Journal of Chromatography, 369 (1986) 244–247
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 954

Note

Chromatographic evaluation of the binding of haemoglobin to polyanionic polymers

V. BARBEROUSSE, D. SACCO and E. DELLACHERIE*
Laboratoire de Chimie-Physique Macromoléculaire, UA CNRS 494 ENSIC, 1, rue Grandville, 54042 Nancy Cedex (France)
(Received July 15th, 1986)

The interactions between haemoglobin and polyanionic compounds have been extensively investigated and several methods have been proposed for determining the stoichiometry of the complexes and their binding constants. However, these methods are generally suitable only for low-molecular-weight ligands, since they are often based on a good separation between macromolecules and small compounds[1,2].

It is now known that some polyanionic polymers, by interaction with haemoglobin, cause a decrease in its affinity for oxygen[3–5] and that the extent of this effect depends on various parameters such as the nature of the ionic groups, their concentration on the polymers, etc. These charged polymers can then be regarded as macromolecular allosteric effectors, whose association with haemoglobin, by giving rise to high-molecular-weight complexes with low affinity for oxygen, could be utilized in the field of blood substitutes[6].

Such complexes between haemoglobin and macromolecular polyanions have already been studied by methods such as sedimentation[3,4] and X-ray scattering[3], but this type of investigation is rather complicated as it requires special equipment. On the other hand, the information obtained from oxygen-binding determinations[4] is not easy to interpret as, during the oxygen equilibrium experiments, the conformation of haemoglobin changes from the deoxy to the oxy form.

In this paper, we report the results of an high-performance liquid chromatographic (HPLC) study of the complexation of polyanionic polysaccharides with oxyhaemoglobin, by the Hummel and Dreyer method[7]. This report essentially deals with polysaccharide sulphates such as dextran sulphate, whose interactions with haemoglobin were recently studied by a potentiometric technique[5], and λ carrageenan, but the same method could be applied to any other charged polymer.

EXPERIMENTAL

Materials

Human haemoglobin was prepared from outdated blood, according to the usual method[8]. It was deionized and freed from organic phosphates by passage through a column of Ultrogel AcA-202 (IBF, France; linear fractionation range 1000–15000; exclusion limit 22000) at pH 8.9, 0.1 *M* sodium chloride.

0021-9673/86/$03.50

Dextran sulphate was synthesized from dextran T40 (Pharmacia, Sweden; $\bar{M}_w \approx 40000$, $\bar{M}_n \approx 26000$), as described by Ricketts[9]. It contained 17% sulphur which corresponds to about 1.9 sulphate groups per glucose unit; it was in the sodium salt form and its $\bar{M}_n$ was evaluated as 54500.

λ carrageenan was obtained from Sigma (St. Louis, MO, U.S.A.). It was essentially in the calcium salt form and contained 10.6% sulphur, which corresponds to 0.88 sulphate groups per glucoside unit. Its molecular weight was not known.

All products were dissolved in 0.05 *M* Tris buffer.

Equipment

A Waters Assoc. Model ALC 200 liquid chromatograph equipped with a M 600 A pump, a M 440 UV detector and an U6K injector was used. The size-exclusion column (60 cm × 0.75 cm I.D.), Ultropac TSK G3000 SW, (linear fractionation range: 1000–300000) was obtained from LKB (Sweden).

Conditions

For the determination of the stoichiometries of polymer–haemoglobin complexes, we used the Hummel and Dreyer method, as already described for the study of drug–protein interactions[10]: thus, we equilibrated the size-exclusion column with a solution of haemoglobin considered as the ligand and injected small amounts of polymer solutions.

The concentration of haemoglobin in the eluent (0.05 *M* Tris buffer) being fixed at a defined value, mixtures in the same eluent of polysaccharide sulphate at a constant concentration and of haemoglobin at increasing concentrations were injected (100 μl). The chromatograms thus obtained showed the presence of protein–polymer complex in the void volume, followed by a negative or positive peak at the retention volume of haemoglobin, which represents the amount of protein bound to the polymer. By plotting the area of this peak *versus* the excess (relative to the eluent concentration) of haemoglobin, and by interpolating to zero, the excess of haemoglobin corresponding to the exact amount of protein bound to the polymer could be determined (internal calibration[6]). By increasing the haemoglobin concentration in the eluent and keeping constant that of the polymer in the injection, the average stoichiometry of the haemoglobin–polymer complex at saturation was determined. The concentration of dextran sulphate in the injections was 0.27 g/l which corresponds to $5 \cdot 10^{-6}$ *M* (with $\bar{M}_n = 54500$), and that of λ carrageenan was 0.56 g/l. The concentration of haemoglobin in the eluent, determined by the Drabkin method[11], was between 0.5 and 15 μM, *i.e.*, between 0.32 and 0.97 g/l. Detection was made at 254 nm, and the flow-rate was 1 ml/min. The experiments were carried out at room temperature.

RESULTS AND DISCUSSION

Fig. 1 shows the internal calibration curve obtained for the interaction of haemoglobin with dextran sulphate, for an haemoglobin concentration in the eluent of 1.8 μM at pH 7. The interpolation at 0 gives the amount of haemoglobin, n_{Hb}, bound to the amount of dextran sulphate injected, $n_{DS} = 5 \cdot 10^{-10}$ mol, and leads to the

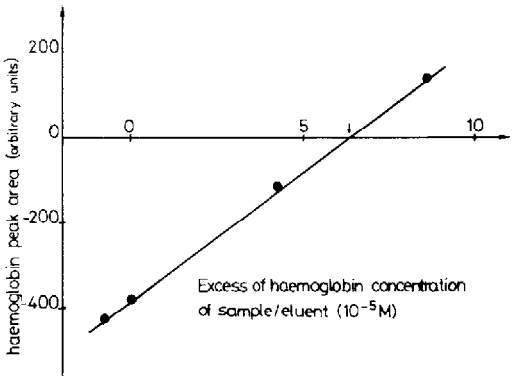

Fig. 1. Internal calibration for binding of oxyhaemoglobin to dextran sulphate. Eluent: 1.8 μM of haemoglobin in 0.05 M Tris buffer pH 7. Injection: 100 μl of mixtures of dextran sulphate (0.27 g/l) and haemoglobin at increasing concentrations in 0.05 M Tris buffer pH 7, 25°C.

mean molar ratio:

$$\bar{r} = \frac{n_{Hb}}{n_{DS}} = \frac{6.4 \cdot 10^{-9}}{5 \cdot 10^{-10}} = 12.8$$

$\bar{r}$ corresponds to the average number of haemoglobin molecules bound to one molecule of dextran sulphate under the concentration conditions used. The same internal calibration was performed for various haemoglobin concentrations in the eluent, and by plotting the different values calculated for $\bar{r}$ as a function of the haemoglobin concentration in the eluent a curve (Fig. 2) was obtained from which the average molar stoichiometry, $\bar{s}$, of the saturated haemoglobin–dextran sulphate complex was calculated, *i.e.*, $\bar{s} = 14$ (haemoglobin tetramer per dextran sulphate).

To appreciate the effect of pH on the ability of dextran sulphate and haemoglobin to form a complex, the same experiments were performed at various pH val-

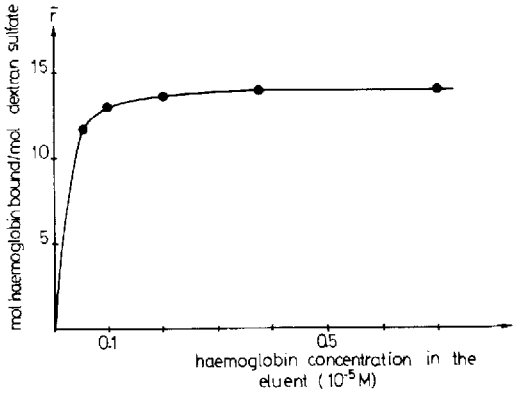

Fig. 2. Binding of oxyhaemoglobin to dextran sulphate as a function of the haemoglobin concentration in the eluent (0.05 M Tris buffer pH 7). Conditions as in Fig. 1.

ues. The average molar stoichiometries of the saturated complexes are 14, 13.4 and 8.75 at pH 7, 7.2 and 7.5, respectively. The results show that, as expected, when the pH increases, the average amount of haemoglobin molecules complexed with one molecule of dextran sulphate decreases, which corresponds to the deprotonation of some of the haemoglobin amino groups involved in the ionic interactions.

In order to compare the relative capacity of different polyanionic polysaccharides to form a complex with haemoglobin, a new parameter, $\bar{s}'$, was calculated, defined as the average amount of haemoglobin molecules bound to one molecule of sulphated glucoside monomer in the saturated complex. Thus, for dextran sulphate (17% sulphur) at pH 7, $\bar{s}'$ is equal to $9 \cdot 10^{-2}$, which means that, at saturation, the complex is formed on average by the association of 9 molecules of haemoglobin with 100 sulphated glucoside units. In the case of λ carrageenan (10.6% sulphur), at pH 7, $\bar{s}' = 6 \cdot 10^{-2}$, which shows that when the concentration of sulphate ions linked to the polymer decreases its capacity to form a complex with haemoglobin is reduced.

Other similar quantifications are now being carried out with dextran carboxylates and phosphates. The results of these studies should lead us to define the best polyanionic polysaccharide to be used in association with haemoglobin in the field of blood substitutes[6], the aim being to find a polymer with as low a charge as possible, but able to lead to a complex with haemoglobin as stable as possible under the conditions (pH and ionic strength) of the physiological media.

REFERENCES

1 A. Chanutin and E. Hermann, *Arch. Biochem. Biophys.*, 131 (1969) 180.
2 A. J. R. Costello, W. E. Marshall, A. Omahi and T. O. Henderson, *Biochim. Biophys. Acta*, 427 (1976) 481.
3 K. Ruckpaul, H. Rein, G.-R. Jänig, W. Pfeil, O. Ristau, G. Damaschun, H. Damaschun, J.-J. Müller, H.-V. Pürschel, J. Behlke and W. Scheler, *Stud. Biophys.*, 34 (1972) 81.
4 G. Amiconi, L. Zolla, P. Vecchini, M. Brunori and E. Antonini, *Eur. J. Biochem.*, 76 (1977) 336.
5 D. Sacco and E. Dellacherie, *FEBS Lett.*, 199 (1986) 254.
6 P. Menu, P. Labrude, Q. T. Nguyen, J. Floquet and C. Vigneron, *Bull. Soc. Pharm. Bordeaux*, 123 (1984) 161.
7 J. P. Hummel and W. J. Dreyer, *Biochim. Biophys. Acta*, 63 (1962) 530.
8 E. Dellacherie, F. Bonneaux, P. Labrude and C. Vigneron, *Biochim. Biophys. Acta*, 749 (1983) 106.
9 C. R. Ricketts, *Biochem. J.*, 51 (1952) 129.
10 B. Sébille, N. Thuaud and J.-P. Tillement, *J. Chromatogr.*, 167 (1978) 159.
11 D. Drabkin, *Am. J. Med. Sci.*, 217 (1949) 710.