Immunochemistry of Sperm Whale Myoglobin. X. Regions Responsible for Immunochemical Cross-Reaction with Finback Whale Myoglobin. Some General Conclusions Concerning Immunochemical Cross-Reaction of Proteins*

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ABSTRACT: Detailed studies were carried out on the immunochemical cross-reaction of finback whale myoglobin (FMb) with antisera to sperm whale myoglobin (SpMb) and of SpMb with antisera to FMb. The two apoproteins were subjected to cleavage at the methionyl peptide bonds, and the specific interactions of the resultant peptides were studied with homologous and heterologous antisera. With antisera to SpMb, the cross-reaction of FMb ranged between 34 and 41% relative to homologous reaction. The cross-reaction of SpMb with antisera to FMb was higher (55–58%). The N-terminal and central peptides from each protein gave, in each case, an immune precipitate with the respective homologous antiserum, while the tail peptide showed inhibitory activity toward the homologous reaction. Fragment S1–55 (from SpMb) failed to react with antisera to FMb and, in the converse situation, peptide F1–54 (from FMb) reacted only slightly (5 or 6%) with SpMb antisera.The reaction of the central peptide from SpMb (*i.e.*, S56–131) with antisera to FMb was of comparable magnitude (56–61%) to its reaction with homologous antisera. Conversely the cross-reaction of the central peptide of FMb (*i.e.*, F55–129) with antisera to SpMb was much lower (14–19%). The C-terminal fragments, S132–153 and F130–151, showed interesting inhibitory behaviors. Fragment F130–151 showed no cross-reaction with antisera to SpMb. However, the reverse did not hold since fragment S132–153 exhibited considerable inhibitory activity of the cross-reaction of FMb with antisera to SpMb. The immunochemical studies were accompanied by extensive peptide mapping on tryptic hydrolysates of each protein and its three respective fragments to determine regions of similarities in sequence. The findings suggest that the cross-reaction of SpMb with antisera to FMb is almost enitirely effected through the two antigenic reactive regions located within the sequence 56–131 of SpMb. Conversely, the cross-reaction of FMb with antisera to SpMb takes place through a reactive region(s) in the central peptide F55–129, and another major contribution is due to a reactive region within the sequence 137–149 of FMb. Only a minimal contribution is due to the N-terminal segment F1–54. From these findings it was concluded that immunochemical cross-reactions of two proteins is not necessarily effected through corresponding antigenic regions on the surface of the two proteins. This behavior may be highly complicated by any differences in conformation between the two proteins that may take place as a result of evolutionary amino acid replacements.

Structural and immunochemical relationships of globular proteins have often been studied by determining their immunochemical cross-reaction. Such studies have usually neglected the effect of conformational changes that might take place upon evolutionary amino acid replacement. It is now well established that the antigenic reactivity of globular proteins is very much influenced by changes in conformation (Atassi, 1967a; Habeeb, 1967; Atassi and Skalski, 1969) and the primary antibody response to a globular protein is directed against its native three-dimensional structure (Atassi and Thomas, 1969). Also, it has recently been shown that immunochemical cross-reaction of closely related proteins is not always in direct relationship to sequence similarity (Atassi *et al.*, 1970b). Conformational changes may take place upon evolutionary amino acid replacement (Atassi, 1970; Atassi *et al.*, 1970a; Habeeb and Atassi, 1971) and must be taken into account as a strongly influencing variable. Therefore, immunochemical cross-reaction of proteins is a function of similarity in sequence of antigenic reactive regions, and also close conformations of these regions are required for appropriate fit onto the antibody combining site. It is hard, therefore, to derive conclusions about similarity of antigenic reactive regions in two cross-reacting proteins. This difficulty may be overcome by studying interaction of peptides isolated from corresponding regions of the two proteins. Conformational factors do not enter when studying the cross-reaction of long peptides since these are largely unfolded (Singhal and Atassi, 1970; Epand and Scheraga, 1968; Atassi and Singhal, 1970a).

In a previous report (Atassi and Saplin, 1966) it was shown that finback whale myoglobin cross-reacted with antisera to sperm whale myoglobin. In the present work, antisera against FMb[1] have been prepared and show an appreciable cross-reaction with SpMb. Since information concerning the

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[1] Abbreviations that are not listed in *Biochemistry 5*, 1445 (1966), are: Mb, metmyoglobin; ApoMb, apomyoglobin; SpMb, the major chromotographic component no. 10 of sperm whale Mb obtained by CM-cellulose chromatography (Atassi, 1964); FMb denotes the major chromatographic component, no. 7, obtained by CM-cellulose chromatography (Atassi and Saplin, 1966). Both SpMb and FMb were completely homogeneous by starch gel or acrylamide gel electrophoresis.

antigenic structure of sperm whale myoglobin, reported in several previous communications from this laboratory, represents the most advanced such knowledge for a globular protein, it would be of great value to determine the immunochemical cross-reaction of peptides isolated from sperm whale and finback whale myoglobins. This approach will make it possible to locate the regions in the two proteins that are responsible for the cross-reaction and gain an insight into the effect of a given amino acid substitution on the reactivity of a well-defined antigenic reactive region in a globular protein.

Materials and Methods

Metmyoglobins and Apomyoglobins. Myoglobins used in the present studies were in the met form. Sperm whale myoglobin used in these studies was the major chromatographic component, 10 (SpMb), obtained by CM-cellulose chromatography (Atassi, 1964). Also, the major chromatographic component, 7 (FMb), of finback whale myoglobin (Atassi and Saplin, 1966) was used here. Both SpMb and FMb were homogeneous by starch or acrylamide gel electrophoresis. The apoproteins were prepared from SpMb and FMb by a procedure similar to that described for apohemoglobin (Atassi and Skalski, 1969).

Cleavage at the Methionine Sites and Separation of the Peptides. Cleavage at the methionine sites was accomplished by reaction of apomyoglobin with cyanogen bromide (Gross and Witkop, 1962). Separation of the resulting fragments was carried out by gel filtration on appropriate grades of Sephadex (Edmundson, 1963). The purity of the peptides was confirmed by peptide mapping.

Antisera. Antibodies against the present myoglobins were raised both in rabbits and in goats. Procedure for the preparation of antisera in rabbits and in goats has already been described (Atassi, 1967b). Antisera from individual animals were kept and studied separately and were stored in 8-ml portions at $-40°$. Goat antisera G1 and G4 and rabbit antiserum 77 were against SpMb. Rabbit antisera F6 and F8 were against FMb.

Analytical Methods. Immunochemical methods employed here (*i.e.*, agar double diffusion, quantitative precipitin, absorption, and inhibition experiments) have already been described in detail (Atassi and Saplin, 1968). Results of inhibition experiments were expressed in per cent inhibition, *i.e.*, $100 \times$ (total precipitate with antigen $-$ total precipitate in presence of peptide)/total precipitate with antigen. Concentrations of protein and peptide solutions were based on their nitrogen contents which were calculated from their amino acid compositions (see Table I). Determinations of nitrogen contents of protein or peptide solutions were carried out by a micro-Kjeldahl procedure (Markham, 1942) and by using Nessler's reagent standardized with ammonium sulfate. At least three replicate analyses were done on each solution, and they varied $\pm 0.5\%$ or less. Optical densities were determined with a Zeiss PMQ II spectrophotometer. Amino acid analysis of acid (110°, 22 or 72 hr in double-distilled, constant-boiling HCl, three times nitrogen-flushed evacuated sealed tubes) and alkaline (saturated $Ba(OH)_2$; Ray and Koshland, 1962) hydrolysates was carried out on a BioCal BC-200 amino acid analyzer. Tryptic digestion and peptide mapping were carried out as described elsewhere (Atassi and Saplin, 1968). The peptide maps were stained with ninhydrin and/or with specific stains for various amino acids (Easley, 1965).

Results

Cleavage of Sperm Whale ApoMb at the Methionine Sites. Preparation of highly purified ApoMb peptides obtained by cleavage at the methionine sites has been described in detail elsewhere (Atassi and Saplin, 1968). The specific interaction of these peptides with several antisera to SpMb was also determined (Atassi and Saplin, 1968).

Cleavage of Finback Whale ApoMb at the Methioine Sites and Characterization of the Peptides. Procedures for preparation and characterization of these peptides were similar to those already described for the corresponding sperm whale ApoMb peptides. In Table I, the amino acid compositions of the finback whale ApoMb peptides are given, together with their molecular weights and nitrogen contents, and these values are compared with those for the corresponding sperm whale ApoMb peptides. To avoid ambiguity, the peptides used here will not be denoted by numbers or letters but will be designated by their location in the primary structure. For the peptides derived from sperm whale ApoMb, the position of a peptide in the sequence will be preceded by S, and for those from finback whale ApoMb, the letter F is used.

Similarities in primary structure between SpMb and FMb were revealed by peptide mapping of trypic hydrolysates of the two proteins and of their corresponding cyanogen bromide fragments. Peptides were revealed by staining with one of (or appropriate combinations of) the following stains: ninhydrin, Pauly stain for histidine and tyrosine, α-nitroso-β-naphthol for tyrosine, Sakaguchi stain for arginine, and platinic iodide stain for methionine-containing peptides. From peptide maps of the two proteins, the following peptides in SpMb were unchanged in FMb: 35–42, 46–47, 48–50, 78–87, 88–96, 97–98, 119–133, 134–139, 140–145, and 146–147. Mapping of the peptides in the tryptic hydrolysates of fragments F1–54 and S1–55 confirmed that the following portions of SpMb were unchanged in FMb: 35–42 and 46–50. Peptide mapping of the core fragments S56–131 and F55–129 confirmed that the following sequences of SpMb were unaltered in FMb: 78–98, possibly 99–102, and 119–131. The maps of the tryptic peptides in the C-terminal fragments S132–153 and F130–151 were different only in the position of one spot corresponding to sequence 148–153 in SpMb. In addition, spots were cut out and eluted from lightly stained maps (0.05% ninhydrin in ethanol) and the solutions were centrifuged (5000 rpm, 30 min) and freeze-dried. The peptides from fragments S132–153 and F130–151 had identical amino acid compositions except that peptide 148–153 in SpMb had the following composition: Glu, 1.93; Gly, 2.01; Leu, 0.98; Tyr, 1.04; peptide 146–151 in FMb had the composition: Glu, 1.97; Gly, 1.88; Leu, 1.00; Phe, 1.02. It was therefore concluded that fragments S132–153 and F130–151 differed only in one location (*i.e.*, position 151 in SpMb) where tyrosine-151 in SpMb was substituted by phenylalanine in FMb (Figure 1).

Immunochemical Cross-Reactivity of the Myoglobins. In agar double diffusion with antisera to SpMb, FMb gave a weak precipitin line that spurred with the line due to SpMb. The spur appeared as a continuation of the SpMb line. Conversely, with antisera to FMb, a weak precipitin line was given by SpMb. In each case a spur was formed and appeared as a continuation of the line due to FMb. This partial immunochemical similarity was confirmed by quantitative precipitin analysis. With antisera to SpMb, a partial reaction was shown by FMb (ranging between 34 and 41% relative to the homologous reaction). Previously, it was shown by

TABLE I: Amino Acid Composition of FMb Peptides Obtained by Cleavage at Methionine Peptide Bonds.[a]

	N-Terminal Peptide			Core Peptide			C-Terminal Peptide				
Amino Acid	Found	Nearest integer	Corresp SpMb peptide	Found	Nearest integer	Corresp SpMb peptide	Found	Nearest integer	Corresp SpMb peptide	Totals of FMb peptides	FMb Composition
Trp	1.88	2	2	—	0	0				2	2
Lys	7.24	7	5	10.13	10	10	3.91	4	4	21	20
His	4.58	5	4	5.95	6	8	—			11	11
Arg	1.02	1	2	1.21	1	1	1.04	1	1	3	3
Asp	4.12	4	3	5.92	6	3	2.03	2	2	12	12
Thr	2.02	2	2	2.68	3	3	—			5	5
Ser	1.96	2	2	2.65	3	4	—			5	5
Glu	5.92	6	9	8.53	8 (or 9)	7	3.15	3	3	17	17
Pro	1.28	1	1	2.85	3	3	—			4	4
Gly	4.25	4	3	5.21	5	6	1.91	2	2	11	11
Ala	6.05	6	4	8.96	9	10	3.08	3	3	18	18
Val	1.89	2	5	3.87	4	3	—			6	6
Ile	2.86	3	2	3.65	4	6	0.89	1	1	8	9
Leu	5.80	6	7	7.92	8	8	2.87	3	3	17	17
Tyr	0.14	0	0	0.802	1	1	0.77	1	2	2	2
Phe	2.26	2	3	3.01	3	2	1.83	2	1	7	7
Met (as HS-Lact)	0.89	1	1	0.76	1	1		—		2	2
Totals		54	55		75	76		22	22	151	151
Calcd mol wt		6136	6414		8265	8266		2540	2556	17,521	17,504
Nitrogen content (%)		17.35	17.03		16.95	18.29		17.09	16.98		

[a] Results are expressed in moles of amino acid per mole of peptide and represent the average of four analyses (two 22-hr and two 72-hr hydrolyses) for each peptide. Values of serine and threonine have been obtained by extrapolation to zero hydrolysis time. Tryptophan was determined from duplicate alkaline hydrolyses. Composition of corresponding SpMb peptides was obtained from Edmundson (1963) and Atassi and Thomas (1969).

132 151
S132-153: Asn- Lys- Ala- Leu- Glu-Leu- Phe- Arg- Lys- Asp- Ile- Ala- Ala- Lys- Tyr- Lys-Glu- Leu- Gly- *Tyr*- Gln- Gly-

130 151
F130-151: (Asn, Lys)(Ala, Leu, Glu, Leu, Phe, Arg)(Lys)(Asp, Ile, Ala, Ala, Lys)(Tyr, Lys)(Glu, Leu, Gly, *Phe*, Gln, Gly)

FIGURE 1: Amino acid sequence of fragment S132-153 from SpMb (Edmundson, 1965) and the composition of the tryptic peptides of F130-151 from FMb (present data). Peptide maps of the tryptic hydrolysates of fragments S132-153 and F130-151 were identical, except for one spot which corresponded in SpMb to sequence 148-153, and this in FMb had phenylalanine instead of tyrosine. For experimental details, see text. It may therefore be concluded that tyrosine-151 in SpMb is replaced by phenylalanine (position 149) in FMb.

TABLE II: Immunochemical Cross-Reaction of Sperm Whale and Finback Whale Myoglobins.

Antiserum[b]	SpMb	FMb
Reaction with Antisera to SpMb[a]		
G1	100	41.5
G4	100	33.8
77	100	40.9
Reaction with Antisera to FMb[a]		
F6	57.6	100
F8	54.9	100

[a] Values represent the percentage of precipitation at equivalence relative to homologous reaction as 100%. Results were obtained from three independent determinations which varied ±1.4 or less. [b] G1 and G4 are goat antisera and 100 is a rabbit antiserum, each to SpMb; F6 and F8 are rabbit antisera to FMb.

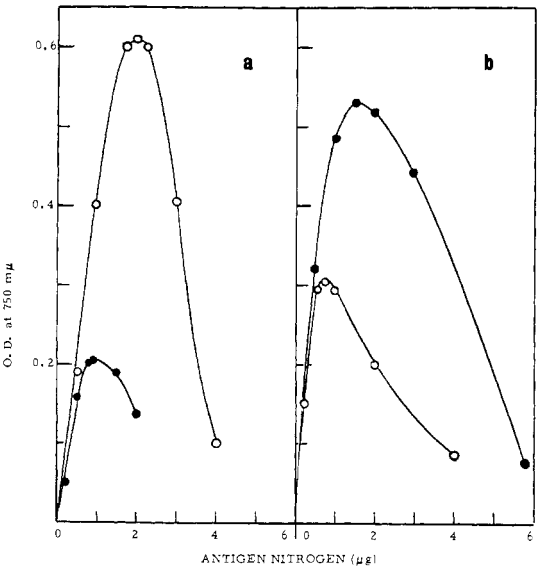

FIGURE 2: Homologous precipitin reactions and cross-reactions of SpMb (○) and FMb (●). Part a shows cross-reaction of FMb, with goat antiserum G4 (which had been diluted with an equal volume of 0.15 M NaCl) to SpMb, together with the reaction of the homologous antigen. Part b shows reaction of rabbit antiserum F6 (also prediluted 1:1) to FMb with homologous antigen and its cross-reaction with SpMb. E_{750} indicates the amount of protein in immune precipitate as determined by the Folin method (Lowry *et al.*, 1951).

complement fixation experiments (Atassi and Saplin, 1966) that FMb cross-reacted 34% with one rabbit antiserum to SpMb. With antisera to FMb, the cross-reaction of SpMb was slightly higher (55–58% relative to homologous antigen) than the reverse cross-reaction. Figure 2a gives an example of the cross-reaction of FMb with one antiserum to SpMb and in Figure 2b the reactions of these two proteins with one antiserum to FMb are shown. Table II summarizes the reactions of SpMb and FMb with several antisera against each of the two proteins.

Immunochemical Reactivity of the Peptides with the Homologous Antisera. A. REACTION OF SpMb PEPTIDES WITH ANTISERA TO SpMb. The specific immunochemical interaction of these peptides with antisera to SpMb has already been investigated in great detail (Atassi and Saplin, 1968) and will only be outlined briefly here. In the SpMb system, peptides S1–55 and S56–131 give specific immune precipitates. This has been shown exhaustively by a variety of precipitation and absorption experiments with antisera to SpMb (Atassi and Saplin, 1968). On the other hand, peptide S132–153 exhibits inhibitory activity for the precipitation of SpMb with its antisera (Atassi and Saplin, 1968). Table III summarizes the activities of these peptides with antisera G1, G4, and 77 relative to reaction of each antiserum with SpMb.

B. REACTION OF FMb PEPTIDES WITH ANTISERA TO FMb. The N-terminal fragment F1–54 and the core fragment F55–129 each gave a precipitate with both antisera F6 and F8 to FMb. It was, however, necessary to confirm that the precipitates were in fact specific immune precipitates and not a phenomemon of nonspecific precipitation. With preimmune sera (*i.e.*, sera from rabbits F6 and F8 before immunization with FMb) no precipitation was obtained with peptide F55–129. Peptide F1–54 gave very little (1–2%) nonspecific precipitation with the preimmune sera, and a correction for this was made when specific immune precipitates for this peptide were calculated. Further evidence that the precipitates with peptides F1–54 and F55–129 were genuine immune precipitates was derived from quantitative absorption experiments.

TABLE III: Reaction of Sperm Whale Myoglobin Peptides with Homologous Antisera: Per Cent Activity Relative to Reaction of Antiserum with SpMb.[a]

Peptide	Antiserum G1	Antiserum G4	Antiserum 77
1–55[b]	27.6	8.2	25.6
56–131[b]	62.6	80.5	49.0
132–153[c]	21.0	13.3	21.0
Total	111.2	102.0	95.6

[a] Values for G1 and G4 are obtained from Atassi and Saplin (1968) and those for antiserum 77 are from Atassi and Thomas (1969). [b] These peptides give specific immune precipitates with antisera to SpMb; the values here therefore represent maximum precipitation by peptide relative to precipitation at equivalence by SpMb. [c] This peptide inhibits the reaction of SpMb with its antisera and the values here represent maximum per cent inhibition by the peptide of the precipitin reactions of SpMb.

TABLE IV: Relative Amounts of Precipitation Formed by F1–54 and F55–129 with Homologous Antisera: Per Cent Precipitation Relative to Reaction of Antiserum with FMb.[a]

Antiserum	Peptide F1–54	Peptide F55–129
F6	14.1 ± 0.9	61.3 ± 1.2
F8	7.7 ± 0.6	75.0 ± 1.0

[a] The percentage of precipitation was derived from maximum precipitation by peptide relative to precipitation at equivalence by FMb. Results were derived from three independent analyses.

TABLE V: Maximum Inhibitory Activities by Peptides F129–151 and S132–153 of Homologous and Heterologous Reactions: Maximum Per Cent Inhibition[a] and Peptide/Antigen Molar Ratios.[b]

Peptide:		F130–151		S132–153			
Antigen:	SpMb	FMb		SpMb[c]		FMb	
			Molar Ratio[b]		Molar Ratio[b]		Molar Ratio[b]
Antiserum	% Inhib	% Inhib[a]	Pept/Antigen	% Inhib[a]	Pept/Antigen	% Inhib[a]	Pept/Antigen
F6	0	8.2	12.8	0		14.6	11.5
F8	0	12.3	6.9	0		11.2	6.5
G1	0	0		21.0	4.1	17.5	5.2
G4	0	0		13.5	13.4	13.0	15.3
77	0	0		21.0	6.8	16.5	7.9

[a] Results are expressed in maximum per cent inhibition by the peptide of the precipitin reactions of SpMb and FMb with various homologous and heterologous antisera. Results represent the average of three determinations which ranged ±1.2% or less. [b] The peptide/antigen molar ratios given are the values of these ratios at 50% of maximum inhibition. [c] These values were obtained from Atassi and Thomas (1969).

For each peptide, the amount of reaction obtained with a given antiserum was quantitatively equivalent to the decrease in reactivity of the absorbed serum with the homologous antigen. An example of such an absorption experiment is shown in Figure 3. The reactivity of these peptides with antisera F6 and F8 is shown in Table IV. In contrast with these two peptides, fragment F130–151 did not give immune precipitates with antisera to FMb but did inhibit the reaction of FMb with its antisera. The inhibitory activity of peptide F130–151 with antisera F6 and F8 is shown in Table V.

It may be pointed out here that in FMb, as in SpMb, all three fragments were immunochemically reactive with homologous antisera. Although the reactivity of a given peptide varied with the antiserum, the core peptide F55–129 was, invariably, the immunochemically dominant portion of the molecule.

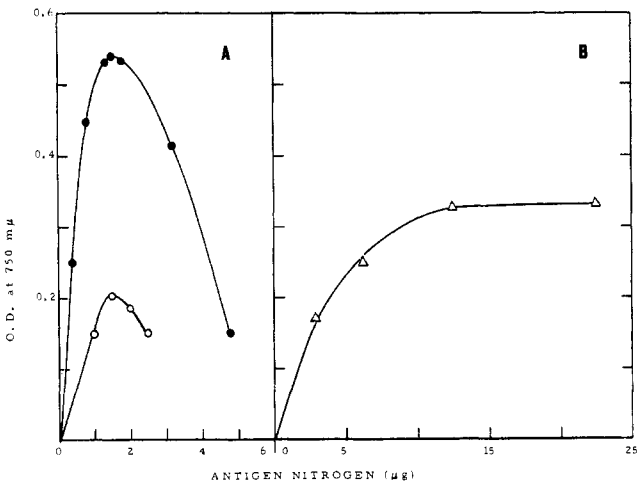

FIGURE 3: Precipitin reaction and absorption experiment on peptide F55–129 with rabbit antiserum F6. Part A shows the reaction of FMb with antiserum which had been absorbed with peptide F55-129 (○); this is compared with its reaction with unabsorbed serum (●) which had been diluted 1:1 with 0.15 M NaCl (to account for the volume of peptide solution used in absorption experiment). In part B, the precipitin reaction of peptide F55-129 with antiserum which had been diluted 1:1 with 0.15 M NaCl is shown (△). Notice that peptide F55–129 precipitated at maximum 61.3% relative to FMb and absorption with this peptide decreased the reactivity of the antiserum with FMb by 62.6%. This was strong evidence that the precipitate obtained with peptide and antiserum was a true immune precipitate.

Immunochemical Reactivity of the Peptides with Heterologous Antisera. A. CROSS-REACTION OF FMb PEPTIDES WITH ANTISERA TO SpMb. Finback whale Mb peptides F1–54 and F55–129 gave specific immune precipitates with antisera to SpMb. That these precipitates were in fact specific immune precipitates was confirmed by lack of precipitation of these peptides with preimmune sera and by quantitative absorption experiments. Table VI summarizes the cross-reactions of peptides F1–54 and F55–129 with three antisera to SpMb. It can be seen that the N-terminal peptide F1–54 had, with a given antiserum, a consistently lower cross-reaction than the core peptide F55–129. Also in Table VI, the results of quantitative absorption experiments with these peptides demonstrate that, for each peptide with a given antiserum, the amount

TABLE VI: Cross-Reaction of Finback Whale Myoglobin Peptides F1–54 and F55–129 with Antisera to Sperm Whale Myoglobin: Per Cent Precipitation Relative to Reaction of Antiserum with SpMb.

	% Ppt by Peptide[a]		Reaction of Antiserum with SpMb after Absorption with Peptide[b]	
Antiserum	F1–54	F55–129	F1–54	F55–129
G1	4.7	19.1	93.4	79.6
G4	6.3	13.9	92.0	85.0
77	5.3	16.8	93.8	83.1

[a] Values were obtained from maximum precipitation by peptide and precipitation at equivalence by SpMb. [b] Values were derived from precipitation at equivalence by SpMb with sera previously absorbed with peptide relative to its reaction at equivalence with the corresponding unabsorbed serum. Results represent the average of three determinations, and they varied ±1.4% or less.

of antibody removed accounted entirely for the decrease in reactivity of the absorbed serum with the homologous antigen.

The C-terminal peptide F130–151 gave no immune precipitates with antisera to SpMb over a wide range of peptide concentration. Also, this peptide did not inhibit the precipitin reaction of SpMb with its antibodies (Table V) even when a 400 molar excess of peptide was employed relative to SpMb. In addition, peptide F130–151 did not inhibit the cross-reaction of FMb with antisera to SpMb or the cross-reaction of SpMb with antisera to FMb (Table V).

B. Cross-Reaction of SpMb Peptides with Antisera to FMb. The N-terminal fragment of SpMb, *i.e.*, S1–55, did not show any cross-reaction with antisera to FMb. Thus no immune precipitation was obtained and the peptide also failed to inhibit the reaction of FMb with its antisera, even at several hundred molar excess of peptide relative to FMb. The core fragment of SpMb, on the other hand, S56–131, showed a substantial cross-reaction (56 and 60%) with antisera F6 and F8. The specificity of this cross-reaction was also confirmed by absorption experiments. With a given antiserum, the amount of antibody precipitated by peptide S56–131 was quantitatively equivalent to the decrease in reactivity of the peptide-absorbed antiserum with the homologous antigen (*i.e.*, FMb). Table VII summarizes the results of cross-reactions of peptides S1–55 and S56–131 with antisera to FMb.

The reactions of the tail peptide of SpMb, S132–153, were unique. It did not give immune precipitates but had interesting inhibitory activities. First, peptide S132–153 did not inhibit the cross-reaction of SpMb with antisera to FMb but inhibited the reaction of FMb with its own antisera. Its "cross-inhibitory" activity against FMb was not limited to the reaction of FMb with its own antisera, but it also inhibited the cross-reaction of this protein with the antisera to sperm whale Mb. Table V summarizes the results of the inhibitory activities by this peptide of the precipitin reaction of SpMb and FMb with the homologous and heterologous antisera.

TABLE VII: Cross-Reaction of Sperm Whale Myoglobin Peptides S1–55 and S56–131 with Antisera to Finback Whale Myoglobin: Per Cent Precipitation Relative to Reaction of Antiserum with FMb.[a]

	Peptide S1–55	Peptide S56–131	
Antiserum	% Ppt by Peptide[b]	% Ppt by Peptide	Reaction of Peptide-Absorbed Antiserum with FMb
F6	0	60.4 ± 1.1	39.2
F8	0	55.8 ± 1.2	42.8

[a] The values of per cent precipitation were derived from maximum precipitation by peptide and precipitation at equivalence by FMb, and they were obtained from three independent determinations. [b] This peptide also possessed no inhibitory activity with these sera.

Discussion

The cross-reactions of the present two proteins with the heterologous antisera were not equal. Sperm whale Mb cross-reacted more strongly with antisera to FMb than the reverse situation. Interpretation of this is not straightforward since immunochemical cross-reactions of intact proteins are complicated by the fact that the antibody response is directed against the native three-dimensional structure of a protein antigen (Atassi and Thomas, 1969), and the antigenic reactivity of native proteins is highly influenced by changes in their conformations (Atassi, 1967a; Habeeb, 1967; Andres and Atassi, 1970).

Homogeneity of the peptide fragments obtained from cleavage of sperm whale ApoMb at the methionine sites has already been exhaustively confirmed and reported in detail (Atassi and Saplin, 1968). Homogeneity of the present corresponding fragments from finback whale ApoMb was confirmed by peptide mapping on heavily loaded chromatograms, by obtaining single symmetrical peaks in two consecutive runs, after gel filtration of each peptide at least five times through the sephadex Columns, and finally by obtaining a true plateau in the precipitin reaction, with each precipitating peptide, in the region of peptide excess. A true plateau with a precipitating peptide would not be possible if the immune precipitate were in fact caused by contamination with undegraded antigen (Atassi and Saplin, 1968).

The N-terminal peptide S1–55 in SpMb carries one reactive region located within the sequence 8–30 (Atassi and Singhal, 1970b; Singhal and Atassi, 1971). In the three-dimensional structure of SpMb, this region of the molecule occupies the corner between helices A and B (Kendrew *et al.*, 1961). In the corresponding fragment of FMb (*i.e.*, F1–54), most of the differences in primary structure seem to be located within the sequence 1–30. It is, therefore, not surprising that fragment S1–55 failed to react with antisera to FMb, despite the fact that this peptide usually possesses substantial (up to 28%) reaction with antisera to SpMb. On the other hand, in the converse situation, peptide F1–54 showed a slight cross-reaction (5 or 6%) with the three SpMb antisera tested here. It appears, therefore, that in the cross-reaction of SpMb with antisera to FMb, the N-terminal sequence S1–55 does not contribute to this cross-reactivity. However, in the reverse case (*i.e.*, cross-reaction of FMb with antisera to SpMb), the N-terminal segment F1–54 may contribute slightly to the interaction.

Fragment S56–131, in SpMb, reacts strongly with homologous antisera (Atassi and Saplin, 1968) and appears to carry two reactive regions (Atassi and Singhal, 1970b). It is significant that the cross-reactions of this peptide with antisera to FMb were comparable in magnitude to its reactivity with antisera to SpMb. Conversely, the cross-reaction of the central peptide of FMb, F55–129, with antisera to SpMb, although appreciable (14–19% with the present antisera), was much lower than that of the corresponding cross-reaction of the sperm whale Mb peptide with the antisera to FMb. The portions which have identical sequences, that could be identified by peptide mapping of tryptic hydrolysates of the two proteins as well as of the two fragments, corresponded to sperm whale Mb sequences 78–98 and 119–131. Within the sequence 79–96 in SpMb one antigenic reactive region is present (Atassi and Saplin, 1968; Atassi and Thomas, 1969), and this explains the substantial cross-reactions of peptides S56–131 and F55–129 with the antisera to the heterologous antigen. Another reactive region has been reported in sequence 56–131 in SpMb. This region is located C-terminal to tyrosine-103 (Atassi, 1968) and is centered around arginine-118 (Atassi and Thomas, 1969; Atassi and Singhal, 1970b). The

TABLE VIII: Comparison of the Cross-Reaction of Sperm Whale Myoglobin and the Total Reaction of Its Peptides with Antisera to FMb: Per Cent Cross-Reaction Relative to Reaction of Antiserum with FMb.

Anti-serum	Peptide S1–55[a]	Peptide S56–131[a]	Peptide S132–153[b]	Total	Cross-Reaction of SpMb
F6	0	60.4	0	60.4	57.6
F8	0	55.8	0	55.8	54.9

[a] Data are obtained from Table VII. [b] These results are from Table V.

major portion of this reactive region in SpMb will be located on peptide 103–118, which is one of the insoluble tryptic peptides (Edmundson, 1963) and consequently not revealed in the peptide maps. The strong reaction of the SpMb peptide, S56–131, with antisera to FMb suggests strong similarities of the sequence 103–118 in SpMb to the corresponding region of FMb. That these regions may not be entirely identical is suggested by the weaker reaction of the FMb peptide, F55–129, with antisera to SpMb.

The behaviors of the C-terminal fragments, S132–153 and F130–151, were quite interesting. Whereas fragment F130–151 showed no cross-reaction with antisera to SpMb, the reverse did not hold. Thus fragment S132–153 exhibited considerable inhibitory activity (14–18%) of the cross-reaction of FMb with antisera to SpMb. In the sequence 132–153 of sperm whale Mb, only one reactive region exists, C-terminal to arginine-139 (Atassi and Thomas, 1969), but does not include the C-terminal dipeptide (Atassi, 1966) and is located around tyrosines-146 and -151 (Atassi, 1968). The results of peptide mapping of the tryptic hydrolysates of fragments S132–153 and F130–151 indicate that, except for one substitution, the two fragments have identical sequence. The difference was at position 151 (tyrosine) of sperm whale Mb. The amino acid at the corresponding location (position 149) in finback whale Mb was phenylalanine. The location of this substitution was quite fortuitous since it occurs, in sperm whale myoglobin, within a reactive region. Since the reaction of sperm whale Mb with antisera to SpMb (or with antisera to FMb) cannot be inhibited by the heterologous fragment F130–151, it may be concluded that the substitution of tyrosine by phenylalanine within the reactive region impairs the efficiency of this region to interact with the antibody combining site. It is likely that, since the antibody specificity was originally directed against a region which carried a tyrosine residue, the phenolic OH of tyrosine may play some active part through hydrogen bonding with the antibody combining site. This was also suggested from the results of nitration of tyrosines-151 and-146 (Atassi, 1968). On the other hand, the reaction of FMb with antisera to FMb, or with antisera to SpMb, can be inhibited by the heterologous fragment S132–153, indicating that a reaction through the tail region of FMb does take place and is well inhibited by a tyrosine-containing reactive peptide. It is, therefore, quite clear that the presence of phenylalanine at position 149 in FMb does not destroy its ability to interact through the C-terminal reactive region with an antibody combining site originally directed against a region that contained tyrosine at that location. The interaction of FMb with the heterologous antisera cannot be inhibited by a phenylalanine-containing reactive peptide but is favorably displaced by a peptide containing tyrosine at that location. All these findings suggest that intact sperm whale Mb does not bind with heterologous antisera through the tail region but finback whale Mb will bind through its tail region with heterologous antisera.

It is noteworthy that the foregoing conclusions may be further confirmed by addition of the reactivities of the various fragments to determine if these will account for the total cross-reaction of the intact protein. In Table VIII, it can be seen that the cross-reaction of sperm whale Mb with antisera to finback whale Mb is entirely accounted for by the reaction of fragment S56–131 with these antisera. On the other hand, the cross-reaction of FMb with antisera to SpMb cannot be accounted for entirely by the total reactivities of its three fragments with these antisera (Table IX). However, as mentioned previously, since the cross-reaction of FMb with these antisera can be inhibited by the tail fragment (S132–153) from sperm whale Mb, it may be reasoned that FMb must have cross-reacted through its tail region, although the free peptide F130–151 will not possess this ability to cross-react. It is clear that the conformation of the intact protein imparts upon this region a mode of folding appropriate for its binding with the antibody combining site, despite the fact the phenylalanine has substituted the tyrosine at position-151 of sperm whale Mb. This may not be achieved with the free peptide F130–151, which therefore exhibits no activity with antisera to SpMb. In fact, when the inhibitory activity by peptide S132–153 of the cross-reaction of FMb is added to the reactivities of FMb peptides, F1–54, and F55–129, the total is in very satisfactory agreement, for a given antiserum to SpMb, to the cross-reaction of FMb itself (Table IX).

In conclusion, factors and regions responsible for the immunochemical cross-reaction of two proteins have been extensively investigated. The cross-reaction of sperm whale Mb with antisera to FMb is almost entirely effected through the two antigenic reactive regions located within the sequence 56–131 of sperm whale Mb. Conversely, the cross-reaction of FMb with antisera to sperm whale Mb takes place through a reactive region(s) within the sequence 55–129 of FMb. Another major contribution to the cross-reaction comes from a reactive region within the sequence 137–149 in FMb (corresponding to sequence 139–151 in sperm whale Mb), and only a minimal contribution to the cross-reaction is due to the N-terminal segment F1–54. A reactive region (with homologous antisera) is known to be present within sequence 8–30 in SpMb. Also the present data confirm that tyrosine-151 in SpMb is located within an antigenic reactive region. Its substitution by phenylalanine removes the reactivity of this region, in the free peptide, with the homologous antisera (*i.e.*, those directed against a tyrosine-containing region in that location). The substitution, however, has only little effect on the reactive efficiency of this region within the intact protein (FMb), clearly pointing to the importance of long-range interactions in conferring an appropriate folding on the reactive region.

Some general conclusions could also be made, here, concerning the immunochemical cross-reaction of two proteins. In the cross-reaction of one protein with antisera to the other, a given antigenic reactive region on this protein may take an active part, while its corresponding structural counterpart on the other protein may not even participate in the reverse cross-reaction. When corresponding regions on the two proteins do participate in the parallel cross-reactions, they

TABLE IX: Comparison of the Cross-Reaction of FMb and the Total Reaction of Its Peptides with Antisera to SpMb: Per Cent Cross-Reaction Relative to Reaction of Antiserum with SpMb.

Antiserum	Peptide F1–54[a]	Peptide F55–129[a]	Peptide F130–151[b]	A Total	B Cross-Reaction of FMb	Difference B − A	Inhibition[b] of FMb Reaction by S132–153
G1	4.7	19.1	0	23.8	41.5	17.7	17.5
G4	6.3	13.9	0	20.2	33.8	13.6	13.0
77	5.3	16.8	0	22.1	40.9	18.8	16.5

[a] Data are obtained from Table VI. [b] These inhibition results are from Table V.

may well not do so with equal affinity. This behavior may be highly complicated by any differences in conformation between the two proteins that may take place as a result of evolutionary amino acid substitutions (Atassi, 1970; Atassi *et al.*, 1970a,b). Thus, if the antibody response against an immunogenic region in one protein can accommodate a substitution in the corresponding region of a second cross-reacting protein, the reverse may not always be true. It may be relevant to point out here that, as previously mentioned (Atassi and Saplin, 1968), one or more *reactive regions* from different parts of the molecule that may be distant in sequence but close in the three-dimensional structure may together be incorporated into a single reactive *site*. Accommodation of an amino acid substitution in a reactive region by cross-reacting antisera will be dependent on the nature of the substitution, on the primary structure around the substitution both in the immediate region and in approaching folds that may constitute a *reactive site*, and finally on the conformation of the reactive region or the reactive site.

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