THE ANTIGENIC STRUCTURE OF MYOGLOBIN AND INITIAL CONSEQUENCES OF ITS PRECISE DETERMINATION

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INTRODUCTION*

Knowledge of the molecular features responsible for the antigenicity of certain parts of native protein molecules lies at the basis of understanding in molecular terms the cellular events of the immune response. The majority of antigens associated with many immunological disorders are proteins, and therefore, defining the antigenic sites of these protein antigens will be critical for molecular elucidation of the mechanisms of these disorders. From a purely chemical perspective, the interaction between protein antigens and their antibodies and the elegant specificity of this recognition phenomenon remains one of the most fascinating and challenging frontiers in biochemistry.

Progress in this field has been very slow, and the elucidation of the entire antigenic structure of a protein had frustrated many attempts. Considerable chemical and technical factors were responsible for the slow progress in this field, and these have already been discussed in detail.[1,2] Determination of the antigenic structure of a protein is primarily a chemical challenge of immense proportions.[1,2] In retrospect, protein chemistry was not sufficiently advanced in the early 1960s to undertake the precise definition of the antigenic sites of a protein. In fact, we have often found it necessary, during the course of our studies on the antigenic structure of myoglobin (Mb) and lysozyme, to devise new chemical modification and cleavage reactions, as well as other approaches, in order to surmount certain obstacles. In addition, conceptual problems may have inadvertently made such an attempt appear unnecessary since it was believed[3] that "antigenic determinants of protein antigens were relatively large."

In 1975, I reported[1] the first precise determination of the entire antigenic structure

* Abbreviations: ApoMb, apomyoglobin; Mb or MbX, metmyoglobin, used here to imply the major chromatographic component No. 10 obtained from crystalline sperm whale Mb by CM-cellulose chromatography;[8] Hb, the major chromatographic component of human adult hemoglobin.

of a protein which was that of a sperm whale Mb. This represented the culmination of intensive research over an 11-year period. When the work commenced, little if anything was known about antigenic sites in proteins in relation to their shape, number, location, distribution, structure, and whether their boundaries will be diffuse or well defined in discrete structural locations for a given protein. The extent of dependency, if any, of the structure and location of an antigenic site on the immunized species was not known. Moreover, the role of conformation of a native protein antigen and the interrelationship of the primary and three-dimensional features in antigenicity were unknown. It was not clear whether the immune response was directed against the intact protein or against fragments thereof subsequent to its in vivo proteolysis. Also, degrees of immunogenicity were often assigned to various amino acid side chains by extrapolation from amino acid polymers. In addition, the molecular features responsible for the immunochemical cross-reaction of proteins were little understood. Our determination of the antigenic structure of Mb (and very recently that of lysozyme) has answered these and many other questions with surprising accuracy. Many of the observations and findings first made from our Mb work[1,4] have since become established concepts that have been confirmed with a variety of other proteins. However, it is necessary to stress here the caution that this is a very complex field, and hence, proper understanding of the immunochemistry of proteins requires the elucidation of the complete antigenic structures of a few native proteins.[4] Already, our precise determination of the entire antigenic structure of lysozyme[5] has shown fascinating differences between these two proteins.

Sperm whale Mb was chosen as our first model because when we started early in 1963, it was the only protein whose three-dimensional structure was completely determined,[6] and its amino acid sequence was soon to be completed.[7] It is a protein composed of 153 amino acid residues linked in a single polypeptide chain which is folded into a highly helical structure and which carries a single heme group. All our studies have been performed on the major chromatographic component No. 10 which we isolated[8] from the crystalline protein. This component was homogeneous by starch gel, polyacrylamide gel, and disc electrophoresis. The antisera employed were raised in goats or in rabbits and were early course antisera obtained from a single bleeding via the same immunization schedule.[9] The antisera from the individual animals were not mixed, but were studied separately.

PRECISE DETERMINATION OF THE ENTIRE ANTIGENIC STRUCTURE

I had considered at the outset that, due to the inherent limitations of each chemical approach, the antigenic structure of a protein cannot be deduced by the exclusive application of a single chemical approach. Our strategy, in fact, relied on five approaches[4] which first enabled us to achieve the precise determination of the entire antigenic structure of Mb[1] and which we subsequently found to be equally effective in scoring a similar achievement on lysozyme.[5] These approaches were

1. To study the effect of conformational changes on the immunochemistry of the protein
2. To study the immunochemistry and conformation of chemical derivatives of Mb specifically modified at appropriate amino acid locations
3. To isolate and characterize immunochemically reactive fragments that can quantitatively account for the total immunochemical reaction of the native protein
4. To study the effect of chemical modification of selected amino acid locations on the immunochemistry and conformation of immunochemically reactive peptides

5. After hopefully narrowing down each of the antigenic sites by approaches (1 to 4) to a conveniently small region, the final delineation would rely on studying the immunochemistry of synthetic peptides corresponding to many overlaps around this region.

It is critical to note that each of these chemical approaches has very serious shortcomings. The application, usefulness, and shortcomings of these approaches to protein immunochemistry have recently been discussed in considerable detail.[1,2,10] It is also necessary to stress here that none of these approaches by itself is capable of yielding the full antigenic structure. We invariably used the results from one approach to confirm and correct those from the others. The complete structure is a composite, logical coordination of all the information.

The extent of influence of conformational factors on the immunochemical behavior of Mb was considered early in our work.[11] The ideal situation for studying this effect is to devise derivatives in which conformational changes are intentionally inflicted upon the protein and in a manner that does not covalently alter an antigenic site. It was shown that the heme group is not part of an antigenic site in Mb and hemoglobin.[12,13] The heme group may be removed from the hemoprotein causing a conformational as well as an immunochemical change. However, reconstitution of the apoprotein with heme yields a Mb preparation which is immunochemically identical with the native protein.[11] Artificial myoglobins were therefore prepared[11] by recombination of ApoMb with various metalloporphyrins and modified hemes[11,14] and their immunochemistry and conformation studied. Except for Cu-Mb, immunochemical differences were observed between the derivatives and the native protein.[11] On the basis of the coordination tendencies of the various metals, we predicted[11] conformational changes in all the artificial Mb prepared with metalloporphyrins, except for Cu-Mb. These conformational alterations, as well as those imposed by the modification of the side chains of the heme, were subsequently confirmed by exhaustive optical rotary dispersion and circular dichroism measurements.[15] The excellent agreement between the immunochemical results and the conformational measurements strongly indicated that conformational changes in a protein can influence its antigenic reactivity,[11,15] since in these derivatives the modification was clearly outside an antigenic site. It is noteworthy that our studies[16] on artificial, human hemoglobins prepared with various modified and metalloporphyrins showed changes in immunochemical reactivity which were in excellent agreement with the conformational measurements.

It is relevant to point out in this connection that our subsequent studies with lysozyme[17-19] and with Mb[20,21] demonstrated that conformational changes will not always influence the immunochemical properties. The effect will rather depend on the protein and on the nature and extent of the conformational change.[17-19] The results with artifical Mb derivatives demonstrated that, under appropriate conditions, immunochemical methods may be employed as a powerful tool to monitor conformational changes in proteins.[11]

Immunochemical studies on homogeneous, specifically modified protein derivatives which suffer no conformational changes will yield valuable information on delineation of antigenic sites.[9,22-25] Many critical precautions have to be observed and have been discussed previously in detail.[1,2,10] Chemical modification reactions of proteins have been extensively reviewed very recently.[10]

Mb was subjected to a large number of chemical modifications in our laboratories. Figure 1 shows schematically the locations of the modified residues in the various Mb derivatives. At least 29 well-purified and characterized derivatives, modified between them at a total of 23 amino acid locations, have so far been prepared (all for the first time) and their conformation and immunochemistry studied (Table 1). The effective-

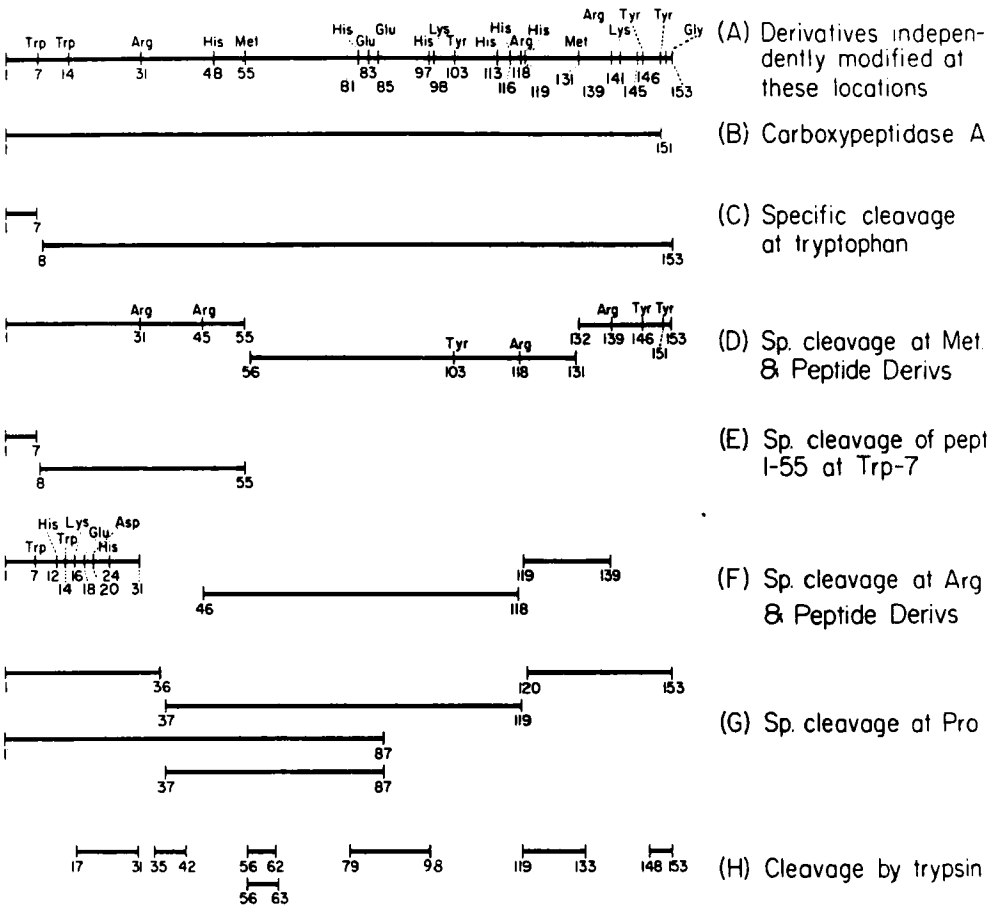

FIGURE 1. Schematic diagram showing the amino acid locations that we have modified in different derivatives of Mb (i.e., the residues indicated were not all modified in one derivative). These represent 29 well-purified and well-characterized derivatives, modified between them at a total of 23 amino acid locations, and whose immunochemistry and conformations were studied in detail (heme derivatives are not shown here). The figure also shows schematically the peptides isolated and studied in our work. Before cleavage at the methionine[26], tryptophan[27], or arginine[28] peptide bonds, we first carefully determined[9,22,23,30] that, except for Arg-118, these residues were not located in antigenic regions in Mb. Cleavage at these locations would not be expected, therefore, to rupture any antigenic regions. In fact, the total of the antigenic reactivities of peptides 1-55, 56-131, and 132-153 accounted for all (95 to 105% of the immunochemical reaction of Mb.[2,24] Also, the total reaction of peptides 1-36, 37-119, and 120-153 accounted for 98 to 101% of the immune reaction of Mb.[29] Delineation of antigenic regions in immunochemically reactive peptides was achieved by specific chemical modifications of the amino acid locations indicated for a given peptide in different derivatives (i.e., not all at once). (From Atassi, M. Z., *Immunochemistry*, 12, 423, 1975. With permission.)

ness of the chemical modification approach often provided us with the necessity to devise new, specific chemical modification reactions for the solution of certain problems.

It is not possible to derive the complete antigenic structure of a protein by employing chemical modification studies alone. The approach is extremely time-consuming and will yield, if applied systematically, a broad type of delineation deduced from the involvement or otherwise of certain residues in the antigenic sites. Furthermore, the approach is limited by the inavailability of chemical modification reactions for all the amino acids and by the inability to modify at will those amino acids which can participate in chemical reactions. A supporting approach in the study of protein antigens is based on the likely ability of an intact antigenic reactive region to interact with an

TABLE 1

Summary of Immunochemical Behavior of Mb Derivatives Chemically Modified at Certain Amino Locations[a]

Reaction	Residues modified	Immunochemical change[b]	Conformational change	Conclusion[c]		Ref.
				Residues in antigenic region	Residues not in antigenic region	
Periodate oxidation, 2 molar excess, pH 5, 0°C	Met-55 and -131	None	None		Met-55 and -131	9
Carboxyethylation: β-propiolactone, pH 3.0, 0°	Met-55 and -131	None	None		Met-55 and -131	30, 86
2-Hydroxy-5-nitrobenzyl bromide	Trp-7	None	None		Trp-7	22
	Trp-7 and -14	Large decrease	Large	Conclusions not possible		
Tetranitromethane, pH 8, 22°C	Tyr-103, -146, and -151	Reaction decreases 14—20%	None	Tyr-146 and -151	Tyr-103	33
Cyclohexanedione	Arg-31 or -139	None	None		Arg-31 or -139	23
	Arg-31 and -139	None	None		Arg-31 and -139	
	Arg-31, -118, and -139	Rn decreases 18—30%	None	Arg-118	Arg-31 and -139	
Acetic anhydride	All - NH_2 groups	Reactivity abolished	Large	Conclusions not possible		44
3,3-Tetramethylene glutaric anhydride						
55—60 molar excess, 0°C	16 Lysine residues	Reactivity abolished	Large	Conclusions not possible		87
10 molar excess, 0°C	Lys-98	Rn decreases 14—21%	None	Lys-98		20
	Lys-98 and -140	Rn decreases 18—19%	None	Lys-98	Lys-140	20
	Lys-98, -140, and -145	Rn decreases 15—20%	Very small	Lys-98	Lys-140 and -145	20
Reduction by diborane, −10°C	Glu-83 and -85	None	None		Glu-83 and -85	38, 88, 89
Activation with carbodiimide followed by coupling with:						
Glycine methyl ester	Glu-83 and -85	None	None		Glu-83 and -85	25, 39
Histidine methyl ester	Glu-83 and -85	Large decrease	Large			

TABLE 1 (continued)

Summary of Immunochemical Behavior of Mb Derivatives Chemically Modified at Certain Amino Locations[a]

Reaction	Residues modified	Immunochemical change[b]	Conformational change	Conclusion[c]		Ref.
				Residues in antigenic region	Residues not in antigenic region	
Diazonium-1H-tetrazole	Extensive	Reactivity abolished	Large	Conclusions not possible		15
Carboxymethylation with ICH_2COOH	His-119	Reaction decreases 11—14%	None	His-119		21
	His-48 and -97	Reaction decreases 18—19%	None	His-97	His-48	
	His-81 (or -82) and -116	Reaction decreases 18—21%	None	His-116	His-81 (or -82)	
	Lys-145, His-48 and -113	Reaction decreases 18—20%	None	His-113	His-48 and Lys-145	
	His-48, -113 and -116	Reaction decreases 22-31%	Present	No conclusions made		
	Val-1, His-48, -113, and -116	Reaction decreases 30%	Present	No conclusions made		
Carboxypeptidase A	C terminal amino acid removed	None	None		C terminal amino acid	44
	C terminal dipeptide removed	None	None		C terminal dipeptide	

[a] Several antisera to MbX were usually employed in studying the immunochemistry of each derivative. In addition, antisera (usually two or three) against almost each derivative were usually prepared and studied. It is not possible to give all this detailed information in a single table. The results herein, therefore, present only a sketchy outline of these exhaustive investigations. For more details, the original references may be consulted.

[b] The values given or statements made describe the reaction of the derivative with antisera to MbX and therefore refer to the percent decrease in reaction relative to MbX. The range of values takes into consideration all the antisera studied. However, for a given antiserum, our experimental variation was usually ± 1.1% or less.

[c] The conclusions summarized in this table are only those that pertain to delineation of the antigenic reactive sites. Therefore, in derivatives that suffer conformational changes, implication of residues in antigenic reactivity is not straightforward and therefore none was attempted.

From Atassi, M. Z., *Immunochemistry of Proteins*, Vol. 2, Atassi, M. Z., Ed., Plenum Press, New York, 1977, 77. With permission.

antibody when it is isolated free from the intact protein molecule. However, this approach is fraught with complications which can limit its utility and, if certain precautions are not observed, erroneous conclusions will be formulated. The limitations of this approach have previously been outlined[1] and recently analyzed in more detail, together with a review of chemical cleavage reactions of proteins.[10]

Cleavage of Mb (or ApoMb) was carried out at the methionine,[26] tryptophan,[27] arginine,[28] and proline[29] peptide bonds and also by tryptic hydrolysis.[26] Before cleavage at the methionine, tryptophan, or arginine residues, we first carefully determined that the methionine residues,[9,30] Arg-31, -45, and -139,[23] as well as Trp-7,[22] were not located in an antigenic region in Mb. This precuation is critical[26] so that destructive scission will not inadvertently happen within an antigenic region. The peptides studied are summarized schematically in Figure 1.

Conformational studies on the proline and arginine peptides of Mb[31,32] revealed that these peptides were indeed greatly unfolded in solution, a prediction which was made originally[26] from the immunochemical findings. If these long peptides are so much unfolded in solution, then it is obvious to expect that the shorter peptides (whose conformation has not been studied and whose immunochemistry has been investigated) will be even more unfolded in solution. The conformational studies of the peptides were critical for our understanding of their immunochemical behavior.

When immunochemically reactive peptides are obtained, delineation of the reactive regions on these peptides may be achieved to a great extent by comparing the immunochemical reactivities of peptides that share considerable overlaps. However, the study of peptides which share considerable overlaps, although a most effective approach, should be expected to yield only a limited narrowing down of antigenic regions in the protein. In order to achieve further delineation of antigenic regions accurately, other supplementary approaches are necessary. These are confined[33] to two choices: (1) shortening the reactive peptide or, (2) making derivatives of it. The advantages and shortcomings of each of these choices have been discussed in detail.[1,2,4]

The delineation of antigenic sites in immunochemically reactive peptides is best achieved by specific chemical modification of selected amino acid residues in the reactive peptide.[33] This will avoid complications arising from shortening the peptide. By this approach, the appropriate mode of folding of a reactive region is more likely to be retained upon limited and specific modification of a peptide than upon removal of what might well be a critical orientating influence of a segment in that peptide.[33]

By application of the aforementioned approaches, it was possible to achieve delineation of the antigenic sites in Mb to within 8 to 10 residues. Further delineation by chemical methods could not be pursued due to the fact that in each case this would have required modification of hydrophobic or nonpolar amino acids which is, of course, not possible. However, having reached a chemical delineation down to such a conveniently small size, precise narrowing down of the reactive regions was achieved by the organic synthesis and immunochemical studies of peptides corresponding to various parts of the regions. It is necessary here to caution that if synthesis precedes the orderly chemical narrowing down, it will clearly be wasteful and in fact can lead to erroneous conclusions.

In our studies, 26 different peptides representing various parts of each reactive region, as well as control reference peptides corresponding to nonantigenic locations, have been synthesized, purified, characterized, and their immunochemistry studied. The structures of all the peptides synthesized in our laboratories for the delineation of the various reactive regions are shown in Figure 2. For the present purposes, the immunochemistry of these peptides is best discussed with their respective regions. This is done in the following section dealing with the delineation of each of the antigenic sites.

This section gives a very brief outline of the first antigenic structure of a protein to be completed.[1] Readers desiring more detail may consult a very comp

Region	Peptides Synthesized	No. of Residues	M. W.
Controls (non-antigenic)	1 6 Val-Leu-Ser-Glu-Gly-Glu	6	614.7
	80 87 Gly-His-His-Glu-Ala-Glu-Leu-Lys	8	872.2
	121 127 Gly-Asn-Phe-Gly-Ala-Asp-Ala	7	650.7
	127 133 Ala-Gln-Gly-Ala-Met-Asn-Lys	7	718.9
Region 1	15 23 Ala-Lys-Val-Glu-Ala-Asp-Val-Ala-Gly	9	858.9
	16 23 Lys-Val-Glu-Ala-Asp-Val-Ala-Gly	8	801.9
	15 22 Ala-Lys-Val-Glu-Ala-Asp-Val-Ala	8	802.0
	15 21 Ala-Lys-Val-Glu-Ala-Asp-Val	7	730.9
Region 2	54 63 Glu-Met-Lys-Ala-Ser-Glu-Asp-Leu-Lys-Lys	10	1178.4
	56 63 Lys-Ala-Ser-Glu-Asp-Leu-Lys-Lys	8	919.0
	57 63 Ala-Ser-Glu-Asp-Leu-Lys-Lys	7	789.9
	54 62 Glu-Met-Lys-Ala-Ser-Glu-Asp-Leu-Lys	9	1050.2
	56 62 Lys-Ala-Ser-Glu-Asp-Leu-Lys	7	789.9
Region 3	92 100 Ser-His-Ala-Thr-Lys-His-Lys-Ile-Pro	9	1018.3
	93 100 His-Ala-Thr-Lys-His-Lys-Ile-Pro	8	931.2
	94 100 Ala-Thr-Lys-His-Lys-Ile-Pro	7	794.1
	93 99 His-Ala-Thr-Lys-His-Lys-Ile	7	834.1
Region 4	108 120 Ser-Glu-Ala-Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro	13	1495.9
	109 120 Glu-Ala-Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro	12	1408.8
	112 120 Ile-His-Val-Leu-His-Ser-Arg-His-Pro	9	1095.4
	113 120 His-Val-Leu-His-Ser-Arg-His-Pro	8	982.2
	113 119 His-Val-Leu-His-Ser-Arg-His	7	885.1
Region 5	146 153 Tyr-Lys-Glu-Leu-Gly-Tyr-Glu-Gly	8	957.2
	147 153 Lys-Glu-Leu-Gly-Tyr-Glu-Gly	7	794.0
	146 151 Tyr-Lys-Glu-Leu-Gly-Tyr	6	771.9
	145 151 Lys-Tyr-Lys-Glu-Leu-Gly-Tyr	7	900.1

FIGURE 2. Peptides synthesized in our laboratory[36,41,42,43,48,60] for final delineation of the antigenic sites of Mb. Having narrowed down the antigenic regions on the protein molecule by chemical methods to a conveniently small size, the precise delineation was achieved by the organic synthesis and immunochemical studies of various parts of each region. Control peptides representing sequences 1 to 6, 80 to 87, 121 to 127 and 127 to 133 were also synthesized and used as controls in the immunochemical studues since they are not parts of antigenic sites.[1,2]

review[2] or original articles cited here. On the other hand, the initial description of our work[1] offers a convenient and concise account. For convenience, assembly of the information for each antigenic region will be presented concisely in a step-by-step manner. This facilitates the process of logical deduction, but it should be obvious that the results were not derived in this fashion.

Site 1. Delineation of the Antigenic Region in Sequence 1 to 55

In intact Mb, it was demonstrated that Met-55 (as well as Met-131),[9,30] Arg-31 (as well as Arg-139),[23] His-48,[21] and Trp-7[22] were not parts of antigenic reactive regions. Cleavage at the two methionine residues[26] yielded three fragments which, between them, carried the entire antigenic reactivity of the native antigen.[2,26] In peptide 1-55, Arg-31 and Arg-45 were not present in a reactive region.[23] This, together with the finding that peptide 35 to 42 did not interact with antisera to MbX,[26] indicated that the sequence 31 to 45 was not part of a reactive region. This was confirmed by the finding that peptides 1-55,[26] 1-36,[29] and 1-31[28] possessed, with a given antiserum, equal antigenic reactivity. It was, therefore, concluded that the entire sequence 31 to 55 was outside a reactive region.[28] The approximate location of the reactive region was indicated from the finding that peptide 17 to 31 possessed some inhibitory activity.[26] Peptide 1 to 7, obtained by cleavage at Trp-7 by periodate,[27] did not inhibit the reaction of Mb with its antisera,[26] suggesting that sequence 1 to 7 was not part of a reactive region, in agreement with results from modification of Trp-7, and this was unequivocally confirmed by the finding that the shortened ApoMb (i.e., sequence 8 to 153) exhibited equal reactivity to that of ApoMb.[26] Therefore, it was concluded that the antigenic reactive region in sequence 1 to 55 must be located within sequence 8 to 30.[28] It was subsequently demonstrated from studies on derivatives of peptide 1 to 31 that the modification of His-12 and His-24,[34] or of Trp-7 and Trp-14[35] did not influence the antigenic reactivity of peptide 1-31. This enabled us to conclude that the reactive region resides in the sequence 15 to 23.[35] The conclusion was confirmed by the findings[35] that succinylation of Lys-16 completely abolished the reaction of peptide 1-31 with antisera to MbX, and that reduction of Glu-18 and possibly Asp-20 also yielded an immunochemically unreactive peptide.[35] Therefore, the reactive region must be contained within the sequence 15 to 23.

The precise delineation of the region was achieved by studying the immunochemical interactions of synthetic peptides (Figure 2) corresponding to its various parts.[1,36] The results showed that the single antigenic region in sequence 1 to 55 of Mb occupies the structure 16 to 21 invariably, +1 or 0 residue on one side of this sequence depending on the antiserum.

The antigenic reactive region, therefore, has extraordinary sharpness and exhibits a narrow extent of variability expressed in a "displacement" or "shift" by one residue to one side or the other from one antiserum to the next. In another way, it may be stated that the reactive region on sequence 1 to 55 of Mb is located on segment 16 to 21 invariably, +1 or 0 residue on one end only depending on the antiserum. This interesting individual variation would be completely undetected if antisera were pooled and the immunochemical properties of the mixture studied, as is often the practice. Also, if a mixture of antisera is used, the sharpness of the reactive region would be mostly obscured, and it would then appear to have more diffuse boundaries.

In the three-dimensional structure of Mb,[6,37] the present reactive region is poised on the bend between helices A and B (Figure 5).

Site 2. The Antigenic Region in Sequence 54 to 87

Of direct relevance here is the immunochemistry of peptides 1-87 and 37-87.[29] The antigenic reactivity of peptide 1-87 was appreciably higher than that of peptide 37-87. The difference between the reactivities of the two peptides was accou

by the equal reactions of any of peptides 1-55, 1-36,[29] or peptide 1 to 31.[28] The preceding section has shown that the segment 23 to 55 was conclusively not part of a reactive region. Therefore, in peptide 37-87 the reactive region must fall within (but will not include all of) sequence 56 to 87.

It was shown that Met-55 (and Met-131) was not part of antigenic region in Mb.[9,30] Similarly, from results with Mb derivatives, it was found that modification of Glu-83 and Glu-85[25,38,39] or His-81 and His-48[21] did not influence the antigenic reactivity of the protein, showing that these residues were not part of an antigenic region in Mb. The antigenic region on segment 37 to 87 must then be situated within the sequence 56 to 80. The approximate location of the antigenic region was suggested from the finding that the tryptic peptide 56-63 had appreciable inhibitory activity.[4] Unfortunately, the sequence 64 to 87 represents one of the insoluble core peptides of Mb[40] and therefore could not be studied immunochemically. However, from the inhibitory behavior of peptide 56 to 63 and the fact that His-64 is inaccessible and coordinates with the iron of the heme group which is not part of an antigenic site, His-64 would be at or near the end of the antigenic region which will then fall within sequence 56 to 63.

Final delineation was achieved by studying the immunochemistry of several synthetic peptides corresponding to various parts of the region.[41] These synthetic peptides comprised sequences 54 to 63, 56 to 63, 57 to 63, 54 to 62, and 56 to 62 (see Figure 2). For each of the antisera studied, the reactive region comprises accurately the entire sequence 56 to 62. In the three-dimensional structure, the segment 56 to 62 of Mb occupies a highly accessible surface location on the corner between helices D and E (Figure 5). The small size of the antigenic region (seven residues) is analogous to the size of the preceding antigenic region which comprised segment 16 to 21 (six residues). However, unlike the region 16 to 21, the antigenic region 56 to 62 has shown no shift with the antisera so far studied.

Site 3. The Antigenic Region in Sequence 83 to 103

The antigenic reactivity of peptide 56-131 accounted with the five Mb antisera tested for 50 to 81% of the total antigenic reactivity of MbX.[26] The reaction of peptide 37-119 was equal, with a given antiserum, to that of peptide 56-131.[29] Since the reaction of peptide 37-87 with a given antiserum accounted for about one third of the total reaction of either peptide 56-131 or peptide 37-119,[29] this suggested the presence of, quite likely, two or more reactive regions within the sequence 88 to 131. Alternatively, of course, a single, highly reactive antigenic region (accounting for 31 to 48% of the total reaction of Mb) could be present. A long peptide from this region (i.e., peptide 79-98) was found[26] to possess substantial inhibitory activity which approximately accounted, with a given antiserum, for about a third of the reactivity of peptide 56-131. This indicated the presence of an antigenic reactive region situated approximately around the segment 79 to 98. From the immunochemistry of Mb derivatives modified at His-81[21] or at Glu-83 and Glu-85,[25,38,39] it was concluded that these three residues were not part of the antigenic region. On the other hand, carboxymethylation of His-97[21] or acylation of Lys-98[20] in Mb, which induced no conformational changes, led in each case to a decrease (19 to 20%) in antigenic reactivity. Clearly, therefore, His-97 and Lys-98 are part of a reactive region. Furthermore, His-93 is buried and coordinates to the iron of the heme group.[6,37] Since the heme group is not part of an antigenic site in Mb,[11] it may also be concluded that His-93 most likely cannot be part of a reactive region. The findings, therefore, indicated that one reactive region is present in this part of the molecule and must reside within, but may not include all of the nine residues in sequence 94 to 102 since nitration of Tyr-103 in Mb or in peptide 56-131 does not alter their respective antigenic reactivities.[33]

The precise delineation was derived from studying the immunochemistry of synthetic peptides (Figure 2) corresponding to sequences 92 to 100, 93 to 100, 9

to 99.[42] With a given antiserum, the four peptides 92-100, 93-100, 94-100, and 93-99 possessed equal inhibitory activites towards the reaction of MbX with its antisera. It is significant that the inhibitory activity by any of the peptides compared well with the decrease in immune reaction of Mb upon modification of Lys-98 or of His-97. The antigenic region, therefore, occupies accurately the sequence 94 to 99. No "shift" was observed with the antisera so far studied.[42] In the three-dimensional structure, this reactive region is located on the bend between helices F and G (Figure 5).

Site 4. The Antigenic Region in Sequence 104 to 120

Of the Mb derivatives modified at various arginine residues, only Arg-118 was found to be present in a reactive region ,[23] whereas derivatives modified at one or both of Arg-31 and Arg-139 were immunochemically identical with native Mb. Also, from modification of Arg-31 and Arg-45 in peptide 1-55, Arg-118 in peptide 56-131, and Arg-139 in peptide 132 to 153, it was shown[23] that only Arg-118 was part of a reactive region. The other three arginines were not located in an antigenic region. The immunochemical reaction of the region was completely abolished by modification Arg-118. The location of the region was firmly established by the findings from carboxymethyl Mb derivatives that His-113, His-116, and His-119 were also essential parts of this reactive region.[21] Clearly, a reactive region exists within the segment 104 to 119 and occupies at least the six-residue span 113 to 119. Therefore, organic synthesis of appropriate lengths around residues 113, 116, 118, and 119 should yield the solution. However, no chemical information was available concerning the immunochemical participation of the segment 104 to 112. The nonpolar nature of the amino acids in this part precluded their chemical modification. The peptide 103-118 is known to be insoluble.[40] Because of these technical problems, this reactive region was in fact the last to be delineated.

In our synthetic scheme (Figure 2), therefore, in addition to peptides 113-119 and 113-120, we synthesized and studied[43] three more peptides whose length was gradually increased to the left in three steps, in the hope that one or more of these peptides would be soluble. Fortunately, the synthetic peptides 112-120, 109-120, and 108-120 were soluble. If any part of the segment 104 to 112 makes a contribution to the reactive region, then that would have been readily revealed by these peptides. Immunochemical studies on the synthetic peptides[43] determined that the antigenic region in sequence 103 to 120 occupies accurately the entire segment 113 to 119, and that it shows no "shift" with the antisera so far studied. This region, in the three-dimensional structure of Mb, is located on the end of helix G and part of the bend between helices G and H (see Figure 5).

Site 5. The Antigenic Region in Sequence 120 to 153

A schematic diagram in Figure 3 gives a step-by-step assembly of our findings and presents an example of the strategy of approach. Early in our work,[44] it was demonstrated that removal of the C terminal dipeptide from MbX did not influence its immunochemical properties. Also, from Mb derivatives oxidized or carboxyethylated at Met-131 (as well as Met-55), it was concluded[9,30] that this residue was not part of an antigenic region in intact Mb. The lack of immunochemical reactivity by peptide 119-133[26] confirmed the aforementioned results and in addition suggested that the entire segment 119 to 133 did not carry a reactive region. This deduction was conclusively established by the finding that the two peptides 120-153 and 132-153 possessed, with a given antiserum, identical antigenic reactivities.[29] Modification of Mb at Arg-139 by cyclohexanedione had no effect on its antigenic reactivity, and this was confirmed by the finding that modification of Arg-139 in peptide 132-153 did not alter the antigenic reactivity of the peptide.[23] An independent support for this came from the result that peptide 119-139 had no immunochemical reactivity indicating that ir

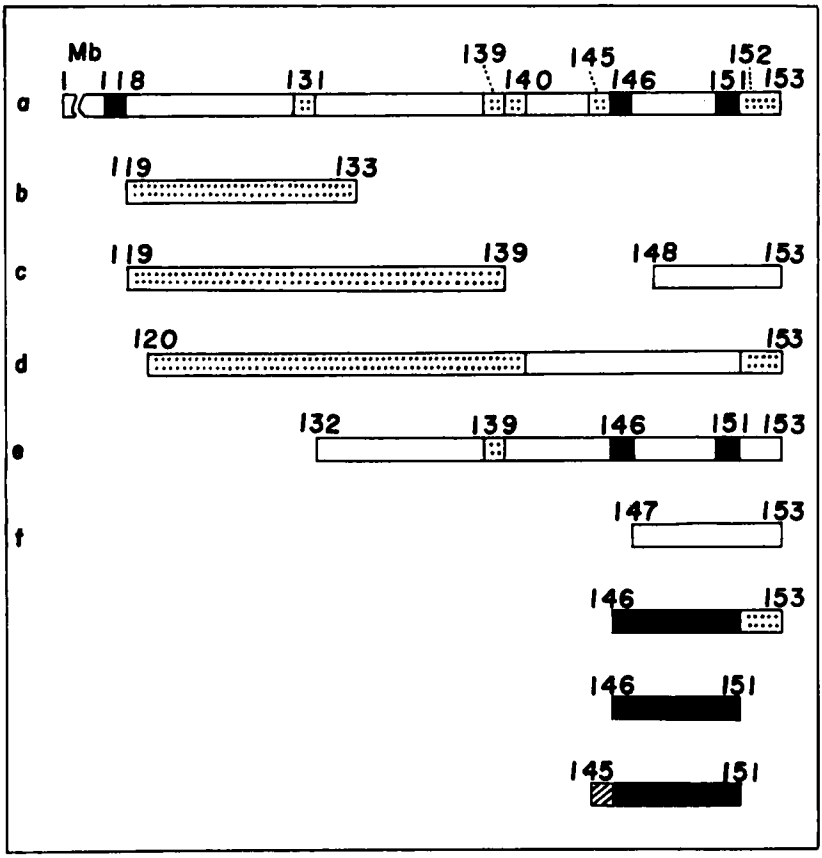

FIGURE 3. Schematic diagram providing an example of a step-by-step representation of our strategy. It shows the steps by which the antigenic region in sequences 120 to 153 was narrowed down to its precise boundaries. Solid blocks represent segments and residues that are part of an antigenic region. Dotted parts represent segments and residues that have been shown to be outside a reactive region. The precise delineation of the antigenic region was derived from the following findings: (a) Removal of the C terminal dipeptide from the intact protein did not influence its antigenic reactivity.[44] Modification of Met-131 in Mb by periodate oxidation[9] or by carboxyethylation,[30] Arg-139 by cyclohexanedione,[23] or Lys-140 and Lys-145 by acylation with 3,3-tetramethyleneglutaric anhydride,[20] did not influence the antigenic reactivity of the respective Mb derivatives. On the other hand, Arg-118 was shown to be within an antigenic region.[23] Nitration of Tyr-146 and Tyr-151 in Mb showed them[33] to be located in an antigenic region. (b) Peptide 119-133 was virtually nonreactive[26] with antisera to Mb. (c) Peptide 119-139 was also entirely nonreactive.[28] Therefore, it can be concluded from (a), (b), and (c) that sequence 119 to 139 is outside the antigenic region.[28] Since residues 140 and 145 are also not part of the antigenic region, the latter must reside within sequence 146 to 151.[20] Peptide 148-153 exhibited a small inhibitory activity toward reaction of ApoMb (and not Mb) with antisera to Mb.[26] (d) Reactions of peptides 120-153 and 132-153 were quantitatively identical,[29] confirming again that sequence 120 to 131 was not part of an antigenic region. (e) Again modification of Arg-139 in peptide 132-153 did not influence the antigenic reactivity of the peptide,[23] whereas nitration of Tyr-146 and Tyr-151 abolished its activity entirely.[33] (f) The synthetic peptides 145-151, 146-151, and 146-153 had comparable inhibitory activities toward reaction of MbX with its antisera.[45] With one antiserum only, peptide 145-151 had significantly higher activity than peptides 146-151 and 146-153. With all the antisera, peptide 147-153 did not inhibit the MbX immune reaction.[46] The antigenic region therefore occupies sequence 146 to 151 entirely.[46] Lys-145 may play an active role with some antisera and therefore is shown striped. (From Koketsu, J., and Atassi, M. Z., *Biochem. Biophys. Acta.*, 328, 289, 1973. With permission.)

sequence 119 to 139 carried no reactive region.[28] In contrast, nitration of Tyr-146 and Tyr-151 in Mb effected a decrease in its antigenic reactivity, and nitration of the same two tyrosine residues in peptide 132-153 abolished the antigenic reactivity of the peptide entirely.[33] The loss of the contribution due to reaction of peptide 132-153 upon modification of the tyrosines accounted quantitatively for the decrease in antigenic reactivity of nitrated intact MbX.[33] These findings suggested that the antigenic region is located around Tyr-146 and Tyr-151. Subsequently results from derivatives of Mb acylated at Lys-140 or Lys-145[20] showed that these two residues were outside the antigenic region. Since the span 141 to 144 is too short to carry a reactive region, these results led us to the conclusion[20] that the antigenic reactive region in sequence 120 to 153 comprises the entire segment 146 to 151.

It can be seen from the aforementioned discussion that a very accurate delineation has been achieved solely by chemical methods. Several observations, which double checked each other, were used for each deductive step before these were assembled to yield the last conclusion. Final support for this conclusion was derived from studying the immunochemistry of various synthetic parts of the region.[45] The synthetic peptides corresponded to the sequences 146 to 153, 147 to 153, 146 to 151, and 145 to 151 (Figure 2). Immunochemical studies with several antisera to MbX clearly showed that the antigenic region in sequence 120 to 153 comprises the entire sequence 146 to 151 and may include Lys-145 with some antisera. This slight "shift" by one residue to one side or the other of an invariable part was observed distinctly in the reactive region 16 to 21. In the three-dimensional structure, this portion of Mb is located on the end of the helix H and part of the randomly coiled *C* terminal pentapeptide (Figure 5).

Do the Five Antigenic Sites Account for the Entire Reaction of Mb and Are They Immunochemically Independent?

A vital evidence in the determination of the entire antigenic structure of a protein is to show unequivocally that the precisely delineated antigenic sites will account for the full immunochemical reactivity of the intact native protein. If this criterion cannot be fulfilled, then the antigenic sites are either incorrectly delineated or, alternatively, one or more antigenic sites have been missed in the delineation. Since the final antigenic structure is derived from putting together a vast amount of information that is obtained from numerous experiments and a variety of approaches, each input must be confirmed by one or more finding obtained by other approaches. The ultimate test will obviously be in demonstrating that all the antibody reactivity is accounted for by the sites thus determined. It is therefore relevant to give here briefly the quantitative contribution by the five antigenic regions of Mb to the total immunochemical reactivity of the native protein.

Since it is obvious from the foregoing brief analysis that no single approach is technically capable of yielding the entire antigenic structure, this critical question cannot and indeed should not be answered by a single technique. Thus, the reactivity of a given antigenic region in the protein is usually derived from:

1. The decrease in reaction upon chemical modification in one or more derivatives of intact Mb of an essential residue (and in the absence of conformational changes) located in that region
2. The reaction of two or more peptides with varying overlaps
3. The reaction of derivatives of these peptides that had been chemically modified at essential amino acid locations
4. The immunochemical reactivity of the synthetic sites

It is critical here to stress that the three peptides 1-55, 56-131, and 132-152 accounted

with a given antiserum for 95 to 105% of the total antigenic reactivity of Mb.[2,26] Also, the peptides 1-36, 37-119, and 120-153 accounted for 98 to 101% of the reactivity of Mb with a given antiserum.[29] It is necessary, therefore, to bear in mind that the antigenic reaction of the intact protein is still fully represented in each of these two sets of peptides. This makes quantitations obtained from derivatives of any of these peptides unambiguous. Evidence relating to retention of full reaction of peptide 1-87 in its two subfragments 1-36 and 37-87 has also been discussed.

The contribution to total antigenic reactivity of the five antigenic regions as derived from chemical modification and cleavage approaches is summarized in Table 2. The final reactivity contribution of an antigenic region is derived from the average of the values obtained by various methods. The total of the averages of the reaction values for the five respective regions accounted with antisera to MbX for all (95 to 105%) of the antigenic reactivity of Mb (Table 2). It is noted that the reaction values of an antigenic region by different methods were essentially equal with a given antiserum, which is very reassuring.

Another approach to quantitative accounting for the total reactivity of Mb may be attempted through addition of the independent inhibitory activities of the five synthetic regions. The results are shown in Table 3. It can be seen that with goat antisera G1, G3, G4, and G19, the combined inhibitory activity of the five synthetic regions was 89, 93, 90, and 94%, respectively (average value, 92%). For rabbit antisera 11, 77, 80, and 100, these values were 90, 89, 93, and 91% (average value, 91%). The improved activities in Part B of the table were obtained with the IgG fractions of the antisera which accounted for 96 to 98% of the total immune reaction of the whole antiserum. Similar improvements in inhibitory activities of peptides upon the employment of the IgG fraction of the antiserum have been observed with other immune systems such as lysozyme[46-50] and bovine serum albumin[51-53] and may be due to proteolysis of the inhibitory peptides in the whole antiserum. The ability of the synthetic regions to account for 90 to 94% of the total immune reaction of Mb by inhibition was further confirmed by studies on immunoadsorbents using two different approaches.

We first employed serial elution by the synthetic antigenic regions of specific ^{125}I-labeled MbX-antibody bound on an MbX-Sepharose® immunoabsorbent.[54] The amount of antibody displaced by a synthetic antigenic region agreed well with the expected reactivity of the region with that antiserum. Antibodies to each region were isolated by elution with the respective region and then rebound (after removal of excess peptide) on Mb-Sepharose®. When these antibodies were redisplaced by the same region as shown in Table 4, a portion of the antibody (ranging 18 to 26% of total) remained on the immunoadsorbent and was displaced only at pH 2.0. In this case, the antibody bound on the immunoadsorbent was originally isolated by specific elution with its own antigenic region. Since that region could not completely redisplace the same antibody after it was once again rebound on the Mb-immunoadsorbent, it is clear that the remaining antibody fraction that was displaceable only at low pH was not directed against some yet unidentified regions. In serial elution with the five synthetic regions (Table 5), the total amount of antibody (from antiserum G1) displaced by the five synthetic regions accounted (after correction for the fraction of site-antibody that is not redisplaceable by that site) for 95.2 to 101% (average value 98.1%) of the antibody to MbX. Furthermore, the findings in Table 4 clearly indicate that these reactive regions are immunochemically independent.[54] Any immunochemical interaction between antigenic regions that are distant in sequence but close in three-dimensional structure (previously suggested by Atassi and Saplin[26]), if it occurs, must be quite minimal for this antigen in its early course antisera. However, the situation may be different on prolonged immunization, and this is now being investigated in our laboratory. Also, we have recently shown that antigenic reactive sites as described by Atassi and

TABLE 2

Contribution to Total Antigenic Reactivity of the Five Antigenic Sites as Derived from Chemical Modification and Cleavage Results

Region	Reactivity Derived from	Percent reaction relative to Mb		
		Antiserum G3	Antiserum G4	Antiserum 77
1	Reaction of peptide 1—55[a]	29.1	8.2	25.6
	Reaction of peptide 1—36[a]	28.2	5.0	25.6
	Reaction of peptide 1—31[b]	27.6	5.6	25.0
	Modification of Lys-16 in peptide 1—31[b]	27.6	5.6	25.0
	Modification of Glu-18 and Asp-20 in peptide 1—31[b]	24.6	5.6	21.0
	Average	27.4	6.0	24.4
2	Difference between reactions of peptide 1—87 and 1—55[a]	21.7	31.5	13.8
	Difference between reactions of peptide 1—87 and 1—36[a]	22.6	34.7	13.8
	Reaction of peptide 37—87[a]	22.2	34.7	11.8
	Average	22.2	33.6	13.1
3	Modification of His-97 in Mb[c]	18.7	18.0	—
	Modification of Lys-98 in Mb[d]	17.0	17.8	18.1
	Average	17.9	17.9	18.1
4	Modification of His-113 in Mb[c]	17.8	21.9	—
	Modification of His-116 in Mb[c]	17.9	21.2	—
	Modification of Arg-118 in Mb[e]	17.5	27.6	31.1
	Modification of Arg-118 in peptide 56-131[e]	18.6	23.8	25.7
	Average	17.9	23.6	28.4
5	Modification of Tyr-146 and Tyr-151 in Mb[f]	—	14.9	18.5
	Reaction of peptide 120—153[a]	18.8	13.9	23.4
	Reaction of peptide 132—153[a]	18.3	13.5	21.0
	Modification of Tyr-146 and Tyr-151 in peptide 132—153[b]	18.3	13.5	20.0

TABLE 2 (continued)

Contribution to Total Antigenic Reactivity of the Five Antigenic Sites as Derived from Chemical Modification and Cleavage Results

Region	Reactivity Derived from	Percent reaction relative to Mb		
		Antiserum G3	Antiserum G4	Antiserum 77
	Average	18.5	14.0	20.7
Total of averages 1-5		103.9	95.1	104.7

[a] Values from Atassi and Singhal.[29]
[b] Values from Singhal and Atassi.[28]
[c] Values from Atassi et al.[21]
[d] Values from Atassi et al.[20]
[e] Values from Atassi and Thomas.[23]
[f] Values from Atassi.[23]

From Atassi, M. Z., *Immunochemistry of Proteins*, Vol. 2, Atassi, M. Z., Ed., Plenum Press, New York, 1977, 77. With permission.

TABLE 3

Quantitative Accounting of the Synthetic Sites Individually and Jointly for the Total Antigenic Reactivity of Myoglobin

Region(s)	Maximum inhibition of the MbX reaction (%)							
	Goat antisera				Rabbit antisera			
	G1	G3	G4	G19	11	77	80	100
Inhibition by the Individual Regions								
Region 1	17.4	10.2	9.3	—	—	16.8	—	—
Region 2	20.0	15.0	9.6	—	—	15.1	—	—
Region 3	16.4	16.7	16.2	15.8	—	14.3	—	—
Region 4	16.7	19.9	14.0	9.3	—	10.9	—	—
Region 5	13.2	6.6	9.8	—	—	19.4	—	—
Total inhibition	83.7	68.4	58.9	—	—	76.5	—	—
Joint Inhibition by a Combination of All the Regions[a]								
Regions (1 + 2 + 3 + 4 + 5)[a]	89.3	92.7	89.9	94.4	90.0	88.5	93.1	90.6

[a] These inhibition results were obtained with the IgG fraction of the antiserum by adding about 300 molar excess of each synthetic region relative to MbX at equivalence. Regions 1 through 5 were added at 2 hr intervals to the IgG fraction of the antiserum, and after the addition of region 5, the mixture was allowed to stand at room temperature for 2 hrs and at 0°C overnight. MbX was then added in an amount that was necessary to achieve equivalence with the respective antibody. The IgG fractions accounted for 96 to 98% of the total immune reaction of the whole antiserum.

From Atassi, M. Z., *Immunochemistry of Proteins*, Vol. 2, Atassi, M. Z., Ed., Plenum Press, New York, 1977, 77. With permission.

TABLE 4

Elution of Specific Antibodies from Mb-Immunoadsorbent by Other Antigenic Sites*

Antibodies to 15—21		Antibodies to 56—62		Antibodies to 94—100		Antibodies to 113—120		Antibodies to 145—151	
Displaced by	cpm	Displaced by	cpm	Displaced by	cpm	Displaced by	cpm	Displaced by	cpm
0.15 *M* NaCl, pH 7.0	58	0.15 *M* NaCl, pH 7.0	62	0.15 *M* NaCl, pH 7.0	64	0.15 *M* NaCl, pH 7.0	66	0.15 *M* NaCl, pH 7.0	79
94—100	56	15—21	56	15—21	58	15—21	63	15—21	78
113—120	53	94—100	58	145—151	58	145—151	65	94—100	85
145—151	59	113—120	58	113—120	61	94—100	70	113—120	80
56—62	49	145—151	64	56—62	69	56—62	60	56—62	76
15—21	2350	56—62	2110	94—100	1720	113—120	3690	145—151	2150
Gly-HCl (pH 2.0)	480 (18.4)†	Gly-HCl (pH 2.0)	520 (22.4)†	Gly-HCl (pH 2.0)	495 (26.0)†	Gly-HCl (pH 2.0)	740 (18.6)†	Gly-HCl (pH 2.0)	510 (20.8)†

* See footnotes in Table 5

† This represents the percent of site-antibody (after correction for the background shown) that could not be displaced by an excess of free site and was removed only at low pH.

Results from Atassi, M. Z. and Koketsu, J., *Immunochemistry*, 12, 741, 1975.

TABLE 5

Serial Displacement of Antibodies from Mb-Immunoadsorbent by Employment of the Five Antigenic Sites

	Experiment 1				Experiment 2			
Peptide	Order in elution schedule	Net cpm of eluent[a]	Ab eluted(%)	Ab bound[b](%)	Order in elution schedule	Net cpm of eluent[a]	Ab eluted(%)	Ab bound[b](%)
15—21	1	3030	17.5	20.7	1	2304	17.1	20.3
56—62	5	2690	15.5	19.0	5	2476	18.4	22.5
94—100	2	2820	16.2	20.4	3	1798	13.4	16.9
113—120	3	3060	17.6	20.9	4	2118	15.7	18.6
145—151	4	2730	15.7	20.0	2	1890	14.0	16.9
Gly-HCl, pH 2.0	6	3034	17.5	—	6	2874	21.4	—
Total Specific antibody bound[b]				101				95.2

Note: Mb-immunoadsorbent, carrying ^{125}I-antibody from goat antiserum G1, was packed in a column (0.5 × 4 cm) and was washed with saline (1*l*) until 0.5 m*l* of effluent counted less than 100 cpm (background 26—36 cpm). After incubation with and subsequent elution of each peptide solution, the column was washed with 0.15 *M* NaCl, pH 7.0 (until 0.5 m*l* of effluent counted between 50 to 100 cpm) before elution with the next peptide.

[a] These values (each representing the average of triplicate determinations) have been corrected for counts of background and the average counts of the saline controls before and after each peptide.

[b] The total per cent antibody eluted has been corrected for the amount of each site-antibody that could not be displaced by an excess of the site as determined in Table 3.

Data are from Atassi, M. Z. and Koketsu, J., *Immunochemistry*, 12, 741, 1975 and have been recalculated here to correct for the irreversible binding given in Table 3.

Saplin[26] made up of spatially adjacent residues on the surface topography of the protein that are distant in sequence do exist in hen egg white lysozyme.[47-50,55-59]

Finally, in more recent studies,[60] the synthetic antigenic sites were coupled to Sepharose® and the fraction of ^{125}I-labeled antibody (from goat antisera G3 and G4) that could be bound directly by each immunoadsorbent site was measured (Table 6). The total antibody bound by the five synthetic sites accounted for 99% of the antibodies to MbX.

The full accountability for total antibody by the synthetic sites in immunoadsorbent studies compares very well with their efficiency in the inhibition of the reaction of MbX with its antibodies. Also, of course, the immunoadsorbent approach takes into account any nonprecipitating antibodies if they had not been detected in inhibition studies. Bearing in mind the shortcomings of each of the approaches employed in the quantitation process, the accountability obtained is in fact remarkable. The results summarized in Tables 2 to 6 revealed that for a given antigenic region, its share of the total antigenic reactivity varied with the antiserum.[4]

Special Features of the Antigenic Structure

Our precise determination of the entire antigenic structure of sperm whale Mb has enabled us to formulate many general conclusions concerning approaches to and the molecular features of protein antigenicity.[1,2] This section summarizes only the main features of the antigenic structure of Mb.

Five antigenic reactive regions are present in the native protein (Figure 4 and 5) and are situated on:

Site 1 — Sequence 16 to 21, + 1 or 0 residue on one side only of this segment,

TABLE 6

Total Binding of Myoglobin Antibodies (from Goat Antisera G3 and G4) by Immunoadsorbents of the Synthetic Five Antigenic Sites, Two Nonantigenic Parts, and a Nonsense Peptide[a]

	Amount of ^{125}I-antibody bound			
	Antibodies from G3		Antibodies from G4	
Immunoadsorbent carrying	cpm bound[b]	% Ab bound	cpm bound[b]	% Ab bound
Myoglobin	65,260	100	11,620	100
Site 1	14,830	22.7	1,090	9.4
Site 2	20,250	31.0	3,550	30.6
Site 3	7,590	11.6	2,520	21.7
Site 4	14,150	21.7	2,580	22.2
Site 5	7,588	11.6	1,680	14.5
Total for sites 1 through 5		98.6		98.4
Peptide 1—6	0	0	0	0
Peptide 121—127	0	0	0	0
Nonsense peptide[a]	0	0	0	0

[a] Nonsense peptide: Glu-Ser-Ser-Gly-Thr-Gly-Ile; a structure that does not exist in Mb.

[b] These values have been corrected for the amount bound by the nonsense peptide which was 1 to 2% of the amount bound by Mb.

Results from Twining, S. S. and Atassi, M. Z., unpublished results.

Region	Structure and Location	No. of Residues
Region 1	15 16 21 22 (Ala)-Lys-Val-Glu-Ala-Asp-Val-(Ala)	6 (or 7)
Region 2	56 62 Lys-Ala-Ser-Glu-Asp-Leu-Lys	7
Region 3	94 99 Ala-Thr-Lys-His-Lys-Ile	6
Region 4	113 119 His-Val-Leu-His-Ser-Arg-His	7
Region 5	145 146 151 (Lys)-Tyr-Lys-Glu-Leu-Gly-Tyr	6 (or 7)

FIGURE 4. Primary structures of the five antigenic sites of sperm whale Mb. Residues in parentheses are part of the antigenic site only with some antisera. Thus for site 1, the reactive region invariably occupies sequence 16 to 21 and with some antisera Ala-15 is part of the region (which will then correspond to sequence 15 to 21) while with other antisera Ala-22 is an essential part of the region (which will then correspond to sequence 16 to 22). This site occupies either six or seven residues depending on antiserum. For sites 2, 3 and 4 no such "displacement" or "shift" to one side or the other has been observed (at least with the antisera so far studied). In the case of site 5, Lys-145 can be part of the antigenic region only with some antisera and this site will therefore comprise six or seven residues, depending on the antiserum. (From Atassi, M. Z., *Immunochemistry*, 12, 423, 1975. With permission.)

depending on the antiserum. This antigenic region exhibits a certain degree of "shift" or "displacement" and minor variability in size (limited to ± 1 residue only) from one antiserum to the next. Its location in the three-dimensional structure is on the bend between helices A and B.

Site 2 — Sequence 56 to 62, on the bend between helices D and E. This reactive region has exhibited no variability in size with the antisera so far studied.

Site 3 — Sequence 94 to 99, on the bend between helices F and G.

Site 4 — Sequence 113 to 119, on the end of helix G and only part of the bend GH.

Site 5 — Sequence 146 to 151 (+ lysine 145 with some antisera). This reactive region is situated on the end of helix H and part of the randomly coiled C terminal pentapeptide.

The primary structures of the five antigenic reactive regions are shown in Figure 4. The locations of the reactive regions in the three-dimensional structure of native Mb are shown in a schematic diagram in Figure 5.

We have previously cautioned[1,43] against the likely formulation of an erroneous conclusion that every bend constitutes a reactive region. No such statement is made or implied here, and indeed examination of Figure 5 immediately reveals that the bends B-C, C-D, and E-F do not carry reactive regions. Also, region 4 (i.e., 113 to 119) is located mostly on a helical portion.

The antigenic reactive regions are surprisingly small (6 to 7 residues) and possess sharp boundaries. They may exhibit limited variability in boundaries w

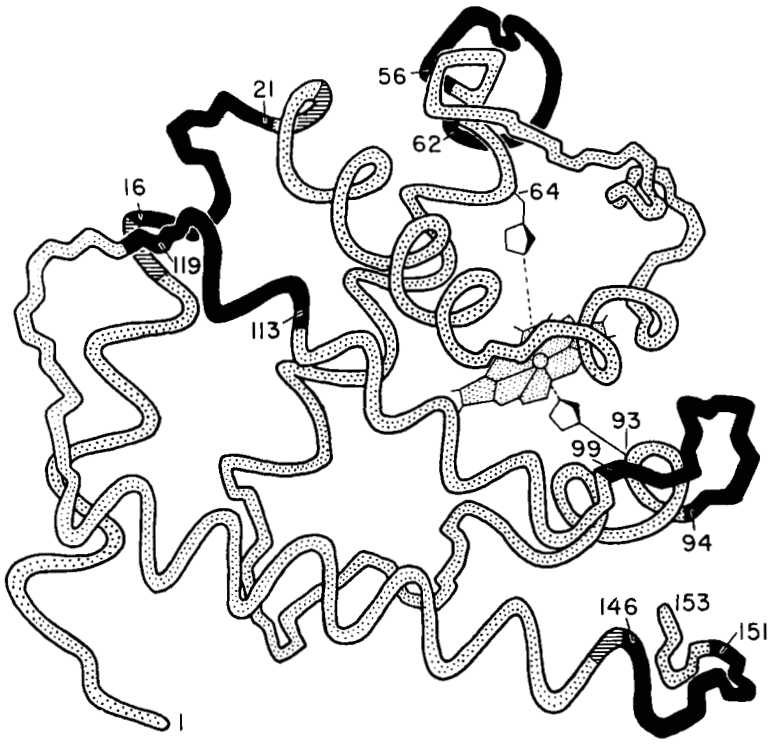

FIGURE 5. A schematic diagram showing the mode of folding of Mb and its antigenic structure. The solid black portions represent segments which have been shown to comprise accurately entire antigenic sites. The striped parts, each corresponding to one amino acid residue only, can be part of the antigenic site with some antisera. The dotted portions represent parts of the molecule which have been shown exhaustively to residue outside antigenic sites. (From Atassi, M. Z., *Immunochemistry*, 12, 423, 1975. With permission.)

sera which, when it exists, will be ± 1 residue. The size, surface locations, and shape of these reactive regions make them quite accessible for binding with antibody combining sites.

The types of amino acids present in the reactive regions is to be expected for their surface locations.[4] Lysine is present in four regions (Figure 4) and in the fifth, arginine is present. Three out of five reactive regions contain aspartic acid or glutamic acid or both. Two regions contain histidine. From this and demonstrated detrimental effect of appropriate modifications of these polar residues on the antigenic reactivity, it may be concluded[1,4] that interactions of the Mb-reactive regions with antibody must be predominantly polar in nature. Stabilizing effects are contributed by hydroxy and nonpolar amino acids through hydrogen bonding and hydrophobic interactions.[4] The sequence and three-dimensional structural features that confer immunogenicity on these regions are not too clear.

Any immunochemical interaction between antigenic reactive regions that are distant in sequence, but close in three-dimensional structure to form antigenic reactive sites (previously suggested by Atassi and Saplin[26]), has been difficult to investigate.[1] In subsequent studies,[54] we showed that this type of interaction, if it occurs, must be quite minimal for this antigen in its early course antisera. However, the situation could be different on prolonged immunization and we are investigating this possibility. In this antigen, antigenic regions and antigenic sites are synonymous. At equivalence no more

than an average of four antibody molecules can sterically fit on the protein, even though five antigenic sites are present in Mb.[9]

The affinity of an antigenic site or its share of the total reactivity of Mb may vary with the antiserum. However, with all the antisera studied so far (at least eight), an antigenic site is invariably an antigenic site, but its potency or efficiency varies with the individual animal immunized.[4] Significantly, antibodies produced in both rabbits and goats to native Mb recognized the same antigenic sites on Mb.

The findings that purely conformational changes in Mb will influence its reaction with antisera to the native protein[11,15] and the immunochemical results on numerous peptide fragments have enabled us to conclude[23] that the primary antibody response, at least in early course antisera, is directed against the native three-dimensional structures of proteins.

An intact antigenic site free of extraneous nonreactive residues would usually react less in solution than when it is isolated as part of a longer peptide.[4,36,41,48] The nonreactive parts may assist the achievement of the correct folding for binding of the antigenic site with antibody combining site.[26] On the other hand, recent evidence[36,41,43] has revealed that nonreactive parts composed of bulky residues linked to an antigenic site may exert a detrimental effect on the reactivity due to unfavorable steric or conformational effects.

ANTIGENICITY OF THE SITES IS INHERENT IN THEIR THREE-DIMENSIONAL LOCATION

Having determined precisely the entire antigenic struture of Mb, which revealed that the antigenic sites are located in conformationally distinct, continuous portions of the polypeptide chain, we proceeded to investigate some of the factors responsible for the antigenicity of the sites. Our findings that rabbit and goat antibodies recognize the same five antigenic sites on sperm whale Mb[1,2] suggested that the antigenecity of the sites is inherent in their three-dimensional location and is independent of any sequence identities between the injected Mb antigen and the Mb of the immunized host. Our recent studies have strongly confirmed this conclusion and are described below.

Antigenic Sites Structurally Identical to the Corresponding Locations in Rabbit Mb: Autoreactivity of Rabbit Antibodies to Sperm Whale Mb

In view of the conservation of the overall three-dimensional structure among globin chains and occasionally extensive homologies in their primary structures, we investigated[61] whether, in responding to sperm whale Mb, the host animal will make antibodies to regions of the sperm whale Mb molecule which are similar or identical to the corresponding regions in the animal's own Mb. The recent availability of the primary structure of rabbit Mb[62] afforded us the opportunity for such studies.

A comparison of the primary structures of sperm whale Mb and rabbit Mb shows that they have identical chain lengths (153 amino acids) and differ in sequence at 22 amino acid locations, only 5 of which fall within the boundaries of the antigenic sites recognized by rabbit antibodies to sperm whale Mb. In Figure 6 the primary sequences of the five antigenic sites of sperm whale Mb are shown together with the corresponding regions from rabbit Mb. The amino acid replacements which occur in the corresponding rabbit Mb sequences are $Ala^{18} \rightarrow Gly$ and $Val^{21} \rightarrow Leu$ in region 15 to 22, $Arg^{118} \rightarrow Lys$ in region 113 to 119, and $Lys^{145} \rightarrow Gln$ and $Tyr^{151} \rightarrow Phe$ in region 145 to 151. Two regions, 56 to 62 and 94 to 99, are identical in both myoglobins.

The close similarity of the two myoglobins in these regions was surprising to us in view of the belief that an animal would not make antibodies to regions of a protein antigen which were identical to equivalent regions in the animal's own protein.[63] These

SITE 1 of	15							22
Sperm-Whale Mb	Ala	Lys	Val	Glu	Ala	Asp	Val	Ala
Rabbit Mb	Gly	Lys	Val	Glu	Ala	Asp	Leu	Ala
SITE 2 of	56						62	
Sperm-Whale Mb	Lys	Ala	Ser	Glu	Asp	Leu	Lys	
Rabbit Mb	Lys	Ala	Ser	Glu	Asp	Leu	Lys	
SITE 3 of	94					99		
Sperm-Whale Mb	Ala	Thr	Lys	His	Lys	Ile		
Rabbit Mb	Ala	Thr	Lys	His	Lys	Ile		
SITE 4 of	113						119	
Sperm-Whale Mb	His	Val	Leu	His	Ser	Arg	His	
Rabbit Mb	His	Val	Leu	His	Ser	Lys	His	
SITE 5 of	145						151	
Sperm-Whale Mb	Lys	Tyr	Lys	Glu	Leu	Gly	Tyr	
Rabbit Mb	Gln	Tyr	Lys	Glu	Leu	Gly	Phe	

FIGURE 6. A diagram showing the primary structures of the five antigenic sites of sperm whale Mb and the corresponding regions of rabbit Mb. The sequences shown occupy identical positions in the respective protein chains. Identical positions having different amino acids in the two chains are indicated by blocks. (From Kazim, A. L., and Atassi, M. Z., *Biochim. Biophys. Acta*, 494, 277, 1977. With permission.)

similarities also suggested that, barring any drastic conformational differences, rabbit antibodies to sperm whale Mb could also react with rabbit Mb. Since both rabbit and goat antibodies to sperm whale Mb recognize identical antigenic sites (shown in Figures 4 and 5) on the sperm whale Mb molecule,[1,2] goat antibodies to sperm whale Mb may also be expected to react with rabbit Mb.

It was most significant that rabbit Mb cross-reacted extensively with rabbit antisera to sperm whale Mb.[61] Although rabbit Mb did not give immune precipitates with either rabbit or goat antisera to sperm whale Mb, it was quite effective in inhibiting the quantitative precipitin reaction of sperm whale Mb with both rabbit or goat antibodies to sperm whale Mb (Table 7). The large inhibitions obtained with these antisera indicate that rabbit Mb interacts effectively with both rabbit and goat antisera to sperm whale Mb, but the affinity for rabbit Mb varied with the antiserum (Table 7). The reactivity of rabbit Mb with antisera to sperm whale Mb was also measured by examining the ability of an immunoadsorbent of rabbit Mb to bind antibodies from antisera to sperm whale Mb.[61] Table 8 summarizes the results with antisera from two rabbits (77 and 80) and one goat (G4). A substantial portion of the antibodies to sperm whale Mb could be absorbed by the rabbit Mb adsorbent.

The extensive ability of rabbit Mb to interact with antibodies to sperm whale Mb leads to a consideration of the sites through which the rabbit Mb may interact with these antisera. [61] Regions 56 to 62 and 94 to 99 are identical in both Mb (Figure 6), and unless their conformations have been altered through amino acid replacements not located in these regions, these two sites would be expected to react completely. ORD and CD studies[64] have revealed no conformational differences between sperm whale Mb and rabbit Mb. With respect to regions 15 to 22 and 113 to 119, our previous studies have shown that the binding of the Mb antigenic sites with antibodies is primarily effected through polar interactions, with nonpolar amino acids providing more of a stabilizing role through hydrophobic interactions. Based on these considerations and on the conservative nature of the amino acid substitutions in reg

TABLE 7

Inhibitory Activities of Rabbit Mb in the Sperm Whale Mb-Anti-Sperm Whale Mb Precipitin Reaction

Antiserum	Maximum inhibition (%)	Molar ratio (R Mb/SW Mb)*
77	72	19
G3	76	12
G4	90	3

Note: Antisera were raised against sperm whale Mb in rabbits (77) and goats (G3, G4). Inhibition values are expressed as maximum per cent inhibition by rabbit Mb of the precipitin reaction of sperm whale Mb with the antisera shown. Each value is an average of at least three determinations which varied ± 1.5% or less.

* These values represent the rabbit Mb per sperm whale Mb molar ratios at 50% of the maximum inhibition. R Mb, rabbit Mb; SW Mb, sperm whale Mb.

From Kazim, A. L. and Atassi, M. Z., *Biochim. Biophys. Acta*, 494, 277, 1977. With permission.

TABLE 8

Summary of Immunoadsorbent Studies by Rabbit Mb of Rabbit Antibodies Against Sperm Whale Mb

Antiserum	Anti-sperm whale Mb adsorbed by sperm whale Mb — Sepharose®* (%)	Anti-sperm whale Mb adsorbed by R Mb — Sepharose®* (%)
77	100	42.9
80	100	65.8
G4	100	49.6

Note: Antisera are the same as those referred to in Table 6 except No. 80 which is a rabbit antiserum to sperm whale Mb. Values are expressed as per cent of the total antibodies adsorbed by a rabbit Mb adsorbent relative to those bound by a sperm whale Mb adsorbent as 100%.

* These values have been corrected for nonspecifically adsorbed protein retained on a Lysozyme-Sepharose® adsorbent.

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113 to 119, these two regions in rabbit Mb would not be expected to be completely unreactive. However, it cannot be excluded that subtle and undetectable conformational changes in these regions directed by these replacements as well as by replacements elsewhere in the rabbit Mb molecule, could diminish the affinity of these sites for their respective antibodies. Although Lys-145 is included in the antigenic site 145 to 151, it plays only a marginal role with some antisera and of those studied is required only for the reaction of the free peptide 145-151 with antiserum G4, but is not part of the reactive region in native sperm whale Mb.[45,20] Therefore, the replacement of Lys-145 by glutamine in rabbit Mb should be without consequence to the reactivity of this region in native rabbit Mb. However, Tyr-151 has been shown to be absolutely essential for the reactivity of this region, and its replacement by phenylalanine completely

obliterates its reactivity.[65] Rabbit Mb is, therefore, not expected to react with antibodies to sperm whale Mb through region 145 to 151. The expected diminished reactivities for regions 15 to 22 and 113 to 119, together with the complete unreactivity of region 145 to 151, would account for the aforementioned lack of total cross-reactivity of these two Mb.

The foregoing studies clearly indicate that rabbits respond to immunization with sperm whale Mb by producing antibodies that are directed against regions of the sperm whale Mb molecule which are both identical (regions 56 to 62 and 94 to 99) and closely similar (regions 15 to 22 and 113 to 119) to the corresponding sequences in the rabbit's own Mb. Furthermore, these antibodies will extensively cross-react with rabbit Mb through these equivalent regions. As mentioned above, both rabbits and goats make antibodies directed against regions of the sperm whale Mb molecule which are identical in their structure and locations.[1,2,36,41-43,45] Pertinent in this regard is that hen egg white lysozyme, the second protein whose antigenic structure has been completely determined,[5,50] (also see following article) is also recognized through identical antigenic sites by both rabbit and goat antisera. In a pilot experiment, we have also observed that goat Mb is extremely effective in inhibiting the precipitin reaction of sperm whale Mb with goat antisera to sperm whale Mb.[64] Although the sequence of goat Mb is not available for comparison at the present time, we predict that similar identities exist between the antigenic sites of sperm whale Mb and the corresponding regions of goat Mb. It is apparent, therefore, that the antigenicity of at least some regions of Mb is characteristic for the Mb molecule and is not related to any sequence differences between the injected Mb and that of the immunized host.[61,66]

These findings are in contrast to conclusions from studies on hemoglobin,[63] that the antigenic sites of proteins are not present at sequence positions that are identical in the immunized host and, from chemically polymerized cytochrome c,[67] that protein antigenic sites are not peculiar to the protein antigen, but are defined by the animal species in which the antibody response is elicited. In this regard, immunization of rabbits with heterologous cytochrome c from various species consistently produced antibodies that reacted considerably with the homologous, rabbit cytochrome c to a large extent.[68] Although the antigenic structures of these cytochromes c are not known, the authors overlooked the implication that the rabbits responded to regions on the injected heterologous cytochrome c which are identical to the rabbit cytochrome c. More recent studies by these authors[67] indicated that in immunization with chemically polymerized, heterologous cytochrome c into two species, the antibody responses were directed to regions which differed in sequence and also to a region identical in both the immunogen and the host cytochrome c. The persistent response to this invariable region supports our concept of structurally inherent antigenic sites and is not consistent with the conclusion that the locations of protein antigenic sites are not peculiar to the protein, but are defined by the species in which the antibody response is elicited. It is relevant to stress here that, as observed for sperm whale Mb, both rabbits and goats also recognize the same antigenic sites on hen egg white lysozyme.[5,50] However, it should be cautioned that these studies on cytochrome c may not be applicable to those of Mb. Cytochrome c is frequently observed to be weakly antigenic or entirely nonimmunogenic in its native monomeric state. The high degree of chemical cross-linking in the polymers used to enhance the antibody response to this protein might have not only altered its antigenic potency, but also the sites recognized as being antigenic in the immunized host.

It is worthwhile to note here that although Mb is an intramuscular protein believed to be unexposed to the immune system, its presence in normal serum has been demonstrated.[69] In fact, there is increasing evidence to indicate that antigens previously thought to be sequestered from the immune system are indeed present in the circulation,[70] although in low amounts.

The induction of autoreactive antibodies to "sequestered" antigens by immunization with a cross-reacting antigen is not unprecedented. In fact, it has been suggested that suppressive and tolerizing influences on the immune response to autologous proteins may be circumvented by stimulating helper effects specifically with cross-reacting antigens.[70] However, the lack of knowledge concerning the molecular structures and locations of protein antigenic sites has led to uncertainty.

Autoantibodies Produced by Immunization with Rabbit Mb

The above finding that the antigenicity of the Mb sites is independent of any sequence differences between the injected Mb and the Mb of the immunized host,[61] led us to the reasoning[71] that immunization of rabbits with rabbit Mb should lead to the production of antibodies against this protein.

Our recent studies[71] clearly demonstrate that rabbits, when immunized with rabbit Mb, do make antibodies against this autologous protein. The results are outlined in Figures 7 and 8 and are summarized here:

1. No significant binding of ^{125}I-rabbit Mb occurred with the preimmune sera (Figure 7).

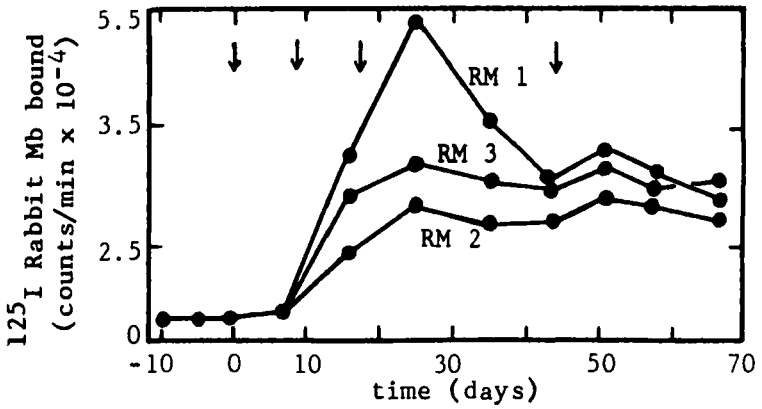

FIGURE 7. Screening of ^{125}I-rabbit Mb binding by antisera obtained from serial bleedings of three rabbits (RM 1, RM 2, and RM 3) immunized with rabbit Mb. Arrows indicate times of immunization. (From Kazim, A. L. and Atassi, M. Z., *Immunochemistry*, 15, 67, 1978. With permission.)

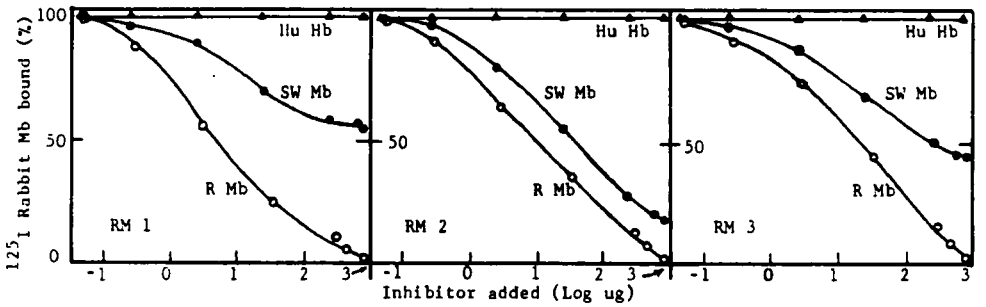

FIGURE 8. Inhibition of ^{125}I-rabbit Mb binding to autoantibodies by unlabeled rabbit Mb, sperm whale Mb and human Hb. The antisera used in these assays were obtained from the 25-day bleedings. In each case, the amount of antisera present bound 20 to 30% of the total ^{125}I-rabbit Mb added in the absence of competing, unlabeled protein. (From Kazim, A. L. and Atassi, M. Z., *Immunochemistry*, 15, 67, 1978. With permission.)

2. Sera from immunized rabbits show a dramatic increase in the binding of ^{125}I-rabbit Mb, the appearance of binding being consistent with the "lag" observed in humoral responses (Figure 7).
3. The binding is associated entirely with the immunoglobins of these antisera as demonstrated by the quantitative ability of the goat anti-rabbit antibody (with specificity only for heavy and lights chains of rabbit immunoglobins) to precipitate labeled antigen bound by these antisera.
4. The binding of ^{125}I-rabbit Mb could be completely inhibited by unlabeled rabbit Mb and partially by unlabeled sperm whale Mb — a related cross-reacting protein (Figure 8). No inhibition was obtained by human hemoglobin at comparable concentrations (Figure 8), indicating that the inhibitions by rabbit Mb and sperm whale Mb were specific and not due to nonspecific protein effects.
5. Two of the three rabbits immunized gave characteristic precipitin curves upon reactions of antisera taken 25 days after the initial immunization with rabbit Mb.
6. The fact that all three rabbits responded to rabbit Mb, either with precipitating or nonprecipitating antibodies, and the similarities in the overall profiles of the response curves (Figure 7) indicate that these rabbits do not represent isolated cases of responses to this autologous antigen.

The Mb used in these studies was the major chromatographic component of the combined muscle extracts of two unrelated rabbits and had an amino acid composition identical to that of rabbit Mb isolated in England.[62] Therefore, it is unlikely that this Mb preparation differs from the major Mb component of these immunized rabbits. Accordingly, we believe these antibodies to be true autoantibodies.

The antisera had somewhat low antibody titres (approximately 0.25 mg/mℓ) which may be accounted for[71] by the formation of complexes between these antibodies and circulating Mb. The Mb content of normal human serum has been reported to be 6 to 85 ng/mℓ.[69] However, the small size of Mb facilitates its fast renal clearance[72] which suggests a somewhat higher rate of entrance into the circulation than is reflected by the net concentration in the serum. By analogy with the human system, the binding of these rabbit antibodies to this small, yet constant supply of circulating Mb would enable the antibodies bound to escape detection. The possibility of more direct attenuating influences on the antibody response should not, however, be overlooked.

Our studies on the antigenic structure of sperm whale Mb showed that antibodies to this protein elicited by immunization with sperm whale Mb emulsified in Freund's complete adjuvant were directed against its native three-dimensional structure.[23] Furthermore, as mentioned earlier, the reactivity of these antibodies with sperm whale Mb were shown to be extremely sensitive to slight conformational distortions intentionally imposed on the native structure of sperm whale Mb by modifications which were clearly outside antigenic sites.[11,15] Similiar observations have been made for lysozyme.[73] Obviously, emulsification in Freund's adjuvant did not alter the native conformation of these proteins. We therefore expect that the autoantibodies to rabbit Mb (obtained by immunization with emulsions of rabbit Mb in Freund's complete adjuvant) are similarly directed against the native conformation of rabbit Mb.

The efficiency of cross-reaction by sperm whale Mb with rabbit Mb autoantibodies, as judged by the comparable concentrations of sperm whale and rabbit Mb required for maximal inhibition of the binding of ^{125}I-rabbit Mb (Figure 8), indicates that the cross-reacting antigenic sites on rabbit Mb to which these antibodies are directed exist in an identical or nearly identical form in sperm whale Mb.[71] These cross-reacting sites are, of course, expected to be located away from regions which carry amino acid replacements between the two myoglobins. This would be especially true of regions carrying nonconservative amino acid replacements which have alterations in their electro-

static characteristics, since we have previously shown that polar interactions predominate over nonpolar interactions in the binding of antigenic sites to their antibodies.[1,4] Sperm whale Mb, however, did not completely inhibit the binding of ^{125}I-rabbit Mb to these anti-rabbit Mb antibodies, although for one antiserum (RM2) the inhibition was greater than 80% (Figure 8). The individual variations in the extent of inhibition by sperm whale Mb with these antisera probably reflect differences in the relative amounts of antibodies directed against sites on rabbit Mb which have cross-reacting counterparts on sperm whale Mb.[71]

The inability of sperm whale Mb to completely cross-react with the antisera to rabbit Mb indicates[71] that antigenic sites peculiar to rabbit Mb are undoubtedly recognized, and these sites are not necessarily excluded from being located at similar structural positions to those of sperm whale Mb. Similarities in the primary structure between the two myoglobins in the regions of the antigenic sites of sperm whale Mb,[61] and the ability of rabbit Mb to react extensively with rabbit antibodies to sperm whale Mb have been mentioned above. However, there is no assurance that antibodies directed against the same structural locations in rabbit Mb, but bearing the new amino acid substitutions, will react with sperm whale Mb.

Immunization with autologous proteins has been previously known to induce autoantibody responses. For example, autoreactive antibodies to thyroglobulin have been observed upon immunization with autologous thyroglobulin in Freund's complete adjuvant or heterologous thyroglobulin in the absence of adjuvant.[70] Immunization with structurally altered autologous proteins has also been shown to result in autoantibody formation.[74] For example, rabbits immunized with papain-digested autologous γ-globulins formed autoreactive precipitating antibodies to these proteins. Similarly, rabbits did not respond to native rabbit cytochrome c, but made antibodies to highly chemically polymerized rabbit cytochrome c preparations.[75] However, such findings were not substantiated by these authors in subsequent trials.[67]

The potential for autorecognition of rabbit Mb is obviously present, and this potential is expressed when rabbits are immunized with rabbit Mb.[71] Furthermore, "structural alterations" of rabbit Mb were not required in order to induce these autoantibodies. Regardless of whether or not the autoantigenic sites of rabbit Mb lie in the same structural locations as the antigenic sites of sperm whale Mb, these autoantigenic sites are clearly not dependent on sequence differences between the immunogen and the corresponding host protein. The relative ease with which autoantibodies to Mb were induced suggests that autoimmunity to Mb may play a role in the pathologenesis of muscle diseases.

Prediction and Synthesis of Two Antigenic Sites in Hemoglobin by Extrapolation from the Mb Antigenic Structure

Our concept that the antigenicity of protein antigenic sites is inherent in their three-dimensional locations has received a strong support from our recent prediction and synthesis of two antigenic sites of hemoglobin by extrapolation from the Mb antigenic structure.[66]

Our previous studies had shown that the structure of antigenic sites of proteins is directed by their amino acid sequences as well as by the three-dimensional arrangement of the participating amino acid residues.[1,56,57,59,76-79] The results obtained during our delineation of the entire antigenic structure of sperm whale Mb also suggested that the antigenic sites of other myoglobins are located at similar structural positions in their respective chains.[65] Since the primary antibody response to a native protein is directed against its native three-dimensional structure,[23] we were prompted to examine whether conformationally homologous, yet more complex, globins would possess antigenic sites at similar locations in their three-dimensional structure.[66] The many structural

features which the α and β chains of Hb share with Mb[80] made the Hb molecule particularly suitable for examining whether it is possible to extrapolate, and thereby predict, the antigenic sites of a large, multisubunit protein from those of a smaller member of the same protein family.

In a very recent study,[66] we have focused on the Mb antigenic site 1 (region 15 to 22). The sequences and structural locations of the corresponding regions in the α and β chains of human Hb are shown in Figure 9. To examine whether these extrapolated regions of the α and β chains (α 15 to 23, β 16 to 23) were immunochemically active, they were synthesized and their immunochemical reactions with antisera to human Hb studied.[66] Antigenic site 1 of sperm whale Mb invariably included the sequence 16 to 21 with all antisera tested.[36] However, some antisera also required either Ala-15 or Ala-22 for full reactivity. Accordingly, antigenic site 1 was taken for the purpose of extrapolation to comprise the sequence 15 to 22 in order to accommodate the individual variabilities of different antisera. Sequence 15 to 22 of sperm whale Mb occupies the helix positions A13 through B3 and is located at the highly exposed bend between helices A and B.[81,82] (Figure 9 explains structural notations). The three-dimensional counterpart of the Mb antigenic site 1 in the Hb α chain also spans residues α 15 to 22.[80] In our studies, we chose to extend this region to include Glu-23 in order to increase the solubility of the synthetic peptide α 15 to 23 in aqueous solvents. The Hb β chain varies in its alignment with the α chain in this region by not having the corresponding AB1 and B1 positions.[80] Therefore, we extended the synthetic region of the β chain to Val-23 in order to compensate for the deficiency in length which would occur from a precise structural extrapolation of this region. By examination of the three-dimensional structures of human deoxyhemoglobin[83] and of horse oxyhemoglobin,[80] these regions (α 15 to 23 and β 16 to 23) are not involved in either chain-heme or chain-chain interactions in the native Hb molecule, and they occupy highly accessible positions in their respective α and β chains in the native Hb tetramer. Thus, these regions were considered[66] ideally suited for examining whether the structural extrapolation of protein antigenic sites is possible.

We found[66] that the synthetic peptides were unable to inhibit the precipitin reaction of Hb with its antisera, even at molar excess of 1000-fold, either individually or in combination. This was not entirely unexpected. Although the synthetic antigenic sites of Mb inhibit its immune reaction quantitatively, other workers have reported that Hb fragments will not inhibit the Hb immune reaction.[63] Also, antibodies to a region

Residue Location:	A_{13}	A_{14}	A_{15}	A_{16}	AB_1	B_1	B_2	B_3	B_4	B_5
Site 1 of Mb:	15 Ala	Lys	Val	Glu	Ala	Asp	Val	22 Ala		
Hb α (15-23):	15 Gly	Lys	Val	Gly	Ala	His	Ala	Gly	23 Glu	
Hb β (16-23):	16 Gly	Lys	Val	Asn	...	...	Val	Asp	Glu	23 Val

FIGURE 9. Diagram showing the sequence and structural location of antigenic site 1 of sperm whale Mb and the corresponding regions of the adult human Hb α and β chains. For the letter/number notation used to designate the structural locations of the amino acid residues, and for the alignment of the Hb α and β chains with sperm whale Mb see Dickerson and Geis.[82] (From Kazim, A. L. and Atassi, M. Z., *Biochem. J.*, 167, 275, 1977. With permission.)

around Val-6 in Hb S, obtained from anti-Hb S by an immunoadsorbent of the synthetic peptide β 1-13 of Hb S, accounted for only about 5% of the total antibodies to Hb S. Furthermore, the peptide, which was initially used to obtain this antibody population by immunoadsorption, was not fully effective in displacing Hb S bound to these antibodies.[84] These results may in part be accounted for by the large size of Hb and conceivably an increased number of antigenic sites and the decreased affinity which immunochemically active peptides in a nonnative conformation exhibit for their antibodies.[26] These observations suggested to us that, not withstanding the structural alterations which would occur upon being covalently coupled to Sepharose,® quantitative immunoadsorption would be the most suitable approach for studying the immunochemical activities of these extrapolated peptides.[66]

The immunoadsorption studies (Table 9) showed[66] that these peptides are immunochemically active with antisera to native Hb, accounting for a significant portion of the reactivities of Hb and of the respective α and β chains. The immunochemical activities of these peptides are further illustrated by comparing them to the activity of a reference peptide, α 1-15, which does not fall within the boundaries of an antigenic site that could be predicted from Mb. This peptide showed no significant immunochemical activity.[66] The calculated sum of the reactivities of the α and β chains was greater than that of Hb. Similar observations have recently been made by others[85] and were attributed to cross-reactivities between the chains.

It is therefore evident that by extrapolation of the structural location of antigenic site 1 of sperm whale Mb, we were able to predict and confirm antigenic sites for a larger, more complex member of the same protein family.[66] Based on these results, we stated[66] that when similar extrapolations of antigenic sites of Mb are made to other homologous proteins, the predicted regions will also be shown to be immunochemically active. However, we cautioned that not all structural counterparts of the antigenic sites of one protein are expected to be immunochemically active in a homologous protein.

TABLE 9

Quantitative Immunoadsorption of ^{125}I-Antihemoglobin Antibodies

Immunoadsorbent	Amount antibody bound (cpm)	Relative to HbA (%)	Relative to Chain (%)
HbA	98010	100.0	
α chain	82140	83.8	
β chain	57800	59.0	
α (15—23)	10840	11.1	13.2
β (16—23)	7130	7.28	12.3
α (1—15)	133	0.14	0.16

Note: Results represent the average of three determinations which varied ± 1.5% each. They have also been corrected for protein nonspecifically bound to lysozyme-Sepharose.® This correction amounted to no more than 1 to 2% of the total counts bound to Hb-Sepharose.® Amount of labeled antibody applied to each immunoadsorbent was 102,000 cpm (1.099×10^{-12} mol). Peptides on the immunoadsorbents were present in a vast molar excess (approximately 600,000) relative to the labeled antibody applied.

From Kazim, A. L. and Atassi, M. Z., *Biochem. J.*, 167, 275, 1977. With permission.

Thus, in the tetrameric hemoglobin molecule, for example, obstructions due to subunit interactions and conformational adjustments effected by amino acid substitutions (both within and outside the predicted regions), as well as by the influence of neighboring subunits, may alter the antigenic expression of these regions. For similar reasons, new antigenic sites on individual subunits or combinations thereof, without antigenic counterparts in the Mb molecule, should not be unexpected in the hemoglobin molecule.

CONCLUSION

We had previously noted[1] that the conclusions and concepts derived from our determination of the entire antigenic structure of Mb, will most likely be expanded and refined when antigenic structures of other native proteins are accurately determined. Already, our precise elucidation of the antigenic structure of lysozyme (see the following article) has enabled us to discard the concept that antigenic sites will always be composed of continuous portions of the primary structure. This concept, applicable correctly to the antigenic sites in Mb, is not applicable to lysozyme in which each of the antigenic sites constitutes spatially adjacent residues that are generally distant in sequence circumscribing part of the surface topography of the molecule and behaving functionally as if they are in direct peptide-bond linkage.[5,47-50,56,59] A simple molecular generalization is therefore unlikely to be formulated at the present time, and restraint should be exercised in this matter. The antigenic structures of many more proteins need to be determined in order to comprehend the common molecular features and the diversity of protein antigenic sites. The position of our knowledge now is not unlike the situation when the first amino acid sequence of a protein was determined or when the first three-dimensional structure of a protein was unraveled.[1] There is truly much that we have learned, but there is yet much more that needs to be discovered. Work in this field will continue to pose great challenges, the conquest of which will greatly enrich protein chemistry.

The precise determination of the entire antigenic structure of Mb provided us with the first completed protein antigen model which we have begun exploiting for answering many vital questions at the molecular, cellular, and immunobiological level. We have shown here, by comparing the antigenic structure of sperm whale Mb with the primary structure of rabbit Mb, that the locations of antigenic sites of this protein are not dependent on sequence differences between the immunizing and host Mb. Also, the ability of rabbit antisera against sperm whale Mb to cross-react with rabbit Mb and the immunogenicity of rabbit Mb in rabbits may implicate the participation of autoreactive clones in the antibody response to heterologous proteins. This may in fact be a general phenomenon with protein antigens and serves to emphasize the need for caution and critical evaluation of approaches relying on the notion that antigenic sites on a protein are located in parts of the molecule that are different in sequence from the corresponding protein in the immunized host.

Furthermore, these observations, together with our successful extrapolation of the antigenic structure of sperm whale Mb to human hemoglobin, demonstrate that the antigenicity of certain regions of a native protein molecule is primarily dependent on the uniqueness of their conformational locations. This property will be independent of whether or not such sites are recognized as being autologous or present on immunochemically unrelated proteins. We would like to refer to these antigenic sites as "structurally inherent antigenic sites."

The utility of "structurally inherent" antigenic sites to the immune system is that the overall complementarity between the shape of the antibody combining site and the antigenic site can be maintained with minimal amino acid replacements in the combin-

ing site and without a drastic overhaul. This affords a biological advantage in that it may reduce the information load and time lag necessary for recognition and antibody response to protein antigens.

ACKNOWLEDGMENTS

The work was supported by a grant (AM 18920) from the Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service and by a grant (AI 13181) from the National Institute of Allergy and Infectious Diseases. The early stages of the work were done during the author's tenure of an Established Investigatorship of the American Heart Association.

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