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SUMMARY

The proton and oxygen affinity was studied of both human hemoglobin A and A₂ over the pH region 5.5-9.0. This study revealed the following:
1. The Bohr effect of hemoglobin A is identical to that of hemoglobin A₂.
2. There is no difference in the oxygen affinity between the two hemoglobins.
3. The oxygen binding curves of hemoglobin A and A₂ are identically affected by 2,3-diphosphoglycerate.
4. His G19(117)ᵦ in hemoglobin A has an abnormally high pK value of about 7.8 in both oxy- and deoxyhemoglobin.

INTRODUCTION

Hemoglobin A₂ (Hb A₂) is one of the minor human hemoglobins occurring in the red cell hemolysate; it has two α chains identical to those of the major component Hb A. The non α chains, the so called β chains, differ from the β chains of Hb A at 10 sites in the amino acid sequence. Among these differences there are 3 replacements of polar residues: Glu B₄(22)ᵦ is replaced by Ala, His G18(116)ᵦ by Arg and His G19(117)ᵦ by Asn.

Since the functional behavior of hemoglobin is closely related to its structure, Hb A₂ has been the subject of several studies. These studies have all presented evidence that in the region between pH 6.6 and 7.6 the Bohr effect, i.e. the mutual dependence of the oxygen and proton affinity of hemoglobin, is the same for both hemoglobins. They disagree, however, on whether Hb A₂ would show a higher affinity for oxygen than Hb A or not. Eddison et al. have found that both proteins exhibit the same oxygen affinity, while Huisman et al. and Santa reported that Hb A₂ has a considerably higher oxygen affinity than normal Hb A.

Abbreviation: Hb, hemoglobin.
Recently, we have presented evidence that of the two histidines which the β chain has in excess to the δ chain, His G19 is titratable and His G18 masked; since Hb A and Hb A2 have identical Bohr effect, this His G19 should have the same pK in both oxy- and deoxyhemoglobin A.

In view of this and the above-mentioned discrepancy concerning the oxygen affinity of Hb A2 and Hb A, we have investigated both the pH dependence of the oxygen affinity and the oxygen-linked proton affinity of the two hemoglobins in the pH region 5.5–9.

**MATERIALS AND METHODS**

Human hemoglobin was prepared from whole blood by the toluene method. The two components A and A2 were isolated by chromatography on DEAE-Sephadex, type A50, as described earlier. The isolation procedure is a modification of the method of Bernini. The collected fractions were dialysed against distilled water and subsequently deionized by repeated passing through a mixed bed ion-exchange column (Amberlite IRA 400 and IR 120). H+ titrations were carried out using the continuous automatic titration equipment described earlier. Isoionic solutions of oxyhemoglobin A2 (pHiso = 7.60) were titrated to pH 6 and subsequently deoxygenated by passing argon through the titration vessel. Hb A2 was deoxygenated at this pH, as the oxygen affinity at the isoionic pH is much higher than at pH 6. Titration results obtained after deoxygenating Hb A2 at pH 7.60 were not reproducible enough. This was probably due to only partial deoxygenation of the solution in the titration vessel. After deoxygenation the titration curve of deoxyhemoglobin A2 was recorded up to pH 9. At this pH, by passing CO through, the deoxy form was converted into the carboxy form; subsequently the titration curve was recorded down to pH 6. Deoxyhemoglobin was converted into carboxyhemoglobin instead of into oxyhemoglobin in order to avoid oxidation into methemoglobin. The same procedure was applied for Hb A. As usual no differences could be detected in control experiments between the titration curves of oxy- and carboxyhemoglobin.

Titrations were carried out at an ionic strength of 0.1 (KCl) at 25 °C. The hemoglobin concentrations were 8·10⁻⁴ M (heme basis). The methemoglobin contamination amounted to only a few percent in all samples studied. Oxygen binding equilibria were measured spectrophotometrically. The oxygen binding curves were calculated applying the relations given by Benesch et al. Oxygen was added to the tonometer (volume 1 l) by injecting air using normal syringes. 0.05 M phosphate buffers were used below pH 8; above this pH we used borax buffers. The hemoglobin concentrations were about 5·10⁻⁵ M.

All hemoglobin concentrations were measured spectrophotometrically on the carboxy derivatives using a value for the extinction coefficient per mole heme, ε, of 14 000 at 540 nm.

**RESULTS AND DISCUSSION**

In Fig. 1 the pH vs $Z_H$ curves are shown for the deoxy and carboxy form of hemoglobin A and A2. $Z_H$ represents the mean proton charge of the protein with the isoionic point ($Z_H = 0$) as reference point for counting. $Z_H$ is calculated per tetramer.
The figure shows that, over the pH region considered, the curves for carboxy- and deoxyhemoglobin A₂ lie above the curves for carboxy- and deoxyhemoglobin A, respectively. The results for Hb A are identical with those reported earlier. The difference in protons bound (ΔZH) is about 2 at low pH and 3.5 at high pH (see also Fig. 2, where this difference in protons bound is plotted vs pH). This increase in difference in ZH is due to the fact that, of the two histidines Gl9 and Gl8, only the first is titratable. Since His Gl9 β can compensate for the positive charge of Arg Gl8 δ, Zmax (the maximum positive proton charge) will be equal for both proteins. As a result, the presence of Glu at position B4 in the β chain, where the δ chain has an Ala residue, is found as the difference in charge of about 2 at low pH.

Fig. 1. H⁺ titration curves of Hb A (triangles) and Hb A₂ (circles) at 25 °C and I = 0.1 (KCl). Filled symbols refer to the deoxy form, open symbols to the oxy form.

Fig. 2. Difference in protons bound (ΔZH) between the carboxy form of Hb A and Hb A₂ (▲) and between the deoxy forms of the two hemoglobins (○); I = 0.1 (KCl); temperature, 25 °C.

Fig. 2 reveals further that the titration behavior of His Gl9 is very similar in both carboxy- and deoxyhemoglobin and that in both forms its imidazole group has an abnormally high pK value of about 7.8. This similar behavior in both states is in agreement with the crystallographic data of Perutz and co-workers, which have shown that this residue forms a saltbridge with Glu B8(26)β both in carboxy- and deoxyhemoglobin (Perutz, M. F., personal communication). Due to this saltbridge the pK of His Gl9 will be elevated in both forms.

Fig. 3 shows the difference in protons bound (ΔZ) between the deoxy and carboxy form of the two hemoglobins. The plot reflects the change in proton affinity of deoxyhemoglobin upon ligation. Since an identical Bohr effect for the two proteins, as presented in Fig. 3, does not mean that they would also show the same oxygen affinity, we measured the log ϕₚ₀ vs pH curves. The results presented in Fig. 4 indicate that hemoglobin A and A₂ have not only the same Bohr effect but also identical oxygen affinity when measured under the same conditions. This observation is supported by the oxygen binding equilibrium studies in the presence and absence of 2,3-
Fig. 3. Difference in protons bound ($\Delta Z$) between the deoxy and carboxy form of Hb A$_a$ (●) and Hb A ($\triangle$); $I = 0.1$ (KCl), temperature, 25 °C.

Fig. 4. pH dependence of the oxygen affinity, as expressed in terms of log $p_{50}$, for Hb A ($\triangle$) and Hb A$_a$ (●). The experiments were carried out at room temperature.

diphosphoglycerate. This polyanion binds strongly to the deoxy form only and has therefore a remarkable influence on the oxygen affinity of adult human hemoglobin$^{12,13}$. The binding site for 2,3-diphosphoglycerate is on the dyad axis at the entrance of the central cavity, where the negative charges of the 2,3-diphosphoglycerate anion can form saltbridges with positively charged groups on the $\beta$ chains. These groups are very probably the two histidines H21, the two terminal amino groups and the lysines EF 6 (ref. 14). Since hemoglobin A and A$_a$ have these groups in common, it could be expected that the oxygen affinity of both proteins would equally be affected by the presence of 2,3-diphosphoglycerate. The results are presented in Fig. 5 and show that

Fig. 5. Effect of 2,3-diphosphoglycerate on the oxygen affinity of Hb A (triangles) and Hb A$_a$ (circles). Open symbols, without 2,3-diphosphoglycerate; filled symbols, with 2,3-diphosphoglycerate; pH 7.15, 0.01 M Bis Tris; hemoglobin concentration 2.5 $\cdot$ 10$^{-4}$ M (heme basis); 2,3-diphosphoglycerate concentration, 5 $\cdot$ 10$^{-4}$ M.
the shift in oxygen affinity introduced by 2,3-diphosphoglycerate is indeed equal for both Hb A and A2. Summarizing, we can say that from the data presented in the last three figures it can be concluded that Hb A and A2 are indistinguishable as far as the oxygen binding features are concerned.

The difference in affinity reported for the two hemoglobins\(^3\),\(^4\) might be explained by assuming that 2,3-diphosphoglycerate, which can only partially be removed from the hemolysate by dialysis\(^1\),\(^6\), was still present in the isolated Hb A and A2 fraction though at different concentration, resulting in pseudo-heterogeneity in the oxygen affinity of the two proteins. In all our experiments 2,3-diphosphoglycerate is routinely removed by deionizing the solutions using the mixed bed ion-exchange column\(^1\).

The equal oxygen affinity of both hemoglobins indicates that the \(\beta\) and \(\delta\) chain when combined with the \(\alpha\) chain have the same functional behavior.

Finally, we want to say something about the Bohr groups of Hb A. Up to now the amino group of the terminal valine of the \(\alpha\) chain and the imidazole group of the terminal histidine of the \(\beta\) chain have been identified as Bohr groups. Together, they probably account for 70% of the total Bohr effect\(^1\),\(^7\). The group responsible for the remaining 30% has not yet been identified. As far as the \(\beta\) chain is concerned, the only residues remaining as possible candidates for this group are His NA2, His FG4 and His EF1. From these residues, His FG4 is the most likely one, since it is in close contact with Tyr C6(41)\(\alpha\) at the \(\alpha_\beta\) interface\(^1\), which undergoes large changes upon oxygenation. Measurement of the Bohr effect of Hb Malmö, in which His FG4 is replaced by Asn\(^19\) could be conclusive in this respect. If this hemoglobin showed a normal Bohr effect, then the still missing Bohr group should be looked for among the histidines of the \(\alpha\) chain; one of these histidines, His H5(122)\(\alpha\), is proposed by Perutz\(^1\) as a possible Bohr group.

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REFERENCES