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Prediction and Confirmation by Synthesis of Two Antigenic Sites in Human Haemoglobin by Extrapolation from the Known Antigenic Structure of Sperm-Whale Myoglobin

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The complete antigenic structure of sperm-whale myoglobin was previously determined in our laboratory. By structural analogy with myoglobin, two regions in human haemoglobin were predicted to comprise antigenic sites. One region was on the α-chain [α-(15–23)] and the other on the β-chain [β-(16–23)]. These two regions were synthesized, purified and characterized, and their immunochemistry was studied. Each peptide was able specifically to bind considerable amounts of haemoglobin antibodies. In a set of homologous proteins, barring any drastic conformational or electrostatic inductive effects exerted by the substitutions, and allowing for obstruction due to subunit interaction, the determination of the antigenic structure of one protein may serve as a useful starting model for the others.

Previous reports from this laboratory have demonstrated conclusively that the structures of antigenic sites of proteins are directed by their amino acid sequences as well as by the three-dimensional arrangement of the participating amino acid residues (Habeeb & Atassi, 1971; Atassi, 1975; Atassi *et al.*, 1976*a*,*b*; Lee & Atassi, 1976). Delineation of the complete antigenic structure of sperm-whale myoglobin showed its antigenic sites to consist of continuous portions of its primary sequence and to be located at specific highly exposed positions in its three-dimensional structure (Atassi, 1975). Our results also suggested that the antigenic sites of other myoglobins are located at similar structural positions in their respective chains (Atassi & Saplin, 1971). Since the primary antibody response to a native protein is directed against its native three-dimensional structure (Atassi & Thomas, 1969), we were prompted to examine whether conformationally homologous but more complex globins possess antigenic sites at similar locations in their three-dimensional structure. The many structural features that the α- and β-chains of haemoglobin share with myoglobin (Perutz *et al.*, 1968) make the haemoglobin molecule particularly suitable for examining whether it is possible to extrapolate, and thereby predict, the antigenic sites of a large multisubunit protein from those of a smaller member of the same protein family.

In this study we have focused on the myoglobin antigenic site 1, which occupies the sequence 15–22 in the myoglobin molecule (Atassi, 1975). The sequences and structural locations of the corresponding regions in the α- and β-chains of human haemoglobin are shown in Fig. 1. To examine whether these extrapolated regions of the α- and β-chains [α-(15–23), β-(16–23)] are immunochemically active, they were

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Residue location:	A_{13}	A_{14}	A_{15}	A_{16}	AB_1	B_1	B_2	B_3	B_4	B_5
Site 1 of myoglobin:	$Ala_{(15)}$	Lys	Val	Glu	Ala	Asp	Val	$Ala_{(22)}$		
Haemoglobin α-(15-23):	$Gly_{(15)}$	Lys	Val	Gly	Ala	His	Ala	Gly	$Glu_{(23)}$	
Haemoglobin β-(16-23):	$Gly_{(16)}$	Lys	Val	Asn	...	...	Val	Asp	Glu	$Val_{(23)}$

Fig. 1. *Diagram showing the sequence and structural location of antigenic site* 1 *of sperm-whale myoglobin and the corresponding regions of the haemoglobin α- and β-chains*
For the letter/number notation used to designate the structural locations of the amino acid residues, and for the alignment of the haemoglobin α- and β-chains with sperm-whale myoglobin, see Dickerson & Geis (1969).

synthesized and their immunochemical reactions with antisera to human haemoglobin studied.

Experimental

Human haemoglobin A_1 was prepared as previously described (Atassi, 1964), and the α- and β-chains were prepared as described by Bucci & Fronticelli (1965); these preparations were homogeneous by polyacrylamide disc-gel electrophoresis. Solid-phase peptide synthesis and purification of the crude peptides on CM-Sephadex C-50 were performed as previously described (Koketsu & Atassi, 1973, 1974). Proteins and peptides were coupled to CNBr-activated Sepharose 4B by the procedure of March *et al.* (1974). The amounts of protein or peptide coupled to Sepharose were quantified by amino acid analysis of acid hydrolysates of the conjugates and were in the range 1.5–2.2 mg of protein or peptide per ml of packed volume.

Antisera to haemoglobin A_1 were raised in goats as previously described (Atassi, 1967). Antibodies specific for haemoglobin A_1 were prepared by immunoadsorption of the antisera on haemoglobin A_1–Sepharose, elution of the specifically bound protein with 5M-guanidine hydrochloride, pH8.5, and dialysis of the eluted fractions for 24h against five changes of 0.15M-NaCl/0.01M-sodium phosphate buffer, pH7.2. Antibodies specific for haemoglobin A_1 were radioactively labelled with ^{125}I (New England Nuclear, Boston, MA, U.S.A.) by the chloramine-T procedure of Hunter & Greenwood (1962). Quantitative immunoadsorption was performed as follows. Samples of Sepharose conjugates (0.25ml each, packed volume) were equilibrated by gentle rotation for 4h at 22°C and then for 18h at 4°C with 1% (w/v) bovine serum albumin in 0.15M-NaCl/0.01M-sodium phosphate buffer, pH7.2. This was followed, after washing with the buffer, by a similar equilibration with 0.1% non-immune goat immunoglobulin G in 1% albumin dissolved in the same buffer. Operations were carried out in plastic columns (1cm × 6cm) fitted with porous Teflon discs. After extensive washing with the buffer, the columns were equilibrated as above with portions (0.5ml) of ^{125}I-labelled antibody in immunoglubulin G/albumin. The immunoadsorbents were washed extensively with buffer until 1ml of effluent contained between 300 and 500c.p.m. of radioactivity. The bound ^{125}I-labelled antibody was then eluted with 5M-guanidine hydrochloride, pH8.5, and the radioactivity in the effluent fractions determined in a Packard γ-scintillation spectrometer without further preparation. A control adsorbent carrying hen's-egg-white lysozyme and a reference adsorbent (see the Discussion section) carrying the peptide α-(1–15) (with phenylalanine substituted for tryptophan-14) were used under identical conditions to monitor non-specific binding of the labelled antibodies. Procedures for quantitative precipitin and inhibition reactions, peptide 'mapping' and amino acid analysis have been described previously (Atassi & Saplin, 1968).

Results

The peptides obtained after chromatography on CM-cellulose C-50 were homogeneous in heavily loaded peptide 'maps'. Amino acid compositions of the pure peptides were: for α-(15–23), Glu, 1.16; Gly, 2.87; Ala, 2.17; Val, 1.01; Lys, 1.17; His, 0.78; for β-(16–23), Asp, 2.17; Glu, 1.00; Gly, 0.97; Val, 2.80; Lys, 1.10; for α-(1–15), Asp, 2.08; Thr, 1.02; Ser, 0.83; Pro, 1.10; Gly, 0.93; Ala, 3.10; Val, 1.98; Leu, 1.05; Phe, 0.92; Lys, 1.97. For each peptide, the composition agreed well with that expected from its sequence.

The synthetic peptides did not inhibit the quantitative precipitin reaction of haemoglobin with its antisera, when studied at molar excess relative to haemoglobin of 1000-fold, either individually or in combination.

On an immunoadsorbent, the α- and β-chains adsorbed large amounts of the antibodies relative to haemoglobin (Table 1). Both the α-(15–23) and β-(16–23) peptides were also effective in adsorbing considerable amounts of antibodies relative to their

Table 1. *Quantitative immunoadsorption of ^{125}I-labelled anti-haemoglobin antibodies*

Results represent the average of three determinations which varied by ± 1.5% each. They have also been corrected for protein non-specifically bound to lysozyme–Sepharose as explained in the text. This correction amounted to no more than 1–2% of the total radioactivity bound to haemoglobin–Sepharose. The amount of labelled antibody applied to each immunoadsorbent was 102000c.p.m. (1.099pmol) [peptides on the immunoadsorbents were present in a vast molar excess (approx. 600000) relative to the labelled antibody applied].

Immunoadsorbent	Amount of antibody bound (c.p.m.)	Percentage relative to haemoglobin A	Percentage relative to chain
Haemoglobin A	98000	100	—
α-Chain	82100	83.8	—
β-Chain	57800	59.0	—
α-(15–23) peptide	10800	11.1	13.2
β-(16–23) peptide	7130	7.28	12.3
α-(1–15) peptide	133	0.14	0.16

respective α- or β-chains, as well as to haemoglobin itself. The amounts of antibody bound to the reference peptide, α-(1–15), were negligible. The calculated sum of the reactivities of the α-(15–23) and β-(16–23) peptides accounted for 18.3% of the immunochemical activity of haemoglobin.

Discussion

Through the use of overlapping synthetic peptides during the final delineation of the antigenic sites of sperm-whale myoglobin to their precise boundaries, we showed that antigenic site 1 invariably included the sequence 16–21 with all antisera tested (Koketsu & Atassi, 1974). However, some antisera also required either alanine-15 or alanine-22 for full reactivity. Accordingly, site 1 was taken here to consist of the sequence 15–22 in order to accommodate the individual variabilities of different antisera. Sequence 15–22 of sperm-whale myoglobin occupies the helix positions A13–B3 and is located at the highly exposed bend between helices A and B (Watson, 1969; also see Fig. 1 for the explanation of structural notations).

The three-dimensional counterpart of the myoglobin antigenic site 1 in the haemoglobin α-chain also spans residues α-(15–22) (Perutz *et al.*, 1968). In our studies we chose to extend this region to include glutamic acid-23 to increase the solubility of the synthetic peptide α-(15–23) in aqueous solvents. The haemoglobin β-chain varies in its alignment with the α-chain in this region by not having the corresponding AB1 and B1 positions (Perutz *et al.*, 1968). Therefore we extended the synthetic region of the β-chain to valine-23 to compensate for the deficiency in length that would occur from a precise structural extrapolation of this region. By examination of the three-dimensional structures of human deoxyhaemoglobin (Fermi, 1975) and of horse oxyhaemoglobin (Perutz *et al.*, 1968) these regions [α-(15–23) and β-(16–23)] are not involved in either chain–haem or chain–chain interactions in the native haemoglobin molecule. In fact, similarly to the location of site 1 in myoglobin, they occupy highly accessible positions in their respective α- and β-chains in the native haemoglobin tetramer, and are ideally suited for examining whether the structural extrapolation of antigenic sites is possible.

The inability of these synthetic peptides to inhibit the precipitin reaction of haemoglobin with its antisera, even at a molar excess of 1000-fold, either individually or in combination, was not entirely unexpected. Although the synthetic antigenic sites of myoglobin inhibit its immune reaction quantitatively, other workers have reported that haemoglobin fragments will not inhibit the haemoglobin immune reaction (Reichlin, 1972). Also, antibodies to a region around valine-6 in haemoglobin S, obtained from anti-(haemoglobin S) by an immunoadsorbent of the synthetic peptide β-(1–13) of haemoglobin S, accounted for only about 5% of the total antibodies to haemoglobin S. Furthermore the peptide initially used to obtain this antibody population by immunoadsorption was not fully effective in displacing haemoglobin S bound to these antibodies (Young *et al.*, 1975). These results may in part be accounted for by the large size of haemoglobin, and conceivably by the increased number of antigenic sites and the decreased affinity that immunochemically active peptides in a non-native conformation exhibit for their antibodies (Atassi & Saplin, 1968). These observations suggested to us that, notwithstanding the structural alterations that would occur on being covalently coupled to Sepharose, quantitative immunoadsorption would be the most suitable approach for studying the immunochemical activities of these extrapolated peptides.

The results of the immunoadsorption studies (Table 1) show that these peptides are immunochemically active with antisera to native haemoglobin, accounting for a significant portion of the reactivities of haemoglobin and of the respective α- and β-chains. The immunochemical activities of these peptides are further illustrated by comparing them with the activity of a reference peptide, α-(1–15), which does not fall within the boundaries of an antigenic site that could be predicted from myoglobin. This peptide showed no significant immunochemical activity. The calculated sum of the reactivities of the α- and β-chains was greater than that of haemoglobin. Similar observations have been made by others (Tan-Wilson *et al.*, 1976) and were attributed to cross-reactivities between the chains.

The results presented here clearly indicate that, by extrapolation of the structural location of antigenic site 1 of sperm-whale myoglobin, we were able to predict and confirm antigenic sites for a larger more complex member of the same protein family. On the basis of these results we feel confident that, when similar extrapolations of antigenic sites of myoglobin are made to other homologous proteins, the predicted regions will also be shown to be immunochemically active. However, it should be noted that not all structural counterparts of the antigenic sites of one protein are expected to be immunochemically active in a homologous protein. Thus in the tetrameric haemoglobin molecule, for example, obstructions caused by subunit interactions, and conformational adjustments effected by amino acid substitutions (both inside and outside the predicted regions) as well as by the influence of neighbouring subunits, may alter the antigenic expression of these regions. For similar reasons, new antigenic sites on individual subunits or combinations thereof, without antigenic counterparts in the myoglobin molecule, should not be unexpected in the haemoglobin molecule.

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