Interaction of a macromolecular polyanion, dextran sulfate, with human hemoglobin

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Interactions of dextran sulfate with amino groups of oxy- and deoxyhemoglobin were followed by both potentiometric measurements between pH 6 and 7.3 and oxygen-binding studies. The uptake of protons observed upon addition of dextran sulfate to hemoglobin shows that the interaction with the deoxy form is strong and that the main site is probably located in the phosphate-binding β-cavity, whereas the interaction with the oxy form is more diffuse, probably with a great number of relatively weak binding sites. The influence of dextran sulfate on the oxygen dissociation curve of hemoglobin confirms these findings, as the effect of the polymer is to lower hemoglobin affinity for oxygen to a great extent, which proves that it stabilizes the deoxy form more strongly than the oxy one.

Dextran sulfate *Oxyhemoglobin* *Deoxyhemoglobin* *Potentiometry* *Oxygen binding*

1. INTRODUCTION

It is known that organic and inorganic anions such as phosphate [1–4], carboxylate [5,6] and sulfate [7] bind both oxy- and deoxyhemoglobin more or less strongly: these interactions result in a reduced oxygen affinity of the protein and at the same time in a more pronounced Bohr effect [5]. Using the X-ray diffraction technique, Arnone [8] and Arnone and Perutz [9] have shown that the binding site of DPG and IHP to deoxyhemoglobin is located in the central cavity between the two β-chains of the tetramer. Bucci et al. [10–12] have demonstrated that the interaction of carboxylic effectors with hemoglobin in solution can be studied by measuring the amount of protons absorbed upon the complexation. Using a similar method, Kilmartin [13,14] and De Bruin et al. [15–17] have studied the interaction of DPG and IHP with deoxy- and oxyhemoglobin and their influence on the Bohr effect.

The interactions of low-M_r polyanions with hemoglobin are now relatively well characterized. Little is known, however, about the effect of organic macromolecular anions. It has been shown by Ruckpaul et al. [18] that heparin, a natural sulfated polysaccharide, interacts with hemoglobin, lowering its oxygen affinity. The stoichiometry of the heparin-oxyhemoglobin complex under defined conditions was determined by oxygen-binding experiments, small-angle X-ray scattering and sedimentation analysis. Similar studies have been reported by Antonini et al. [19] on both heparin and dextran sulfate (average M_r 8000, 2.3 mmol sulfate per g). These authors observed that both macromolecular polyanions had an identical effect on the oxygen affinity of hemoglobin, and found the same molar stoichiometry for their complexes with ox-

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Abbreviations: DPG, 2,3-diphosphoglycerate; IHP, *myo*-inositol hexaphosphate; BHC, benzene hexacarboxylate

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yhemoglobin. However, no information was given on the possible sites of interaction on hemoglobin.

This paper describes the study, by a potentiometric technique, of the complexes resulting from the interaction between dextran sulfate and deoxy- and oxyhemoglobin. An average stoichiometry is proposed for the corresponding complexes and the localisation of the possible interaction sites is discussed.

2. MATERIALS AND METHODS

Human hemoglobin was prepared from outdated blood by lysing the washed red cells according to [20]. It was deionized and freed from organic phosphates by passage through a column of AcA 202 Ultrogel (IBF-France; linear fractionation range 1000–15000; exclusion limit 22000) at pH 8.9, 0.1 M NaCl.

Dextran sulfate was synthesized from T40 dextran (Pharmacia; $\bar{M}_w \simeq 40000$, $\bar{M}_n \simeq 26000$) as described by Ricketts [21]. It contained 17% S, which corresponds to about 1.9 OSO_3^- groups per glucose unit. It was in the Na salt form and its $\bar{M}_n$ was evaluated at 54500.

All pH measurements and acid-base titrations were carried out according to Bucci [10] with a Metrohm 632 pH meter, 614 Impulsomat and E 655 microburet equipped with a Metrohm EA 147 microglass electrode.

All experiments were performed in 0.05 N NaCl at 25°C. Deoxygenation of hemoglobin and of all titration reagents was carried out by first applying a vacuum to remove oxygen, then flushing nitrogen through the solutions. Spectrophotometric analysis was used to confirm the state of ligation of the solutions before and after titrations. pH measurements were carried out in a dry box under a nitrogen stream for anaerobic titrations of deoxyhemoglobin.

The concentration of hemoglobin was determined as methemoglobin cyanide according to Kaplan [22].

The pH of hemoglobin solutions was adjusted to the required value by adding 0.01 N HCl or NaOH solutions. The oxygen-binding experiments and the determination of P_{50} were performed spectrophotometrically with a tonometer, as described [20].

3. RESULTS AND DISCUSSION

3.1. *Interaction with oxyhemoglobin*

Fig.1 shows proton uptake on addition of dextran sulfate to oxyhemoglobin at different pH values. Above pH 7.3 proton uptake was no longer observed. At the other pH values, the amount of absorbed protons was significant, indicating that the two products interact with each other. Fig.2 shows the pH dependence of the proton uptake per mol hemoglobin (ΔH^+) for various molar ratios n of dextran sulfate to oxyhemoglobin. All the curves exhibit the same profile, showing a decrease in proton uptake when the pH increases from 6 to 7.3. Contrary to the findings obtained with low-M_r effectors [12,14,15,17], no maximum was observed in the pH range 6.5–7, and moreover the amount of absorbed protons was much higher. This means that dextran sulfate binds to more oxyhemoglobin amino groups with pK_a below 7.3 than the small effectors, and that other binding sites are involved.

It is also clear that, since there is no well-defined equivalence point in the binding isotherms (fig.1), the interaction of dextran sulfate with oxyhemoglobin is weak, and consequently no precise binding stoichiometry can be determined. Below pH 7, it appears that in addition to a relatively

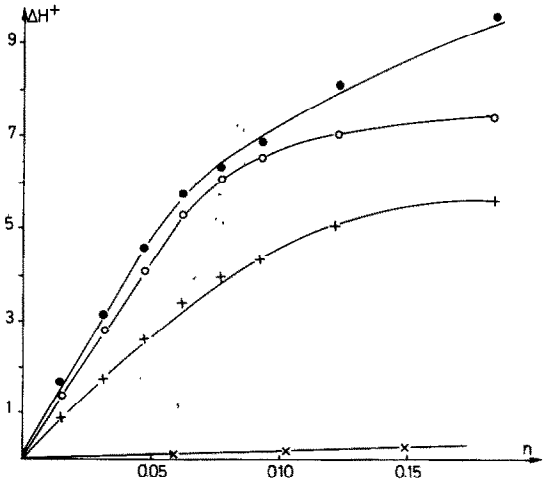

Fig.1. Proton uptake per tetramer upon binding of dextran sulfate to oxyhemoglobin at various pH values. (●) pH 6, (○) pH 6.6, (+) pH 6.95, (×) pH 7.3; hemoglobin concentration 1.4×10^{-5} M on tetramer basis; n, molar ratio of dextran sulfate ($\bar{M}_n \simeq 54500$) to oxyhemoglobin; 0.05 N NaCl; temperature 25°C.

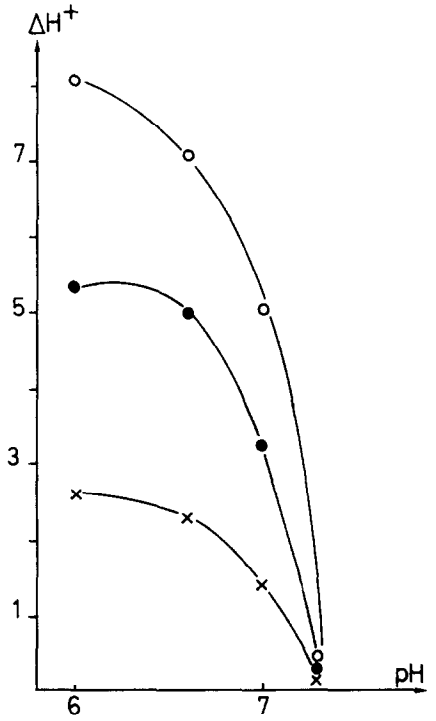

Fig.2. Dependence on pH of ΔH^+ for various values of the molar ratio of dextran sulfate to oxyhemoglobin (n); (×) n = 0.025, (●) n = 0.060, (○) n = 0.125. All conditions as in fig.1.

strong binding site, other weaker binding sites are present.

All these results globally concord with those obtained with small polyanionic effectors. They show, however, that the macromolecular nature of dextran sulfate, the length and flexibility of its chain, and the regular distribution of the negative charges along it offer other binding possibilities with oxyhemoglobin, possibly induced by cooperative effects.

3.2. *Interaction with deoxyhemoglobin*

The proton uptake on addition of dextran sulfate to deoxyhemoglobin at various pH values is shown in fig.3. Except at pH 6, where the measurements were disturbed by precipitation phenomena provoked by increasing concentrations of dextran sulfate, all the curves exhibit a sharp break point after which no more protons were bound by deoxyhemoglobin. If one assumes that this reflects the existence of a single strong binding

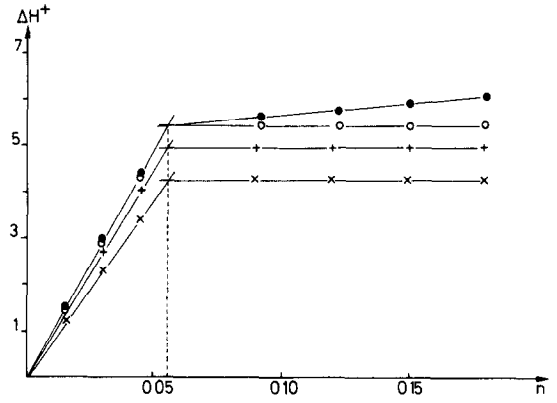

Fig.3. Proton uptake per tetramer upon binding of dextran sulfate to deoxyhemoglobin at various pH values. (●) pH 6, (○) pH 6.60, (+) pH 6.95, (×) pH 7.30. All conditions as in fig.1.

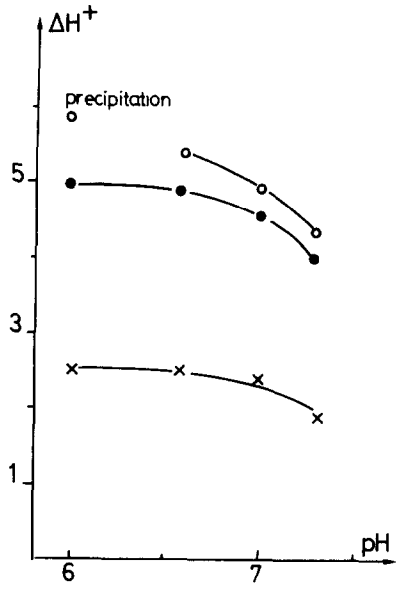

Fig.4. Dependence on pH of ΔH^+ for various values of the molar ratio of dextran sulfate to deoxyhemoglobin (n); (×) n = 0.025, (●) n = 0.050, (○) n = 0.125. All conditions as in fig.1.

site on deoxyhemoglobin, then the average molar stoichiometry of the complex can be calculated at the sharp break of the titration curve. With $\bar{M}_n \simeq$ 54500 for dextran sulfate, this was estimated at 18 (hemoglobin tetramer per dextran sulfate), and it can be seen that it is not pH dependent in the range pH 6.6–7.3. On the other hand, the number of

protons taken up by the protein depends slightly on the pH and its variation is shown in fig.4 for various molar ratios n of dextran sulfate to deoxyhemoglobin. The maximum average number of bound protons was found to be 5.3: this corresponds to the formation of the saturated complex ($n > 0.055$) at pH 6.6. As dextran sulfate has a pK_a smaller than 2, the proton uptake observed is only due to the protein, and thus it appears that the number of deoxyhemoglobin amino groups interacting with dextran sulfate is higher than in the case of DPG [14,16], IHP [17] and BHC [12] where ΔH^+_{max} was always found to be close to 2. Moreover, in our experiments the pH dependence of ΔH^+_{max} is weaker.

For small anionic effectors it has been postulated [10] that proton uptake by deoxyhemoglobin was due to pK shifts of only 3 pairs of amino groups located in the phosphate-binding β-cleft [8,9], i.e. the β1 valine, the β2 histidine and the β143 histidine residues. pK_a values were assigned to these groups in the presence of Cl^- ([12] and references therein) as follows: a pK_a of about 5.6 was assigned to the β143 histidines, 6.8 to the β1 valines and 7.6 to the β2 histidines. As in our experiments, the maximum number of protons absorbed by the interaction at a pH near 7 is 5.3, and since the binding appears to be strong, it is reasonable to assume, firstly, that all the β-cavity amino groups are involved in the observed proton uptake and give rise to a very strong interaction and, secondly, that other amino groups located outside the cavity can also participate in the binding in a cooperative manner because of the proximity of the anions present on the polymeric chain. Thus, one cannot dismiss the possibility of the involvement of other histidine residues [23] whose pK would be shifted upon interaction with the charged dextran, but we have no evidence for this.

In order to interact in the cavity, the polymer must be able to penetrate into it, permitting several successive sulfate groups to form salt bridges with the amino groups. As our dextran sulfate is slightly branched (5% of α_{1-4} linkages), it possesses a certain number of sulfated extremities (about 10) which can interact in the β-cavity sufficiently freely. On the other hand, it can be seen on Dreiding models that a loop formed with several successive sulfated glucose units (~1.9 sulfate groups per unit) is able to penetrate into the β-cavity. These two possibilities can thus explain on the one hand the observed value of ΔH^+_{max}, and on the other, the calculated average stoichiometry of the complex.

The oxygen-binding properties of hemoglobin in the presence of dextran sulfate saturating concentrations were also studied at various pH and the results are shown in fig.5 (Bohr effect). It appears that in the presence of the polymeric effector, the steepness of the Bohr effect curve is greater than that of stripped hemoglobin. This means that the effector binds preferentially to deoxyhemoglobin and that the resulting complex is more stable than that formed with oxyhemoglobin. This confirms the data of the potentiometric studies, which demonstrated a stronger interaction between deoxyhemoglobin and dextran sulfate, despite the fact that with oxyhemoglobin the number of binding sites is higher.

On the other hand, at pH 6.95 the maximum ef-

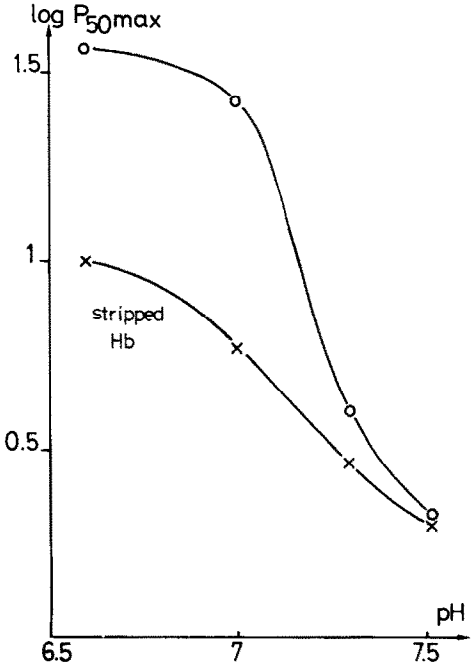

Fig.5. Effect of pH on the oxygen affinity of hemoglobin in the presence of saturating concentrations of dextran sulfate ($\bar{M}_n \simeq 54500$), i.e. 0.13 mol per mol hemoglobin; hemoglobin concentration 1.4×10^{-5} M on tetramer basis; 0.05 N NaCl; pH 6.95, 25°C. (×) Stripped hemoglobin; P_{50} in Torr.

fect on P_{50} is higher with dextran sulfate than with DPG (P_{50} (max) = 13 Torr), which means that the polymer binds to deoxyhemoglobin more strongly than the small effector does. As it is known that the higher the density of effector negative charges, the stronger the interaction, this finding can confirm that dextran sulfate indeed interacts inside the β-cavity, by means of several sulfated glucose units.

In conclusion, dextran sulfate is capable of binding hemoglobin and the interaction with deoxyhemoglobin is very strong, which results in a lowering of the oxygen affinity of the protein. On the basis of the similarities between our results and those reported for DPG, IHP or BHC, it is likely that the main site of interaction with deoxyhemoglobin is located in the β-cavity whilst other weaker interactions are formed due to the presence on the polymeric chain of numerous anions which are thus close to the amino groups of the protein.

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