Structurally inherent antigenic sites

Localization of the antigenic sites of the α-chain of human haemoglobin in three host species by a comprehensive synthetic approach

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The antigenic structure of the α-chain of human haemoglobin was studied by a synthetic approach consisting of the synthesis of a series of consecutive overlapping peptides that together systematically represent the entire primary structure of the protein. This approach enabled the identification of a full profile of immunochemically active α-chain peptides and the localization of its major 'continuous' antigenic sites. Antibodies to haemoglobin raised in each of three different species (goat, rabbit and mouse) recognize similar sites on the α-chain. Further, the molecular locations of these sites coincide with α-chain regions extrapolated from antigenic sites of the conformationally similar myoglobin molecule. These findings support our earlier proposed concept of 'structurally inherent antigenic sites', namely that antigenicity is conferred on certain surface regions of proteins by virtue of their three-dimensional locations. Thus the antigenic sites of conformationally related proteins are likely to have similar molecular locations.

Previously we reported the prediction and confirmation by synthesis (Kazim & Atassi, 1977a) of two antigenic sites on human haemoglobin, one each on its α- and β-chains, by extrapolation of the three-dimensional location of an antigenic site of sperm-whale myoglobin (Atassi, 1975). The finding that antigenic sites on immunochemically unrelated members of the same protein family can exist in conformationally homologous regions gave considerable support to our proposal of 'structurally inherent antigenic sites' (Atassi & Kazim, 1978), namely that the antigenicity of protein antigenic sites is derived largely from their conformational locations in the respective protein chains (Atassi & Kazim, 1978). Although the individual α- and β-subunits of haemoglobin (Hb) have similar overall tertiary structures to myoglobin (Mb), its tetrameric subunit structure endows Hb with unique conformational properties that contrast with those of the monomeric Mb molecule (Perutz *et al.*, 1968). Therefore the presence of structurally inherent antigenic sites on Hb and Mb do not preclude these proteins from having antigenic sites at dissimilar three-dimensional positions.

In order to identify other continuous (see the Discussion section for explanation of nomenclature) antigenic sites on Hb, we devised (Kazim & Atassi, 1980b) a novel and comprehensive synthetic approach that would localize antigenic sites occurring throughout the α- and β-chains of the Hb molecule. This approach consists of studying the immunochemical activities of a series of overlapping synthetic peptides that encompass the entire primary structure of the protein chain (Fig. 1). The rationale for this approach is presented in detail in the Discussion. In a preliminary communication (Kazim & Atassi, 1980b) we reported the application of this strategy to the α-chain of Hb that enabled identification of the full profile of immunochemically active synthetic peptides recognized by goat antisera to Hb. In the present paper we report additional details of the synthesis of these peptides, our findings with antisera to Hb raised in other animal species, and the localization of the antigenic sites to fairly narrow regions of the α-chain.

Experimental

Human adult Hb (cyanmet-HbA) was prepared by chromatography on CM-cellulose as previously described (Atassi, 1964). The α- and β-chains of Hb were obtained by treatment of carbonmonoxy-Hb

Abbreviations used: Hb, haemoglobin; Mb, myoglobin; IgG, immunoglobin G.
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with p-hydroxymercuribenzoate (Bucci & Fronti-celli, 1965), followed by repeated chromatography on CM-cellulose or DEAE-cellulose (Geraci et al., 1969) until they were homogeneous by polyacryl-amide-gel electrophoresis. After isolation of the subunits their thiol groups were regenerated with mercaptoethanol (Geraci et al., 1969).

Solid-phase synthesis (Merrifield, 1963) of the α-chain peptides (see Fig. 1), from the known primary structure of Hb (Braunitzer et al., 1961; Hill & Konigsberg, 1962), was performed by procedures similar to those described by Stewart & Young (1969) and as employed for the synthesis of myoglobin antigenic sites (Koketsu & Atassi, 1973, 1974). After cleavage from the resin and removal of the amino acid side-chain protecting groups, the peptides were purified by ion-exchange chromato-graphy on CM-Sephadex (C-50) or DEAE-Sepha-dex (A-50) as previously described (Koketsu & Atassi, 1973, 1974). The purity of the peptides thus obtained was examined by high-voltage paper electrophoresis. The compositions of each peptide was verified by amino acid analysis (Atassi & Saplin, 1968). For the determination of tryptophan, peptide 11–25 was hydrolysed with 3 M-toluene-p-sulphonic acid containing 0.2% 3-(2-aminoethyl)-indole, as described by Liu & Chang (1971).

Proteins and peptides were coupled to CNBr-activated Sepharose CL-4B (March et al., 1974). The amounts of proteins and peptides used in the coupling reactions were 3 mg/ml and 1 mg/ml packed volume respectively. These afforded adsorb-ents that had proteins and peptides at 2.4 and 0.5 mg/ml packed volume respectively.

Antisera to HbA were raised in goats, rabbits and outbred mice by a previously described immuniza-tion schedule (Atassi, 1967). The IgG fractions of these antisera, completely free of haptoglobin, were prepared as previously described (Gray et al., 1969; Kazim & Atassi, 1980a). The pure IgG fractions were labelled with ^{125}I by a chloramine-T procedure (Hunter & Greenwood, 1962). The specific radio-activities of the labelled IgG fractions were 10–20 μCi/μg.

The immunochemical activities of the protein and peptide fractions were determined by quantitative immunoadsorbent titrations of fixed amounts of ^{125}I-labelled immune IgG and various amounts of peptide or protein adsorbents as previously de-scribed for myoglobin antigenic sites (Twining & Atassi, 1979). Non-specific adsorption was deter-mined by titrating equivalent volumes of uncoupled Sepharose and bovine serum albumin–Sepharose under identical conditions. The values of ^{125}I-labelled immune IgG bound by the various adsorbents and reported in Table 2 were corrected for this non-specific binding.

Inhibition studies were employed to further

confirm the specificity of antibody binding to peptide adsorbents. In these studies, the appropriate adsorb-ents and labelled IgG fractions were allowed to react as above in the presence of either Hb (1 mg/ml) or an unrelated protein, bovine serum albumin (1 mg/ml). After correction for non-specific binding, the amount of radioactivity bound in the presence of Hb (inhibited values) was expressed as a percentage of that bound in the presence of bovine serum albumin (uninhibited values).

Specific antibodies were also isolated, and labelled with ^{125}I, on the peptide adsorbents (Matzku & Zoller, 1977). The activities of these peptide-specific antibodies were confirmed by binding studies with Hb, with α-chain, with the peptide–adsorbent origin-ally employed for the isolation of the antibodies and in some cases with adsorbents of other peptides.

Results

Synthesis, purification and characterization of the peptides

The structures of the synthetic α-chain peptides are shown in Fig. 1. It should be noted that, since tryptophan is somewhat unstable under the acidic conditions used during synthesis, tryptophan-14 was replaced by phenylalanine in peptide 1–15 but not in peptide 11–25. The penultimate location of trypto-phan in peptide 1–15 would have required its exposure to several additional acidic cycles than in peptide 11–25, which, it was feared, would result in an excessive accumulated loss. The substitution of tryptophan by phenylalanine in immunochemically active peptides has been shown to be permissible in previous studies on the antigenic structure of lysozyme (Lee et al., 1976). Furthermore, trypto-phan-14 is located in the overlapping region between peptides 1–15 and 11–25, and thus if phenylalanine were an unsuitable replacement for tryptophan any immunochemical activity due to this region would be detected by peptide 11–25. In addition, cysteine-104 was replaced by serine in peptides 91–105 and 101–115 in order to avoid difficulties associated with the oxidation of thiol groups (dimerization etc.).

After cleavage and deprotection, each peptide gave by ion-exchange chromatography a major chromatographic component that migrated as a single spot in high-voltage paper electrophoresis. That the major components were the desired products was confirmed by amino acid analysis. The results of the amino acid analyses of the deprotected and purified synthetic peptides are given in Table 1. The compositions found for these peptides agree well with the expected values. In peptide 81–95, how-ever, there was a complete loss of proline. This may be attributed perhaps to the lability of this particular Asp–Pro peptide bond to acidic conditions such as the HBr/trifluoroacetic acid used to cleave the

Peptide

1-15:   Val-Leu-Ser-Pro-Ala-Asp-Lys-Thr-Asn-Val-Lys-Ala-Ala-Phe-Gly

11-25:   Lys-Ala-Ala-Trp-Gly-Lys-Val-Gly-Ala-His-Ala-Gly-Glu-Tyr-Gly

21-35:   Ala-Gly-Glu-Tyr-Gly-Ala-Glu-Ala-Leu-Glu-Arg-Met-Phe-Leu-Ser

31-45:   Arg-Met-Phe-Leu-Ser-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His

41-55:   Thr-Tyr-Phe-Pro-His-Phe-Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val

51-65:   Gly-Ser-Ala-Gln-Val-Lys-Gly-His-Gly-Lys-Lys-Val-Ala-Asp-Ala

61-75:   Lys-Val-Ala-Asp-Ala-Leu-Thr-Asn-Ala-Val-Ala-His-Val-Asp-Asp

71-85:   Ala-His-Val-Asp-Asp-Met-Pro-Asn-Ala-Leu-Ser-Ala-Leu-Ser-Asp

81-95:   Ser-Ala-Leu-Ser-Asp-Leu-His-Ala-His-Lys-Leu-Arg-Val-Asp-Pro

91-105:   Leu-Arg-Val-Asp-Pro-Val-Asn-Phe-Lys-Leu-Leu-Ser-His-Ser-Leu

101-115: Leu-Ser-His-Ser-Leu-Leu-Val-Thr-Leu-Ala-Ala-His-Leu-Pro-Ala

111-125: Ala-His-Leu-Pro-Ala-Glu-Phe-Thr-Pro-Ala-Val-His-Ala-Ser-Leu

121-135: Val-His-Ala-Ser-Leu-Asp-Lys-Phe-Leu-Ala-Ser-Val-Ser-Thr-Val

131-141: Ser-Val-Ser-Thr-Val-Leu-Thr-Ser-Lys-Tyr-Arg

Fig. 1. *Primary structures of the synthetic overlapping peptides of the α-chain*
The numbers refer to the locations of residues in the α-chain. Regions that overlap with the preceding and following peptides are underlined. Note that Trp-14 is replaced by phenylalanine in peptide 1–15, and Cys-104 by serine in peptides and 91–105 and 101–115. See the text for details.

peptide from the Merrifield resin. On the other hand, peptide 91–105, which contains the same Asp–Pro bond, gave the expected composition, indicating that this bond was unaffected by the identical procedures used in the preparation of this peptide. Possibly the C-terminal location of proline in peptide 81–95 might have contributed to the lability of this bond.

Binding studies of ^{125}I-labelled antibodies on protein and peptide adsorbents

In quantitative immunoadsorbent titrations of each of the ^{125}I-labelled immune IgG preparations [see Fig. 2 in Kazim & Atassi (1980b) for a representative titration], Hb–Sepharose invariably showed the highest immunochemical activity (Table 2). This is expected, since the antisera were prepared against Hb. At saturation (plateau values), the activities of the α-chain adsorbents were in the range 50–60% of those maximally attained by the Hb–adsorbent (Table 2). This indicates that, on immunization with Hb, antibodies capable of reacting with the α-chain are not particularly immunodominant over those reacting with the β-chain, at least in the representatives of the three animal

species studied. Further, their relative expression will probably vary somewhat even between animals of a given species. For example, the activity of the α-chain relative to Hb ranged from 51.4% to 61.8% for rabbits H-2 and R-91 respectively (Table 2). We have demonstrated (Krco *et al.*, 1980, 1981*a,b*, 1982) that the immune responses in mice to the α- and β-chains of Hb are under separate genetic control. This is therefore an additional factor that will influence the relative expression of antibodies to the α- and β-chains. In the present studies, however, antisera from five outbred mice were pooled, and for these pooled antisera antibodies to both the α- and β-chain were present.

The titrations with immunoadsorbents of the synthetic peptides differentiated the peptides into two categories. With some peptides, the amount of radioactivity bound remained constant throughout the entire range of the titration. For these peptides, the amounts of ^{125}I-labelled antibody bound, after correction for non-specific binding, did not exceed 200 c.p.m. (Table 2) and were: for the goat antiserum, peptides 1–15, 21–35, 31–45, 61–75, 71–85, 91–105 and 131–141; for the rabbit and mouse antisera, peptides 1–15, 21–35, 31–45, 41–55,

Table 1. *Summary of the results of amino acid analyses of the synthetic peptides*

The values reported are the averages for three or more analyses. The expected values are shown in parentheses. The values of serine and threonine were obtained by extrapolation to zero hydrolysis time from 22h, 48h and 72h hydrolyses.

Amino acid	Amino acid composition (residues/molecule)													
Peptide ...	1–15	11–25	21–35	31–45	41–55	51–65	61–75	71–85	81–95	91–105	101–115	111–125	121–135	131–141
Asp	2.08 (2)				1.01 (1)	0.98 (1)	3.94 (4)	3.93 (4)	1.93 (2)	1.97 (2)	0.91 (1)	1.01 (1)	1.06 (1)	
Thr	1.02 (1)		0.87 (1)	2.95 (3)	0.85 (1)		0.90 (1)						0.92 (1)	2.01 (2)
Ser	0.83 (1)	1.04 (1)	3.20 (3)	1.02 (1)	1.85 (2)	1.00 (1)		1.85 (2)	1.79 (2)	1.92 (2)	1.95 (2)	0.96 (1)	2.77 (3)	2.89 (3)
Glu					1.05 (1)	1.12 (1)			0 (1)			1.07 (1)		
Pro	1.10 (1)			1.97 (2)	0.92 (1)			0.96 (1)		1.13 (1)	1.10 (1)	2.02 (2)		
Gly	0.93 (1)	4.20 (4)	2.07 (2)		1.14 (1)	3.13 (3)								
Ala	3.10 (3)	3.90 (4)	2.95 (3)		1.06 (1)	2.81 (3)	4.11 (4)	3.03 (3)	2.00 (2)		3.10 (3)	4.04 (4)	2.14 (2)	
Val	1.98 (2)	1.00 (1)			0.85 (1)	2.00 (2)	3.04 (3)	1.08 (1)	1.12 (1)	1.95 (2)	0.98 (1)	1.01 (1)	2.87 (3)	1.99 (2)
Met			0.81 (1)	1.12 (1)				0.91 (1)						
Leu	1.05 (1)		2.15 (2)	0.97 (1)	0.85 (1)		1.00 (1)	1.92 (2)	3.04 (3)	4.20 (4)	5.30 (5)	2.04 (2)	2.14 (2)	1.00 (1)
Tyr		0.95 (1)	0.91 (1)	0.98 (1)	0.87 (1)					1.12 (1)		0.93 (1)	0.97 (1)	0.88 (1)
Phe	0.92 (1)		0.94 (1)	2.94 (3)	2.10 (2)	0.94 (1)			2.07 (2)	0.91 (1)			0.97 (1)	
His		1.10 (1)		0.95 (1)	2.00 (2)		0.92 (1)		0.97 (1)	1.13 (1)			1.03 (1)	
Lys	1.97 (2)	2.11 (2)		1.11 (1)		3.20 (3)	1.10 (1)	0.90 (1)			1.90 (2)	2.18 (2)		1.08 (1)
Trp														
Arg		0.95 (1)	0.95 (1)	0.99 (1)					0.93 (1)	0.87 (1)				1.01 (1)

61–75, 71–85, 111–125 and 131–141. These peptides were considered to be inactive with these antisera. Alternatively, other peptides bound increasing amounts of radioactivity and the amounts of radioactivity bound by these peptides eventually levelled off at 'plateau' or maximum values. These peptides were clearly active and were: for the goat antiserum, peptides 11–25, 41–55, 51–65, 81–95, 101–115, 111–125 and 121–135; for the rabbit and mouse antisera: peptides 11–25, 51–65, 81–95, 91–105, 101–115 and 121–135. It should be noted that the titration curves for adsorbents of Hb and of the α-chain rose more sharply and attained a maximal value at lower amounts than those of the immunoadsorbents of the peptides. This is consistent with the observation that peptides, even though active, will have predominantly random conformations and thus diminished affinities for their antibodies (Atassi & Saplin, 1968). Further, a considerable proportion of a peptide on the immunoadsorbent is expected to have been rendered inactive owing to attachment of the peptide via side chains essential for binding with antibody, and to steric inaccessibility (Twining & Atassi, 1979; Atassi *et al.*, 1979). The binding of antibodies by these peptide–adsorbents, however, was completely inhibited by the addition of free uncoupled Hb to the reaction (results not shown). Furthermore, antibodies isolated on peptide–adsorbents bound only to peptide, Hb and α-chain, but did not bind to unrelated protein–adsorbent. These findings leave little doubt that antibodies reacting with the peptide adsorbents are specific for Hb.

It should be cautioned that the calculated sum of the activities of the peptides with each of the antisera is significantly larger than the activities of the α-chain itself. However, these results must be considered in the context of the structures of the peptides and their overlaps, which were designed to detect antigenic sites located in the overlapping regions between adjacent peptides (see the Discussion section). Thus adjacent overlapping peptides that have similar activities may be expressing identical specificities. The activities of overlapping peptides are therefore not additive. The activities of the peptides, and the localization of the antigenic sites that they represent, are considered in the Discussion section.

Discussion

Outline of the strategy

A key approach in the delineation of protein antigenic structures consists of immunochemical studies of overlapping peptide fragments obtained by chemical and enzymic cleavage of the parent protein (Atassi, 1972). This approach, when properly employed, can yield valuable information on the

Table 2. *Summary of quantitative radioimmunoadsorption studies of* ^{125}I-*labelled IgG*

The data here represent a summary of the 'plateau' values of the binding obtained from quantitative immuno-adsorbent titrations. The total amounts added of ^{125}I-labelled immune IgG of antisera G-2, H-2, R91 and M1–M5 were respectively 248000, 430000, 617000, and 222000 c.p.m.

Animal species in which anti-Hb antisera were raised ...	Goat (G2)		Rabbit				Mouse (M1–M5*)	
			H-2		R-91			
Sepharose adsorbent	^{125}I-labelled anti-Hb bound (c.p.m.)	Reaction relative to α = 100%	^{125}I-labelled anti-Hb bound (c.p.m.)	Reaction relative to α = 100%	^{125}I-labelled anti-Hb bound (c.p.m.)	Reaction relative to α = 100%	^{125}I-labelled anti-Hb bound (c.p.m.)	Reaction relative to α = 100%
Hb	19278	—	46339	—	36635	—	26876	—
α-chain	11721	100	23818	100	22671	100	17008	100
1–15	<200	<1.7	<200	<0.8	<200	<0.9	<200	<1.2
11–25	670	5.7	4032	16.9	3341	14.7	4859	28.6
21–35	<200	<1.7	<200	<0.8	<200	<0.9	<200	<1.2
31–45	<200	<1.7	<200	<0.8	<200	<0.9	<200	<1.2
41–55	1455	12.4	<200	<0.8	<200	<0.9	<200	<1.2
51–65	1678	14.3	521	2.2	<200	<0.9	542	3.2
61–75	<200	<1.7	<200	<0.8	<200	<0.9	<200	<1.2
71–85	<200	<1.7	<200	<0.8	<200	<0.9	<200	<1.2
81–95	3958	33.8	2159	9.1	2273	10.0	1964	11.5
91–105	<200	<1.7	4505	18.9	3748	16.5	5249	30.9
101–115	1011	8.6	4144	17.4	4933	21.8	4509	26.5
111–125	3303	28.2	<200	<0.8	<200	<0.9	<200	<1.2
121–135	3043	26.0	15185	63.8	22971	101.3	15736	92.5
131–141	<200	<1.7	<200	<0.8	<200	<0.9	<200	<1.2

* This is a pool of antisera from five outbred mice that had been immunized with Hb.

number of antigenic sites, their locations and their relative contributions to the activity of the native protein (Atassi, 1972). However, the approach has many limitations, and these have been previously discussed in detail (Atassi, 1977). An alternative to the use of cleavage procedures for obtaining over-lapping peptides is their synthesis. The advantages that can be realized from the synthesis of peptides for immunochemical studies have been previously discussed (Atassi, 1975, 1978; Kazim & Atassi, 1980b). Indeed, the use of synthetic peptides has been invaluable in the elucidation of the antigenic sites of myoglobin (Atassi, 1975), lysozyme (Atassi, 1978) and albumin (Atassi *et al.*, 1979), and was originally proposed as a separate approach for determining protein antigenic structures once the sites had been narrowed down to a conveniently small size (Atassi, 1975).

In each of these proteins the peptides were invariably designed to include residues that had been identified from chemical modification and frag-mentation studies as being antigenically important. For haemoglobin, however, little or no information was available about its antigenically important residues or the locations of its antigenic sites.

Therefore we introduced a strategy (Kazim & Atassi, 1980b) for studying the antigenic structure of haemoglobin (or indeed any other protein) that involved the systematic dissection of the haemo-globin subunits by a series of overlapping synthetic peptides encompassing the entire structures of the α- and β-chains. It was considered that the sizes of the synthetic peptides should be consistent with the number of residues expected for an antigenic site [e.g. five to seven residues for myoglobin (Atassi, 1975) and lysozyme (Atassi, 1978)] as well as having a high probability of possessing most (or all) of a site should it happen to fall in the overlap region between adjacent peptides. Peptides consisting of 15 residues and overlapping at their *N*- and *C*-termini by five residues each with adjacent peptides were considered adequate for these purposes [Fig. 1; also see Fig. 1 in Kazim & Atassi (1980b) for a schematic representation of this synthetic strategy]. It should be mentioned that this approach is restricted to the localization of continuous antigenic sites [i.e. those whose reactivity can be preserved in a continuous portion of the polypeptide chain, e.g. myoglobin antigenic sites (Atassi, 1975)]. Discon-tinuous antigenic sites, such as those of lysozyme
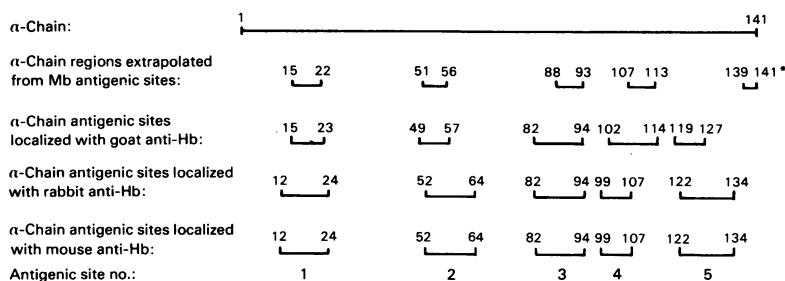
α-Chain: 1   141

α-Chain regions extrapolated   15 22   51 56   88 93   107 113   139 141*
from Mb antigenic sites:

α-Chain antigenic sites   15   23   49 57   82   94 102   114 119 127
localized with goat anti-Hb:

α-Chain antigenic sites localized   12   24   52   64   82   94 99 107   122   134
with rabbit anti-Hb:

α-Chain antigenic sites localized   12   24   52   64   82   94 99 107   122   134
with mouse anti-Hb:

Antigenic site no.:   1   2   3   4   5

Fig. 2. *Schematic diagram of the α-chain, α-chain regions that have been extrapolated from the five antigenic sites of sperm-whale Mb, and the antigenic sites of the α-chain that were localized in the present work with goat, rabbit and mouse antisera to Hb*

The numbers refer to the locations of the residues in the α-chain. The asterisk (*) indicates that, owing to differences in the sizes of the polypeptide chains of Mb and the α-subunit, the antigenic site Mb 145–151 does not really have a full structural counterpart in the α-chain. See the text for details.

(Atassi, 1978), will not be detected unless a given peptide possesses most of the residues of that site which make the major contribution to its binding energy.

Analysis of immunochemical activities of the peptides

The activities of the peptides in quantitative immunoadsorbent titrations were quite similar for the goat, rabbit and mouse antisera studied, there being an identical correspondence between the rabbit and mouse antisera (Table 2). This is consistent with our previous findings that the locations of protein antigenic sites have not been dependent on the immunized species (Kazim & Atassi, 1977b; Twining et al., 1980). The observed variations in the relative expression among and within these animal species of antigenic sites represented by the α-chain peptides (Table 2) are not unusual (Atassi, 1975). The active peptides represent immunodominant (more than 1–2% of the antibodies) antigenic sites of the α-chain of Hb. Peptide 121–135 deserves particular attention, since its activity in some instances was virtually equal to that of the α-chain itself (R-91, M1–M5; Table 2). At present we have no satisfactory explanation for this high activity. It is pertinent, however, that this binding was fully inhibited by free α-chains, suggesting that, perhaps as a result of the manner in which they are immobilized, adsorbents of α-chains are incapable of binding their full complement of antibodies.

Peptides that were seemingly inactive in the titration assay (Table 2) were further studied by attempting to isolate from adsorbents of these peptides antibodies specific for Hb. By using large excesses of unlabelled immune IgG, traces of Hb-specific antibodies could be isolated from that of peptide 1–15. Although the amounts of these antibodies were not quantified, they account for less than 1–2% of the specific antibodies, since no binding could be detected with this peptide in the titration assay. This indicates the presence of a trace antibody response directed to this region of the α-chain, and suggests that other apparently inactive regions of the α-chain may also be recognized by amounts of antibodies below the detection level of these methods. In previous studies on myoglobin (Atassi, 1975) and lysozyme (Atassi, 1978) a 'general background response' to regions between their respective antigenic sites was found to account for 0.1–1.4% of the total antibodies to these proteins. Similarly, Dean & Schechter (1979) have reported the isolation of a subpopulation of anti-Hb antibodies directed against the region α129–141, but which represents less than 1% of the total anti-Hb antibodies. Also, Takagaki et al. (1980) have reported the detection of antibodies to a continuous region of hen egg-white lysozyme (residues 38–54) that account for about 0.1–1% of the total anti-lysozyme antibodies. Thus the presence of trace antibody responses to regions interspersed among immunodominant sites would appear to be a general phenomenon for protein antigens. Although the regulation of the relative immunodominance of antigenic sites in proteins is poorly understood, the methodology for producing monoclonal antibodies introduced by Kohler & Milstein (1975) should permit amplification in vitro of what would ordinarily constitute trace antibody responses in vivo. The specificities and regulation of such responses could then be more readily studied.

Localization of the antigenic sites on the α-chain

For localizing antigenic sites within an active peptide we consider antigenic sites to consist of seven residues, and for a peptide to bind antibodies

of that specificity it must possess at least five of the seven site residues. Of course, exceptions to this rule can be expected where the absence of a single residue may abolish reactivity altogether. Similar activities of adjacent peptides may be due to antigenic sites lying in their overlapping regions. Accordingly, five antigenic sites on the α-chain that are recognized by goat, rabbit and mouse antisera to Hb were tentatively assigned to reside within, but not necessarily include all of, the general regions shown in Fig. 2. The site within region 12–24 recognized by goat antisera can be further localized to the region 15–23 since we had previously confirmed the presence of an antigenic site in this region by using goat antisera to Hb (Kazim & Atassi, 1977a). It should be kept in mind that the precise boundaries of these sites have not as yet been determined, and they are intentionally enlarged here in order to increase the probability of a correct assignment. Further studies with synthetic peptides overlapping within the assigned regions are required to determine the boundaries. Upon such refinement of the data, however, small shifts in the boundaries of these sites should not be unexpected (Koketsu & Atassi, 1973, 1974; Atassi, 1975).

Inspection of the three-dimensional structure of Hb (Perutz et al., 1968; Fermi, 1975) reveals that three of the five antigenic sites localized here for the α-chain contain residues that participate in $\alpha_1-\beta_1$ (sites 4 and 5) or $\alpha_1-\beta_2$ (site 3) subunit interactions, and are therefore potentially obstructed in the Hb dimer and tetramer. This indicates that the immune recognition of the Hb occurs, at least in major part, at the level of its individual subunits. Our studies on the genetic control in mice of the immune response to Hb (Krco et al., 1980, 1981a,b, 1982) have shown that the immune response to the α- and β-subunits in Hb are under separate genetic control. However, it has been reported (Tan-Wilson et al., 1976; Crowley et al., 1980) that a minor subpopulation of antibodies can occur in goat antisera to Hb that binds only to Hb (probably the αβ-dimer) and not to either of the isolated subunits. It is likely therefore that, on immunization, Hb is recognized predominantly at the level of individual subunits and to a much smaller degree in higher states of subunit association.

Previous attempts, with Hb variants, to localize the antigenic sites of Hb resulted in the tentative identification of only two regions, around residues 15 and 16 and residue 68, as contributing to antigenic sites on the α-chain (Reichlin, 1975). In the present analysis, residues 15 and 16 lie within a localized antigenic site. Residue 68, however, does not fall within any of the localized antigenic sites. At this level of delineation we cannot exclude the possibility that residues outside of the regions assigned to the antigenic sites but in close spatial proximity to the sites in the native α-chain influence, to various degrees, their reactivities. In this context, we have described (Atassi & Kazim, 1980; Kazim & Atassi, 1980c) antigenic sites as existing in a steric and electronic equilibrium with neighbouring residues, and their reactivities will therefore be affected by changes or perturbations in these environmental residues (Twining et al., 1980).

Structural inherency of antigenic sites

We have previously mentioned that we were able, by extrapolation of the conformational location of Mb antigenic site 1 (Mb15–22), to predict and confirm by synthesis two antigenic sites (α15–23 and β16–23) on Hb (Kazim & Atassi, 1977a). Subsequent to these studies and to our proposal of structurally inherent antigenic sites (Atassi & Kazim, 1978), the antigenic structure of Mb (Atassi, 1975) was extrapolated to the conformationally similar soya-bean leghaemoglobin molecule (Hurrell et al., 1978). Each of the extrapolated regions were found to be immunochemically active with an antiserum to leghaemoglobin. It was of interest then to compare the locations of the antigenic sites localized here for the α-chain with those precisely determined for the Mb molecule (Atassi, 1975). A structural extrapolation of the five antigenic sites of Mb to the α-chain indicates that the regions 15–22, 51–56, 88–93, 107–113 and 139–141 occupy conformational locations analogous to the five Mb antigenic sites: 15–22, 56–62, 94–99, 113–119 and 145–151. There is almost a complete coincidence, in terms of their locations, of the localized α-chain antigenic sites with the regions extrapolated from Mb (Fig. 2). With the exception of the region 139–141, all of the regions extrapolated from Mb fall within the localized antigenic sites on the α-chain.

These observations give overwhelming support to our proposal for the structural inherency of antigenic sites in a family of proteins (Atassi & Kazim, 1978). Other evidence has recently been reviewed in detail (Atassi, 1980). The term 'structurally inherent antigenic sites' was used to refer to antigenic sites whose antigenicities were determined by the uniqueness of their conformational locations, and could be present on immunochemically unrelated members of a protein family. The α-chain is 12 residues shorter than Mb, and therefore site 5 (region 145–151) of Mb does not have a counterpart in the α-chain. However, it should also be noted that antigenic site 5 on the α-chain does not have a counterpart antigenic site in Mb. We have previously cautioned that new antigenic sites, unique to Hb and its α- and β-chains, and without counterparts in the Mb molecule, should not be unexpected (Kazim & Atassi, 1977a). Clearly, unique shape and structural factors in a given protein may also

contribute to the recognition and selection of new antigenic sites.

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