Haemoglobin binding with haptoglobin

Localization of the haptoglobin-binding site on the α -chain of human haemoglobin

A. Latif KAZIM and M. Zouhair ATASSI*

Department of Immunology, Mayo Medical School, Rochester, MN 55901, U.S.A.

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A synthetic approach was employed to identify the haptoglobin-binding site on the α -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 peptides were synthesized (α 1–15, α 11–25, α 21–35, α 31–45, α 41–55, α 51–65, α 61–75, α 71–85, α 81–95, α 91–105, α 101–115, α 111–125, α 121–135 and α 131–141), and their ability to bind human haptoglobin was studied. Only peptide α 121–135 bound haptoglobin significantly. On this basis we conclude that the haptoglobin-binding site on the α -chain of haemoglobin resides within, but does not necessarily encompass all of, the region α 121–135.

Haptoglobins (Hp) are a group of genetically polymorphic serum glycoproteins that form extremely stable complexes with haemoglobin (Hb) (Polonovski & Jayle, 1939). Such complexes are, in fact, considered to be essentially irreversible under physiological conditions (Nagel & Gibson, 1971). Thus the molecular interactions between Hb and Hp in these complexes are among the strongest of all known non-covalent protein–protein interactions. Owing to the unusual stability of these complexes, the molecular features of these interactions are of particular interest with regard to an understanding of the general nature of interacting surfaces among proteins. In view of the fact that Hp binds to isolated α - and β -chains of Hb (Chiancone *et al.*, 1968; Nagel & Gibson, 1971; Terpstra & Smith, 1976; Tsapis *et al.*, 1976; Hwang & Greer, 1979; Kazim & Atassi, 1980a), it is logical and less complex to first identify the Hp-binding site on the individual α - and β -chains and then to determine how these chains together bind to Hp to form such stable complexes.

Recently, we (Kazim & Atassi, 1980b) introduced a synthetic strategy for the localization of protein-binding sites of the continuous type [i.e. those that can be preserved in a continuous segment of the protein chain (Atassi, 1978; Atassi & Smith, 1978)]. This strategy, first applied to the localization of the antigenic sites on the α -chain of human Hb (Kazim & Atassi, 1980b), involves the synthesis of peptides of appropriate length and which, taken

together, represent the entire protein chain. Each peptide is suitably overlapped with the peptides preceding and following it in order to avoid inadvertent scission of a binding site. It was determined (Kazim & Atassi, 1980b) for Hb chains that peptide chain lengths of fifteen residues and overlaps of five residues were optimal in terms of synthetic load, the ability to narrow down binding sites to a convenient size and to maintain full activity should the binding site fall within a region of overlap. This synthetic approach is schematically depicted in Fig. 1. We have applied this strategy to localize the binding site(s) on the Hb chains for Hp. This initial paper describes our findings for the α -chain.

Experimental

Synthesis and purification of the α -chain peptides have already been reported (Kazim & Atassi,

Fig. 1. A schematic representation of the synthetic strategy employed for localizing the Hp-binding site on the α -chain of Hb (depicted by the unbroken line, 1–141).

The synthetic overlapping peptides shown each consist of 15 residues, except α 131–141. Consecutive peptides overlap by five residues. Numbers refer to the locations of residues in the primary structure (Braunitzer *et al.*, 1961; Hill & Konigsberg, 1962) of the α -chain. See the text for details.

Abbreviations used: Hb, haemoglobin; Hp, haptoglobin.

* To whom correspondence and requests for reprints should be addressed.

Fig. 2. Quantitative solid-phase radiometric titrations of ${}^{125}\text{I}$ -labelled Hp with adsorbents of Hb, its α -chain and the synthetic overlapping peptides

(a) Shows titration curves which were obtained by reaction of a fixed amount of ${}^{125}\text{I}$ -labelled Hp (49 300 c.p.m.) incubated in duplicate with various amounts of Sepharose adsorbents (proteins and peptides at 2.4 and 0.4 mg/ml packed volume respectively) in a final reaction volume of 400 μl . The reaction medium consisted of phosphate-buffered saline (0.15 M-NaCl/0.01 M-phosphate buffer, pH 7.2), and contained 0.4% (w/v) highly purified haptoglobin-free non-immune goat immunoglobulin G to minimize non-specific binding. After shaking for 18 h at 23°C, the adsorbents were washed three times (3 ml each) with phosphate-buffered saline and the ${}^{125}\text{I}$ -labelled Hp bound was quantified on a gamma counter. Equivalent amounts of unconjugated Sepharose and bovine serum albumin-

1980b). Human Hp (type 2-1), ${}^{125}\text{I}$ -labelled Hp (20 $\mu\text{Ci}/\mu\text{g}$), human Hb (cyanomethaemoglobin A) and the α -chain of Hb were prepared as previously described (Kazim & Atassi, 1980a). Proteins and peptides were coupled to CNBr-activated Sepharose CL4B. The amounts of proteins and peptides used in the coupling reactions were 3 mg/ml and 1 mg/ml (packed volume) respectively. These afforded adsorbents that had proteins and peptides at 2.4 and 0.5 mg/ml packed volume respectively. The binding of Hp to Hb, its α -chain and the synthetic peptides was studied by quantitative solid-phase titrations of fixed amounts of ${}^{125}\text{I}$ -labelled Hp with various amounts of the Sepharose adsorbents. The radiometric titration and inhibition procedures were identical with that previously used to demonstrate the binding of ${}^{125}\text{I}$ -labelled Hp to solid-phase Hb and its α - and β -chains (Kazim & Atassi, 1980a).

Results and discussion

Quantitative titration studies (Fig. 2a) indicated that, under the conditions of this binding assay, 72% of the total added ${}^{125}\text{I}$ -labelled Hp was maximally bound by the Hb adsorbent in the 'plateau' region. This agrees with the previous finding that there is a substantial retention of the Hb-binding capacity of Hp on radioiodination of the latter (Kazim & Atassi, 1980a). Although the binding profile of the α -chain adsorbent rises less steeply than that of Hb, it eventually attained the same maximum binding value as for Hb. This finding was also previously reported (Kazim & Atassi, 1980a), and the differences in the binding profiles for the Hb and

Sepharose were used as controls for estimating non-specific background binding. The values reported are uncorrected for non-specific binding to these control adsorbents. See Kazim & Atassi (1980a) for further details. The symbols refer to adsorbents of Hb ($\blacktriangledown$); α -chain (O); $\alpha 121-135$ ($\blacktriangle$); $\alpha 11-25$, $\alpha 91-105$ and $\alpha 101-115$ ($\blacksquare$); all other peptides and including the control adsorbents ($\bullet$). (b) Double-reciprocal analysis of the data shown in (a) for adsorbents of Hb ($\blacktriangledown$), its α -chain (O) and the synthetic peptide $\alpha 121-135$ ($\blacktriangle$). Values for ${}^{125}\text{I}$ -labelled Hp bound were corrected for non-specific binding by subtracting the amount of radioactivity bound to an equivalent amount of the control adsorbents. The lines shown are those obtained from least-squares regression analysis of the data. The correlation coefficients (r) for these lines are: 0.959 (Hb), 0.998 (α -chain) and 0.999 ($\alpha 121-135$). The amounts of ${}^{125}\text{I}$ -labelled Hp that would be bound to infinitely large amounts of adsorbents were obtained from the intercept of the ordinate and are 35 700 c.p.m. (Hb), 38 500 c.p.m. (α -chain) and 38 700 c.p.m. ($\alpha 121-135$).

α -chain adsorbents probably reflect the lower binding affinity for Hp of the α -chain relative to that of Hb (Chiancone *et al.*, 1968; Nagel & Gibson, 1971; Tsapis *et al.*, 1976; Hwang & Greer, 1979; Kazim & Atassi, 1980a). Double-reciprocal analysis (Fig. 2b) of the binding curves for Hb and α -chain adsorbents indicate that both adsorbents would bind the same maximal amounts (intercepts of the ordinate) of ^{125}I -labelled Hp.

Of the 14 synthetic peptides of the α -chain studied, only the adsorbent of $\alpha 121$ –135 bound Hp significantly. Adsorbents of the remaining peptides showed very little ($\alpha 11$ –25, $\alpha 91$ –105, and $\alpha 101$ –115) or no ($\alpha 1$ –15, $\alpha 21$ –35, $\alpha 31$ –45, $\alpha 41$ –55, $\alpha 51$ –65, $\alpha 61$ –75, $\alpha 71$ –85, $\alpha 81$ –95, $\alpha 111$ –125 and $\alpha 131$ –141) binding activity toward Hp. The binding profile for $\alpha 121$ –135 (Fig. 2a) indicates that, under these conditions, a plateau was not achieved and would have required impractically large amounts of adsorbent in order to do so. This is not surprising, since, even though active, this peptide, by analogy with adsorbents of other active peptides (Twining & Atassi, 1978, 1979; Kazim & Atassi, 1980c), would be expected to bind to Hp with less affinity than the equivalent conformationally distinct region within the native α -chain. The double-reciprocal transformation of the binding curve for the adsorbent of $\alpha 121$ –135, however, indicates that, at infinitely large amounts of adsorbent, the maximum amount of Hp bound would be the same as that of Hb and the α -chain itself (Fig. 2b). It is difficult to ascertain at present whether the very small net amounts of ^{125}I -labelled Hp bound by adsorbents of $\alpha 11$ –25, $\alpha 91$ –105 and $\alpha 101$ –115 represent significant binding.

Although $\alpha 121$ –135 binds Hp with less apparent affinity than does the α -chain, it is the only α -chain peptide that interacts significantly with Hp. Furthermore, this binding could be inhibited by free α -chain, whereas unrelated proteins were non-inhibitory, indicating that $\alpha 121$ –135 and α -chain compete for the same binding site on Hp. Thus it may be concluded that residues within the sequence $\alpha 121$ –135 are by far the major contributors to the binding energy of the α -chain to Hp. This does not imply, of course, that residues outside of this region are not capable of influencing the interaction of the α -chain with Hp. We have previously emphasized that protein-binding sites exist as integral portions of the entire molecule and are therefore subject to chemical and conformational influences exerted from regions outside the sites but that are transmitted to the sites through mutual interactions (Atassi & Kazim, 1980; Kazim & Atassi, 1980d; Twining *et al.*, 1980). Thus Hwang & Greer (1979) have observed that, on removal of three C-terminal residues of the α -chain (Lys-139, Tyr-140 and Arg-141), there is a decrease over one order of magnitude in the affinity of α -chain

for Hp. This is most likely due to transmitted effects on the chemical and conformational integrity of the Hp-binding site resulting from the loss of these residues.

From the present data, the Hp-binding site on the α -chain can be considered as residing within, but not necessarily encompassing all, the residues of the region $\alpha 121$ –135. The region $\alpha 121$ –135 is entirely in the H helix (Perutz *et al.*, 1968; Fermi, 1975) of the α -chain, and the residues that come into direct contact with Hp may be only those lying on an accessible surface of the helix. Since neither of the adjacent overlapping peptides, $\alpha 111$ –125 and $\alpha 131$ –141, showed any binding activity whatsoever, the Hp-binding site residing in $\alpha 121$ –135 appears to be localized around the central region of the peptide. Thus the Hp-binding site on the α -chain seems to be reasonably small and discrete and not to involve the extensive areas of contact suggested by Tsapis *et al.* (1979).

The locations of the region $\alpha 121$ –135 in the α -subunit and Hb($\alpha\beta$) dimer are shown in plates 1(a) and 1(b). As previously stated, the Hp-binding site on the α -chain (Plate 1a) is probably localized within this region. These models, therefore, are not intended to depict the exact boundaries of the site. They do, however, indicate the approximate location of the binding site, and the susceptibility of this site to influences from neighbouring regions (see Atassi & Kazim, 1980; Kazim & Atassi, 1980d). Plate 1(b) shows the relative location of the site in the Hb($\alpha\beta$) dimer. Although residues of the β -chain that bind to Hp have not been identified, this part of the model suggests their likely location(s). Regions on the β -chain that are spatially adjacent to the site on the α -chain are the most likely candidates, particularly in view of the proposal that Hp binds to the $\alpha\beta$ dimer of Hb (Nagel & Gibson, 1971). Verification of this must await the results with the synthetic β -chain peptides. It should be cautioned, however, that Plate 1(b) depicts the deoxy-Hb dimer, which is thought not to bind to Hp and which is somewhat conformationally different from the liganded forms of the dimer.

The successful application of this synthetic strategy, initially for the localization of antigenic sites on the α -chain (Kazim & Atassi, 1980b), and here for the localization of its Hp-binding site, should also prove useful in the localization of the Hp-binding site on the β -chain.

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References

- Atassi, M. Z. (1978) *Immunochemistry* **15**, 909–936
- Atassi, M. Z. & Kazim, A. L. (1980) *Biochem. J.* **187**, 163–172
- Atassi, M. Z. & Smith, J. A. (1978) *Immunochemistry* **15**, 609–610
- Braunitzer, G., Gehring-Muller, R., Hilschmann, N., Hilse, K., Hobom, G., Rudloff, V. & Wittman-Liebold, B. (1961) *Z. Physiol. Chemie* **325**, 283–286
- Chiancone, E., Alfsen, A., Ioppolo, C., Vecchini, P., Finazzi-Agro, A., Wyman, J. & Antonini, E. (1968) *J. Mol. Biol.* **34**, 347–356
- Fermi, G. (1975) *J. Mol. Biol.* **97**, 237–256
- Hill, R. J. & Konigsberg, W. J. (1962) *J. Biol. Chem.* **237**, 3151–3156
- Hwang, P. K. & Greer, J. (1979) *J. Biol. Chem.* **254**, 2265–2270
- Kazim, A. L. & Atassi, M. Z. (1980a) *Biochem. J.* **185**, 285–287
- Kazim, A. L. & Atassi, M. Z. (1980b) *Biochem. J.* **191**, 261–264
- Kazim, A. L. & Atassi, M. Z. (1980c) *Biochem. J.* **187**, 661–666
- Kazim, A. L. & Atassi, M. Z. (1980d) *Biochem. J.* **191**, 673–680
- Nagel, R. N. & Gibson, Q. H. (1971) *J. Biol. Chem.* **246**, 69–73
- Perutz, M. F., Muirhead, H., Cox, J. M. & Goaman, L. C. G. (1968) *Nature (London)* **219**, 131–139
- Polonovski, M. & Jayle, M. F. (1939) *C.R. Séances Soc. Biol. Ses Fil.* **129**, 454–457
- Terpstra, F. A. & Smith, D. B. (1976) *Can. J. Biochem.* **54**, 1011–1015
- Tsapis, A., Rogard, M., Alfsen, A. & Mihaesco, C. (1976) *Eur. J. Biochem.* **64**, 369–372
- Tsapis, A., Blouquit, Y., Buezard, Y., Mihaesco, C. & Rosa, J. (1979) *C.R. Séances Soc. Biol.* **73**, 314–320
- Twining, S. S. & Atassi, M. Z. (1978) *J. Biol. Chem.* **253**, 5259–5262
- Twining, S. S. & Atassi, M. Z. (1979) *J. Immunol. Methods* **30**, 139–151
- Twining, S. S., Lehmann, H. & Atassi, M. Z. (1980) *Biochem. J.* **191**, 681–697

EXPLANATION OF PLATE 1

Computer-generated space-filling model of the α -subunit and $\alpha\beta$ dimer showing the relative location of the region $\alpha 121-135$

(a) A view of the α -subunit of Hb (1) showing the region $\alpha 121-135$ (2). As cautioned in the text, the Hp-binding site is located within $\alpha 121-135$ and does not necessarily encompass the entire region. (b) A view of the Hb $\alpha\beta$ dimer from the same perspective as that in (a). The α - (1) and β - (3) subunits are shown in their deoxy state and therefore serve only to illustrate their overall relationship to one another. The region $\alpha 121-135$ (2) is partially obscured by the β -subunit, but this does not exclude the possibility that conformational rearrangements on binding to Hp may expose the obscured residues, making them available for binding.