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Haemoglobin Binding with Haptoglobin

UNEQUIVOCAL DEMONSTRATION THAT THE β-CHAINS OF HUMAN HAEMOGLOBIN BIND TO HAPTOGLOBIN

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Haptoglobin binding to haemoglobin and its isolated α- and β-chains was studied by use of a highly sensitive solid-phase radiometric assay. As expected, adsorbents of haemoglobin bound ^{125}I-labelled haptoglobin more efficiently than did adsorbents of the α-chain. However, unexpectedly, adsorbents of the β-chain were found to be essentially identical with those of the α-chain in their ability to bind haptoglobin. These results demonstrate, unequivocally, the ability of β-chains to bind to haptoglobin, and indicate that this assay is particularly convenient and useful for studying haptoglobin interactions with haemoglobin and its α- and β-chains.

Since the discovery of haptoglobins in serum by Polonovski & Jayle (1938), the interaction of these proteins with haemoglobin and its α- and β-chains has been studied by a variety of experimental approaches (for review see Putnam, 1975). Although the affinity of haptoglobin for α-chains is generally reported to be somewhat less than that for haemoglobin, its affinity for isolated β-chains is thought to be very low. Indeed, the very detection of complexes of haptoglobin with β-chains appears to depend on the experimental technique that is employed (see the Results and Discussion section). An inability to monitor haptoglobin–β-chain interactions therefore presents a potential difficulty for studying the molecular features of haptoglobin–haemoglobin interactions.

During the course of our studies on the antigenic structures of myoglobin (Twining & Atassi, 1979), lysozyme (Lee & Atassi, 1977*a*,*b*), serum albumin (Atassi *et al.*, 1979) and haemoglobin (Kazim & Atassi, 1977) we have utilized a highly sensitive solid-phase radioactive adsorbent titration technique capable of detecting relatively weak interactions. The sensitivity of this experimental technique makes it highly appropriate for its application to the study of haptoglobin. In initial studies reported here we demonstrate the ability of Sepharose adsorbents of haemoglobin, as well as each of its α- and β-chains, to bind radioiodinated haptoglobin efficiently. The convenience of this technique suggests that it will be particularly useful for studying haptoglobin–β-chain interactions.

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Experimental

Haptoglobin (type 2–1) was prepared from human serum according to the procedures of Waks & Alfsen (1966). The haptoglobin preparation was free of contamination by haemoglobin as judged by the absence of a detectable reaction in Ouchterlony immunodiffusion with goat antisera to human haemoglobin. Immunoelectrophoresis of the haptoglobin with goat antisera to human serum gave a single arc (see Fig. 1).

Adult human haemoglobin (CN-MetHbA) was the major chromatographic component obtained by chromatography on CM-cellulose as previously described (Atassi, 1964). The α- and β-chains of haemoglobin were isolated by treatment of haemoglobin with *p*-hydroxymercuribenzoate followed by chromatography on CM-cellulose (Bucci & Fronticelli, 1965). The chains were further purified by rechromatography on CM-cellulose or DEAE-cellulose until they were entirely homogeneous by polyacrylamide-gel electrophoresis. After isolation of the pure subunits, their thiol groups were regenerated with 2-mercaptoethanol (Geraci *et al.*, 1969). Spectrophotometric scanning of heavily loaded disc electrophoresis gels indicated that the α-chain had a purity greater than 99.98% (being contaminated with β-chain to the extent of less than 0.02%), and that the β-chain was better than 99.99% pure. The molecular weights of haemoglobin and its α- and β-chains were taken to be 64400, 15700 and 16500 respectively.

Immunoglobin G was prepared from the sera of non-immune goats by precipitation with $(NH_4)_2SO_4$

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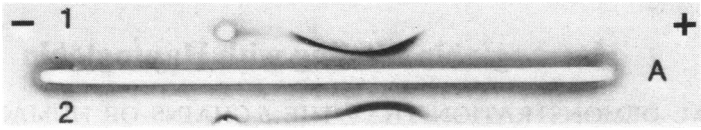

Fig. 1. *Immunoelectrophoretic pattern of the human haptoglobin preparation (wells 1 and 2) obtained with a goat antiserum to human serum (trough A)*
For experimental details see the text.

(33%, w/v), followed by ion-exchange chromatography on DEAE-cellulose (Gray *et al.*, 1969).

Haptoglobin was radioiodinated with ^{125}I by the method of Hunter & Greenwood (1962), with a chloramine-T/haptoglobin ratio of 2:1 (w/w). Precipitation with 10% (w/v) trichloroacetic acid indicated that 97% of the radioactivity in the preparation was bound to protein. The specific radioactivity of the ^{125}I-labelled haptoglobin was estimated to be 15–16 μCi/μg.

Haemoglobin, its isolated α- and β-chains or bovine serum albumin (4× crystallized; ICN Pharmaceuticals, Irvine, CA, U.S.A.) were coupled to CNBr-activated Sepharose CL-4B (March *et al.*, 1974). The amounts of haemoglobin, its α- and β-chains and bovine albumin coupled to Sepharose were respectively 3.15, 0.85, 0.95 and 2.1 mg per ml of packed volume.

Quantitative solid-phase titrations of ^{125}I-labelled haptoglobin with the Sepharose adsorbents were performed as follows. Various amounts of Sepharose adsorbents (0.8–50 μl, packed volume) were incubated by shaking with a fixed amount of ^{125}I-labelled haptoglobin (19600 c.p.m.) in 0.15 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.2, in 10 mm × 75 mm glass test tubes. The reaction mixture (350 μl total volume) contained a protein 'carrier' consisting of non-immune goat immunoglobin G (3 mg/ml) to minimize non-specific adsorption. After incubation for 18 h at 23°C, unbound ^{125}I-labelled haptoglobin was removed by washing the adsorbents three times (3 ml each) with 0.15 M-NaCl/0.01 M-sodium phosphate buffer. Bound radioactivity was then measured quantitatively on a Packard γ-scintillation spectrometer. The amount of non-specific adsorption occurring was evaluated by titrating equivalent volumes of uncoupled Sepharose or bovine serum albumin–Sepharose under identical conditions.

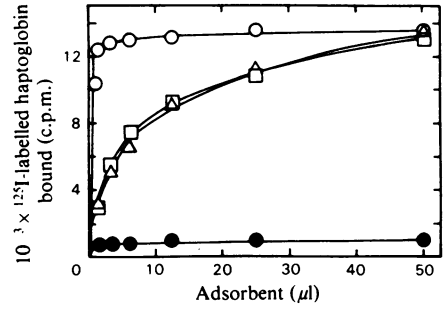

Fig. 2. *Solid-phase radiometric titration of a fixed amount of ^{125}I-labelled haptoglobin* (19600 *c.p.m.*) *with various amounts of Sepharose adsorbents of haemoglobin* (○), α-chain (△), β-chain (□) *and albumin or uncoupled Sepharose* (●)
For the various adsorbents, the amounts of protein coupled per ml (packed volume) of Sepharose were: 3.15, 0.85, 0.95 and 2.1 mg for haemoglobin, α-chain, β-chain and albumin respectively. See the text for details.

Results and Discussion

The results of quantitative titration studies of a fixed amount of ^{125}I-labelled haptoglobin with increasing amounts of the various adsorbents is shown in Fig. 2. As expected, the curve for binding of ^{125}I-labelled haptoglobin by haemoglobin rises sharply and attains a plateau value at relatively low amounts of haemoglobin. In contrast, the amount of non-specific binding to equivalent volumes of bovine serum albumin–Sepharose, which was identical with that bound to uncoupled Sepharose, was negligible and accounted for only 4–5% of the total radioactivity added. The differences in the binding profiles for haemoglobin and bovine serum albumin illustrate the specificity of the observed ^{125}I-labelled haptoglobin binding by haemoglobin. When corrected for non-specific binding by bovine serum albumin–Sepharose, the amount of ^{125}I-labelled haptoglobin maximally bound by haemoglobin–Sepharose accounts for 62% of the total reactivity added. This indicates that there is a substantial retention of the haemoglobin-binding ability of haptoglobin on radioiodination.

The binding curve for the α-chain–Sepharose adsorbent rises less steeply than that of haemoglobin, but eventually attains the same maximum value as for haemoglobin. Surprisingly, the binding curve for the β-chain–Sepharose adsorbent was

essentially identical with that of the α-chain–Sepharose. This binding reflects a very definite interaction of β-chains with haptoglobin, and under the conditions of the assay both the α- and β-chains were found to have the same apparent affinities for haptoglobin, although less than that of haemoglobin.

When the amounts of ^{125}I-labelled haptoglobin bound are corrected for non-specific binding to equivalent amounts of bovine serum albumin–Sepharose, the following amounts of the proteins coupled to Sepharose are required for half-maximal binding: haemoglobin, 0.03 pmol; α- and β chains, 0.30 pmol. These values indicate about a 10-fold difference in the amounts of haemoglobin and its α- and β-chains required to attain half-maximal binding. It should be noted that the binding of haptoglobin by a given subunit cannot be accounted for by contamination with haemoglobin or the other subunit. A contamination of at least 10% or higher would be required in order to yield these results. The α- and β-chain preparations utilized here had a far greater degree of purity (see the Experimental section). Also, the binding of haptoglobin to the immobilized β-chain is unlikely to be due to the exposure, in the process of coupling, of haptoglobin-binding sites otherwise absent from the free β-chain, since this binding was fully (90%) inhibited by a 4.2-fold excess of free β-chain. This excess was comparable with the excess of free α-chain required to achieve similar inhibition of haptoglobin binding to α-chain–Sepharose, or of free haemoglobin for inhibition of binding to haemoglobin–Sepharose.

From results with ultracentrifugation (Chiancone *et al.*, 1968) and fluorescence-quenching (Nagel & Gibson, 1967; Chiancone *et al.*, 1968) techniques, there is general agreement that haptoglobin exhibits a very high affinity for liganded forms of haemoglobin, and a somewhat lower affinity for isolated α-chains. However, there is less agreement on the ability of haptoglobin to bind isolated β-chains. Although ultracentrifugation studies (Chiancone *et al.*, 1968) have demonstrated complex-formation with haptoglobin of high concentrations of β-chains, only very small fluorescence-quenching values have been observed with this chain (Chiancone *et al.*, 1968; Nagel & Gibson, 1967). These results may reflect an inability of β-chains to quench haptoglobin fluorescence even when bound. These findings have led to the proposal that haptoglobin interacts very weakly, if at all, with isolated β-chains.

The solid-phase radiometric assay described in the present paper is a convenient method for obtaining the large molar excesses of β-chains (relative to haptoglobin) that are apparently required for detectable complex-formation with haptoglobin. Indeed, in these studies, the binding of haptoglobin by adsorbents of α- and β-chains are indistinguishable and attain maximal values equivalent to that of haemoglobin. We believe this to be the first unequivocal demonstration of the binding to haptoglobin by isolated β-chains and hope that it resolves the conflict concerning the ability of haptoglobin to bind to β-chains.

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