate analyses were performed on each solution and they varied ±0.5%. Optical rotatory dispersion measurements were done in a Cary Model 60 spectropolarimeter, and results were treated as previously described (Atassi and Singhal, 1970a). 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 subtractive Edman degradation according to the procedure described by Konigsberg and Hill (1962).

Results

Blocked and Deblocked Derivatives Obtained by Reaction with Diketene or Tetrafluorosuccinic, Maleic, or Citraconic Anhydrides. Since several reagents are available for reversible blocking of amino groups, it was necessary to evaluate the suitability of these reagents with regard to specificity, ease of removal, homogeneity of the masked and unmasked derivatives, and change in their conformations and their biological activities. The specificity of the four reagents studied here has been investigated in detail elsewhere (Habeeb and Atassi, 1970). In the blocked derivatives no free amino groups that could react with fluorodinitrobenzene could be detected (Table II). It has already been shown (Habeeb and Atassi, 1970) that reaction with any of these reagents leads to modification of some hydroxyl groups as well as the complete modification of all the amino groups. This was not investigated here. Completely blocked derivatives migrated as single, very negatively charged bands on starch gel electrophoresis. Also, none of these derivatives exhibited any antigenic reactivity with antisera to MbX (Table I). Deblocking of acetoacetylated Mb (from reaction with diketene) for 16 hr resulted in

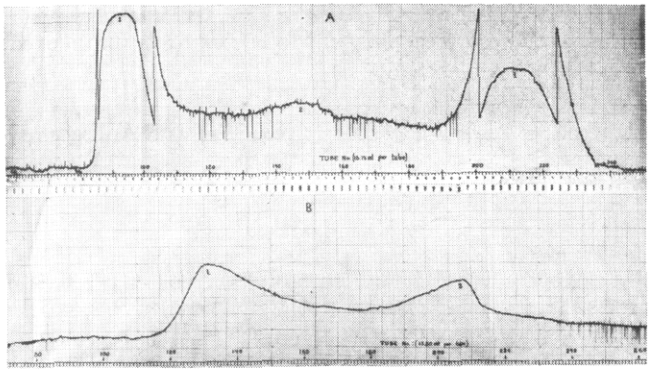

FIGURE 1: Gel filtration patterns of the tryptic hydrolysate of CT-Mb. Section A shows the filtration pattern of the tryptic hydrolysate of G-25. Section B shows the pattern of peak 2, from section A, on refiltration on Sephadex G-75. For experimental details see text.

TABLE II: Optical Rotatory Dispersion Parameters of Mb and Derivatives with Masked or Unmasked Amino Groups.

Protein	No. of Free Amino Groups	$[m']_{233}$	$[m']_{199}$	b_0
Mb	20	−9320	+46,360	−417
TFSu-Mb	0.2	−6700	+32,360	−301
ML-Mb	0.1	−6500	+31,400	−290
Deblocked TSFu-Mb	13.0	−7980	+38,540	−350
Deblocked ML-Mb	18.2	−8830	+42,650	−385
Deblocked CT-Mb	20.0	−9350	+46,500	−418

a preparation which had appreciable amounts of insoluble protein. The soluble fraction showed little or no reversion to native MbX when tested by starch gel electrophoresis. Only 10% of amino groups were deblocked and the antigenic reactivity of the soluble fraction with antisera to MbX was less than 10% relative to the homologeous reaction. Deblocking for 35 to 48 hr resulted in preparations that were completely insoluble and therefore were unsuitable for immunochemical studies.

Deblocking of TFSu-Mb for 4 days resulted in the unmasking of 65% of all amino groups. The preparation was extremely heterogeneous on starch gel electrophoresis with one band (amounting to about 30% of the total) migrating like MbX, and five additional bands were more negatively charged. The antigenic reactivity of the deblocked preparation with antisera to MbX was 61% (Table I). Reversion to the native Mb conformation was also not achieved as seen in Table II.

Maleylation seemed to offer a more promising approach. Deblocking for 92 hr in 0.2 N acetic acid at room temperature resulted in a preparation that had 91% of its amino groups free. On starch gel electrophoresis the preparation gave four bands; the major one, migrating like MbX, accounted for about 60% of the total. The three other bands were more negatively charged indicating incomplete demaleylation. The antigenic reactivity of the deblocked preparation with antisera to MbX was 93% (Table I). Its conformational parameters resembled, but were not identical with, those of MbX (Table II).

Deblocking of CT-Mb was carried out at pH 3.75 and 30°. Complete deblocking of amino groups was achieved within 4 hr. Starch gel electrophoresis of deblocked material gave a single band migrating like MbX. In heavily loaded gels a trace (1%) of a more negatively charged band with mobility of 2.2 (relative to MbX = 1) was obtained. The antigenic reactivity of the preparation with antisera to MbX was 100% relative to the homologous reaction (Table I). The conformational parameters of the deblocked preparation were identical with those of MbX (Table II). From the foregoing experiments it may be concluded that citraconic anhydride is the most satisfactory of the reversible amino group blocking reagents investigated here. Similar conclusions were obtained by Habeeb and Atassi (1970) in a very extensive and careful study using these reagents and employing lysozyme as the protein model. Therefore, citraconic anhydride was employed here for blocking of the amino groups in Mb followed by specific cleavage with trypsin at arginine peptide bonds and subsequent isolation and deblocking of the fragments.

Cleavage at Arginine Peptide Bonds and Separation of the Peptides. On tryptic digestion of CT-Mb at pH 8.2 (see Experimental Section) 4.38 moles of NaOH was consumed per mole of Mb. This suggested that cleavage might have taken place essentially at four peptide bonds. Reaction of the tryptic digest with fluorodinitrobenzene showed the presence of 4.60 free amino groups per mole of digested Mb. After deblocking at pH 3.5, 20° for 6 hr, the number of free amino groups was 25.1 groups/mole of digested Mb. This confirmed that the blocking groups had remained on the protein during hydrolysis with trypsin and that they were completely removed upon deblocking. Peptide mapping of the tryptic hydrolysate, which had been deblocked, showed six spots. In contrast, Mb controls consumed 18–19 mole of NaOH/mole of protein and peptide mapping of the tryptic hydrolysate showed at least 24 spots. These results suggested that cleavage at lysine peptide bonds was, most likely, arrested. N-Terminal determination on the deblocked tryptic digest showed that the following amino acids occupied amino-terminal positions: valine (N terminal of Mb), leucine, phenylalanine, histidine, and lysine. These were in equimolar proportions except for lysine which was in a molar ratio of 1.48. All these data suggested that a complete and virtually specific cleavage had taken place at arginine peptide bonds in Mb. Therefore, separation of the fragments was next carried out. Figure 1A shows the gel filtration pattern of the tryptic hydrolysate of CT-Mb on Sephadex G-25. Three peaks were obtained. Peak 3A was further purified by reapplication on the same column three times. Peaks 1A and 2A were applied on Sephadex G-75. Again, peak 1 (from G-25) emerged in the void volume on Sephadex G-75 and was purified by three reapplications on G-75 columns. Peak 2A was further resolved into two components on G-75 (*i.e.*, 1B and 2B) as shown in Figure 1B. Peaks 1B and 2B were purified by four reapplications on the G-75 columns. All these peptides were pure by peptide mapping. Reaction with fluorodinitrobenzene showed that the masking groups had been completely removed from all amino groups in each peptide (see Table III).

Amino acid analysis was carried out on these peptides and the results are shown in Table III. From the known amino acid sequence of Mb (Edmundson, 1965), the location of these peptides in the primary structure of Mb can be identified unequivocally. Peak 1A (which also emerged in the void volume around tube 90 in Figure 1B) corresponded to sequence 1–31. Peak 3A corresponded to sequence 148–

TABLE III: Amino Acid Composition, Molecular Weight, and Nitrogen Contents of Various Peptides from Apomyoglobin Cleaved at Arginine.[a]

Amino Acid	Peptide 119–139		Peptide 1–31		Peptide 46–118	
	Found	Calcd	Found	Calcd	Found	Calcd
Asp	2.85	3	1.95	2	0.90	1
Thr	0.11		0.13		3.78	4
Ser	0.15		0.88	1	3.82	4
Glu	2.10	2	5.15	5	8.05	8
Pro	0.93	1	0.08		2.12	2
Gly	3.04	3	3.12	3	3.23	3
Ala	4.07	4	3.20	3	8.10	8
Val	0.08		4.96	5	2.91	3
Met	0.90	1	0.13		1.11	1
Ile	0.12		1.90	2	5.85	6
Leu	1.91	2	3.86	4	9.20	9
Tyr	1.10		0.08		0.98	1
Phe	1.97	2	0.16		1.88	2
Lys	0.87	1	0.95	1	11.93	12
His	0.91	1	1.90	2	8.07	8
Arg	1.06	1	1.12	1	0.95	1
Trp			*b*	2		
Total number of residues		21		31		73
N Terminal[c]	His (0.91)	His	Val (0.95)	Val	Phe (0.93)	Phe
Free amino groups	1.96	2	2.03	2	12.95	13
Molecular weight	2245		3470		8228	
Per cent N content	18.72		17.36		17.87	

[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 for 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.

153 and must have arisen from cleavage at lysine-147 with trypsin. This suggests that either lysine-147 was not modified by reaction with citraconic anhydride or it was more easily deblocked. Peak 1B corresponded to sequence 46–118 and peak 2B corresponded to sequence 119–139. Peptide 32–45 was not located or isolated probably because it does not possess any ultraviolet absorption as might be expected from its sequence. The C-terminal arginine peptide 140–153 has already been shown above to have suffered cleavage at lysine-147. Peptide 140–147 was not located and will be expected to have very little ultraviolet absorption.

It is relevant to mention here that the position of peptide 1–31 in the elution pattern from Sephadex G-75 or even G-25 was not expected from its size. Thus, this peptide which contains 31 amino acid residues should not elute before peptide 46–118 (73 amino acids). This observation suggested to us that peptide 1–31 was highly aggregated. On calibrated (Atassi and Caruso, 1968) Sephadex G-200 columns, peptide 1–31 eluted in the void volume indicative of a molecular weight that exceeded 250×10^3. These results are discussed later.

Immunochemical Reactivities of the Peptides. PEPTIDE 1–31. This peptide gave a precipitate with all the three sera tested here. However, since this peptide exhibited a high degree of aggregation and low solutility, it was necessary to confirm that the precipitate obtained with each antiserum was a true immune precipitate and did not arise from nonspecific precipitation. This was clarified by control experiments in which the peptides were reacted with sera withdrawn from each animal before immunization with MbX (*i.e.*, preimmune sera) A precipitate was in fact obtained with each preimmune serum and peptide 1–31 which accounted to 20–25% (depending on the serum) relative to reaction of the peptide with the respective antiserum proper. The presence and amount of the immune precipitate given by peptide 1–31 were further confirmed by absorption experiments. Upon absorption of each antiserum with an amount of peptide necessary to give maximum precipitation, the reactivity of the serum supernatant toward MbX was suppressed relative to the unabsorbed serum. The decrease was quantitatively equivalent to the amount of corrected immune precipitate brought down by peptide 1–31 at maximum. Figure 2 shows reaction of this peptide (after correction for nonspecific precipitate) with antiserum G3, and the reactivities with antisera G3, G4, and 77 are listed in Table IV.

PEPTIDE 46–118. This was also a precipitating peptide. Control experiments with preimmune sera showed only little (5–8%) nonspecific precipitation. Quantitative absorption experiments showed that this peptide removed an amount of serum reactivity with MbX quantitatively equivalent to the amount of corrected immune precipitate. Figure 2 shows an example of reaction of this peptide with antiserum G3, after correction for the small amount (6% at maximum) of precipitate obtained with the preimmune serum from the same goat. The reactivity of this peptide with antisera G3, G4, and 77 is summarized in Table IV. It can be seen that this peptide possessed strong reactivity ranging between 44% (with anti-

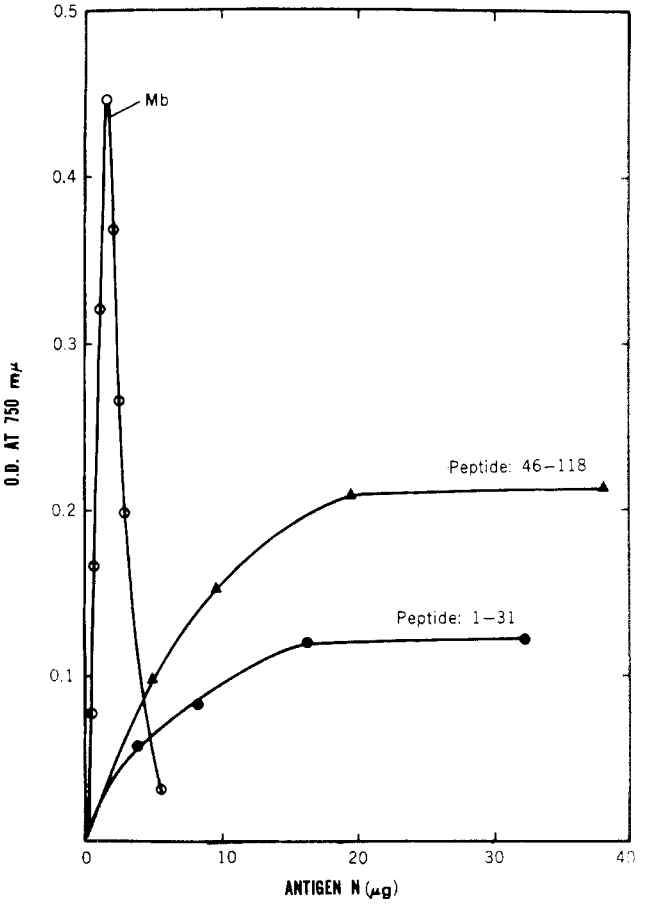

FIGURE 2: Quantitative precipitin analyses of MbX (○) and peptides 1–31 (●) and 46–118 (▲) with antiserum G3. For assay, precipitates were dissolved in 0.5 ml of 0.5 N NaOH and then determined with the Folin–Lowry method (Lowry *et al.*, 1951).

serum 77) and 74% (with antiserum G4) relative to precipitation with MbX at equivalence.

PEPTIDE 119–131. With all the sera tested, this peptide showed only some slight inhibitory activity. The maximum per cent inhibition ranged between 3% (with antiserum G4) and 5% (with antiserum G3). The molar ratios of peptide to antigen at 50% of maximum inhibition ranged between 26 and 51. The results of the inhibitory activity of this peptide with antisera G3, G4, and 77 are summarized in Table V.

TABLE IV: Relative Amounts of Precipitation Formed by Peptides 1–31 and 46–118 with Various Myoglobin Antisera.

Antiserum	% Ppt Rel to Reaction of Antiserum with MbX[a]	
	Peptide 1–31	Peptide 46–118
G3	27.6 (6.3)	47.6 (12.8)
G4	5.6 (9.7)	74.1 (2.3)
77	25.6 (8.6)	44.1 (13.6)

[a] The values of per cent precipitation of peptide are relative to reaction of each antiserum with MbX and are based on the total nitrogen values in the precipitates at maximum precipitation. Values represent the average of three analyses and varied ±1.3% or better. Values in parentheses represent the molar ratios of peptides to antigen at their precipitin maxima.

TABLE V: Inhibitory Activity of Peptide 119–139.

Antiserum[b]	Max. Inhibit Act.[a]	Molar Ratio at 50% Max. Inhibn[c]
G3	4.7	25.7
G4	3.3	50.9
77	3.9	33.7

[a] Results are expressed in maximum per cent inhibition of the precipitin reaction of MbX by the peptide. [b] See Table I. [c] Values represent peptide to antigen molar ratio at 50% of the maximum inhibition.

Discussion

Complete reaction of the amino groups was obtained with each of the reagents used here. Accessibility of the lysine residues in Mb has also been demonstrated by acetylation (Atassi, 1966). On the other hand, with 3,3-tetramethyleneglutaric anhydride, three lysine residues were not accessible to this reagent (Atassi, 1967b). Habeeb and Atassi (1970) recently showed that reaction with succinic, maleic, tetrafluorosuccinic, or citraconic anhydride or reaction with diketene leads to modification of some hydroxyl groups together with complete modification of the amino groups. Derivatives obtained by reaction of Mb with each of the present reagents showed appreciable conformational changes which were also accompanied by complete loss of antigenic reactivity.

Useful information may be obtained from the present work concerning the reversibility of the removable masking groups. Deblocking of acetoacetylated Mb (from reaction with diketene) gave preparations that were only slightly soluble and the soluble fraction showed appreciable heterogeneity on starch gel electrophoresis with little or no reversion to MbX. This, coupled with the finding that only partial deblocking (10%) of the amino groups and only a small recovery of antigenic reactivity were achieved, made this reagent completely unsuitable for the present immunochemical studies. Deblocking of TFSu-Mb gave preparations that were extremely heterogeneous and showed only partial unmasking (65%) of all amino groups. Also, since reversion to native conformation and complete recovery of antigenic reactivity were not achieved, this reagent was again unsuitable for immunochemical studies. Similarly, the results obtained on deblocking of ML-Mb showed that, although maleylation presented a substantial improvement, it was not entirely satisfactory. The deblocked preparation was heterogeneous and showed incomplete unmasking (91%) of its total amino groups and its antigenic reactivity was lower (93%) relative to the homologous antigen. Freedman *et al.* (1968) showed that a completely maleylated anti-*p*-azobenzenarsonate antibody recovered 88% of the free amino groups on demaleylation for 4 days. However, heterogeneity of the deblocked antibody was not investigated and although recovery of combining sites was complete, the average binding constant was only restored to 72% of its original value. Also, studies with maleylated lysozyme (Habeeb and Atassi, 1970) showed that removal of the

maleyl blocking groups resulted in a grossly electrophoretically heterogeneous preparation, with recovery of 90% of free amino groups, 83% of enzymic activity, and almost all of the reactivity with antisera to lysozyme.

In contrast with the foregoing reagents, derivatives prepared by reaction of Mb with citraconic anhydride gave on deblocking homogeneous preparations with complete (100%) recovery of free amino groups, immunochemical properties, and native conformation.

The usefulness of a reversible reagent depends on the ease of complete removal of the masking groups under conditions that will not lead to denaturation, the homogeneity of the deblocked preparation, and extent of recovery of the biological properties and reversion to native conformation. From the present work it is apparent that these criteria were satisfied only by citraconic anhydride derivatives. It is extremely likely that these conclusions are of wide applicability and are not confined to Mb, since recent extensive investigations with lysozyme (Habeed and Atassi, 1970) gave identical results.

Peptides were isolated by repeated (at least three times) gel filtration until single symmetrical peaks were obtained. The purity of each peptide fragment was confirmed by peptide mapping on heavily loaded chromatograms, by amino acid and N-terminal analyses, and, with the precipitating peptides, by obtaining a true plateau in the region of antigen excess. This would not have been possible if the immune precipitate were caused by some contamination with undegraded antigen (Atassi and Saplin, 1968).

Peptide 1–31 occupies, in the three dimensional structure of the intact protein (Kendrew *et al.*, 1961), all of helix A and part of helix B and the bend AB. This peptide aggregates greatly in solution. It carries large segments which contain predominantly hydrophobic amino acids. In fact, the segment 1–16 is one of the insoluble "core" peptides obtained by tryptic hydrolysis of Mb (Edmundson, 1963). The insolubility of segment 1–16 will explain the aggregation of the longer peptide 1–31. By comparison to previous work, the antigenic reactive region(s) on peptide 1–31 can be further delineated. It has already been shown that tryptophan-7 is not located in a reactive region (Atassi and Caruso, 1968). Also, the N-terminal heptapeptide, isolated by cleavage at tryptophan-7 with periodate (Atassi, 1967c) or by chymotryptic digestion (Crumpton and Wilkinson, 1965), is noninhibitory. In addition, fragment 8–153 was identical in antigenic reactivity with periodate-oxidized (but not cleaved at tryptophan-7) ApoMb (Atassi and Saplin, 1968). Therefore, there is strong evidence that the reactive region on peptide 1–31 is located within the sequence 8–31. In fact an active tryptic peptide from Mb comprising the sequence 17–31 has previously been reported (Atassi and Saplin, 1968). It is also highly significant that the results with peptide 1–31 give information concerning the reactivity of region 32–55. It has already been shown (Atassi and Thomas, 1969) that arginines-31 and -45 are not located in antigenic reactive regions. Similarly, methionine-55 is not in a reactive region (Atassi, 1967a, 1969). However, the gaps between residues 31, 45, and 55 might be sufficiently long so that a reactive region might conceivably exist within these gaps. Such possibility is ruled out completely from previous results and the present data on peptide 1–31. Peptide 35–42 has already been shown to be noninhibitory (Atassi and Saplin, 1968). Table VI compares the relative amounts of quantitative immune precipitates formed by peptides 1–31, 1–36, and 1–55 with three different antisera. It can be seen that, with a given antiserum, the three peptides possessed equal reactivities confirming that the entire segment between residues 31 and 55 is nonreactive. Therefore, it can be concluded that peptide 1–31 (and in fact the whole sequence 1–55) carries one reactive region located around, but need not necessarily include the entire length of, sequence 17–30. This sequence occupies the bend AB and parts of helices A and B. Results obtained with peptides 1–31, 1–36, and 1–55 demonstrate the advantage of studying long and overlapping peptides as a powerful tool for delineation of reactive regions. However, it must be cautioned that problems are often encountered on shortening the active peptide (Atassi and Saplin, 1968; Atassi, 1968) and the results should be interpreted with care if changes in activity are observed upon shortening of the active peptide.

TABLE VI: Relative Amounts of Immune Precipitation Formed by Peptides 1–31, 1–36, and 1–55.

Antiserum	% Ppt Rel to Reaction of Antiserum with MbX[a]		
	Peptide 1–31[b]	Peptide 1–36[c]	Peptide 1–55
G3	27.6	28.2	29.1[c]
G4	5.6	5.0	8.2[d]
77	25.0	25.6	25.1[d]

[a] The percentage of precipitation relative to MbX was derived, for each antiserum, from maximum precipitation by peptide and precipitation at equivalence by MbX. [b] Values obtained from the present work (see Table IV). [c] Obtained from Atassi and Singhal (1970). [d] Values have been determined elsewhere (Atassi and Thomas, 1969).

Peptide 46–118 occupies, in the intact protein, part of the nonhelical segment CD, all of helices D, E, and F, and the bends between them. This peptide gave immune precipitates with antisera to MbX and the extent of its reaction with these sera was substantial ranging from 44% (for rabbit antiserum 77) to 74% (with antiserum G4). It has already been shown (Atassi and Saplin, 1968) that in this region, peptide 79–96 possesses inhibitory activity. Also, arginine-118 is part of an antigenic region in Mb (Atassi and Thomas, 1969). On the other hand, tyrosine-103 is not located in a reactive region (Atassi, 1968). Peptide 46–118, therefore, most likely carries two reactive regions. One is located around the sequence 79–96 and terminates before tyrosine-103. The second reactive region, which starts after tyrosine-103 and is situated around arginine-118, requires more work for further delineation.

Peptide 119–139 comprises, in the native protein, all the nonhelical segment GH and a portion of helix H. This peptide showed only little or no inhibitory activity and is, most likely, inactive in the native protein. In support of this conclusion is the recent finding that peptides 120–153 and 132–153 exhibit equal inhibitory activities (Atassi and Singhal, 1970a). Also, it has been recently demonstrated that arginine-139 (Atassi and Thomas, 1969) and methionine-131 (Atassi, 1967a, 1969) were not part of an antigenic site in Mb. It may therefore be concluded that in the whole sequence 120–153 only one reactive region occurs after arginine-139 and is located around tyrosines-146 and -151 (Atassi, 1968) but does not include the C-terminal dipeptide (Atassi, 1966).

In order to achieve maximum reactivity (precipitation or inhibition) for each peptide, a large amount of the latter is required, relative to MbX. This can be rationalized on a conformational basis, suggesting that only a fraction of the pep-

tide in solution will approach the mode of folding it possesses in the intact protein. The native mode of folding is necessary for appropriate fit onto the antibody combining site (Atassi, 1967d; Atassi and Thomas, 1969). The unfolding of the peptides has been confirmed by our conformational studies on these peptides using optical rotatory dispersion and circular dichroism measurements (Atassi and Singhal, 1970b). Similar conclusions were also derived from studies on peptides obtained by cleavage at the methionine sites (Atassi and Saplin, 1968) and recently by cleavage at the proline sites (Singhal and Atassi, 1970). It is noteworthy that peptide 1–31 which forms an immune precipitate carries only one antigenic reactive region. The formation of the immune precipitate is promoted by the aggregation of this peptide.

In conclusion, the results of the present work show that of the reversible masking reagents for amino groups studied here, only derivatives obtained by reaction with citraconic anhydride gave satisfactory results. Specific cleavage at arginine sites has been obtained by acylation of the amino groups prior to tryptic hydrolysis. After cleavage, the amino groups were quantitatively unmasked yielding unmodified peptides. The immunochemistry of these peptides has been studied and the information obtained was quite valuable for further delineation of reactive regions in Mb, especially within the regions of overlap with previously studied peptides. The results have, therefore, been discussed in the light of previous data obtained from this laboratory on the antigenic structure of Mb.

Acknowledgments

The authors thank Mrs. Marie Perlstein for her expert technical assistance.

References

Atassi, M. Z. (1964), *Nature (London) 209*, 496.
Atassi, M. Z. (1966), *Nature (London) 209*, 1209.
Atassi, M. Z. (1967a), *Biochem. J. 102*, 478.
Atassi, M. Z. (1967b), *Biochem. J. 102*, 488.
Atassi, M. Z. (1967c), *Arch. Biochem. Biophys. 120*, 56.
Atassi, M. Z. (1967d), *Biochem. J. 103*, 29.
Atassi, M. Z. (1968), *Biochemistry 7*, 3078.
Atassi, M. Z. (1969), *Immunochemistry 6*, 801.
Atassi, M. Z., and Caruso, D. R. (1968), *Biochemistry 7*, 699.
Atassi, M. Z., and Saplin, B. J. (1968), *Biochemistry 7*, 688.
Atassi, M. Z., and Singhal, R. P. (1970a), *Biochemistry 9*, 3854.
Atassi, M. Z., and Singhal, R. P. (1970b), *J. Biol. Chem. 245*, 5122.
Atassi, M. Z., and Skalski, D. J. (1969), *Immunochemistry 6*, 25.
Atassi, M. Z., and Thomas, A. V. (1969), *Biochemistry 8*, 3385.
Butler, P. J. G., Harris, J. I., Hartley, B. S., and Liberman, R. (1967), *Biochem. J. 103*, 78P.
Braunitzer, G., Beyreuther, K., Fujiki, H., and Schrank, B. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 5*, 265.
Crumpton, M. J., and Wilkinson, J. M. (1965), *Biochem. J. 94*, 545.
Dixon, H. B. F., and Perham, R. N. (1968), *Biochem. J. 109*, 312.
Edmundson, A. B. (1963), *Nature (London) 198*, 394.
Edmundson, A. B. (1965), *Nature (London) 205*, 883.
Freedman, M. H., Grossberg, A. L., and Pressman, D. (1968), *Biochemistry 7*, 1941.
Habeeb, A. F. S. A., and Atassi, M. Z. (1970), *Biochemistry 9*, 4939.
Kendrew, J. C., Watson, H. C., Strandberg, B. E., Dickerson, R. E., Phillips, D. C,. and Shore, V. C. (9161), *Nature (London) 190*, 666.
Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 3157.
Lowry, O. H., Rosebrough, N. J., and Randall, R. J. (1951), *J. Biol. Chem. 237*, 2493.
Marzotto, A., Pajetta, P., Galzigna, L., and Scoffone, E. (1968), *Biochim. Biophys. Acta 154*, 450.
Marzotto, A., Pajatta, P., and Scoffone, E. (1967), *Biochem. Biophys. Res. Commun. 26*, 517.
Singhal, R. P., and Atassi, M. Z. (1970), *Biochemistry 9*, 4252.