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The antibody response to myoglobin is independent of the immunized species

Analysis in terms of replacements in the antigenic sites and in environmental residues of the cross-reactions of fifteen myoglobins with sperm-whale myoglobin antisera raised in different species

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The recent determination of the entire antigenic structure of sperm-whale myoglobin with rabbit and goat antisera has permitted the examination of whether the antigenic structure recognized by antibodies depends on the species in which the antisera are raised. Also, by knowledge of the antigenic structure, the molecular factors that determine and influence antigenicity can be better understood in terms of the effects of amino acid substitutions occurring in the antigenic sites and in the environmental residues of the sites. In the present work, the myoglobins from finback whale, killer whale, horse, chimpanzee, sheep, goat, bovine, echidna, viscacha, rabbit, dog, cape fox, mouse and chicken were examined for their ability to cross-react with antisera to sperm-whale myoglobin. By immunoadsorbent titration studies with radioiodinated antibodies, each of these myoglobins was able to bind antibodies to sperm-whale myoglobin raised in goat, rabbit, chicken, cat, pig and outbred mouse. It was found that the extent of cross-reaction of a given myoglobin was not dependent on the species in which the antisera were raised. This indicated that the antibody response to sperm-whale myoglobin (i.e. its antigenic structure) is independent of the species in which the antisera are raised and is not directed to regions of sequence differences between the injected myoglobin and the myoglobin of the immunized host. Indeed, in each antiserum from a given species examined, that antiserum reacted with the myoglobin of that species. The extent of this auto-reactivity for a given myoglobin was comparable with the general extent of cross-reactivity shown by that myoglobin with antisera raised in other species. The cross-reactivities and auto-reactivities (both of which are of similar extents for a given myoglobin) can be reasonably rationalized in terms of the effects of amino acid substitutions within the antigenic sites and within the residues close to these sites. These findings confirm that the antigenicity of the sites is inherent in their three-dimensional locations.

The entire antigenic structure of sperm-whale Mb has been precisely determined (Atassi, 1975; or in more detail Atassi, 1977). The native protein has five antigenic sites, each of which consists of six or seven amino acid residues and occupies a continuous conformationally distinct surface part of its polypeptide chain. Such antigenic sites have been termed continuous antigenic sites (Atassi, 1978; Atassi & Smith, 1978). The antigenic structure of sperm-whale Mb was determined with early-course antisera raised in rabbits and goats. An important question that can be asked is whether antibodies raised against sperm-whale Mb in species other than rabbits and goats would recognize the same five antigenic sites. One way to answer this question without resorting to the extensive and time-consuming investigations used previously is to compare the abilities of antisera raised in various species to cross-react with different myoglobins. If the same sites on sperm-whale Mb are recognized by all antisera, then the same general degree of cross-reaction for a given Mb would be expected. In addition, cross-reaction studies with a protein whose antigenic structure is completely known can lead to

Abbreviations used: Mb, myoglobin; Hb, haemoglobulin; IgG, immunoglobulin G.

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insight into the structural factors that determine and influence antigenicity, and should enable the recognition of the effects of amino acid replacements occurring both in the antigenic sites and in the neighbouring residues that form the environment of the sites. The latter were derived in the preceding paper (Kazim & Atassi, 1980).

Materials and methods

Materials

Myoglobins from the skeletal muscle of bovine, sheep, goat, rabbit, finback whale and sperm whale and from the heart muscle of mice (outbred) and chicken were isolated and crystallized, and the major chromatographic components were obtained by CM-cellulose chromatography of the twice-crystallized myoglobins (Atassi, 1964*a*, 1970). Horse Mb from skeletal muscle was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and further purified by CM-cellulose chromatography (Atassi, 1970). Chimpanzee, cape-fox, dog, echidna, killer-whale and viscacha myoglobins were purified by the procedure of Romero-Herrera *et al.* (1976*a*). Human adult Hb was from CM-cellulose chromatography of twice-crystallized Hb (Atassi, 1964*b*). Sepharose CL-4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and CNBr from Pierce Chemical Co. (Rockford, IL, U.S.A.). Rabbit Hb, fraction V, was from Sigma Chemical Co. and bovine serum albumin (three-times crystallized) was obtained from Nutritional Biochemical Corp. (Cleveland, OH, U.S.A.). Purified rabbit IgG and goat IgG were prepared by the procedure already described (Lee & Atassi, 1977). Carrier-free [^{125}I]-iodide was obtained from New England Nuclear (Boston, MA, U.S.A.). All other reagents employed were of analytical grade.

Preparation of antisera and specific antibodies

Early-course antisera (28–30 days) were raised against sperm-whale Mb in goats (G3 and G4), rabbits (77, 80, M8, M9), cats (CTM1 and CTM2), hens (CK1 and CK2), outbred mice (MS2, MS3 and MS5) and a pig (P1) as previously described (Atassi, 1967*a*). The antisera from individual animals were not mixed and were stored separately in small samples at −40°C. Hyperimmune antisera to purified goat IgG or mouse IgG were prepared in rabbits, and antiserum to rabbit IgG was prepared in a goat, by using initially the procedure of Atassi (1967*a*). After 1 month, the animals were given booster injections once a month and bled every 2 weeks. The various bleedings of the rabbits to a given IgG were combined, as were the bleedings from the goat to give anti-(rabbit IgG), anti-(goat IgG) or anti-(mouse IgG) sera, which were used as reagents in double-antibody determinations. Preparation of the IgG fractions from these antisera was performed as previously described (Lee & Atassi, 1977). Specific antibodies to sperm-whale Mb, goat IgG, mouse IgG or rabbit IgG were prepared by immunoadsorption on the appropriate Sepharose–protein adsorbent followed, after extensive washing with phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.2), by displacement with 5 M-guanidinium chloride, pH 8.5, and immediate dilution with 17.5 mM-sodium phosphate buffer, pH 7.2, according to the previously described procedures (Lee & Atassi, 1977). Antibody preparations and immune IgG fractions were radioiodinated with ^{125}I by the chloramine-T method (Hunter & Greenwood, 1962).

Preparation and use of immunoadsorbents

Sepharose CL-4B was activated by CNBr (March *et al.*, 1974) and coupled to Mb from various species, rabbit IgG, goat IgG, mouse IgG, bovine serum albumin, rabbit Hb or human Hb under the optimum conditions for active immunoadsorbents (Twining & Atassi, 1979). The immunoadsorbents contained 1.5–1.8 mg of protein/ml packed volume.

Quantitative immunoadsorbent titration studies were performed with fixed amounts of ^{125}I-labelled specific anti-(sperm-whale Mb) antibodies or ^{125}I-labelled immune IgG fractions and increasing amounts of Mb–Sepharose or control protein–Sepharose in glass tubes by the procedures described previously (Twining & Atassi, 1979). The solvent for the titration studies was 0.1% non-immune rabbit IgG in phosphate-buffered saline. Adsorbents of bovine serum albumin, rabbit Hb and human Hb were used as controls for background binding.

Quantitative immunoadsorbent studies by the double-antibody procedure were performed with unlabelled goat, mouse or rabbit anti-(sperm-whale Mb) sera followed by the appropriate ^{125}I-labelled specific anti-IgG antibodies. The unlabelled antisera to sperm-whale Mb were diluted with phosphate-buffered saline containing 0.1% rabbit Hb, so the final dilution was in the range of 1 : 1000 to 1 : 10000 (v/v), depending on the antibody titre. For titration, increasing amounts (6–100 μl) of the immunoadsorbents were pipetted as a 1 : 1 (v/v) suspension in the phosphate-buffered saline containing 0.1% rabbit Hb into glass tubes, and a portion (10–50 μl) of the diluted antiserum was added to each tube. After rotation for at least 6 h at room temperature, the immunoadsorbents were washed as previously described (Twining & Atassi, 1979). After the last wash, the liquid was drawn off so that the volume of liquid left in the tubes was constant (100 μl). A portion (100 μl) of the appropriate ^{125}I-labelled second antibody [anti-(goat IgG), anti-(mouse IgG) or anti-(rabbit IgG) antibody, 1×10^5–1.5×10^5

c.p.m.] in phosphate-buffered saline containing 0.1% rabbit Hb was added to each tube. The tubes were again rotated and washed as previously described.

Analytical procedures

Absorbance measurements were made with a Zeiss PMQII spectrophotometer. The homogeneity of the myoglobins used was confirmed by disc-gel electrophoresis (Atassi, 1970), and their identity was verified by amino acid analysis of acid hydrolysates (Atassi & Saplin, 1968). The amounts of ^{125}I-labelled antibodies bound on an immunoadsorbent were determined with a Packard γ-scintillation counter.

Results

Comparison of binding results with radioiodinated specific antibodies and the immune IgG fractions

In certain antisera it was desirable to use the immune IgG fraction of the antiserum rather than the specific antibody preparation. Even though it should be expected that this would have no effect on the fraction of antibodies bound in the plateau by a given adsorbent, the possibility was nevertheless investigated with several goat, mouse and rabbit antisera to sperm-whale Mb. Table 1 shows an example of a comparison of the binding results of immunoadsorbents of the various myoglobins with the specific antibody preparation and the immune IgG fraction from a goat antiserum (G3) to sperm-whale Mb. It can be seen that the binding values were essentially the same with the ^{125}I-labelled immune IgG or with ^{125}I-labelled specific antibodies. Accordingly, the use of the IgG fraction presented a considerable saving in time, and the only advantage in the employment of specific antibodies is to conserve on the ^{125}I label.

Table 1. *Comparison of the binding of anti-(sperm-whale Mb), specific antibodies and the immune IgG fraction with various myoglobins attached to Sepharose*

Results are given as percentage of antibody bound to each Mb relative to the label bound by sperm-whale Mb as 100%. They represent plateau binding values obtained by the titration of fixed amounts of ^{125}I-labelled specific antibodies or ^{125}I-labelled immune IgG fraction (from goat antiserum G3) by increasing amounts of each Mb–Sepharose. Values represent averages of four replicate analyses and are corrected for non-specific binding. Non-specific binding to human Hb–Sepharose or bovine serum albumin–Sepharose was 1–3% of the binding to sperm-whale Mb.

	^{125}I-labelled antibodies bound (%)	
Myoglobin	^{125}I-labelled specific antibodies	^{125}I-labelled immune IgG fraction
Sperm whale	100.0	100.0
Finback whale	67.3	64.5
Killer whale	63.2	63.6
Horse	48.5	49.6
Chimpanzee	44.0	41.9
Lamb	34.3	35.7
Goat	38.9	36.0
Bovine	28.7	29.8
Echidna	25.2	21.1
Viscacha	31.7	31.3
Dog	27.5	28.3
Cape fox	27.3	28.7
Chicken	22.3	21.8

Comparison of the single-antibody and double-antibody binding techniques

In view of the fact that these studies employed several rabbit, mouse and goat antisera to sperm-whale Mb, it was desirable to be able to investigate the binding capacity of the various Mb–Sepharose adsorbents by the double-antibody procedure, which would afford an appreciable saving in time. Thus the ^{125}I-labelled IgG fractions of antisera against rabbit IgG in a goat, and against goat IgG or mouse IgG in rabbits, were used as general anti-IgG reagents for the appropriate species in the double-antibody procedure. It was necesary, however, to determine for each anti-Mb serum the range for the linear part of the binding curve, so that, for a fixed amount of labelled second antibody added, the amount of radioactivity (c.p.m.) bound would be proportional to the amount of the first unlabelled antibody bound on to the adsorbent. Figs. 1 and 2 show examples of the linear part of this curve for two antisera (rabbit antiserum M8 and goat antiserum G4) bound to an adsorbent of sperm-whale Mb. The plateau region in which the maximum antibody binding occurred was also determined for each Mb–Sepharose by titrating fixed amounts of antiserum and second antibody with increasing amounts of the Mb–Sepharose adsorbents. The second antibody was used in excess. Typical binding curves are given in Fig. 3 for a goat antiserum (G3) with adsorbents of several myoglobins. Under the conditions used in these experiments, 25μl of the Sepharose adsorbents were required to achieve maximum (plateau) binding (Fig. 3). Table 2 shows a comparison of the degree of cross-reaction obtained by the single-antibody and double-antibody procedures for various Mb–Sepharose adsorbents with two antisera to sperm-whale Mb (goat G3 and rabbit M8). It can be seen that the values of cross-reactivities by the two procedures were in good agreement. It should be noted that all binding values reported here were corrected for non-specific binding to adsorbents of bovine serum albumin, human Hb and rabbit Hb. The amount of non-specific binding was 5% or less

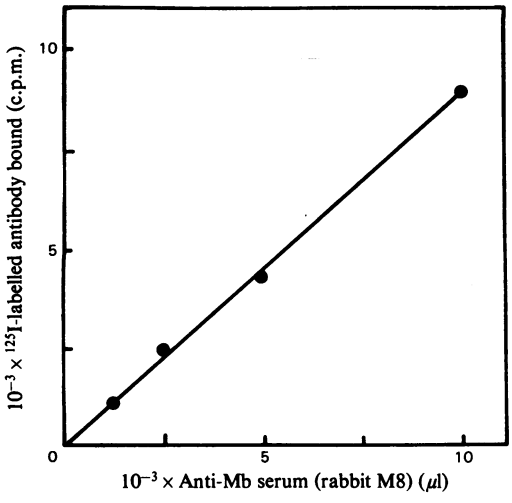

Fig. 1. *Determination of the linear binding region of ^{125}I-labelled goat anti-(rabbit IgG) antibodies*

Increasing amounts of rabbit antiserum M8 were bound to sperm-whale Mb–Sepharose (50μl). After the excess proteins had been washed from the adsorbents, a portion of the ^{125}I-labelled goat anti-(rabbit IgG) antibodies (1.2×10^5 c.p.m.) was added to each tube and allowed to bind. The net binding values are corrected for non-specific binding (<1% by an equal volume of human Hb–Sepharose).

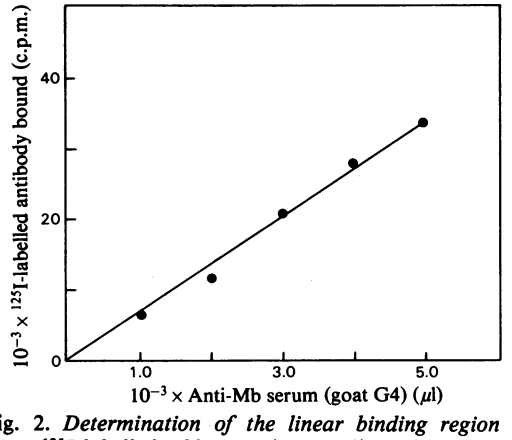

Fig. 2. *Determination of the linear binding region of ^{125}I-labelled rabbit anti-(goat IgG) antibodies*

Increasing amounts of goat antiserum G4 were bound to sperm-whale Mb–Sepharose (50μl). After the excess proteins had been washed from the adsorbents, a portion of ^{125}I-labelled rabbit (anti-(goat IgG) antibodies (1×10^5 c.p.m.) was added to each tube and allowed to bind. The net binding values are corrected for non-specific binding (<5% by an equal volume of rabbit Hb–Sepharose).

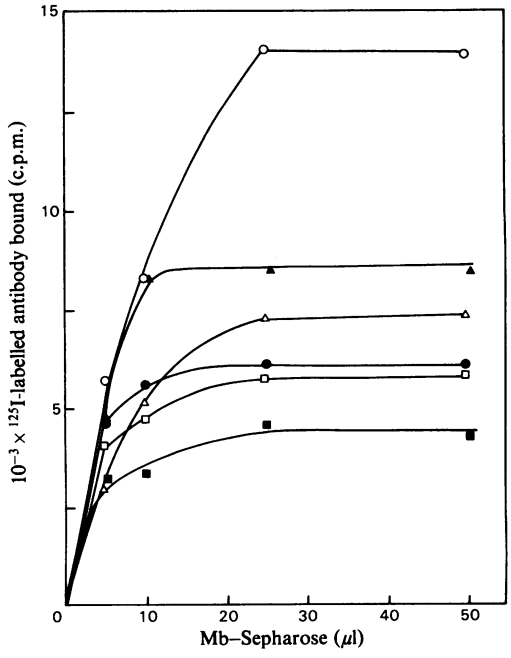

Fig. 3. *Representative titration studies with a fixed amount of unlabelled goat antiserum (G3) to sperm-whale Mb (20×10^{-3} μl) followed by a fixed amount of second antibody [^{125}I-labelled rabbit anti-(goat IgG) antibodies, 1.2×10^5 c.p.m.] with increasing amounts of Mb–Sepharose*

The second antibody was in excess. The net binding was corrected for non-specific binding (<5% relative to sperm-whale Mb by equal volumes of human Hb–Sepharose). ○, Sperm-whale Mb; ▲, finback-whale Mb; △, horse Mb; ●, goat Mb; □, rabbit Mb; ■, viscacha Mb.

relative to the amount of radioactivity bound by the adsorbents of sperm-whale Mb.

Cross-reactions of various myoglobins with antisera to sperm-whale Mb raised in different species

The cross-reactions of the various myoglobins were studied by single-antibody quantitative immunoadsorbent titration experiments. The ^{125}I-labelled immune IgG fraction was used in studies on cat antisera CTM1 and CTM2 and goat antisera G3 and G4. The specific antibody fraction was employed with goat antisera G3 and G4, rabbit antisera M8 and M9, chicken antiserum CK1 and the pig antiserum. The double-antibody technique was employed with rabbit antisera 77, 80, M8 and M9, with goat antisera G3 and G4 and with mouse antisera MS2, MS3 and MS5. Table 3 summarizes the immunochemical cross-reactions for the following 15 myoglobins: sperm-whale, finback-whale,

Table 2. *Comparison of the use of single-antibody and double-antibody techniques in determining the cross-reaction of antibodies to sperm-whale Mb with various myoglobins*

The single-antibody experiments were performed with ^{125}I-labelled immune IgG from the respective serum. The double-antibody experiments employed reaction with an appropriate dilution of unlabelled antibody followed by reaction with ^{125}I-labelled rabbit anti-(goat IgG) antibodies (for goat antiserum G3) or goat anti-(rabbit IgG) antibodies (for rabbit antiserum M8). For details see the text. Values are expressed as percentage of labelled antibody bound to a given Mb relative to that bound on sperm-whale Mb as 100%. Each is the average of triplicates in one experiment representing plateau binding values. All values are corrected for non-specific binding to control human Hb–Sepharose and bovine serum albumin–Sepharose (1–5% relative to sperm-whale Mb).

	^{125}I-labelled antibodies bound (%)			
Antiserum ...	Goat G3		Rabbit M8	
Technique ...	Single antibody	Double antibody	Single antibody	Double antibody
Myoglobin				
Sperm whale	100.0	100.0	100.0	100.0
Finback whale	67.5	65.2	66.5	65.1
Chimpanzee	41.5	38.1	54.0	51.4
Goat	36.0	35.6	39.0	41.9
Bovine	29.9	29.0	43.7	38.9
Viscacha	31.0	28.3	36.7	37.6
Dog	28.1	29.4	39.4	38.2
Echidna	20.9	19.7	37.6	38.2
Rabbit	37.8	39.6	37.9	38.6

killer-whale, horse, chimpanzee, sheep, goat, bovine, echidna, viscacha, rabbit, dog, cape-fox, mouse and chicken Mb. The results are also presented diagrammatically in Fig. 4. The whale myoglobins exhibited the greatest cross-reaction, whereas the Mb of the avian species, chicken, showed the lowest cross-reaction, with the other myoglobins falling between, regardless of the immunized species. In fact, the degree of cross-reaction for a given Mb was virtually independent of the species in which the antiserum was raised. The variations among antisera of various species were no different from the variations with antisera of individual animals within the same species (e.g., for a given Mb cross-reaction, the differences among the four rabbit antisera were comparable with the differences among the antisera of rabbits, goats, cats, pig, mouse and chicken). It is important to note that, in each species, an autoreactivity was observed between the Mb of that species and its own antisera to sperm-whale Mb (Table 4).

Discussion

Antigenic structure of sperm-whale Mb and its selection as a model

The precise elucidation of the entire antigenic structure of sperm-whale Mb has shown that both rabbits and goats make antibodies that recognize the same five antigenic sites on sperm-whale Mb (Atassi, 1975). With these antisera, the five sites account for the entire (100%) antibody response to the whole molecule (Atassi, 1977; Twining & Atassi, 1979). These and other findings (see under 'Cross-reactivity and auto-reactivity of the anti-myoglobin sera' below) suggested that the same molecular features on sperm-whale Mb that are recognized by rabbits and goats as antigenic sites will also be so recognized by other species.

The studies leading to the determination of the antigenic structure of sperm-whale Mb have been very extensive, and it would be prohibitive to duplicate these studies with antisera raised against sperm-whale Mb in many species. However, the antigenic structure, elucidated with rabbit and goat antisera, may be employed as a valuable 'reference model', and antisera raised in other species can be inspected for any departure from the expected behaviour. We have considered several approaches for the application of the 'reference-model' antigenic structure of sperm-whale Mb. One approach relies on the cross-reactions of myoglobins of known covalent structures from different species with antisera to sperm-whale Mb raised in various species. These cross-reactions can be compared with those obtained with rabbit and goat antisera against sperm-whale Mb. Since the sites responsible for the reaction with rabbit and goat antisera are known, then from the numerous myoglobins employed it will be possible to determine whether the expression of a

Table 3. *Cross-reaction of various myoglobins relative to sperm-whale Mb with anti-sperm-whale Mb sera produced in various species*

Results represent plateau binding values by immunoadsorbent titration studies with fixed amounts of antibody and increasing amounts of each Mb–Sepharose. The results were obtained either by single anti-(sperm-whale Mb) antibody titrations (either ^{125}I-labelled immune IgG and/or ^{125}I-labelled specific antibody) and/or by double-antibody [^{125}I-labelled rabbit anti-(goat IgG) antibodies, for goat antisera and ^{125}I-labelled goat anti-(rabbit IgG) antibodies for rabbit antisera] titrations (see the text for details). Values are expressed as percentage of antibody bound in the plateau relative to the amount of label bound by sperm-whale Mb in the plateau as 100%.

		Antibody bound in various sera (%)												
	Species ...	Goat		Rabbit				Chicken	Cat		Pig	Outbred mouse		
Myoglobin	Serum ...	G3*	G4†	77†	80§	M8§	M9§	CK1‖	CTM1¶	CTM2¶	P1¶	MS2‡	MS3‡	MS5‡
Sperm whale		100	100	100	100	100	100	100	100	100	100	100	100	100
Finback whale		67.2	65.3	54.4	64.7	66.8	63.9	70.0	65.9	76.0	71.0	73.0	65.0	57.7
Killer whale		61.0	57.7	50.6	61.9	67.1	59.4	58.2	62.1	68.1	54.9	69.9	59.7	56.5
Horse		49.5	39.2	37.8	52.3	55.6	52.8	52.7	47.9	58.8	51.4	55.6	44.2	52.5
Chimpanzee		45.0	42.9	54.6	52.0	52.7	51.9	44.5	45.0	50.8	45.4	53.8	50.9	46.5
Sheep		38.6	34.5	40.1	40.5	40.1	43.2	30.5	34.7	33.2	32.4	40.0	30.9	30.3
Goat		39.0	28.8	31.1	37.8	40.0	44.0	33.0	35.9	29.6	30.2	38.9	32.1	34.0
Bovine		28.7	24.0	32.8	38.8	40.5	43.4	36.8	29.9	37.2	29.1	34.9	26.1	31.5
Echidna		22.5	26.2	22.2	30.4	37.6	45.8	38.7	31.2	43.5	27.9	35.1	26.7	34.6
Viscacha		30.7	28.8	22.1	29.6	39.0	40.9	33.4	30.6	37.3	31.5	33.1	27.2	29.2
Rabbit		39.4	37.9	40.3	28.8	37.7	41.9	29.8	36.7	26.0	33.2	36.9	29.4	40.6
Dog		27.1	27.2	24.0	22.9	35.4	36.4	34.0	21.5	31.1	22.9	34.8	25.7	31.1
Cape fox		26.4	23.4	26.4	21.6	36.6	35.7	32.3	19.6	27.8	24.2	35.0	24.6	31.0
Mouse		—	22.8	—	—	31.6	—	—	—	—	—	33.5	21.8	30.4
Chicken		21.3	12.4	16.0	30.0	35.5	31.0	20.4	23.0	23.8	24.6	21.4	15.4	18.7

* Average of 11 analyses with ^{125}I-labelled immune IgG fraction (four analyses), specific ^{125}I-labelled antibodies (four analyses) and the double-antibody technique (three analyses).

† Average of three replicate analyses by the double-antibody technique.

‡ Average of five replicate analyses by the double-antibody technique.

§ Average of eight analyses each with ^{125}I-labelled specific antibodies and the double-antibody technique.

‖ Average of seven replicate analyses by specific ^{125}I-labelled antibodies.

¶ Average of seven replicate analyses by ^{125}I-labelled immune IgG fraction.

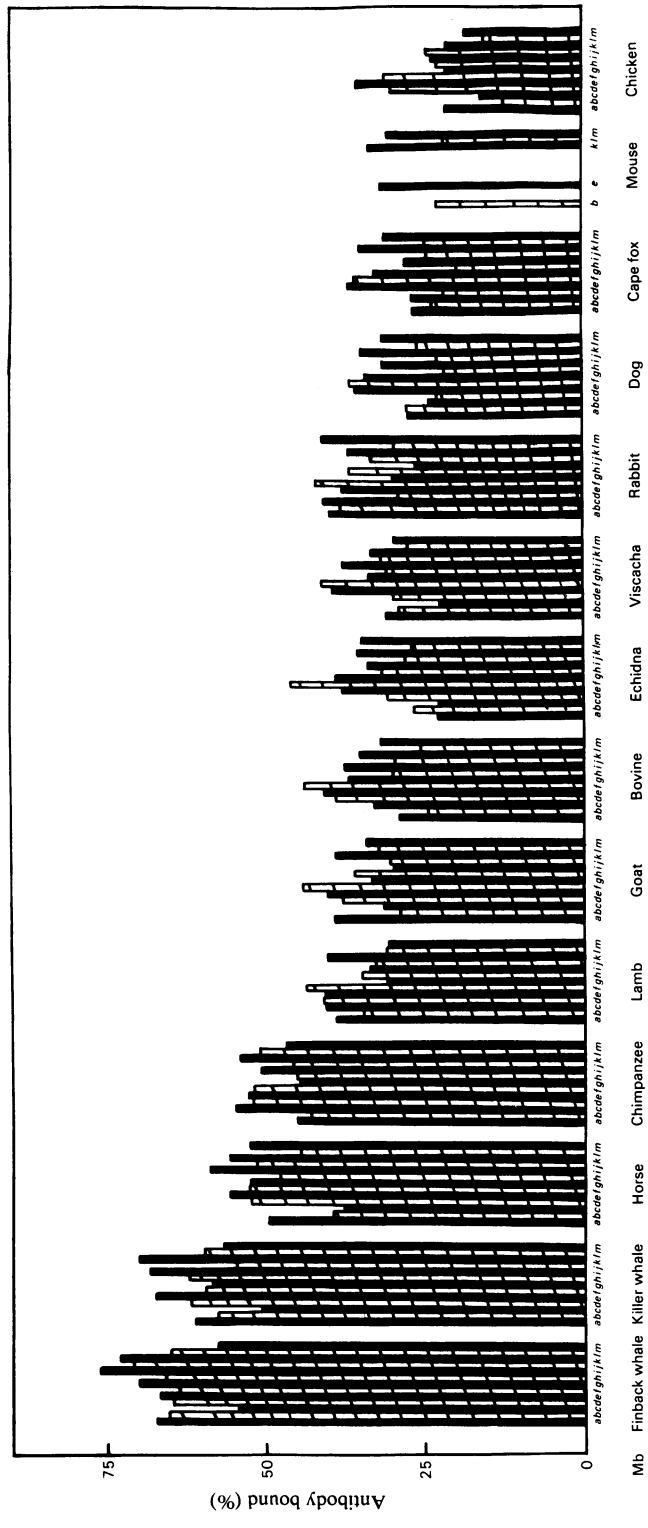

Fig. 4. *Binding of antibodies to sperm-whale Mb raised in goats, rabbits, cats, pig, mouse and chicken with adsorbents of various myoglobins* Values are from Table 3: bar *a*, goat antiserum G3; bar *b*, goat antiserum G4; bar *c*, rabbit antiserum 77; bar *d*, rabbit antiserum 80; bar *e*, rabbit antiserum M8; bar *f*, rabbit antiserum M9; bar *g*, chicken antiserum CK1; bar *h*, cat antiserum CTM1; bar *i*, cat antiserum CTM2; bar *j*, pig antiserum; bar *k*, mouse antiserum MS2; bar *l*, mouse antiserum MS3; bar *m*, mouse antiserum MS5. See Table 3 and the text for experimental details.

Table 4. *Auto-reactivity of anti-(sperm-whale Mb) sera with the animal's own Mb*

Results represent plateau binding values of ^{125}I-labelled antibodies by immunoadsorbent titration studies. Values are expressed as percentage of antibody bound in the plateau relative to the amount bound by sperm-whale Mb in the plateau as 100%.

(A)	Goat antiserum ...	G3	G4		
	Goat Mb	39.0	28.8		
(B)	Rabbit antiserum ...	77	80	M8	M9
	Rabbit Mb	40.3	28.8	37.7	41.9
(C)	Chicken antiserum ...	CK1	CK2		
	Chicken Mb	20.4	25.8		
(D)	Mouse antiserum ...	MS2	MS3	MS5	
	Mouse Mb	33.5	21.8	30.4	

given antigenic site is absent from a given antiserum.

Analysis of methods for studying cross-reactions of proteins

The present studies employed a quantitative immunoadsorbent titration technique reported from this laboratory (Twining & Atassi, 1978; Sakata & Atassi, 1979*a*,*b*). In this approach, a constant amount of ^{125}I-labelled immune IgG or ^{125}I-labelled antibody is titrated with increasing amounts of immunoadsorbent, until there is a complete depletion of antibodies directed to the antigen on the immunoadsorbent (Sakata & Atassi, 1979*a*,*b*). A major advantage of this technique is that it allows the quantitative determination of all antibodies directed to an antigen, including non-complement-fixing and non-precipitating antibodies. Thus, for example, myoglobins of horse, bovine, lamb and goat that do not give immune precipitates with antibodies against sperm-whale Mb (Atassi *et al.*, 1970) are demonstrated in the present work by employing immunoadsorbent titrations to bind considerable amounts of these antibodies.

An amino acid substitution, be it within an antigenic site or in a neighbouring residue, would not necessarily be expected to completely abolish the binding ability of the site with antibodies, but will more frequently influence the overall binding energy of the site (see under 'Factors influencing the reaction of an antigenic site' below). Accordingly, inhibition experiments may, under certain conditions, be incapable of detecting the presence and extent of immunochemical cross-reactions. For example, by inhibition of the Farr assay method, bovine Mb did not inhibit the binding of ^{125}I-labelled sperm-whale Mb with its antibodies (Hurrell *et al.*, 1977). This is due to the fact that the affinity of bovine Mb for the antibodies is too low, since, in the direct binding assay method reported in the present paper, antibodies to sperm-whale Mb can indeed bind with bovine Mb when the homologous antigen is not present. The present work measures quantitatively, in the plateau (i.e. under depleting conditions), all the cross-reacting antibodies in anti-(sperm-whale Mb) sera that can bind with other myoglobins from various species. The immunoadsorbent titration technique employed in the present work makes it possible to achieve this even when the cross-reacting antigenic sites cannot, owing to decreased affinity, effectively compete with the native protein (Kazim & Atassi, 1977*b*; Sakata & Atassi, 1979*a*,*b*; Atassi *et al.*, 1979).

Cross-reactivity and auto-reactivity of the anti-Mb sera

The present findings that, for a given Mb, the cross-reaction values were virtually independent of the host species in which the antibodies are raised, strongly indicate that these antibodies recognize the same five antigenic sites on sperm-whale Mb that are recognized by rabbits and goats. Clearly, then, the antibody response to the antigenic sites is not directed to the locations where the sequences of the injected Mb and the Mb of the immunized host are different. It is important to note that antisera to sperm-whale Mb in a given species showed an auto-reactivity with the Mb of that species (Table 4). The auto-reactivity of rabbit Mb with rabbit antisera to sperm-whale Mb has been reported (Kazim & Atassi, 1977*a*). Furthermore, rabbits immunized with rabbit myoglobin produced auto-antibodies against this protein (Kazim & Atassi, 1978). It was concluded that the antibody response to sperm-whale Mb was not necessarily directed to the parts of the sperm-whale Mb molecule that are different in sequence from the Mb of the immunized host (Kazim & Atassi, 1977*a*, 1978). The present findings clearly confirm these conclusions. These results and our success in inducing autoimmune responses to self-serum albumin (Sakata & Atassi, 1980*b*) indicate that the potential for autoimmune recognition and responses is a general phenomenon basic to the function of the immune system.

The extent of auto-reactivity of the host Mb with the host's antisera to sperm-whale Mb was comparable in magnitude with the cross-reactivity obtained with antisera that are raised in other species (see Table 3). Thus, for example, the extent of auto-reactivity of rabbit Mb with rabbit antisera to sperm-whale Mb was about the same as the cross-reaction of rabbit Mb with anti-(sperm-whale Mb) sera prepared in goat, chicken, cat or pig. Similarly, the cross-reactions of goat Mb were essentially the same regardless of whether the antisera to sperm-whale Mb were raised in goats or in other species. Clearly, the extent of auto-reactivity or cross-reactivity is not dependent on the

immunized species. Therefore the antibody response could not be related to the differences in primary structure between the injected Mb and the Mb of the immunized host.

Overall, these findings strongly confirm our earlier proposal for the 'structural inherency' of protein antigenic sites (for review see Atassi & Kazim, 1978). That is, the antigenicity of the sites is inherent in their three-dimensional locations and is independent of sequence identities between the antigen and corresponding host protein. 'Structurally inherent' antigenic sites have also been identified in human Hb (Kazim & Atassi, 1977*b*) and soya-bean leghaemoglobin (Hurrell *et al.*, 1978) by extrapolation of the three-dimensional locations of the antigenic sites of sperm-whale Mb. And our studies on bovine and human serum albumins (Atassi *et al.*, 1979; Sakata & Atassi, 1980*a*) show that their antigenic sites are located at equivalent structural locations.

Factors influencing the reaction of an antigenic site

It is now well established that the immune response to a protein antigen is directed against its native three-dimensional structure (Atassi, 1967*b*, 1978; Atassi & Skalski, 1969; Atassi & Thomas, 1969; Andres & Atassi, 1970). In the preceding paper (Kazim & Atassi, 1980) and in another recent paper (Atassi & Kazim, 1980) the molecular factors influencing the binding activity of an antigenic site were discussed in detail. It is unnecessary to repeat this treatment here. Briefly, these can be attributed mainly to the chemical and steric effects of substitutions within the antigenic sites and within the residues in the neighbourhood of the sites. The effects of substitutions at once-removed or even more distant locations, although perhaps less frequent, cannot be discounted. Furthermore, substitutions may cause conformational re-adjustments that could influence the reactivity of a site, even though these readjustments may be regional and undetectable in solution by present techniques (Atassi, 1970; Atassi *et al.*, 1970; Habeeb & Atassi, 1971).

It is perhaps useful to consider why the binding ability of the site is not necessarily completely

Table 5. *Antigenic sites of sperm-whale Mb and substitutions within these regions in the other myoglobins*

The amino acid substitutions were based on the sequences given in the references cited: sperm-whale Mb (Edmundson, 1965; Romero-Herrera & Lehmann, 1974), finback-whale Mb (DiMarchi *et al.*, 1978), killer-whale Mb (Castillo *et al.*, 1977), chimpanzee Mb (Romero-Herrera & Lehmann, 1972), horse Mb (Dautrevaux *et al.*, 1969; Romero-Herrera & Lehmann, 1974), sheep Mb (Han *et al.*, 1972; Vötsch & Anderer, 1972), bovine Mb (Han *et al.*, 1970), echidna Mb (Castillo *et al.*, 1978), rabbit Mb (Romero-Herrera *et al.*, 1976*b*), dog Mb (Dumur *et al.*, 1976), cape-fox Mb (Jones *et al.*, 1977) and chicken Mb (Deconinck *et al.*, 1975). Residues in parentheses are part of the antigenic site only with some antisera. For details see Atassi (1975).

		Site 1							
Myoglobin	Residue no ...	15	16	17	18	19	20	21	22
Sperm whale		(Ala)	Lys	Val	Glu	Ala	Asp	Val	(Ala)
Finback whale									
Killer whale		Gly						Leu	
Chimpanzee		Gly						Ile	Pro
Horse		Gly						Ile	
Sheep		Gly							
Bovine		Gly							
Echidna		Gly				Thr		Ile	Thr
Rabbit		Gly						Leu	
Dog/cape fox		Gly				Thr		Leu	
Chicken		Gly						Ile	

		Site 2						
Myoglobin	Residue no. ...	56	57	58	59	60	61	62
Sperm whale		Lys	Ala	Ser	Glu	Asp	Leu	Lys
Finback whale								
Killer whale								
Chimpanzee								
Horse								
Sheep								
Bovine								
Echidna					Ala			
Rabbit								
Dog/cape fox			Gly					
Chicken			Gly					

Table 5 (*continued*)

		Site 3					
Myoglobin	Residue no. ...	94	95	96	97	98	99
Sperm whale		Ala	Thr	Lys	His	Lys	Ile
Finback whale							
Killer whale							
Chimpanzee							
Horse							
Sheep			Asn				
Bovine			Asn				Val
Echidna							
Rabbit							
Dog/cape fox							
Chicken							

		Site 4						
Myoglobin	Residue no. ...	113	114	115	116	117	118	119
Sperm whale		His	Val	Leu	His	Ser	Arg	His
Finback whale								
Killer whale								
Chimpanzee		Gln					Lys	
Horse							Lys	
Sheep						Ala	Lys	
Bovine						Ala	Lys	
Echidna					Gln		Lys	
Rabbit							Lys	
Dog/cape fox		Gln			Gln		Lys	
Chicken		Lys		Ile	Ala	Glu	Lys	

		Site 5						
Myoglobin	Residue no. ...	145	146	147	148	149	150	151
Sperm whale		(Lys)	Tyr	Lys	Glu	Leu	Gly	Tyr
Finback whale								Phe
Killer whale								Phe
Chimpanzee		Asn						Phe
Horse								Phe
Sheep		Gln			Val			Phe
Bovine					Val			Phe
Echidna						Phe		Phe
Rabbit		Gln						Phe
Dog/cape fox								Phe
Chicken						Phe		Phe

destroyed by an adverse substitution, especially when it is outside the site. Antibodies to an antigenic site are heterogeneous in terms of affinity. So, when the binding affinity of an antigenic site is altered by any of the foregoing factors, a certain fraction of antibodies will be excluded from binding, whereas a fraction representing the high-affinity antibodies may still bind but with decreased affinity. The exclusion of a fraction of antibodies from binding will be reflected in a lower reactivity for the altered cross-reacting antigenic site in a homologous protein.

Structural–immunochemical analysis of the individual myoglobins

It is now appropriate to consider the immunochemical cross-reactions of each of the myoglobins, with antisera against sperm-whale Mb, in terms of its structural relationship to sperm-whale Mb. The following treatment is concerned with the major effects that are caused by substitutions within the sites and within the residues in the neighbourhood of the sites. Previous optical-rotatory-dispersion and circular-dichroism studies have ruled out the presence of major conformational differences between the sperm-whale Mb and goat, lamb and bovine Mb (Atassi, 1970) and rabbit Mb (Kazim & Atassi, 1977*a*). However, it should be kept in mind that regional conformational readjustments cannot be excluded, and their effects are hard to evaluate. In the preceding paper (Kazim & Atassi, 1980) we have identified all the environmental residues around

the Mb antigenic sites [within 0.7nm (7.0Å)] to help in understanding the effect of substitutions in these residues on the binding activity of the sites (Kazim & Atassi, 1980). To simplify the treatment, the effects of substitutions in the antigenic sites and in the residues forming the environments of the sites are considered separately below.

Substitution of residues inside the antigenic sites. Substitutions within the antigenic sites account for the major effects on the antigenic reactivity (Atassi & Kazim, 1980; Kazim & Atassi, 1980). Table 5 summarizes, for the 11 myoglobins whose sequences are known (see Romero-Herrera *et al.*, 1978), the residues that are located at equivalent positions to the five antigenic sites of sperm-whale Mb.

For finback-whale Mb, sites 1, 2, 3 and 4 are unaltered relative to the sites of sperm-whale Mb, whereas in site 5 phenylalanine replaces tyrosine at position 151. Substitution of tyrosine by phenylalanine eliminates the reactivity of this site (Atassi & Saplin, 1971; Koketsu & Atassi, 1973). Furthermore, the phenolic hydroxy group of Tyr-151 in sperm-whale Mb is probably important for the conformational integrity of this end of the molecule (Takano, 1977).

Killer-whale Mb has two substitutions in site 1: Ala-15→Gly and Val-21→Leu. Since Ala-15 is part of the site only with some antisera (Koketsu & Atassi, 1974), its replacement by glycine will have some effect only in the reaction with those antisera. The conservative replacement of valine by leucine will also have a slight effect on the binding ability of the site. On the whole, then, these substitutions will cause a slight decrease in the binding capacity of the site. Sites 2, 3 and 4 are unaltered in killer-whale Mb. In site 5 the replacement Tyr-151→Phe will be expected to remove the binding activity of the site.

In chimpanzee Mb the reaction of site 1 will be diminished owing to the substitutions Ala-15→Gly, Val-21→Ile and Ala-22→Pro. Sites 2 and 3 are unaltered. In site 4, the replacement His-113→Gln will cause a decrease in the reactivity of the site, whereas the conservative replacement Arg-118→Lys will have very little effect. In the case of site 5, its reactivity will be virtually eliminated by the replacements Lys-145→Asn and Tyr-151→Phe.

The reactivity of site 1 in horse Mb will be only slightly diminished by the replacements Ala-15→Gly and Val-21→Ile. Sites 2 and 3 are unaltered, whereas in site 4 the replacement Arg-118→Lys will have little or no effect on the reactivity of the site. The binding ability of site 5 is virtually compromised by the replacement Tyr-115→Phe (Atassi & Saplin, 1971).

In sheep and bovine Mb the site-1 replacement Ala-15→Gly will have only a slight effect on the binding ability of the site. Site 2 is unaltered in both myoglobins. In the case of site 3, both myoglobins

Table 6. *Residues neighbouring to antigenic site 1 of sperm-whale Mb that undergo substitution in the other myoglobins and the nature and effect of substitution*

Only the nearest-neighbour residues to sperm-whale Mb sites [within 0.60nm (6.0Å) from a site residue] that undergo substitution in the myoglobins studied here are listed below. For a complete list of all environmental residues within 0.70nm (7.0Å) from a site residue see the preceding paper (Kazim & Atassi, 1980).

Site 1 environment in sperm-whale Mb*		Environmental residue substitutions in other myoglobins									
Environmental residue	Neighbouring site residue(s)	Finback whale	Killer whale	Horse	Chimpanzee	Sheep	Bovine	Echidna	Rabbit	Dog	Cape fox	Chicken
His-12	Ala-15, Lys-16	Asn	Asn	Asn	Asn	Asn	Asn	Lys	Asn	Asn	Asn	Thr
Val-13	Ala-15, Lys-16, Val-17	Ile				Ala	Ala			Ile	Ile	Ile
Gln-26	Ala-22											His
Asp-27	Asp-20			Glu	Glu	Glu	Glu		Glu	Glu	Glu	Glu
Val-66	Val-21, Ala-22	Asn	Asn	Thr	Ala	Asn	Asn	Gly	Asn	Asn	Asn	Gln
Leu-115	Val-17											Ile
Arg-118	Asp-20			Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
Asp-122	Lys-16		Gln			Asn	Asn					
Expected reaction of the site†		Dec.	Dec.	Dec.	Dec.	Gr. Dec.	Gr. Dec.	Sl. Dec.	Dec.	Dec.	Dec.	Gr. Dec.

* For a comprehensive list of neighbouring residues and nearest-atom distances to site residues see the preceding paper (Kazim & Atassi, 1980).

† The qualitative evaluation pertains only to the effects of substitutions in the environmental residues on the reactivity of the antigenic site. The overall effects of substitutions both within the site and in the neighbouring residues are summarized in Table 11. Abbreviations for the reactivities are: Sl. Dec., slightly decreased; Dec., decreased; Gr. Dec., greatly decreased.

Table 7. *Residues neighbouring to antigenic site 2 of sperm-whale Mb that undergo substitution in the other myoglobins and the nature and effect of substitution*
Only the nearest-neighbour residues to sperm-whale Mb sites [within 0.60nm (6.0Å) from a site residue] that undergo substitution in the myoglobins studied here are listed below. For a complete list of all environmental residues within 0.70nm (7.0Å) from a site residue see the preceding paper (Kazim & Atassi, 1980).

Site 2 environment in sperm-whale Mb*		Environmental residue substitutions in other myoglobins										
Environmental residue	Neighbouring site residue(s)	Finback whale	Killer whale	Horse	Chimp-anzee	Sheep	Bovine	Echidna	Rabbit	Dog	Cape fox	Chicken
Ala-22	Lys-62				Pro			Thr				
Gln-26	Lys-56, Ala-57, Ser-58, Glu-59, Leu-61, Lys-62											His
Ile-30	Lys-56, Leu-61											Met
Arg-45	Asp-60	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
His-48	Ser-58											Gly
Ala-53	Lys-56, Ala-57				Asp			Asp	Asp	Asp	Asp	Pro
Glu-54	Lys-56, Ala-57, Ser-58		Asp									Asp
Val-66	Lys-62	Asn	Asn	Thr	Ala	Asn	Asn	Gly	Asn	Asn	Asn	Gln
Expected reaction of the site†		Sl. Dec.	Sl. Dec.	Sl. Dec.	Gr. Dec.	Sl. Dec.	Sl. Dec.	Gr. Dec.	Gr. Dec.	Gr. Dec.	Gr. Dec.	Gr. Dec.

* For a comprehensive list of neighbouring residues and nearest-atom distances to site residues see the preceding paper (Kazim & Atassi, 1980).
† This qualitative evaluation pertains only to the effects of substitutions in the environmental residues on the reactivity of the antigenic site. The overall effects of substitutions both within the site and in the neighbouring residues are summarized in Table 11. Abbreviations for the reactivities are: Sl. Dec., slightly decreased; Gr. Dec., greatly decreased.

Table 8. *Residues neighbouring to antigenic site 3 of sperm-whale Mb that undergo substitution in the other myoglobins and the nature and effect of substitution*
Only the nearest-neighbour residues to sperm-whale Mb sites [within 0.60nm (6.0Å) from a site residue] that undergo substitution in the myoglobins studied here are listed below. For a complete list of all environmental residues within 0.70nm (7.0Å) from a site residue see the preceding paper (Kazim & Atassi, 1980).

Site 3 environment in sperm-whale Mb*		Environmental residue substitutions in other myoglobins										
Environmental residue	Neighbouring site residue(s)	Finback whale	Killer whale	Horse	Chimp-anzee	Sheep	Bovine	Echidna	Rabbit	Dog	Cape fox	Chicken
Lys-42	His-97, Lys-98, Ile-99											Arg
Ser-92	Ala-94, Thr-95, Lys-96, His-97											Thr
Pro-100	Lys-98, Ile-99							Ser				
Ile-101	Ile-99				Val	Val			Val	Val	Val	Val
Tyr-103	Ile-99							Phe				
Leu-149	Ala-94, Thr-95							Phe				Phe
Tyr-151	Ala-94, Thr-95	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe
Expected reaction of the site†		F. to Sl. Dec.	F. to Sl. Dec.	F. to Sl. Dec.	Sl. Dec.	Sl. Dec.	F. to Sl. Dec.	Dec.	Sl. Dec.	Sl. Dec.	Sl. Dec.	Sl. Dec.

* For a comprehensive list of neighbouring residues and nearest-atom distances to site residues see the preceding paper (Kazim & Atassi, 1980).
† This qualitative evaluation pertains only to the effects of substitutions in the environmental residues on the reactivity of the antigenic site. The overall effects of substitutions both within the site and in the neighbouring residues are summarized in Table 11. Abbreviations for the reactivities are: F. to Sl. Dec., Full to slightly decreased; Sl. Dec., slightly decreased; Dec., decreased.

Table 9. *Residues neighbouring to antigenic site 4 of sperm-whale Mb that undergo substitution in the other myoglobins and the nature and effect of substitution*
Only the nearest-neighbour residues to sperm-whale Mb sites [within 0.60nm (6.0Å) from a site residue] that undergo substitution in the myoglobins studied here are listed below. For a complete list of all environmental residues within 0.70nm (7.0Å) from a site residue see the preceding paper (Kazim & Atassi, 1980).

Site 4 environment in sperm-whale Mb*		Environmental residue substitutions in other myoglobins										
Environmental residue	Neighbouring site residue(s)	Finback whale	Killer whale	Horse	Chimp-anzee	Sheep	Bovine	Echidna	Rabbit	Dog	Cape fox	Chicken
Val-13	Leu-115, His-119	Ile				Ala	Ala			Ile	Ile	Ile
Ala-19	His-119							Thr		Thr	Thr	
Asp-27	Val-114, Arg-118			Glu	Glu	Glu	Glu		Glu	Glu	Glu	Glu
Ile-28	Val-114, Leu-115			Val	Val	Val	Val	Val	Val	Val	Val	Val
Arg-31	His-113, Val-114	Ser										
Glu-109	His-113	Asp		Asp		Asp	Asp			Asp	Asp	
Ala-110	His-113, Val-114				Cys							Val
Pro-120	His-116, Arg-118, His-119							Ser		Ser	Ser	Ala
Gly-121	His-119	Ala	Ala			Ser	Ser	Ala				Ala
Asp-122	His-119		Gln			Asn	Asn					
Gly-124	His-116						Ala			His	His	
Gln-128	His-116									Glu	Glu	
Expected reaction of the site†		Dec.	Dec.	Sl. Dec.	Sl. Dec.	Gr. Dec.	Gr. Dec.	Dec.	F. to Sl. Dec.	Gr. Dec.	Gr. Dec.	Dec.

* For a comprehensive list of neighbouring residues and nearest-atom distances to site residues see the preceding paper (Kazim & Atassi, 1980).

† This qualitative evaluation pertains only to the effects of substitutions in the environmental residues on the reactivity of the antigenic site. The overall effects of substitutions both within the site and in the neighbouring residues are summarized in Table 11. Abbreviations for the reactivities are: F. to Sl. Dec., full to slightly decreased; Sl. Dec., slightly decreased; Dec., decreased; Gr. Dec., greatly decreased.

Table 10. *Residues neighbouring to antigenic site 5 of sperm-whale Mb that undergo substitution in the other myoglobins and the nature and effect of substitution*
Only the nearest-neighbour residues to sperm-whale Mb sites [within 0.60nm (6.0Å) from a site residue] that undergo substitution in the myoglobins studied here are listed below. For a complete list of all environmental residues within 0.70nm (7.0Å) from a site residue see the preceding paper (Kazim & Atassi, 1980).

Site 5 environment in sperm-whale Mb*		Environmental residue substitutions in other myoglobins										
Environmental residue	Neighbouring site residue(s)	Finback whale	Killer whale	Horse	Chimp-anzee	Sheep	Bovine	Echidna	Rabbit	Dog	Cape fox	Chicken
Gln-91	Lys-145, Tyr-146, Leu-149					Glu	Glu					
Thr-95	Leu-149, Tyr-151					Asn	Asn					
Ile-99	Tyr-146						Val					
Pro-100	Tyr-146							Ser				
Ile-101	Tyr-146				Val	Val			Val	Val	Val	Val
Ile-142	Lys-145, Tyr-146, Lys-147				Met	Met	Ala	Met				Met
Ala-144	Lys-145, Tyr-146, Tyr-151				Ser		Glu	Thr				Ser
Gln-152	Tyr-146, Lys-147, Tyr-151		His				His					
Expected reaction of the site†		Full	V. Sl. Dec.	Full	Sl. Dec.	Gr.or Comp. Dest.	Gr. or Comp. Dest.	Sl. Dec.	F. or V. Sl. Dec.	F. or V. Sl. Dec.	F. or V. Sl. Dec.	Sl. Dec.

* For a comprehensive list of neighbouring residues and nearest-atom distances to site residues see the preceding paper (Kazim & Atassi, 1980).

† The qualitative evaluation pertains only to the effects of substitutions in the environmental residues on the reactivity of the antigenic site. The overall effects of substitutions both within the site and in the neighbouring residues are summarized in Table 11. Abbreviations for the reactivities are: F. or V. Sl. Dec., full or very slightly decreased; V. Sl. Dec., very slightly decreased; Sl. Dec., slightly decreased; Gr. or Comp. Dest., great or complete destruction.

suffer the replacement Thr-95→Asn, which would cause a decrease in the binding activity of this site in both myoglobins. In addition, bovine Mb has the conservative replacement Ile→Val in this site. For site 4 both myoglobins have the replacements Arg-118→Lys and Ser-117→Ala. The replacement Arg→Lys will have little or no effect, whereas Ser→Ala will cause a considerable decrease in the binding ability of the site. In the case of site 5 both sheep and bovine Mb are expected to be unreactive because of the serious replacements Glu-148→Val and Tyr-151→Phe. In addition, sheep Mb has the drastic replacement Lys-145→Gln.

Echidna Mb has four replacements in site 1 (Ala-15→Gly, Ala-19→Thr, Val-21→Ile and Ala-22→Thr), which will be expected to cause a great decrease in the reactivity of this site. Site 2 is virtually unreactive owing to the drastic replacement Glu-59→Ala. Site 3 remains unaltered. In site 4 the substitutions His-116→Gln and Arg-118→Lys will cause a slight decrease in its reactivity. Site 5 will be unreactive as a result of the replacement Tyr-151→Phe, and it has the additional substitution Leu-149→Phe.

Rabbit Mb has in site 1 two replacements (Ala-15→Gly and Val-21→Leu), which will cause only a slight decrease in its binding ability through that site. Sites 2 and 3 are unaltered by direct replacements, and in site 4 the Arg-118→Lys replacement will have a very small effect on the reaction of that site. Site 5 will be rendered unreactive by the two drastic substitutions Lys-145→Gln and Tyr-151→Phe.

In dog and cape-fox Mb (which have the same primary structure) the reaction of site 1 is considerably diminished by the substitutions Ala-15→Gly, Ala-19→Thr and Val-21→Leu. The reactivity of site 2 will be slightly affected by the replacement Ala-57→Gly. There are no substitutions within the residues of site 3. The reactivity of site 4 is greatly diminished or eliminated by the combined effects of the three substitutions His-113→Gln, His-116→Gln and Arg-118→Lys. In the case of site 5 its reactivity is virtually destroyed by the substitution Tyr-151→Phe.

Finally, chicken Mb is only slightly affected in site 1 by the substitutions Ala-15→Gly and Val-21→Ile, and similarly in site 2 by the substitution Ala-57→Gly. There are no replacements within the residues of site 3. The reactivity of site 4 is completely destroyed by the combined effect of the substitutions His-116→Ala and Ser-117→Glu, as well as the less severe substitutions His-113→Lys, Leu-115→Ile and Arg-118→Lys. Similarly site 5 is rendered unreactive by the substitutions Tyr-151→Phe and Leu-149→Phe.

Table 11. *Summary of the reactivity of the cross-reacting sites in various myoglobins with antibodies to sperm-whale Mb expected on the basis of effects of substitutions in the antigenic sites and in their environmental residues*

The effects of replacements within the antigenic sites are considered together with the effects of replacements in the neighbouring residues. The expected reactivity of the site therefore takes into account the overall combined effects of these, and the results can be expressed here only in relative qualitative terms (see the text for details). The letter notations indicating reactivity of the antigenic sites are used as follows: F, full reactivity (100%) expected for the site; SD, slightly decreased activity (about 75%) expected for the site; D, decreased reactivity (about 50%) expected for the site; GD, greatly decreased reactivity (about 25%) expected for the site; 0, no reactivity expected for the site. The values for cross-reactivity of the myoglobins are based on the assumption that the fractions of antibodies directed to the five antigenic sites are equal in amounts. This is known not to be the case (Atassi, 1975; Twining & Atassi, 1979). However, this approximation may be permissible, since the differences will be expected to even out when the average values for the 13 antisera are taken. The 'expected' and 'found' values of cross-reactivity have a linear correspondence, with a correlation coefficient of 0.986. The 'found' values are average values of reactivity for the 13 antisera shown in Table 3.

	Reactivity of the antigenic sites					Cross-reactivity of the myoglobins	
Myoglobin	Site 1	Site 2	Site 3	Site 4	Site 5	Expected	Found (± S.D.)
Sperm whale	F	F	F	F	F	100	100
Finback whale	SD	SD	F	SD	0	65	66 (5.8)
Killer whale	D	SD	F	SD	0	60	61 (5.4)
Horse	D	D	F	SD	0	55	50 (6.3)
Chimpanzee	D	D	SD	D	0	45	49 (4.1)
Sheep	GD	SD	D	GD	0	35	36 (4.5)
Bovine	GD	SD	D	GD	0	35	33 (5.8)
Echidna	GD	0	SD	D	0	30	32 (7.5)
Rabbit	D	GD	SD	SD	0	45	35 (5.2)
Dog/cape fox	GD	GD	SD	0	0	25	28 (5.3)
Chicken	GD	GD	SD	0	0	25	23 (6.6)

Substitutions in the environmental residues of the sites. The nearest-neighbour residues for each of the antigenic sites of sperm-whale Mb were calculated from the X-ray co-ordinates and are detailed in the preceding paper (Kazim & Atassi, 1980). For the myoglobins studied here, many of the residues making up the environment of a given antigenic site do not change. To economize on space, the nearest-neighbour lists are condensed for the present purposes in Tables 6–9 to include only those residues that undergo substitutions in the myoglobin species examined in the present study. However, for the full profile of the environment of each antigenic site, the reader should refer to the preceding paper (Kazim & Atassi, 1980), especially if analysis of myoglobins other than those examined in the present study is desired. Tables 6–10 therefore list the environmental residues of each antigenic site in sperm-whale Mb that may undergo substitution, and the nature of this substitution, in one or more of the other Mb species. The effects of replacements in the nearest-neighbour residues on the binding ability of the site was evaluated by employing the same considerations outlined above. The systematic analysis of the environmental substitution effects on each site at the residue-by-residue level would be too lengthy to be discussed in the present paper. However, this analysis employs the same rules as those outlined in the preceding section. It was possible to derive estimates of detrimental effects from environmental substitutions on the reactivity of the site and, for the sake of brevity, only the conclusions are given in Tables 6–10.

Reactivity of the sites

By taking together the effects of substitutions inside the antigenic sites and of substitutions in the environmental residues of each site, it was possible to make an estimate of their combined effects. The results are summarized in Table 11. Since the cross-reaction of a given Mb was virtually independent of the species in which the antiserum is raised, it may be permissible for the sake of convenience to obtain the value of average percentage cross-reaction with all the 13 antisera. The value compared reasonably well with the expected cross-reactivity for all the myoglobins. For rabbit Mb the correlation is less satisfactory, suggesting most probably the involvement of other effects discussed above (e.g. effects of once-removed or distant substitutions or conformational readjustments). However, these are, of course, qualitative and subjective estimates, because it is not possible to know precisely the independent contribution of each side chain in an antigenic site to the overall binding energy of the site and then the effect of a direct or an environmental substitution or a conformational readjustment on that binding energy. By operating within these constraints it is possible to correlate, at least in qualitative terms, the expected effects of the substitutions in each of the myoglobins and its actual observed cross-reaction with antisera to sperm-whale Mb.

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