Immunological properties of the dimeric and tetrameric hemoglobins from the mollusc *Scapharca inaequivalvis*

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The dimeric and tetrameric hemoglobins from the bivalve mollusc *Scapharca inaequivalvis* produce highly specific antibodies in rabbits. No antigenic cross-reactivity between the two hemoglobins has been observed despite the presence of highly conserved regions, i.e., the E and F helices, in the dimer and in the α-chain of the tetramer. These results may be explained on the basis of the unusual assembly of *S. inaequivalvis* hemoglobins, in which the E and F helices are not exposed to solvent as in vertebrate hemoglobins, but form a crucial intersubunit contact. The specific antibodies only slightly affect the oxygen binding properties of the respective antigen.

Molluscan hemoglobin *Antibody* *Immunological cross-reactivity*

1. INTRODUCTION

The studies carried out in recent years on the hemoglobins contained in the red cells of Arcid molluscs (e.g., *Anadara satowi, Anadara broughtonii, Anadara trapezia, Scapharca inaequivalvis*) have brought to light interesting structural and functional properties that are shared by all the dimeric (HbI) and tetrameric (HbII) components [1–8]. The two components bind oxygen in a cooperative fashion and are characterized by similar oxygen affinities at neutral pH values ($p_{1/2}$ = 9–10 Torr), both lack the alkaline Bohr effect, but the tetrameric component HbII displays an acid Bohr effect below pH 6.0, a feature that may help the unloading of oxygen under hypoxic conditions which are usually accompanied by an acidification.

The dimeric HbI is of special interest in that it is constructed by two identical chains [2,3], does not undergo ligand-linked association-dissociation reactions, and yet displays a marked cooperativity in oxygen binding ($n_{1/2}$ = 1.5). These homodimers therefore represent an ideal system for the study of cooperativity in hemoglobins [6–8]. The amino acid sequence of three such dimers has been determined (*A. broughtonii* [4]; *A. trapezia* [9]; *S. inaequivalvis*; Petruzzelli et al., in preparation). The differences are limited to at most 15 amino acids in 145 residues. In contrast, the sequence homology with other globins, from both vertebrates and invertebrates, is rather small and amounts to 14–21%. The tetrameric component, HbII, has an $\alpha_2\beta_2$ type of structure which may form polymers upon removal of heme ligands [3,10]. The sequence of the α chains has been determined for *A. trapezia* [5]. Although the overall homology with the dimer chain is not very high (~45%), it reaches 75 and 100%, respectively, in the regions corresponding to the E and F helices.

This paper reports data on the structural relatedness of the two Arcid hemoglobin components obtained by means of a different approach, namely their immunological cross-reactivity. It is well known that immunological methods do not detect all sequence changes, since the antigenicity of a protein reflects primarily its surface [11,12]. However, the surface residues are

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those which vary most commonly in evolution, and antigenicity can be used as a sensitive measure of small differences between closely related proteins. In the case of Arcid hemoglobins this approach has an additional point of interest. The immunological cross-reactivity between HbI and HbII provides a means to probe subunit assembly in view of the presence of both invariant and variable regions along the polypeptide chains.

Specific antibodies to the dimeric and tetrameric hemoglobins of *S. inaequivalvis* have been prepared. In addition to the immunological assays preliminary experiments on the effect of the specific antibody on the oxygen equilibrium of the respective antigen have been performed. There is essentially no antigenic cross-reactivity between HbI and HbII, in line with the unusual assembly of these hemoglobins in which the globin fold is conserved, but the E and F helices form a crucial inter-subunit contact and are not exposed to solvent as in vertebrate hemoglobins [13].

2. MATERIALS AND METHODS

2.1. *Hemoglobins*

Specimens of the mollusc *S. inaequivalvis* were collected in the Middle Adriatic Sea. HbI and HbII were isolated from the hemolysate by ion-exchange chromatography as described in [3]. HbI purified in this way may be contaminated by small amounts ($\leq 5\%$) of HbII; complete purification of HbI has been obtained by means of a Pharmacia FPLC apparatus using the Mono Q HR/5 strong anion exchanger column. The column and the hemoglobin solution were equilibrated with 0.02 M Tris–HCl buffer at pH 8.0. Under these conditions HbI does not stick to the column while the contaminant HbII is retained and is eluted by a linear gradient of the same buffer containing 0.5 M NaCl. Apohemoglobin I (apo-HbI) was prepared by the acid-acetone method in [14] and was stored in the lyophilized state. The concentrations of HbI and HbII were determined on the oxygenated derivative from the optical absorption at 578 nm, using $E^{1\%}_{1cm}$ of 8.70 and 8.60, respectively. Apo-HbI concentration was calculated from the absorbance at 280 nm using $E^{1\%}_{1cm} = 12$ [14]. Acetylation of HbII was performed according to Riordan and Vallee [15].

2.2. *Oxygen equilibria*

The oxygen binding properties were measured at 20°C in the Soret region by the spectrophotometric method of Rossi-Fanelli and Antonini [16]. In the experiments which involved mixing of HbI and anti-HbI in the deoxygenated state, a tonometer equipped with a side arm was used. The measurements were all carried out in $I = 0.2$ M phosphate buffer at pH 7.0.

2.3. *Production of antisera*

Antibodies against HbI and HbII were produced in two adult New Zealand rabbits by injecting subcutaneously 1 ml of antigen (1 mg) mixed with complete Freund's adjuvant each week for three consecutive weeks. A booster injection with 2 mg of antigen was made 10 days after the last inoculation. One week after the booster injection the rabbits were bled from the ear for 7 successive days (20 ml blood/day).

2.4. *Purification of specific anti-HbI and anti-HbII IgG*

The IgG fraction was isolated from the total antiserum according to [17]. From the total IgG fraction the specific antibodies were separated by immunoaffinity chromatography using a column (0.9×15 cm) packed with the corresponding immunogen (HbI or HbII) covalently linked to Sepharose 4B by means of CNBr [18] and equilibrated with 0.05 M phosphate buffer containing 0.1 M NaCl (pH 7.5) (PBS buffer). Aliquots of 5 ml of the total IgG fraction in the same buffer were loaded on the column at a flow rate of 5 ml/h. After elution of a peak of unbound proteins with PBS buffer, aspecifically bound material was removed with 1 M NaCl in PBS buffer. The elution of the specific IgG was carried out with 2 ml of 6 M urea at neutral pH. The urea was removed rapidly from the specific IgG sample by means of a Sephadex G-25 column connected online with the first column and equilibrated with PBS buffer.

2.5. *Immunoreactions*

Immunoelectrophoresis was performed on a thin layer of 1% agar in 0.05 M Veronal buffer at pH 8.6. Electrophoresis of the antigenic components (electric field across the plate of 20 mA for 2 h unless otherwise stated) was followed by a 24 h dif-

fusion step at room temperature [19]. Immunodiffusion was performed according to the method of Outcherlony on 0.75% agarose in PBS buffer containing 0.1% NaN_3. The wells were 5 mm in diameter and placed 4 mm apart. The hemoglobin concentration was 1 mg/ml. The reaction time was 24 h at room temperature.

3. RESULTS

3.1. *Antigenicity of HbI and HbII*

The elution profile of the immunoaffinity chromatography step for the isolation of the specific anti-HbI IgG fraction is shown in fig.1. The total IgG fraction applied to the column of immobilized HbI amounted to 150 mg, ~22 of which were found in the specific IgG fraction after dissociation of the immunocomplex with 6 M urea and elution with PBS buffer.

The relative amounts of aspecific and specific IgG fractions are similar in the anti-HbII antiserum.

3.2. *Immunological assays*

In double diffusion experiments the antiserum against HbI yields well defined precipitation lines with HbI and its apoprotein; no cross-reaction is found between anti-HbI and HbII or acetylated HbII. In a reciprocal way, the antiserum against HbII gives a precipitation reaction with HbII and acetylated HbII, but does not cross-react with HbI and apo-HbI. The results of relevant experiments are shown in fig.2. HbA and sperm whale myoglobin do not precipitate with either antiserum (not shown).

Immunoelectrophoresis of HbI and its specific antibody as expected gives one precipitin line in the cathodic zone of the gel (fig.3E). Surprisingly, anti-HbII, assayed against HbII, shows a main precipitin arc centered around the well containing the antigen and a second arc towards the anode. This latter part of the precipitation zone becomes more intense with increase in electrophoresis time and protein concentration, but disappears when the electrophoretic run is carried out in phosphate buffer at pH 7.5 (fig.3A–D). This complex behavior has to be ascribed to the electrophoretic properties of HbII since the antibody works simply as a specific stain that monitors the electrophoretic mobility of the antigen. Complex electrophoretic

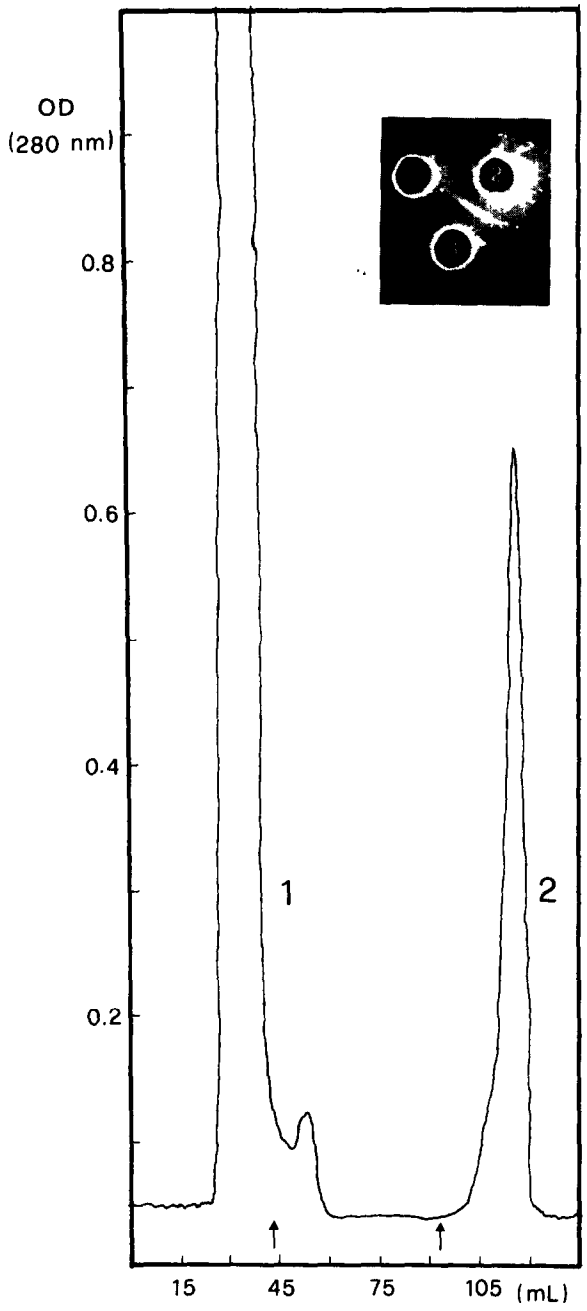

Fig.1. Purification of specific IgG anti-HbI from *S. inaequivalvis* by immunoaffinity chromatography. The chromatography was performed on a 0.9 × 1.5 cm column containing the Sepharose 4B-HbI covalent complex. The arrows denote the points where 1 M NaCl and 6 M urea were added to the column. The inset shows the antigenic activity of peaks 1 and 2 tested by immunodiffusion against HbI (well 3).

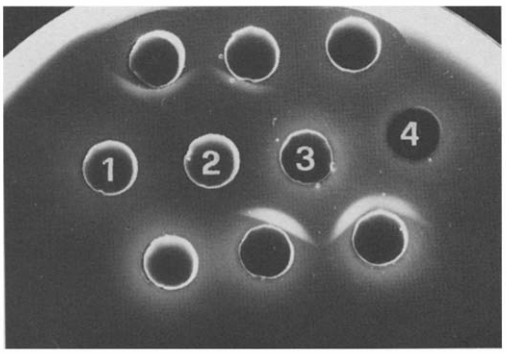

Fig.2. Immunodiffusion test of specific IgG anti-HbI and anti-HbII from *S. inaequivalvis*. Gel: 0.75% agarose in PBS buffer at pH 7.5 containing 0.1% sodium azide. Upper wells: IgG anti-HbI. Lower wells: IgG anti-HbII. (1) HbI, (2) apo HbI, (3) HbII, (4) acetylated HbII.

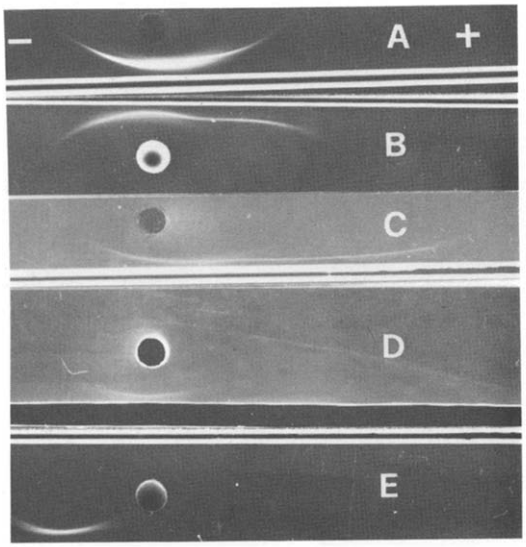

Fig.3. Immunoelectrophoresis of HbI and HbII from *S. inaequivalvis* at different electrophoresis times. HbII (A,B,C) and HbI (E) in 0.05 M veronal buffer (pH 8.6); HbII (D) in 0.05 M phosphate buffer (pH 7.5). Time of electrophoresis (h): (A) 1, (B) 2, (C) 3.5, (D) 3.5, (E) 3.5.

patterns such as those displayed by HbII in veronal buffer may be due either to polymerization of the protein or to its interaction with buffer components and/or the gel matrix [20]. The former possibility was excluded on the basis of sedimentation velocity experiments which provided no evidence for polymerization. On the other hand, it is well known that on the agarose backbone there are negatively charged groups in variable amounts and that these can interact with the protein molecules. In the case of HbA, for example, the interaction occurs with the positively charged residues of the organic phosphate binding pocket [21]. HbII does not bind organic phosphates, but interacts with fairly high affinity with simple anions like Cl^-. This is indicated by the effect of anions on the polymerization of the deoxygenated derivative [3] and by ^{35}Cl NMR measurements on the oxygenated one (in preparation).

3.3. *Effect of specific antibody on the oxygen equilibrium*

The specific antibody was mixed with oxygenated HbI at two IgG to hemoglobin molar ratios, 1:2 and 1:1. The specific antibody brings about a slight decrease in the oxygen affinity and a slight increase in $n_{1/2}$, the effect being larger in the presence of the higher amount of antibody. Under the latter condition an experiment was performed in which the two components were mixed after deoxygenation. The effect on the oxygen-binding parameters was significantly less than in the experiment carried out by mixing the oxygenated solutions (fig.4A).

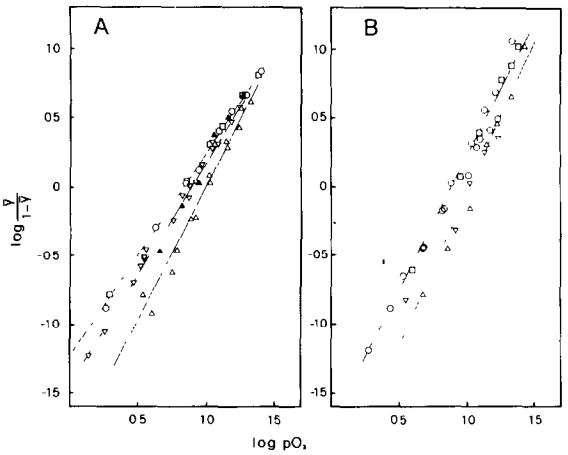

Fig.4. Hill plots of oxygen equilibria of HbI (A) and HbII (B) from *S. inaequivalvis* combined with the corresponding specific antibody. Native protein (○); oxyform mixed with specific IgG, molar ratio (R) = 1:1 (△); R = 2:1 (▽); deoxy-form mixed with specific IgG, R = 1:1 (▲); oxy-form mixed with non-specific IgG, R = 1:1 (□).

As far as HbII is concerned, the specific antibody was mixed with the oxygenated derivative at two IgG to hemoglobin molar ratios, 1:2 and 1:1. Under both conditions the presence of the antibody results in a slight decrease in oxygen affinity and an increase in $n_{1/2}$ (fig.4B).

Fig.4A,B also shows that purified IgG from non-immune rabbits do not affect oxygen binding in any of the conditions described above.

4. DISCUSSION

Both hemoglobin components of *S. inaequivalvis* induce production of substantial amounts of specific antibodies in the rabbit in accordance with the large sequence difference between the hemoglobins from the immunogen and of the immunized organism. In precipitin assays the two molluscan hemoglobins do not cross-react with HbA and sperm whale myoglobin in agreement with the knowledge that sequence changes exceeding 40% usually suffice to eliminate immunological cross-reactivity between genetically homologous proteins [22]. In this light one would expect the two *S. inaequivalvis* hemoglobins to cross-react if the highly homologous regions of HbI and of the HbII α chain, i.e., those corresponding to the E and F helices, were exposed to solvent, while the opposite would be true if they were in the interior of the molecule. The absence of cross-reactivity between the two molluscan hemoglobins indicates that the E and F helices are not exposed to solvent, in accordance with the X-ray crystallography data of Love and collaborators [13]. The X-ray work shows that both clam hemoglobins are made of myoglobin-like subunits which are assembled in an unusual way, with the E and F helices forming an extensive intersubunit contact. This type of assembly provides an important rationale for the conservation of this region in the molecule. It may be recalled that the subunit arrangement of Arcid hemoglobins is 'back-to-front' with respect to that characteristic of vertebrate hemoglobins where the E and F helices are on the outside of the molecule.

The immunological data also confirm that both HbI and HbII are very stable towards dissociation into subunits [3,23]. Thus, even during the immunization process no dissociation takes place as indicated by the absence of cross-reactivity in the assays of the antibodies carried out with dissociated antigens, i.e., apo-HbI [14] and acetylated HbII (unpublished).

The absence of cross-reactivity between multiple components of a hemolysate has been observed by Tan-Wilson et al. [24] in the case of trout hemoglobin components I and IV and by Tichy et al. [25] for the monomeric and dimeric hemoglobins from several *Chironomus* species. In *Chironomus*, where the hemoglobin genes are located on one chromosome, the immunological data have been taken to indicate that the monomer and dimer genes separated early in the evolutionary development of Chironomids.

The specific antibodies do not have a dramatic effect on the oxygen binding properties of the two *S. inaequivalvis* hemoglobins under the conditions described above. In HbI there is a slight decrease in oxygen affinity and a slight increase in $n_{1/2}$ only when HbI is mixed with the antibody in the oxygenated state in accordance with the fact that the oxygenated derivative may be expected to react better than the deoxygenated one with the specific antibody. However, on this basis, linkage relationships demand the oxygen affinity to be increased by antibody binding while the opposite effect is observed. A possible explanation may lie in the polymerization of the immunocomplex, a fact that is suggested also by the increase in cooperativity. In any case, the experiment yields yet another direct proof of the difference in conformation of the oxy- and deoxy-derivatives of these dimeric hemoglobins in addition to previous results by Furuta et al. [26]. These authors have shown that the oxy- and deoxy- forms of the dimeric and tetrameric component of *A. broughtonii* have a different reactivity with sulfhydryl group reagents and with 1-anilinonaphthalene-8-sulfonate, a fluorescent probe of hydrophobic regions in proteins. The binding of the specific antibody to HbII results in a small decrease in oxygen affinity and an increase in cooperativity, as in the case of HbI, and the observed effects may be explained along the same lines. In HbA experiments of this type have been carried out with specific Fab fragments which do not give rise to polymerization of the immunocomplex; their binding to HbA brings about an increase in oxygen affinity, a decrease in cooperativity and a marked asymmetry of the oxygen equilibrium curve [27].

In conclusion, the present data provide evidence for the unusual assembly of Arcid hemoglobins indicated by the X-ray crystallography data. As to the effect of antibody on the oxygen binding properties, the preparation of specific anti-HbI and anti-HbII Fab fragments in high amounts will allow us to perform a more detailed study and a direct comparison with the behavior of HbA.

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