CHANGE OF HUMAN HEMOPEXIN ISOELECTRIC POINT UPON HEME BINDING

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1. Introduction

Hemopexin is the serum β-glycoprotein with a high affinity for heme [1] and its biological and physicochemical properties have been reviewed by Muller-Eberhard and Liem [2]. The detailed characterization of the protein is hampered by the complexity and low yields of the isolation procedures, specially for human hemopexin [3–5].

In an attempt to explain differences in purification procedures of human hemopexin, we present here experimental evidence for a change in its isoelectric point upon heme-binding. It provides information on the change in the conformation of hemopexin induced by its interaction with heme and thus may contribute for understanding the means by which hemopexin fulfills its physiological function in heme transport.

2. Experimental

2.1. *Isolation of a crude hemopexin fraction*

The human serum fractionation technique by rivanol and ammonium sulfate was that of Hayem-Levy and Havez [3], with slight modifications. Ammonium sulfate concentrations were lower. Transferrin elimination was checked by radial immuno-diffusion using partigen Behring.

All operations were carried out at 4°C. The serum came from a pool saved after clinical analysis and was dialysed against NaCl 1.17%. One liter was diluted with one liter of 0.8% NaCl. Successive precipitates obtained under the following conditions were discarded after 90 min centrifugation at 3000 × *g*:

(i) One liter of 2.50% rivanol solution in 0.05 M, pH 8.0, phosphate buffer was added dropwise, under stirring, overnight.

(ii) NaCl to a 5% final concentration, citric acid to adjust pH at 7.0 and ammonium sulfate, to a 0.96 M final concentration, were added.

(iii) Citric acid lowered pH to 3.8.

(iv) Ammonium sulfate was added to 1.31 M final concentration.

Finally a 2.91 M ammonium sulfate precipitate was dialysed against 10 mM Tris–HCl buffer, pH 7.0, in a Biorad '50' biofiber beaker. Protein concentration was adjusted between 10 mg/ml and 20 mg/ml. The resulting fraction was named Fraction A.

2.2. *Displacement chromatography*

Tris–HCl buffer 10 mM, pH 7.0, was used for equilibration and for elution of the column (3.5 × 10 cm) of DEAE–Sephadex A-50 at 4°C. Experimental conditions are given in the legend to fig.1. Elution was followed by absorbance measurements at 280 nm and 414 nm, by the rockett technique of Svendsen and Carsten [6] and by radial immunodiffusion with partigen Behring for hemopexin. Immunoelectrophoresis was performed using whole human serum protein antiserum, specific anti-human haptoglobin antiserum and specific anti-human hemopexin antiserum (fig.2). Haptoglobin concentration was determined by the Hyland method used in clinical analysis. Crossed immunoelectrophoresis was then realised according to Laurell [7]. After analysis, hemopexin containing fractions of displacement chromatography were pooled and named Fraction B.

Abbreviation: Heme, Ferriprotoporphyrin IX

A rabbit antiserum raised against this Fraction B was prepared and used in Laurell technique with a fresh normal, non-hemolysed human serum as antigen. Absence of hemolysis was ascertained by potassium measurement with flame spectrophotometry.

3. Results

After precipitation steps, Fraction A consisted of 10% hemopexin with a yield of 33%.

Figure 1 shows the elution profile of displacement chromatography. Pooled fractions or Fraction B consisted of less retained proteins, whose interactions with the exchanger were weak. The exchanger was not oversaturated since the composition of the plateau was constant. As shown in fig.2 the only contaminant found was haptoglobin, its level was 5% of the whole proteins. The final recovery was 16% of serum hemopexin. Purification was not carried out any further for haptoglobin elimination by a gel-filtration step as described by Hayem-Levy and Havez [3], but haptoglobin was kept as an internal marker for electrophoretic study. Its position in crossed immuno-electrophoresis was ascertained using specific antihaptoglobin antiserum as control for each of the following plates. It migrated in the medium of the plates, and could be distinguished from hemopexin by a weaker precipitation line.

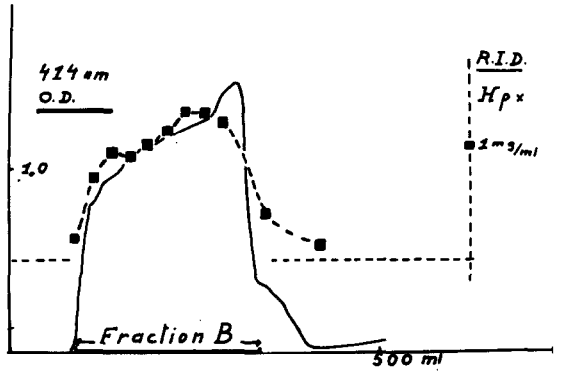

Fig.1. Displacement chromatography on DEAE–Sephadex A-50 of Fraction A. Buffer: Tris–HCl, 50 mM, pH 7.0. Fraction A obtained from 2 liters of serum at a protein concentration of 10–20 mg/ml (i.e., about 300 ml) was deposed. Flow rate: 36 ml/h. Column: 3.5 × 10 cm. Fraction volume: 7.5 ml. (———) Optical density at 414 nm. (– – –■– –) Hemopexin determination by radial immuno-diffusion. Pooled fractions were named Fraction B.

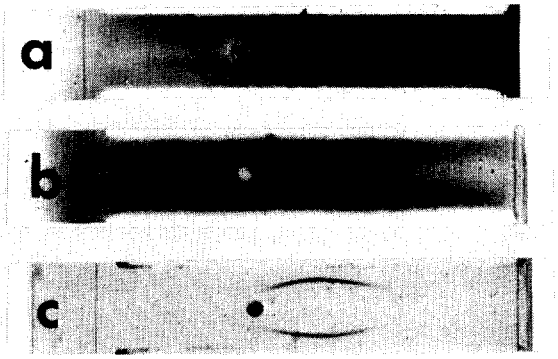

Fig.2. Immunoelectrophoresis of Fraction B. (a) Anti-whole human serum protein antiserum. (b) Specific anti-human haptoglobin antiserum. (c) Specific anti-human hemopexin antiserum.

Figure 3 shows Laurell plates of Fraction B as antigen against anti-whole human serum protein antiserum (fig.3A). The major protein fraction split into two peaks, the one noted 1, faster, and the other one noted 2, slower than haptoglobin after the first dimension electrophoresis.

In order to study this splitting after electrophoretic migration, normal non-hemolysed human serum was used as antigen in the first dimension, with antiserum

Fig.3A

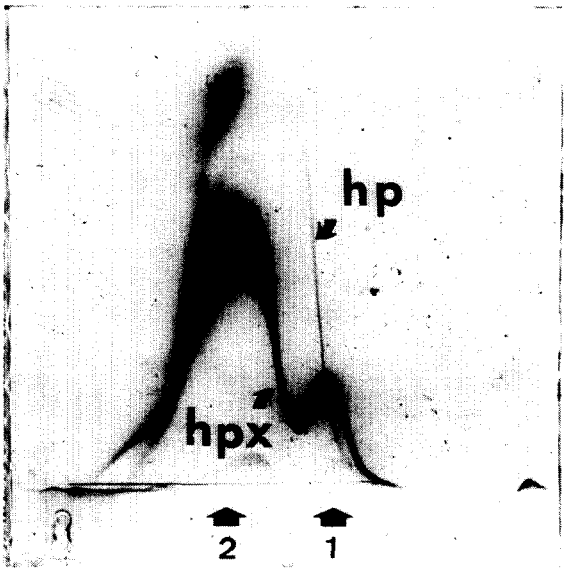

Fig.3B

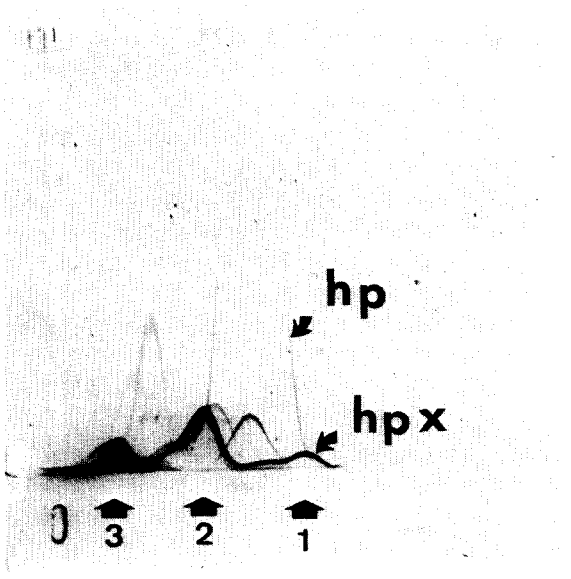

Fig.3C

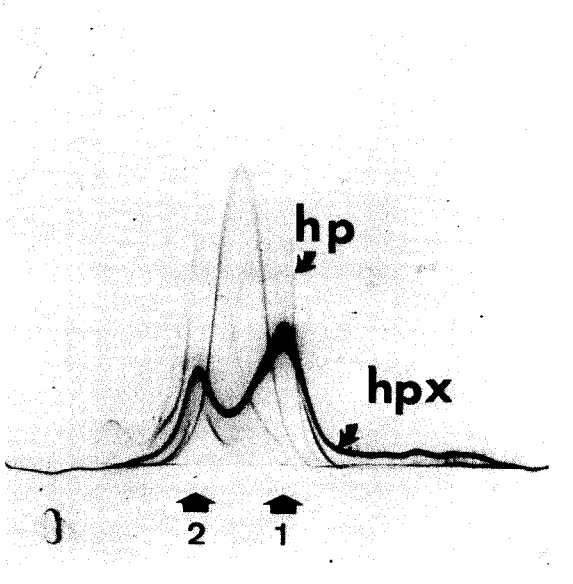

Fig.3. Laurell plates of Fraction B. (Hp) Haptoglobin. (Hpx) Hemopexin. (3A) Antigen: Fraction B. Antiserum: anti-whole human serum protein. (3B) Antigen: Normal, fresh, non-hemolyzed human serum. Antiserum: Antiserum raised against fraction B. (3C) Antigen: Normal, fresh, non-hemolyzed human serum. Heme in equimolecular amount to Hpx was added in the well. Antiserum: Antiserum raised against Fraction B.

raised against Fraction B in the second dimension. Figure 3B shows a major peak noted 2, slower than haptoglobin. Moreover, there is a less important bow still slower and noted 3 on fig.3B, this bow seems to enclose several precipitation lines, one of them is the same for both peak 2 and peak 1. It may be polymers whose high molecular weight hindered migration through gelose.

Finally, in fig.3C, heme was dissolved in the electrophoresis buffer and added in equimolecular amounts with hemopexin to the same serum in the well. It caused firstly, the apraisal of peak 1 of fig.3A, secondly, it abolished peak 3 of fig.3B. In order to keep heme-saturated and heme-depleted hemopexin in the same plate, optimal incubation was not achieved, the only equimolecular heme addition just before migration suffice to show the two forms together.

Thus peak 1 seems to be heme–hemopexin, peak 2 apo–hemopexin and peak 3 polymers.

4. Discussion

These results may provide a means for improving preparation procedure, moreover, they confirm available physico-chemical data.

The isolation method reported by Hayem-Levy and Havez [3] involves the use of precipitation of human serum with rivanol and ammonium sulfate, followed by gel-filtration. Heme-hemopexin and apo-hemopexin are then isolated together. Heme is reported a polymerising agent, and therefore may diminish hemopexin monomer recovery after the gel-filtration step.

In the method of Aisen et al. [4], Cohn fraction IV-7 is submitted to ion-exchange chromatography and then to gel-filtration. These authors obtained monomeric apo-hemopexin; heme-hemopexin was lost.

Hrkal et al. [5] added heme in the course of the fractionation procedure, elution of ion-exchange chromatography was followed by $A_{414\ nm}$ and provided heme-hemopexin and its aggregated form.

Suttnar et al. [8] employed affinity chromatography for human hemopexin, although hemopexin was eluted in three peaks no differences in the purity of the three fractions were found on acrylamide gel electrophoresis, and immunoelectrophoresis.

In each procedure hitherto reported in literature either apo-hemopexin, heme-hemopexin or polymerised

hemopexin was lost and contribute to diminish yield. Data presented here furnish experimental evidence for preexisting of these three states of the molecule in native serum.

Heme is known to facilitate hemopexin aggregation [3]. The present experiments provide evidence that in the presence of a sufficient amount of heme, such aggregates will disappear, if hemopexin has kept its binding properties.

The chief difficulty is to know what exact proportion of heme may be bound by hemopexin in serum after bleeding. Dissociation of the complex was never achieved in the literature. In Hrkal et al. work [1], the affinity constant value for the binding of heme to the single binding center of hemopexin molecule was estimated as $1.9 \times 10^{14} M^{-1}$. It would be of interest to obtain heme-hemopexin on a preparative scale. Although affinity is high, all hemopexin failed to convert into heme-hemopexin in our study. Perhaps it would be necessary to find adequate incubation conditions for such a binding, and to dispose of a non-denaturing technique for preparing human hemopexin as did Bernard et al. for rat hemopexin [9].

This serum β-glycoprotein binds circulating heme and transports it to the liver parenchymal cells [10]. The interaction of heme with hemopexin produces changes in the tertiary structure of the protein [11] which possibly lead to recognition of the heme–hemopexin complex by hepatocytes.

Data reported here may give arguments for two different interactions of hemopexin with heme. The site of interaction of polymers is not necessarily the same as in hemopexin-heme. This could explain why heme seems to be relatively accessible in Morgan et al. work on rabbit hemopexin.

For polymer formation, heme remains on the outside of the molecule, equally accessible to each protein molecule of the complex. This may correspond to the sterically unhindered heme-binding site found by Morgan et al. [12]. For these authors, a relatively open heme-binding site perhaps helps either the recognition of the heme–hemopexin complex by hepatocytes or the degradation of heme once the heme–hemopexin complex has entered the cell.

Polymerization mechanism remains to be explained. It is different from a single aggregation after denaturation, since it is encountered in native serum. It would be a competition mechanism of several hemopexin molecules for one molecule of its ligand. This first site could be responsible for myoglobin and cytochrome *c* binding as shown by Biserte et al. [13].

The second site on the inside of the molecule may correspond to a strong binding, responsible for transfer of heme from ferri-myoglobin and ferri-hemoglobin isolated chains to hemopexin [1].

The intimate mechanism of such a strong binding is not clearly understood yet. It may be important, however, for heme–hemopexin complex not to be contaminated either by apo–hemopexin or by polymers for interpreting physico-chemical data. The relative importance of the three states of hemopexin may provide a tool for metabolic studies and is easy to determine with Laurell technique as reported here.

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