PURIFICATION AND STRUCTURE OF SHEEP HAPTOGLOBIN

Jacques MARTI and Jean MORETTI
Laboratoire de Biochimie des Protéines, Faculté des Sciences, 34060 Montpellier, France

Received 27 April 1976

1. Introduction

Haptoglobin (Hp) is a glycoprotein which is found in the serum of several animals. Its characteristic property is the ability to form with hemoglobin (Hb) a stable equimolecular complex Hp–Hb which possess a peroxidase activity; this property allows a quantitative estimation of Hp [1].

The normal level of Hp varies according to the species: from 0.05 g/l in Bovidae and Cervidae, to 1.2 g/l in human. It always rises in any inflammatory state [2].

In the sheep, Hp is composed of a series of polymeric proteins, whose electrophoretic pattern is very similar to that of human Hp 2-2 [3]. Their mol. wts. range from 10^5 to 10^6 [3]. They are formed in the human by combination in different numbers of two polypeptidic chains called α and β [4]. We have undertaken the purification of sheep Hp in order to study its structure.

We have controlled the purification by using the Hp estimation after each stage. But, as we shall establish later, sheep Hp is easily altered: below pH 4 or above pH 6.5, it looses its ability to combine with Hb. For these reasons, the preparation of this protein in the native state is very difficult. Therefore, after two successive chromatographic purifications, we have used an immunological technique as a last step of purification.

The method is based on the following fact: since the Hp level in normal sheep is very low, a rabbit immune-serum anti-normal-sheep serum contains a very small quantity of γ-globulins anti-haptoglobin. After insolubilisation of these rabbit antibodies, affinity chromatography retains selectively proteins which contaminate the preparation. At the expense of a small loss of Hp, the final product is very pure.

2. Materials and methods

2.1. *Sera*

Sheep (*Ovis aries*) were obtained from Ecole Nationale d'Agriculture de Montpellier. Blood (200 ml per animal) was withdrawn by jugular puncture. From samples were obtained from healthy individuals and six from sheep in an inflammatory state caused by one injection of turpentine oil. Haemolytic sera were discarded.

2.2. *Haptoglobin titration*

The Hp concentration was estimated according to Jayle [1] and expressed as the 'index' defined by the author.

2.3. *Polyacrylamide gel electrophoresis*

The Davis technique [5] was used. For mol. wt. determination, electrophoresis in the presence of SDS was carried out according to Weber and Osborn [6], the samples being heated 5 min at 100°C in the presence of 1% SDS and 1% mercaptoethanol prior to the analysis. Proteins of known mol. wt. were used as markers (Kit Boeringer).

2.4. *Immunochemical methods*

Immunization: Antisera against sheep proteins were prepared by injecting rabbits at weekly intervals successively with 0.15–0.25–0.5 and 1.0 of sheep serum, the first rabbit with normal sheep serum, whose Hp level is very low (0.05 g/l); the second rabbit with

the serum of a sheep in a inflammatory state, whose Hp level was 1.5 g/l. Only this second immuneserum reveals Hp by immunological techniques and was used for this purpose.

Crossed electrophoresis: Sera and protein samples were analysed on glass plates of 9 × 12 cm coated with 1% agarose gel (Agarose A 37, Industrie biologique française) in a barbital buffer, pH 8.6, 0.02 M. The line of precipitation of Hp was specifically revealed using the fact that the immunoprecipitate retains its ability to combine with Hb. After crossed immuno-electrophoresis, the plate was immersed in 30 ml of a M/50 000 Hb solution for 4 h at room temperature, then washed during 5 h with several changes of saline solution. The precipitation line of Hp-Hb appears stained in blue by benzidine.

Preparation of antinormal sheep serum gamma globulins: 20 ml of the first antiserum diluted 2 times in physiological buffer saline (P.B.S) were precipitated with 40% ammonium sulfate. The precipitate was twice washed, dissolved in 10 ml 0.1 M carbonate buffer, pH 8.5, and dialysed against several changes of the same buffer. The protein content was 210 mg (Lowry's method).

Coupling of the gamma globulins to Sepharose 4 B: Cyanogen bromide, acetonitrile, hexamethylenediamine, glutaraldehyde (25% aqueous solution) and sodium thiocyanate were purchased from Merck, Darmstadt. 10 ml of gravity packed Sepharose 4 B (Pharmacia) were activated in 2 M sodium carbonate solution by adding CNBr dissolved in acetonitrile, according to [7]. The degree of activation was controlled by the ability of the gel to bind Blue Dextran (Pharmacia). Immediately after washing the gel, hexamethylene-diamine was coupled to the activated Sepharose according to [8]. At this stage, the efficiency of coupling was tested using the 2,4,6-trinitrobenzene sulphonate colour test [9].

The aminated gel was activated by glutaraldehyde at a concentration of 2.5% in the carbonate buffer, in a total volume of 30 ml. After 10 min, the gel was washed with 300 ml of buffer, then mixed with 20 ml of the antibody preparation containing 210 mg of protein. The suspension was stirred for 45 min at room temperature, after which the gel was washed successively with 300 ml of P.B.S., pH 7.2. We have measured the absorbance at 280 nm of the protein solution before fixation and of the washings. By difference, the apparent yield of coupling was 49%, the amount of insolubilized gamma globulins being 103 mg. The gel was poured into a glass column (1 × 15 cm) and kept in P.B.S. containing 0.1% sodium azide at 4°C until used.

2.5. *Purification of sheep Hp*

35 ml of serum were dialysed against 2 liters 0.05 M acetate buffer pH 4.6, and chromatographed on CMC column (Whatman CM 23) (Pharmacia K 15/90). Elution of Hp was performed by a linear gradient of concentration from 0.05 M to 0.35 M in acetate. The fractions containing Hp were pooled, dialysed and after concentration by freeze-drying, filtered onto a column of Sephadex G 200 (3 × 90 cm) prepared in and equilibrated with 0.02 M acetate buffer, pH 5.25, containing 0.1 M NaCl. Hp was eluted with the same buffer immediately after the void volume.

3. Results

3.1. *Stability of sheep haptoglobin*

In order to test the stability of sheep Hp, we have incubated a partially purified preparation of Hp in different buffers at pH ranging from 2 to 10 during 18 h. The peroxidase activity of the Hp–Hb complex, formed in the conditions of the titration method, was then measured. After incubation at pH between 4 and 6, the 'index' of Hp remains constant. But after incubation below pH 4 or above pH 6, the peroxidase activity is strongly lowered.

3.2. *Purification*

The first chromatography on CM-cellulose removes about 50% of the serum proteins. After gel filtration on Sephadex, the first peak contains Hp and other proteins (fig.1), which represent about 10–15% of the eluted fraction. At the end of these two operations, the amount of Hp recovered is more than 70% of the initial quantity, estimated by its haemoglobin binding capacity.

After affinity chromatography, immunoelectrophoresis shows only one line (fig.2). No impurity is detected by this method. The yield of this last step exceeds 80%.

Elution diagrams of the two first chromatographies are indicated in fig.3 and 4.

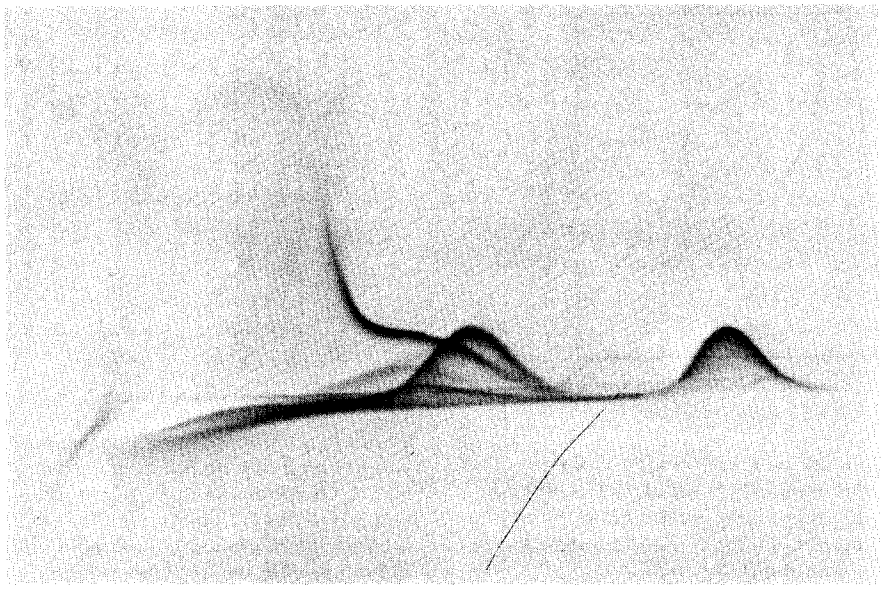

Fig.1. Immunoelectrophoresis of the serum proteins after gel filtration on Sephadex G 200. First migration: 2 h, 15–40 V. Second migration: 18 h, 20 V. The gelose contains 500 μl of rabbit serum anti sheep serum in inflammatory state.

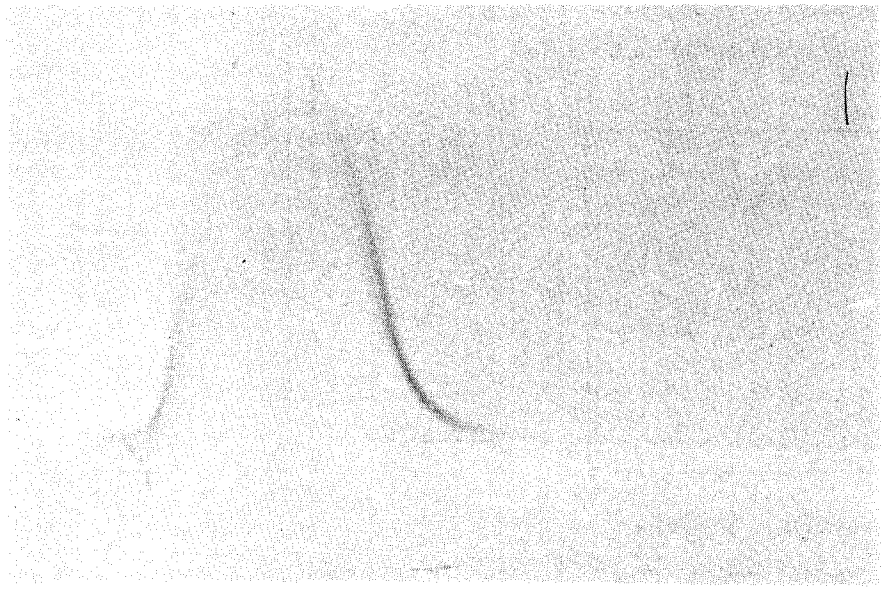

Fig.2. Immunoelectrophoresis of the preparation after affinity chromatography. Conditions are the same as for fig.1. Only Hp is detected, after staining with Coomassie blue.

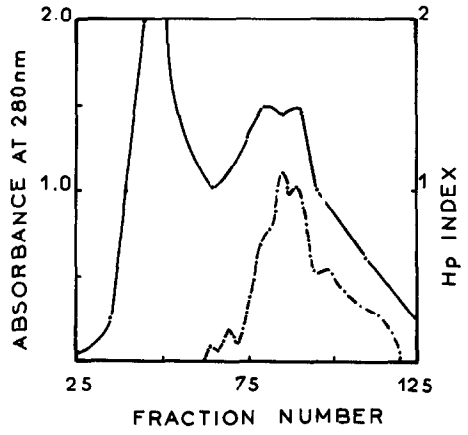

Fig.3. Chromatography of sheep serum on CMC column (2.5 X 50 cm) equilibrated in 0.05 M acetate buffer pH 4.6. Elution by a linear gradient of saline concentration (0.05 M to 0.35) of the same buffer. (——) Absorbance at 280 nm (—·—·) Hp estimation.

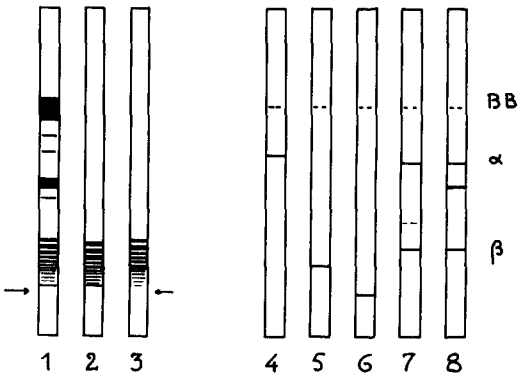

Fig.5. Polyacrylamide gel electrophoresis. First group: 7% acrylamide (1) Sheep serum (Coomassie blue) (2) The same plus Hb (revelation by Benzidine) (3) Pure Hp (Coomassie blue) Second group (4 to 8): gel containing 7% acrylamide plus 0.2% SDS (4) Cytochrome *c*-(13 000) (5) Ovalbumin (45 000) (6) Bovine serum albumin (67 000) Sheep Hp [7] and complex Hp–Hb [8] after reducing cleavage by 1% mercaptoethanol.

3.3. *Structure of Hp*

The electrophoretic pattern of sheep Hp (fig.5) is characterized by a polymeric pattern containing multiple bands (ten at least) and resembling the human Hp 2-2 pattern. The mol. wts. range between 10^5 and 10^6, but we can not give more precise values. The same result is obtained by gel filtration chromatography [3].

After reduction by mercaptoethanol, acrylamide gel electrophoresis of Hp exhibits two bands stained by the Coomassie blue. We shall designate these bands α and β, by analogy with Hp 1-1; only the slowest band (β) is stained by the Schiff reagent: as in human Hp, glycans are present only in the β band.

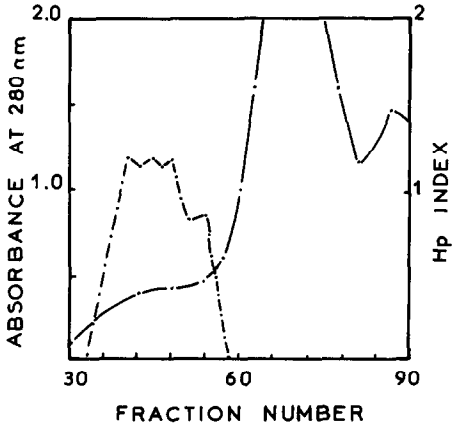

Fig.4. Purification of the Hp containing fractions eluted from CMC column. Chromatography on Sephadex G 200 column (1.5 × 90 cm) in 0.1 M sodium acetate buffer pH 5.25. (——) absorbance at 280 nm. (—·—·) Hp estimation.

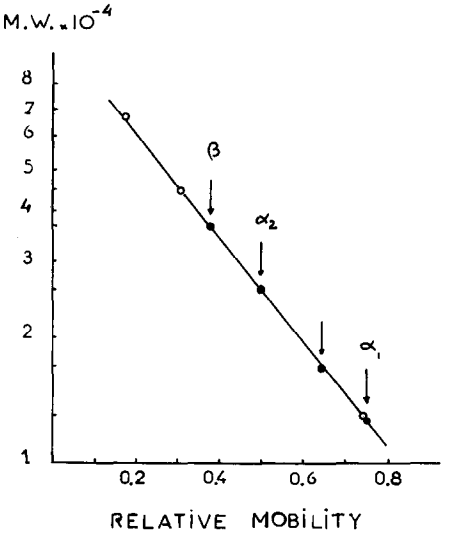

Fig.6. Determination of the mol. wt. of α and β chains by gel electrophoresis in the presence of SDS and mercaptoethanol. The mol. wts. of marker proteins were plotted on a logarithmic scale against their relative mobilities against Bromophenol blue.

The mol. wt. plotted on a logarithmic scale against the relative mobility of the marker proteins gave a straight line (fig.6). From this standard line, the values of mobility correspond to the following mol. wts.: $\alpha = 13\,000 \pm 2000$; $\beta = 37\,000 \pm 2000$.

In one case out of 6, another band is present. Its mol. wt., 26 000 ± 2000, is twice the mol. wt. of the α chain.

4. Discussion

After all types of tissue injury, as after turpentine oil subcutaneous injection, the level of different α and β globulins increases: fibrinogen, orosomucoid and other proteins which are usually called 'acute phase proteins'. In the sheep, an α_2-macroglobulin appears which is very difficult to separate from Hp by the usual methods, because of their phyisicochemical similarities (mol. wt., isoelectric point).

In addition, the instability of Hp makes it necessary to operate only in a limited pH range. Therefore, the affinity chromatography seems to be the best method of purification. Rabbit gamma-globulins containing anti-normal sheep serum antibodies bound to Sepharose retain a small quantity of sheep Hp. But the disadvantage of this small loss (less than 5%) is unimportant compared with the advantage of high degree of purification.

The fixed proteins are removed by elution with a 0.1 M acetate, 3 M Na-thiocyanate buffer, pH 4, after which the column is rapidly equilibrated with P.B.S. and can be used again several times without loss of efficiency: it yields 15 mg of pure Hp at each operation.

Sheep haptoglobin is a polymorphic protein; in this respect it differs from that in most other animals. On the contrary, it exhibits certain analogies with human Hp 2-2.

The peptide chains if the subunits also have mol. wts near to those of the α_2 and β chains of Hp 2-2. The α chain is lighter (13 000 instead of 18 000) as is the β chain (37 000 instead of 40 000). The latter contains the glycans.

A similar study has been made with Hp from goats. According to Travis et al. [10], the β chain exhibits a mol. wt. of 39 000 and the α chain 13 500. Hp from sheep and from goat are therefore closely similar.

Among the 6 sheep sera analysed, we have only once ascertained the presence of two α chains, so called by analogy with the α_1 and α_2 chains of human Hp 2-1. A genetic polymorphism is possible, but we cannot conclude this with sufficient certainty. Travis and Sanders have not detected any polymorphism in the Bovidae (cattle). In sheep, on the contrary, the existence of three phenotypes has been described [11] Our work seems to confirm this latter conclusion.

Acknowledgements

This work was supported by I.N.S.E.R.M. (Grant N° 75.1.054.4). The authors are indebted and very grateful to Professor Jerne for correcting the manuscript.

References

[1] Jayle, M. F. (1951) Bull. Soc. Chim. Biol. 33, 876–881.
[2] Jayle, M. F. and Moretti, J. (1962) Progr. Hemat. 3, 342–359.
[3] Travis, J. C. and Sanders, B. G. (1972) J. Exp. Zool. 180, 141–148.
[4] Connell, G. E., Smithies, O. and Dixon, G. H. (1966) J. molec. Biol. 21, 225–229.
[5] Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404–427.
[6] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[7] March, S. C., Parikh, I. and Cuatrecasas, P. (1974) Anal. Biochem. 60, 149–152.
[8] Cambiaso, C. L., Goffinet, A., Vaerman, J. P. and Heremans, J. F. (1975) Immunochem. 12, 273–278.
[9] Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059–3065.
[10] Travis, J. C., Garza, J. and Sanders, B. G. (1975) Comp. Biochem. Physiol. 51 B, 93–97.
[11] Beisembaeva, R. U., Dzhusupova, R. Z. and Omarov, B. S. (1974) Izv. Akad. Nauk Kaz. SSR, Ser. Biol. 12, 76–78.