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# Haemoglobin binding with haptoglobin

## Localization of the haptoglobin-binding sites on the β-chain of human haemoglobin by synthetic overlapping peptides encompassing the entire chain

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A synthetic approach was employed to identify the haptoglobin-binding sites on the β-chain of human haemoglobin. This approach consists of the synthesis of a series of consecutive overlapping peptides that, together, systematically represent the entire protein chain. Fourteen synthetic peptides (β1–15, β11–25 etc.) were examined for their ability to bind human haptoglobin by quantitative solid-phase radiometric titrations of $^{125}$I-labelled haptoglobin. Of these 14 peptides only peptides β11–25 and β131–146 bound haptoglobin significantly; peptide β21–35 exhibited a small binding activity as a consequence of the overlap with peptide β11–25. On this basis and by examination of the three-dimensional structure of haemoglobin, it was concluded that the β-chain of haemoglobin has two binding sites for haptoglobin that reside in, but do not necessarily encompass all of, the regions β11–25 and β131–146.

## INTRODUCTION

Haptoglobins (Hp) are a group of genetically polymorphic serum glycoproteins that migrate in the $\alpha_2$-globulin regions. They exhibit a high affinity for haemoglobin (Hb), forming exceedingly stable complex (Polnovski & Jayle, 1938) *in vivo* and *in vitro*. In fact, the molecular interactions between Hb and Hp in these complexes are among the strongest of all known non-covalent protein–protein interactions. The interactions of Hp with Hb and its α- and β-chains have been examined by a variety of experimental approaches (for review see Putnam, 1975). It was thought, until recently, that Hp binds very weakly, if at all, to isolated β-chains. However, solid-phase radiometric titration studies have demonstrated unequivocally that the isolated β-chain binds to Hp (Kazim & Atassi, 1980*a*). It is logical and less complex first to identify the Hp-binding site(s) on the indiviudal α- and β-chains and then to determine how these chains together bind to Hp.

A comprehensive synthetic approach, consisting of the synthesis of a series of consecutive overlapping peptides that systematically represent the entire protein chain, initially devised for the localization of protein antigenic sites (Kazim & Atassi, 1980*b*), has also been employed to localize the Hp-binding site on the α-chain of human Hb (Kazim & Atassi, 1981). Application of this strategy to the localization of the continuous antigenic sites of the β-chain is described in an accompanying paper (Yoshioka & Atassi, 1986). These synthetic peptides are employed in the present work to localize the binding site(s) on the β-chain of Hb for Hp.

## MATERIALS AND METHODS

The α- and β-chains of Hb were prepared as described by Bucci & Fronticelli (1965). The α- and β-chain preparations were homogeneous by polyacrylamide-disc-gel electrophoresis, and purity was also confirmed by chain-specific monoclonal anti-Hb antibodies (Yoshioka & Atassi, 1983). Solid-phase peptide synthesis of the β-chain peptides [see Figs. 1 and 2 in Yoshioka & Atassi (1986) for the location and structure of the synthetic β-chain peptides] and purification of the synthetic products were reported in an accompanying paper (Yoshioka & Atassi, 1986). Proteins and peptides were coupled to CNBr-activated Sepharose CL-4B as described by March *et al.* (1974). The adsorbents for the proteins had 2 mg/ml packed volume and those of the peptides 0.5 mg/ml packed volume.

Haptoglobins 2-1 were fractionated from the plasma of healthy adults by modification of method described by Kurosky *et al.* (1976). Namely, after fractionation with $(NH_4)_2SO_4$ at 55% and 33% saturation, the precipitate was solubilized with, and extensively dialysed against, 0.03 M-sodium acetate buffer, pH 4.6. The sample was then applied to a DEAE-cellulose column that had been pre-equilibrated with the same buffer, and unbound proteins were washed through from the column with the same buffer. The Hp fractions were eluted with a linear gradient of 0–0.3 M-NaCl in 0.03 M-sodium acetate buffer, pH 4.6. The fractions containing Hp were pooled and concentrated by ultrafiltration. They were then subjected to gel filtration on Sephadex G-150 in 0.01 M-sodium phosphate buffer, pH 7.2, containing 0.15 M-NaCl, and the eluent fractions were concentrated. The material was characterized as Hp by polyacrylamide-gel electrophoresis, by Hb-binding capacity (benzidine staining) and by Coomassie Brilliant Blue staining, and by agarose-gel immunoelectrophoresis. In immunoelectrophoresis, with anti-(human whole serum) antibody and monospecific anti-(human Hp) antibody, the Hp preparation gave a single precipitin line with each antiserum. Hp was labelled with $^{125}$I by a chloramine-T procedure

Abbreviations used: Hp, haptoglobin; Hb, haemoglobin.

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(Hunter & Greenwood, 1962). The specific radioactivity of radiolabelled Hp preparations was 20–25 $\mu$Ci/$\mu$g.

The binding of Hp to Hb, its $\beta$-chain and the synthetic peptides was studied by quantitative solid-phase titrations of fixed amounts of $^{125}$I-labelled Hp with various amounts of the Sepharose adsorbents according to the procedure described by Twining & Atassi (1979). Non-specific adsorption was determined by control titrations of labelled Hp with equivalent volumes of unconjugated Sepharose, bovine serum albumin–Sepharose and adsorbents of synthetic albumin peptides (antigenic sites; Atassi *et al.*, 1979) under the same conditions.

## RESULTS

Titration of $^{125}$I-labelled Hp with Sepharose-linked adsorbents of the synthetic peptides revealed that some of the $\beta$-chain peptides did indeed bind Hp. Peptides 11–25 and 131–146 exhibited considerable binding activity, and a lower but significant binding was obtained with peptide 21–35. The results of radiometric titrations of Hp with Hb, $\beta$-chain and peptides are shown in Fig. 1. Table 1 summarizes the binding values (in c.p.m. of $^{125}$I-labelled Hp) at 200 $\mu$l volumes of adsorbent. Peptides 1–15, 41–55, 51–65, 61–75, 81–95, 91–105 and 121–135 showed no binding to Hp over the entire range tested. Peptides 31–45, 71–85, 101–115 and 111–125 showed a very slight binding activity (Table 1). In order to determine by titration the maximum binding capacity of the two active peptides, relatively large amounts of their adsorbents would be needed. This plateau binding value was therefore obtained by double-reciprocal analysis of the data shown in Fig. 1. The double-reciprocal plots of the binding values for adsorbents of Hb, the $\beta$-chain and synthetic peptides 11–25 and 131–146 are presented in Fig. 2. From these plots it was determined that the amounts of $^{125}$I-labelled Hp that would be bound to infinitely large amounts of adsorbents (obtained from the intercept of the ordinate) were: Hb, 39000 c.p.m.; $\beta$-chain, 40800 c.p.m.; peptide 11–25, 39200 c.p.m.; peptide 131–146, 39200 c.p.m.

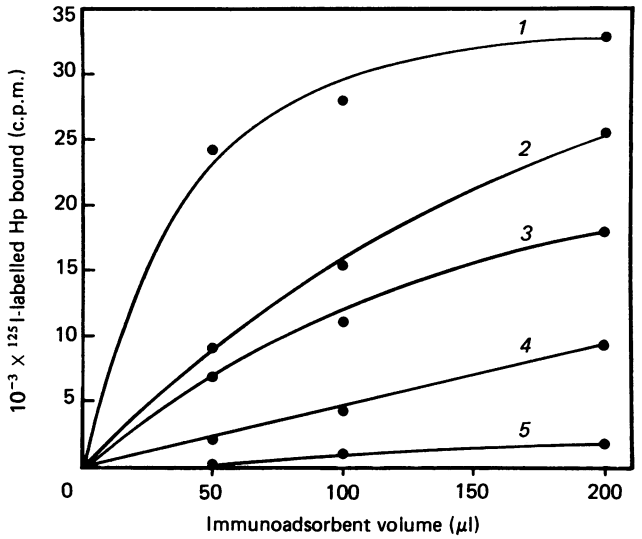


**Fig. 1. Quantitative radiometric titrations of $^{125}$I-labelled Hp with adsorbents of Hb, $\beta$-chain and the synthetic overlapping peptides**

Titrations were carried in triplicate with a fixed amount of $^{125}$I-labelled Hp 2-1 ($5 \times 10^4$ c.p.m.) and increasing amounts of the various Sepharose-linked adsorbents. Control titrations were performed with equivalent amounts of adsorbents of bovine serum albumin, synthetic antigenic sites of bovine serum albumin and unconjugated Sepharose. The values shown have been corrected for non-specific binding (600–1200 c.p.m.) to these control adsorbents. Curve *1*, Hb adsorbent; curve *2*, $\beta$-chain adsorbent; curve *3*, adsorbents of peptide 11–25 or 131–146; curve *4*, adsorbent of peptide 21–35; curve *5*, adsorbents of peptide 31–45, 71–85, 101–115 or 111–125.

**Table 1. Binding of $^{125}$I-labelled Hp to Hb, $\beta$-chain and its synthetic overlapping peptides**

Radiometric titrations were carried out with fixed amounts of $^{125}$I-labelled Hp ($5 \times 10^4$ c.p.m.) and increasing volumes of adsorbents. Values represent the amounts of $^{125}$I-labelled Hp bound by 200 $\mu$l of each adsorbent and were obtained from six replicate analyses, which varied $\pm 1.5\%$ or less. The values have been corrected for non-specific binding (2–3%) by equal volumes of uncoupled Sepharose, Sepharose-linked adsorbents of bovine albumin and synthetic antigenic sites of bovine serum albumin (Atassi *et al.*, 1979).

| Protein or peptide adsorbent | $^{125}$I-labelled Hp bound (c.p.m.) |
|---|---|
| Hb | 33877 |
| $\beta$-Chain | 27428 |
| 1–15 | 0 |
| 11–25 | 18670 |
| 21–35 | 9872 |
| 31–45 | 1640 |
| 41–55 | 0 |
| 51–65 | 0 |
| 61–75 | 0 |
| 71–85 | 2140 |
| 81–95 | 0 |
| 91–105 | 0 |
| 101–115 | 2040 |
| 111–125 | 1930 |
| 121–135 | 0 |
| 131–146 | 18920 |

## DISCUSSION

Previous studies from this laboratory (Kazim & Atassi, 1981), using the overlapping peptide strategy previously introduced (Kazim & Atassi, 1980*b*) for localization of continuous antigenic sites of proteins, resulted in the localization of the binding site on the $\alpha$-chain for Hp. It was found that the $\alpha$-chain binds to Hp through a binding site that resides within, but does not necessarily include all of, the region $\alpha$121–135 (Kazim & Atassi, 1981). Subsequent studies with ten synthetic peptides of gradually decreasing size from the *N*- or the *C*-terminus of peptide $\alpha$121–135 delineated the binding site for Hp on the $\alpha$-chain to occupy the region $\alpha$121–127 (McCormick & Atassi, 1983). This is a region entirely within the H helix of the $\alpha$-chain (from H4 through to H10) (McCormick & Atassi, 1983).

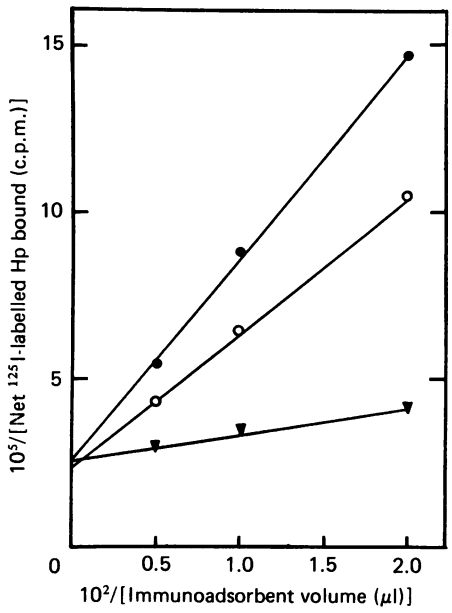


**Fig. 2. Double-reciprocal plots of the binding data shown in Fig. 1 for adsorbent of Hb (▼), adsorbent of the β-chain (○) and adsorbents of peptide 11–25 or 131–146 (●)**

The lines are obtained by least-squares regression analysis of the data. The amounts of $^{125}$I-labelled Hp that would be bound to infinitely large amounts of adsorbents were obtained from the intercepts of the ordinate and are: Hb, 39000 c.p.m.; β-chain, 40800 c.p.m.; peptide 11–25, 39200 c.p.m.; peptide 131–146, 39200 c.p.m.

The results of Hp binding to the 14 overlapping synthetic peptides of the β-chain have shown here that the binding activity of the β-chain resides almost entirely in peptides 11–25 and 131–146. Clearly, the two peptides exhibit lower binding affinity than does the intact β-chain. Since peptide 21–35 also possessed significant binding activity, it can be concluded that the region of the five-residue overlap between peptides 11–25 and 21–35 contains some of the essential contact residues of the binding site. On the other hand, because the binding of peptide 11–25 was much higher than the binding of peptide 21–35, the binding site must extend to the left of the overlap (i.e. to the left of residue 21 rather than to the right of residue 25). Therefore peptide 11–25 carries an intact binding site, whereas peptide 21–35 carries only a part of the same site. Similarly, since the binding peptide 131–146 was preceded by an overlapping peptide that had no binding activity (see peptide 121–135; Table 1), it can be concluded that peptide 131–146 carries all the essential contact residues of this site. The present approach does not permit delineation of the binding regions within β-chain peptides 21–35 and 131–146 to their precise boundaries. Synthesis of a series of peptides representing different overlaps within each of these two indicated regions will be required to determine the precise boundaries of the site, an approach previously developed and employed for protein antigenic sites (Koketsu & Atassi, 1973, 1974*a*,*b*) and more recently for the delineation of the binding site for Hp on the α-chain (McCormick & Atassi, 1983). Finally, it is difficult to determine, at present, whether the very slight amounts of

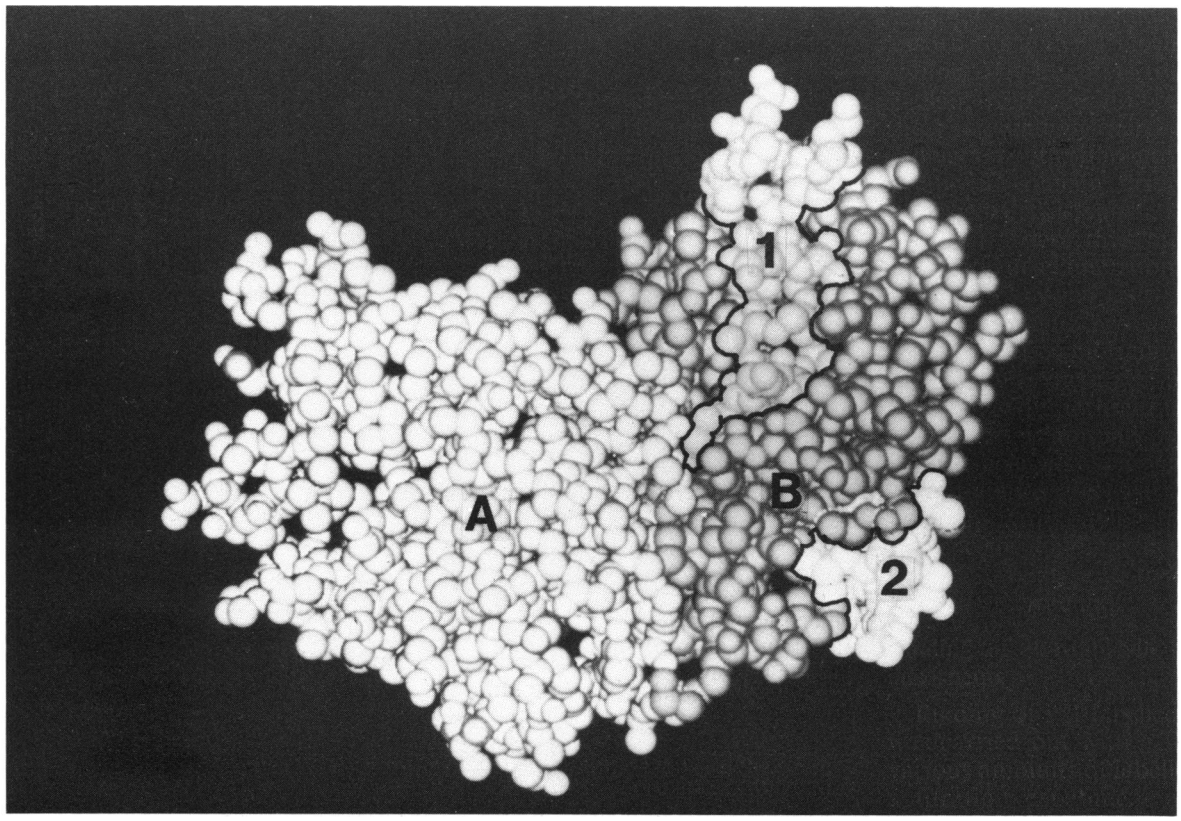


**Fig. 3. Computer-generated space-filling model of the αβ-dimer of Hb showing the relative locations of regions β11–25 and β131–146**

Key: A, α-chain; B, β-chain; 1, region 131–146 of the β-chain; 2, region 11–25 of the β-chain.

labelled Hp bound by peptides 31–45, 71–85, 101–115 and 111–135 represent significant binding.

In the three-dimensional structure of Hb (Perutz *et al.*, 1968; Fermi, 1975), the region 11–25 occupies half of the A helix and almost half of the B helix (from A8 through to A15 and from B1 through to B7). The region 131–146 corresponds to the last two-thirds of helix H and the *C*-terminal non-helical tripeptide segment (H9 through to H21 and HC1 through to HC2). These two binding regions are quite well separated on the surface of the $\beta$-chain (Fig. 3). This will make it very unlikely that they constitute two subsites within a larger single binding site on the $\beta$-chain. Rather, their locations would indicate that they form two independent sites. However, in the $\alpha$-chain binding site for Hp three contact residues (122, 123 and 126) are also contact residues in the $\alpha_1$–$\beta_1$ interface. This would suggest that, in binding to Hp, a major shift or dislocation takes place in the subunit interaction of Hb. This dislocation will be better understood when the exact boundaries of the binding regions on the $\beta$-chain are determined.

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