Antigenic structure of human haemoglobin

Localization of the antigenic sites of the β -chain in three host species by synthetic overlapping peptides representing the entire chain

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A comprehensive synthetic approach is applied here to localize the continuous antigenic sites of the β -chain of haemoglobin. The approach was based on the synthesis and purification of the following consecutive 15-residue peptides (each overlapping by five residues at both ends with the peptides preceding it and following it in the sequence): 1–15, 11–25 etc. Quantitative radiometric titrations of protein and peptide adsorbents were performed with ^{125}I -labelled anti-haemoglobin antibodies from three different host species. The specificity of antibody binding to peptide adsorbents was confirmed by inhibition studies and by the binding specificity of antibodies isolated from peptide adsorbents. These studies established the full profile of antigenic β -chain regions, which was found to be independent of the host species. Five major antigenic sites were localized, and their three-dimensional and structural characteristics are discussed in relation to the immune recognition of haemoglobin and other proteins.

INTRODUCTION

Previously, a novel and comprehensive synthetic approach was introduced by this laboratory (Kazim & Atassi, 1980b) which was designed to localize the full profile of the continuous antigenic sites on a protein molecule. This approach consists of the synthesis and examination of the immunochemical activities of a series of overlapping peptides having uniform size and overlaps and that encompass the entire polypeptide chain, from the beginning to the end. This strategy was first applied to localize the continuous sites recognized by antibodies on the α -chain of human Hb (Kazim & Atassi, 1980b, 1982). More recently, it has been applied for the localization of the sites recognized by T cells on sperm-whale Mb (Bixler & Atassi, 1983, 1984) and hen's-egg lysozyme (Bixler *et al.*, 1984a,b). In the present paper, the application of this strategy to the β -chain of human Hb, and the use of anti-Hb antibodies raised in three different animal species, enabled the localization of the antigenic sites to within fairly small regions on the β -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 with *p*-hydroxy-mercuribenzoate (Bucci & Fronticelli, 1965), followed by repeated chromatography on CM-cellulose or DEAE-cellulose (Geraci *et al.*, 1969) until they were homogeneous by polyacrylamide-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 β -

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 in detail for the synthesis of myoglobin antigenic sites (Koketsu & Atassi, 1973, 1974a). The dinitrophenyl protecting group on the imidazole side chain and the sulphoxide protecting group of methionine were removed by thiolation. The peptides were purified by gel filtration on Sephadex G-25, followed by ion-exchange chromatography on CM-Sephadex (C-50) or DEAE-Sephadex (A-50) as previously described (Koketsu & Atassi, 1973, 1974a,b). Peptide purity was checked by peptide mapping (Atassi and Saplin, 1968), and by amino acid analysis of HCl (Atassi and Saplin, 1968) and 3 M-toluene-*p*-sulphonic acid (Liu & Chang, 1971) hydrolysates.

Antisera to HbA were raised in goats, rabbits and outbred mice by a previously described immunization schedule (Atassi, 1967). The IgG fractions of these antisera, completely free of haptoglobin (Gray *et al.*, 1969; Kazim & Atassi, 1980a), were labelled with ^{125}I (Amersham Corp., Arlington Heights, IL, U.S.A.) by a chloramine-T procedure (Hunter & Greenwood, 1962). The specific radioactivities of the labelled IgG fractions were 16–22 $\mu\text{Ci}/\mu\text{g}$.

The proteins (HbA, β -chain, bovine serum albumin) and the synthetic peptides were coupled to CNBr-activated (March *et al.*, 1974) Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) under the optimum conditions previously described (Twining & Atassi, 1979). The immunochemical activities of the protein and peptide fractions were determined by quantitative immuno-adsorbent titrations (Twining & Atassi, 1979) of fixed amounts of ^{125}I -labelled immune IgG and various amounts of peptide or protein adsorbents. Non-specific adsorption was determined by titrating equivalent

Abbreviations used: Hb, haemoglobin; Mb, myoglobin.

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Fig. 1. Schematic presentation of the synthetic-overlapping-peptides strategy that was employed for the delineation of the continuous antigenic sites of the β -chain of human adult Hb

The strategy relied on the synthesis of the entire polypeptide chain in 15-residue peptides, each overlapping its two adjacent neighbours by five residues on both sides.

Peptide	
1-15:	Val-His-Leu-Thr-Pro-Glu-Glu-Lys-Ser-Ala- <u>Val-Thr-Ala-Leu-Phe</u>
11-25:	<u>Val-Thr-Ala-Leu-Trp-Gly-Lys</u> -Val-Asn-Val-Asp-Glu-Val-Gly-Gly
21-35:	<u>Asp-Glu-Val-Gly-Gly-Glu-Ala-Leu-Gly-Arg</u> -Leu-Leu-Val-Val-Tyr
31-45:	<u>Leu-Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg</u> -Phe-Phe-Glu-Ser-Phe
41-55:	<u>Phe-Phe-Glu-Ser-Phe-Gly-Asp-Leu-Ser-Thr</u> -Pro-Asp-Ala-Val-Met
51-65:	<u>Pro-Asp-Ala-Val-Met-Gly-Asn-Pro-Lys-Val</u> -Lys-Ala-His-Gly-Lys
61-75:	<u>Lys-Ala-His-Gly-Lys-Lys-Val-Leu-Gly-Ala</u> -Phe-Ser-Asp-Gly-Leu
71-85:	<u>Phe-Ser-Asp-Gly-Leu-Ala-His-Leu-Asp-Asn</u> -Leu-Lys-Gly-Thr-Phe
81-95:	<u>Leu-Lys-Gly-Thr-Phe-Ala-Thr-Leu-Ser-Glu</u> -Leu-His-Ser-Asp-Lys
91-105:	<u>Leu-His-Ala-Asp-Lys-Leu-His-Val-Asp-Pro</u> -Glu-Asn-Phe-Arg-Leu
101-115:	<u>Glu-Asn-Phe-Arg-Leu-Leu-Gly-Asn-Val-Leu-Val</u> -Ser-Val-Leu-Ala
111-125:	<u>Val-Ala-Val-Leu-Ala-His-His-Phe-Gly-Lys</u> -Glu-Phe-Thr-Pro-Pro
121-135:	<u>Glu-Phe-Thr-Pro-Pro-Val-Gln-Ala-Ala-Tyr</u> -Gln-Lys-Val-Val-Ala
131-146:	<u>Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala</u> -Leu-Ala-His-Lys-Tyr-His

Fig. 2. Primary structures of the synthetic overlapping Hb β -chain peptides

The numbers refer to the positions of the residues in β -chain. The underlined parts represent the regions on a peptide that overlap with the preceding and following peptides. Note that Trp-15 was replaced by phenylalanine in peptide 1-15, and that Cys-93 and Cys-112 were replaced by serine in peptides 81-95 and 101-115 and by alanine in peptides 91-105 and 111-125. For details see the text.

volumes of uncoupled Sepharose, bovine serum albumin-Sepharose and Sepharose adsorbents of five synthetic peptides of bovine serum albumin (Atassi *et al.*, 1979) 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 (1-3%).

The specificity of antibody binding to peptide adsorbents was confirmed by inhibition studies in which the labelled IgG fractions were allowed to bind as above in the presence of either Hb (1 mg/ml) or an unrelated protein, bovine serum albumin (1 mg/ml).

RESULTS

Synthesis, purification and characterization of the peptides

The synthetic peptides are shown schematically in Fig. 1, and their primary structures are shown in Fig. 2. It should be noted that, since tryptophan is somewhat unstable under the acidic conditions used during synthesis, tryptophan was replaced by phenylalanine in

peptide 1-15 (residue no. 15) but not in peptides 11-25 and 31-45. The terminal location of tryptophan in peptide 1-15 would have required its exposure to many more acidic cycles than in peptides 11-25 and 31-45, which might result in an excessive accumulated loss. The substitution of tryptophan by phenylalanine in immunologically active peptides has been shown to be permissible in previous studies on the antigenic structure of lysozyme (Lee *et al.*, 1976). Furthermore, tryptophan-15 is located in the overlapping region between peptides 1-15 and 11-25, and thus if phenylalanine were an unsuitable replacement for tryptophan any immunological activity due to this region would be detected by peptide 11-25. In addition, cysteine residues 93 and 112 were replaced by serine in peptides 81-95 and 101-115 and by alanine in the overlapping peptides 91-105 and 111-125 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

Table 1. Summary of the amino acid compositions of the synthetic Hb β -chain peptides

The values represent the averages of six or more replicate analyses, at 22, 48 and 72 h of hydrolysis. The values for serine and threonine were obtained by extrapolation to zero hydrolysis time. The expected values are given in parentheses.

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	131-146
Asp			2.09 (2)	1.11 (1)		2.13 (2)	1.81 (2)	1.13 (1)	2.96 (3)	1.03 (1)	3.11 (3)	1.73 (2)			1.04 (1)
Thr		1.95 (2)	1.13 (1)		1.00 (1)	0.97 (1)			1.17 (1)	1.80 (2)			1.05 (1)	1.00 (1)	
Ser		1.09 (1)			1.18 (1)	1.81 (2)		1.11 (1)	0.88 (1)	2.25 (2)		0.99 (1)			
Glu		1.93 (2)	1.01 (1)	2.15 (2)	2.13 (2)	1.00 (1)				1.09 (1)	1.15 (1)	0.89 (1)	1.09 (1)	3.12 (3)	1.18 (1)
Pro		1.15 (1)			0.95 (1)	0.98 (1)	1.95 (2)						2.00 (2)	1.93 (2)	
Gly			2.97 (3)	4.01 (4)		1.03 (1)	2.04 (2)	2.95 (3)	1.99 (2)	1.02 (1)		0.90 (1)	1.04 (1)		1.13 (1)
Ala		2.07 (2)	0.96 (1)	1.00 (1)		1.05 (1)	2.05 (2)	1.67 (2)	0.98 (1)	0.95 (1)	0.89 (1)	1.12 (1)	2.08 (2)	3.10 (3)	3.95 (4)
Val		1.85 (2)	3.90 (4)	2.87 (3)	1.69 (2)	1.09 (1)	1.89 (2)	1.23 (1)			1.15 (1)	2.75 (3)	1.85 (2)	2.87 (3)	3.13 (3)
Met						0.92 (1)	0.81 (1)								
Leu		1.84 (2)	1.03 (1)	3.05 (3)	1.86 (2)	1.06 (1)		1.96 (2)	2.94 (3)	2.97 (3)	2.88 (3)	4.16 (4)	0.88 (1)		0.89 (1)
Tyr				0.88 (1)	0.91 (1)									1.04 (1)	0.84 (1)
Phe		1.05 (1)			3.24 (3)	2.95 (3)		1.00 (1)	1.88 (2)	1.04 (1)	1.03 (1)	0.87 (1)	2.10 (2)		
His		0.94 (1)					1.08 (1)	1.00 (1)	0.94 (1)	1.00 (1)	2.13 (2)		2.09 (2)	0.94 (1)	1.93 (2)
Lys		1.00 (1)					3.12 (3)	3.03 (3)	1.00 (1)	1.89 (2)	1.08 (1)		1.01 (1)	1.00 (1)	2.07 (2)
Trp			1.07 (1)		1.01 (1)							0.87 (1)			
Arg			0.94 (1)		0.88 (1)						0.87 (1)	0.91 (1)			

paper electrophoresis, and was confirmed by amino acid analysis to be the desired product. 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.

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

Quantitative immunoadsorbent titrations were performed with fixed amounts of ^{125}I -labelled antibodies and increasing amounts of adsorbents. Antibodies to Hb were raised in three different host species and examined in terms of their binding profile with all the overlapping peptides. Fig. 3 shows an example of a titration with ^{125}I -labelled goat anti-Hb antibodies, and Table 2 summarizes the binding values in the plateau region with rabbit, goat and outbred-mouse antisera.

The highest binding activity was obtained with Hb, as expected, since the antibodies were raised against the native protein. About 50–60% of anti-Hb antibodies were directed against the β -chain. The fraction of anti-Hb antibodies directed to the individual subunits has already been discussed in detail (Kazim & Atassi, 1980b, 1982). Several peptide adsorbents showed very little (below 3%) or no binding activity throughout the titration range. These were: for the rabbit and goat antisera, peptides 41–55, 51–65, 61–75, 81–95, 91–105 and 121–135; for mouse antisera, peptides 41–55, 51–65, 81–95, 91–105 and 121–135. Also, the antibody-binding activity of peptide 1–15 with the various antisera was quite low (3–4%), relative to the binding by the β -chain. On the other hand, the other peptides exhibited considerable antibody-binding capacity and were therefore unambiguously active. Essentially the same set of peptides was active with rabbit, goat and mouse antisera. These peptides were: 11–25, 21–35, 31–45, 71–85, 101–115,

Fig. 3. Example of a quantitative radioimmunoassay titration of anti-Hb antibodies with peptide adsorbents

For the titration, fixed amounts of ^{125}I -labelled immune IgG (1.95×10^5 c.p.m.), from goat (G2) anti-Hb antisera, were titrated with increasing amounts of adsorbents of Hb, β -chain and the 14 synthetic overlapping β -chain peptides. Each point represents the average of six replicate analyses, which varied $\pm 1.3\%$ or less. For details see the text.

Table 2. Binding of radioiodinated antibodies to synthetic Hb β -chain peptides

The results summarized in this Table represent the 'plateau' binding values obtained by quantitative radioimmunoabsorbent titrations. The values represent the averages for three or more replicate analyses, which varied $\pm 1.3\%$ or less, and have been corrected for non-specific binding to adsorbents of unrelated proteins and peptides (see the text). The amounts of radiolabelled immune IgG used in these titrations were: H-2, 340000 c.p.m.; G2, 195000 c.p.m.; M1-M5 (representing a pool of anti-Hb antisera from five outbred mice), 210000 c.p.m.

Protein or peptide adsorbent	^{125}I -labelled anti-Hb bound					
	Rabbit antiserum (H2)		Goat antiserum (G2)		Mouse antiserum (M1-M5)	
	Anti-Hb bound (Δ c.p.m.)	Binding relative to β -chain (%)	Anti-Hb bound (Δ c.p.m.)	Binding relative to β -chain (%)	Anti-Hb bound (Δ c.p.m.)	Binding relative to β -chain (%)
Hb	39939	—	22710	—	58262	—
β -Chain	25026	100	13810	100	36870	100
1-15	1005	4.0	511	3.7	998	2.7
11-25	8080	32.3	5690	41.2	20790	56.3
21-35	6450	25.8	7021	50.8	18820	51.0
31-45	3270	13.1	1808	13.1	10509	28.5
41-55	527	2.1	0	0	840	2.2
51-65	310	1.2	0	0	570	1.5
61-75	250	1.0	0	0	6320	17.1
71-85	2830	11.3	1736	12.6	31118	84.4
81-95	0	0	0	0	770	2.1
91-105	0	0	0	0	0	0
101-115	3720	14.9	3106	22.5	8943	24.3
111-125	3862	15.4	3090	22.4	27930	75.7
121-135	0	0	310	2.2	0	0
131-146	11262	45.0	8840	64.0	21070	57.1

Fig. 4. Schematic diagram showing the β -chain of Hb, its regions that have been extrapolated from the five antigenic sites of sperm-whale Mb and its antigenic sites that have been localized with goat, rabbit and mouse antisera to Hb

The general areas of the sites are highlighted by shading for clarity. The numbers refer to the locations of the residues in the β -chain. Note that antigenic site 5 of Mb (residues 145–151) does not really have a full structural counterpart in the β -chain. For details see the text. It is not implied that the antigenic sites comprise the entire size of the regions shown, but rather that they fall within these regions.

111–125 and 131–146. In addition, peptide 61–75 was clearly active with mouse antisera. The titration curves show that the active peptides exhibited lower binding affinity, relative to Hb or β -chain. This is due to conformational factors and because a considerable proportion of a peptide on the adsorbent is linked via side chains that might also be essential for binding to antibody (Atassi & Saplin, 1968; Atassi, 1975; Twining & Atassi, 1979; Atassi *et al.*, 1979).

The binding of antibodies to these peptide adsorbents was completely inhibited by the addition of free Hb to the reaction (results not shown) but not by unrelated proteins (bovine serum albumin, hen's-egg lysozyme and ovalbumin). Also, none of the peptides bound antibodies to unrelated proteins. Furthermore, antibodies isolated on peptide adsorbents bound only to peptide, Hb and β -chain, but did not bind to unrelated protein adsorbent. These findings indicated unambiguously that antibodies reacting with the peptide adsorbent are specific for Hb.

It should be cautioned that the activities of the peptides with each of the antisera 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. Thus adjacent overlapping peptides that have similar activities may be expressing identical specificities. The activities of overlapping peptides are therefore not additive. Also, it should be noted that, in coupling to Sepharose, Hb and the isolated α - and β -subunits suffer considerable loss of antibody-binding activity (Kazim & Atassi, 1982). Thus the binding values for Hb and the β -chain shown in Table 2 are lower than the full antibody-binding capacity of these molecules.

DISCUSSION

The determination and synthetic verification of the first antigenic structures of proteins [myoglobin (Atassi, 1975) and lysozyme (Atassi, 1978)] relied on a complex chemical strategy based on five different chemical

approaches (Atassi, 1972). The strategy was time-consuming and required a great deal of chemical and immunological expertise (for reviews see Atassi, 1975, 1977). In order to render localization of 'continuous' protein antigenic sites (i.e. sites comprising residues that are directly linked by peptide bonds; for definitions of antigenic sites see Atassi & Smith, 1978) feasible within a relatively short time, this laboratory introduced (Kazim & Atassi, 1980b, 1982) a comprehensive synthetic strategy consisting of the synthesis of a series of consecutive overlapping peptides having uniform size and overlaps and which encompass the entire protein chain. The β -chain peptides designed here consisted of 15 residues and overlapped at their N- and C-termini by five residues each with adjacent peptides. A schematic presentation of the design is shown in Fig. 1. It should be noted that this strategy has been devised specifically for the localization of continuous antigenic sites. A more detailed discussion of this strategy has already been reported (Kazim & Atassi, 1980b, 1982).

For localization of the antigenic sites, they were considered to consist of seven residues, and for binding with antibody a peptide must contain at least five of the seven site residues. Antibody-binding activities in adjacent peptides may reside in the overlap regions, and such similar activities were confirmed by adsorbent experiments. Five antigenic sites were localized on the β -chain by goat, rabbit and mouse anti-Hb antibodies. The sites reside in, but do not necessarily include all of, the regions shown in Fig. 4. Furthermore, with goat antisera, site 1 had previously been further delineated by synthesis to the region 16–23 (Kazim & Atassi, 1977a). It should be emphasized that the sites are assigned to fall within the regions indicated in Fig. 4 and that their precise boundaries are yet to be determined. In fact, the localized areas are intentionally enlarged in order to avoid errors stemming from narrow assignments at this stage. Further studies with synthetic peptides overlapping within the assigned regions are required to define the boundaries. Upon such refinement of the data, however,

small shifts in the boundaries of these sites should not be unexpected (Koketsu & Atassi, 1973, 1974a; Atassi, 1975).

In the three-dimensional structure of Hb (Perutz *et al.*, 1968; Fermi, 1975), site 2 of the β -chain comprises almost entirely residues involved in the β_1 - α_1 and β_2 - α_1 subunit contacts, whereas most of the residues of site 4 participate in β_1 - α_1 contacts. Also, it has been shown (Kazim & Atassi, 1982) that three of the five antigenic sites of the α -chain contain residues that participate in α_1 - β_1 (sites 4 and 5) or α_1 - β_2 (site 3) subunit interactions. Such site residues are therefore potentially obstructed in the Hb dimer and tetramer. These findings are consistent with our previous conclusion (Kazim & Atassi, 1982), derived from similar studies on the α chain, that the immune recognition of Hb occurs, largely, at the level of its individual subunits.

Other workers have implicated, by the use of Hb variants, β -chain positions 6, 73, 115–116 and 125–126 to reside in antigenic sites (Reichlin, 1975). The present studies show unambiguously that residues, 6, 125 and 126 do not reside in antigenic sites: In all likelihood, also, residue 73 may be found not to reside in an antigenic site when the precise boundaries are determined because, as mentioned above, the assignments here are made intentionally larger than the expected boundaries of the sites. Thus, of the residues implicated by analysis of Hb variants, only residues 115 or 116 are likely to fall within an antigenic site. Similar disagreements were found in the α -chain between assignments postulated by Hb mutant analysis (Reichlin, 1975) and those actually found by the comprehensive synthetic strategy (Kazim & Atassi, 1982). The use of protein variants to localize areas of immunochemical activity has many inherent difficulties and, in general, has not been satisfactory (for reviews see Atassi & Habeeb, 1977; Atassi & Kazim, 1980; Kazim & Atassi, 1980c; Atassi, 1984a), and conclusions derived by this method have mostly been unsubstantiated by synthetic studies (Atassi, 1981; Kazim & Atassi, 1982; Bixler & Atassi, 1983, 1984).

The finding that several of the peptides exhibited very low (below 3%) antibody-binding activity (Table 2) indicated the presence of very low antibody responses to regions outside the five antigenic sites localized above. This was confirmed by adsorption with large excesses of immune IgG followed by radiolabelling on the adsorbent and then elution (Twining *et al.*, 1980; Kazim & Atassi, 1982). Trace amounts of Hb-specific antibodies could be isolated on adsorbents of peptides 1–15, 41–55 and 81–95. Quantification of these antibodies was difficult because of their presence in very low amounts, but they accounted for less than 1–2% of the total anti-Hb specific antibodies. Thus regions of the β -chain that are apparently inactive may be recognized by amounts of antibodies that are below detection level by these sensitive methods. Similarly, previous studies on Mb (Atassi, 1975), lysozyme (Atassi, 1978) and the α -chain of Hb (Kazim & Atassi, 1982) found that 'a general background response' to regions outside their respective antigenic sites accounted for 0.1–1.4% of the total anti-protein antibodies. Clearly, the present findings confirm our previous conclusion (Atassi, 1980) that the presence of trace antibody responses to regions interspersed among immunodominant sites would appear to be a general phenomenon for protein antigens.

It was proposed (Atassi, 1980) that the methodology

for producing monoclonal antibodies (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. Indeed, later findings with Mb (Schmitz *et al.*, 1983; Atassi, 1984a,b) and lysozyme (Metzger *et al.*, 1984) are consistent with the conclusion (Atassi, 1980) that the distribution of antibody specificities in the antisera may be different from that in the hybridomas. Therefore when monoclonal antibodies are used to localize antigenic sites on proteins the relevance of the findings to their biological immunodominance (*in vivo*) should be treated with caution. It would be necessary to confirm the existence of such specificities in the antisera of the parent animal.

The present mapping of the antigenic profile of the β -chain reveals discrete sites that are similarly recognized (although their immunodominance varies) by goat, rabbit and mouse antisera (Table 2). This is in agreement with our previous conclusions that the locations of protein antigenic sites are not dependent on the immunized species (Kazim & Atassi, 1977b; Atassi & Kazim, 1978), and has been found to be the case for Mb (Atassi, 1975; Twining *et al.*, 1980), lysozyme (Atassi, 1978), serum albumin (Atassi *et al.*, 1979; Sakata & Atassi, 1980; Atassi, 1982), the α -chain of Hb (Kazim & Atassi, 1982) and those sites that have been localized on influenza-virus hemagglutinin (Atassi & Webster, 1983; Atassi & Kurisaki, 1984). The present and previous findings (for a review see Atassi, 1984a) do not support the view (Benjamin *et al.*, 1984) that protein antigenic sites represent a 'continuum of recognition' varying with the host species and even with individual animals within a given species.

Previously we were able to predict and confirm by synthesis (Kazim & Atassi, 1977a) two antigenic sites on Hb (α 15–23 and β 16–23) by extrapolation of the conformational location of Mb antigenic site 1 (residues 15–22) (Koketsu & Atassi, 1974a). This led to our proposal of structurally inherent antigenic sites (Atassi & Kazim, 1978). Subsequent to these studies, the antigenic structure of Mb (Atassi, 1975) was extrapolated to the immunochemically unrelated, but conformationally similar, soya-bean leghaemoglobin (Hurrell *et al.*, 1978). The extrapolated regions were immunochemically active with antisera to leghaemoglobin. In the α -chain of Hb, the locations of four of its five sites coincided almost completely with the regions extrapolated from Mb (Kazim & Atassi, 1982). In the present work, the locations of two of the β -chain antigenic sites (sites 1 and 4) coincide almost completely with the structural locations of the corresponding sites of Mb (Fig. 4), whereas the locations of β -chain sites 2 and 3 are unique for the β -chain. Since the β -chain is shorter than Mb, site 5 of Mb could not be properly extrapolated to the β -chain, yet at this level of localization the results indicate that the β -chain possesses an antigenic site near its C-terminal (Fig. 4). It should be noted 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). Unique shape and structural factors in a given protein will be expected to contribute to the recognition and selection of new antigenic sites (Kazim & Atassi, 1977a, 1982). The present findings with the β -chain clearly confirm this expectation.

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