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Dependence on pH of formation and oxygen affinity of hemoglobin S fibers in the presence and absence of phosphates and polyphosphates

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This paper presents data on the effect of phosphates and polyphosphates on the formation of hemoglobin S fiber, and on the Bohr effect of hemoglobin S samples whose concentration was high enough (near 5 mM) in order to form fibers upon deoxygenation. The experiments were performed in 0.2 M Bistris or Tris buffers at 30 ° C in the presence and absence of inositol hexakisphosphate and of 2,3-diphosphoglycerate. Alternatively, 0.2 M phosphate buffers were used without addition of effectors. Under these conditions, few fibers were formed in Tris or Bistris buffers, while extensive fiber formation occurred in the presence of phosphates and polyphosphates. In all cases, increasing pH strongly inhibited fiber formation. At pH 7.5 and above, fibers were not formed in our samples. In the presence of phosphates and polyphosphates fiber formation reduced the oxygen affinity of hemoglobin S with respect to either hemoglobin A or soluble hemoglobin S under similar experimental conditions. The fiber-polyphosphate complexes showed a larger Bohr effect than that in hemoglobin A. In the presence of inositol hexakisphosphate fiber-forming solutions of hemoglobin S liberated as much as six protons per tetramer upon oxygen binding. The increased liberation of protons was probably due to a higher affinity of the effectors for the fibers of hemoglobin S. Very likely the higher affinity was supported by a conformational change of hemoglobin S specific for the fibers.

1. Introduction

Preferential binding of ligands to those conformers of a system for which they have a higher affinity stabilizes those conformers and provides the basis for the allosteric regulation of biological activity of proteins [1].

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Abbreviations: Bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; C_{sat}, concentration of hemoglobin S in the supernatant, after centrifugation of the gels; 2,3-DPG, 2,3-diphosphoglycerate; Hb, hemoglobin; IHP, inositol hexakisphosphate.

In hemoglobin S, fiber formation in the absence of ligands is a special form of preferential binding of the deoxytetramers to themselves. This stabilizes the deoxygenated form of the system thereby decreasing its oxygen affinity. These expectations have been amply confirmed by the findings of Sunshine et al. [2], Gill et al. [3] and Hofrichter [4] which indicated a very low affinity of the fibers for ligands, much lower than that of hemoglobin A or of soluble hemoglobin S.

Fiber formation is an entropy-driven phenomenon, due essentially to hydrophobic interactions [5,6]. There is also an involvement of electrostatic charges, as proven by the pH dependence of C_{sat} [5,7,8]. Furthermore, Bookchin et al. [9] have shown that in the red cells of either homozygous or heterozygous individuals for hemoglobin S, the

Bohr effect is larger than that present in normal red cells.

As reported by Scholberg et al. [10] in the β-subunits of hemoglobin S the $\beta 2$ histidine appears to have a higher pK than in the βA subunits. This would be due to the presence of a valine at $\beta 6$ in the βS subunits which stabilizes a β-bend, which in turn places the $\beta 2$ histidine and the $\beta 7$ glutamic acid residues near enough in order to establish an electrostatic interaction. This conformation is not detectable in soluble hemoglobin S in either the presence or absence of ligands and would be stabilized by the fibers. This phenomenon would make the histidine at $\beta 2$ a new Bohr effect group in the hemoglobin S fibers, which could explain a larger Bohr effect in hemoglobin S.

This paper presents results which attempt to clarify the role played by electrostatic interactions in the functional properties of hemoglobin S at concentrations comparable to those present in vivo in red cells, where fiber formation occurs. The fibers appear to have a higher affinity for polyphosphates than soluble hemoglobin S. This results in an increased Bohr effect, dependent on the type of anions present in the solvent. A specific conformational change of hemoglobin S upon fiber formation may play a key role in this phenomenon.

2. Materials and methods

2.1. Preparation of hemoglobin S

Hemoglobin S was purified in its oxygenated form from blood either homozygous for HbS or heterozygous for HbS/HbC following the procedures of Benesch and Benesch [11] and of Huisman and Dozy [12], respectively. The blood was a generous gift from Dr. S. Charache of the Johns Hopkins University Medical School. The fractions collected during ion-exchange chromatography were checked for purity on cellulose acetate electrophoresis. The pure fractions of hemoglobin S were pooled and concentrated by suction filtration to about 1.0 g/dl. These samples did not contain appreciable amounts of hemoglobins A_2, C or F. After dialysis against distilled water these solutions were recycled in the cold through a cartridge of mixed-bed ion-exchange resin to remove all of the organic and inorganic ions, including 2,3-DPG, from the protein. Aliquots of these samples were dialyzed against the appropriate buffers, and concentrated to 35–40 g/dl by suction filtration. The pH of the concentrated samples was measured at 30 °C; they were quickly frozen as droplets in liquid nitrogen and stored at −120 °C.

2.2. Preparation of hemoglobin A

Normal human blood was obtained from the blood bank of the University of Maryland. Hemoglobin A in its oxygenated from was purified as described for hemoglobin S. The subsequent manipulations paralleled those mentioned for hemoglobin S.

Measurement of pH

These measurements were performed with a Radiometer M26 pH meter, equipped with a Radiometer glass/calomel combination microelectrode. To ensure reproducibility, the equipment was located in a thermostatted room at 30 °C and all of the solutions were equilibrated at 30 °C before measurements.

2.4. Measurement of oxygen binding

Oxygen binding equilibria were measured using the thin-layer apparatus and procedure described by Dolman and Gill [13]. Mixtures of oxygen and nitrogen were used for oxygenating the samples, ranging from 25 to 100% oxygen according to need. The saturation of hemoglobin with oxygen was monitored spectrophotometrically following the absorbance at 560 and 577 nm during a stepwise deoxygenation of the samples. A constant value of the absorbance was taken as indication of equilibrium at each step of the titration. The oxygen tension in the equilibration chamber was measured with an oxygen electrode connected to a Keithley model 174 multimeter with accuracy to 10^{-7} V. All measurements were conducted at 30 °C. Complete oxygenation and deoxygenation of the samples were monitored spectrophotometri-

cally using a procedure similar to that described by Benesch et al. [14]. Each determination was done in duplicate or triplicate, and average values are reported in the various figures. When the deviation was higher than ±5% all determinations are reported. The amount of methemoglobin in the samples was monitored spectrophotometrically before and after experiments. In all cases it was less than 5%.

2.5. Measurement of C_{sat}

The samples used for oxygen binding equilibrium experiments were also used for measuring C_{sat} at 30°C. Samples of 200–300 μl were placed in 1 ml nitrocellulose tubes and covered with mineral oil. A 2 M solution of sodium dithionite was prepared by dissolving weighed amounts of the reagent in the appropriate buffer previously vigorously bubbled with nitrogen to remove dissolved oxygen. With a microsyringe 10 μl dithionite solution were added to the protein under the oil layer, and gently stirred, without disturbing the oil. In separate experiments it was shown that this addition of dithionite did not modify the pH of the solution. The tubes were equilibrated in a water bath at 30°C for 30–60 min and subsequently centrifuged in a swinging-bucket rotor for 90 min at 30°C at 45000 rpm, in a Beckman L5 ultracentrifuge.

After centrifugation the tubes were pierced with a microsyringe under the oil layer and above the solid phase. The liquid hemoglobin supernatant was aspirated and 5–10-μl samples were added to 2 ml of 0.15 M phosphate buffer at pH 7.0, previously equilibrated with carbon monoxide at atmospheric pressure. The concentration of carbonmonoxyhemoglobin was measured spectrophotometrically using $E = 0.868$ cm^2 mg^{-1} at 540 nm. This was taken as the C_{sat} value of the samples. Each sample was at least duplicated and average values are reported.

2.6. Buffers used in the various experiments

Oxygen equilibria and C_{sat} measurements were performed in 0.2 M Bistris buffers between pH 6.5 and 7.3, and in 0.2 M Tris buffers at pH 7.3 and above. The buffers were prepared by titrating the respective bases with HCl, before adjusting their pH to the desired values with NaOH. In this way the concentration of Cl^- was constant at all pH values. Ad hoc experiments proved that these buffers maintained a constant pH of the various hemoglobin solutions during ligand-binding experiments.

2.7. Treatment of the data

The following equation was used to compute the fractional saturation of hemoglobin with ligands from the absorbance values monitored at 560 and 577 nm:

$$Y = \frac{(A_o - A_i)_{560} + (A_i - A_o)_{577}}{(A_o - A_\infty)_{560} + (A_\infty - A_o)_{577}}.$$

where A_i is the absorbance of the sample after equilibration at each step of the titration and A_o the value of the fully oxygenated ones. The subscripts denote the wavelength (in nm) of the observations.

These data were processed by a computer routine capable of producing Hill plots of the data, and of determining the value of $P_{1/2}$ and the slope of the Hill plots for any chosen interval of P_{O_2}.

Alternatively, the software computed the value of the median ligand activity, P_m, as defined by Wyman [15]:

$$\int_0^{P_m} Y \, d \ln(P_{O_2}) = \int_{P_m}^{\infty} (1 - Y) \, d \ln(P_{O_2})$$

where the integrals were computed by trapezoidal rule on either hand-smoothed or computer-smoothed curves. The values of $P_{1/2}$ (obtained from the Hill plots between 30 and 70% saturation of hemoglobin with oxygen) and the values of P_m were very similar, within 2% of each other. For this reason we have used the $P_{1/2}$ values in this work.

3. Results

3.1. Solubility of hemoglobin S

Fig. 1 shows the pH dependence of C_{sat} of hemoglobin S at 30°C, under the various condi-

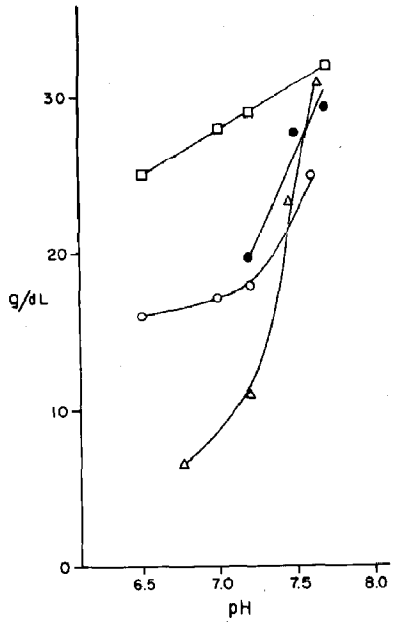

Fig. 1. Dependence on pH of C_{sat} of deoxyhemoglobin S at 30 °C, in the various buffers used for the oxygen absorption measurements. The initial protein concentrations were between 32 and 38 g/dl. (□) 0.2 M Bistris or Tris buffers, (○) 0.2 M phosphate buffers, (△) 0.2 M Bistris or Tris buffers with 5 mM IHP, (●) 0.2 M Bistris or Tris buffers with 5 mM 2,3-DPG.

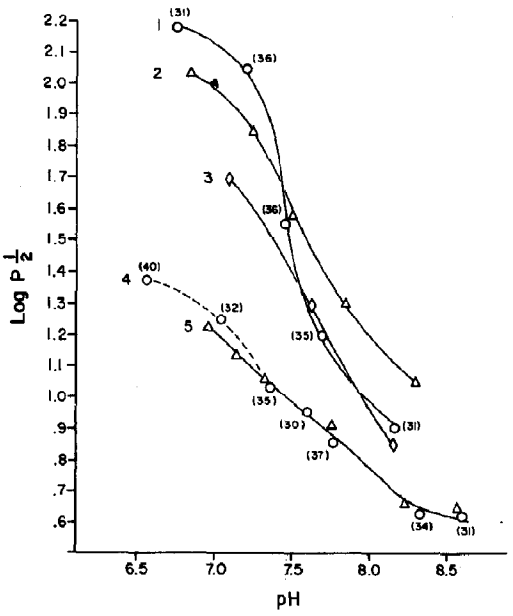

Fig. 2. Bohr effect of hemoglobin A and S at 30 °C in 0.2 M Bistris or Tris buffers. Protein concentrations (in g/dl) of fiber-forming solutions of hemoglobin S are given in parentheses near each experimental point. Values are rounded off to the nearest integer. Curves: 1, HbS with 5 mM IHP; 2, HBA with 5 mM IHP; 3, HbS (5 g/dl) with 1.0 mM IHP; 4, HbS, no effector; 5, HbA, no effector.

tions of oxygen equilibria used.

In Bistris and Tris buffers, gel formation was minimal ($C_{sat} > 25$ g/dl), with a very small pH dependence. The amount of fibers increased in the presence of phosphates and polyphosphates. The lowest value of C_{sat} was reached at pH 6.5 in the presence of equimolar amounts of IHP and hemoglobin with $C_{sat} = 6.5$ g/dl. Equimolar amounts of 2,3-DPG had a similar although less pronounced effect. It may be noted that the fiber-forming effect of polyphosphates ceased at pH 7.5 where the C_{sat} values in the presence and absence of the effectors were very similar. In contrast, in phosphate buffers substantial amounts of fibers were still formed at pH 7.5.

3.2. *The Bohr effect of hemoglobin S fibers*

Fig. 2 shows the Bohr effect of fiber-forming solutions of hemoglobin S in the presence and absence of IHP. It is evident that the presence of fibers reduced the oxygen affinity of the samples. Benesch et al. [16] have reported a concentration dependence of the oxygen affinity of hemoglobin S in these circumstances. In fig. 2, values in parentheses refer to the protein concentration at which the experiments were performed. It may be noted that the middle portion of line 1 (critical for the estimation of the Bohr effect) includes determinations conducted practically at the same concentration, near 36 g/dl. The extreme acid and alkaline ends of the curve were obtained using a lower protein concentration, 31 g/dl. At the acid end solutions at higher protein concentration could not be fully oxygenated even using oxygen at atmospheric pressure. Thus, the value reported for pH 6.6 represents a minimum value of $P_{1/2}$ under those conditions. This implies a probable underestimation of the Bohr effect of the system. For the determinations performed above pH 7.5 protein concentration was not a relevant parameter because fibers were not formed.

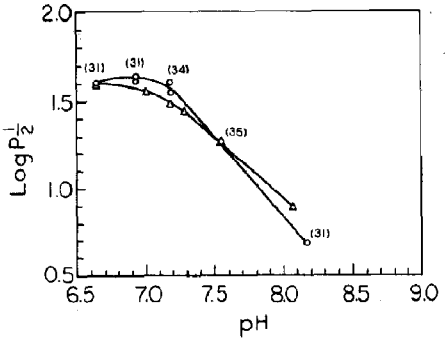

Fig. 3. Bohr effect of hemoglobins A and S at 30 °C in 0.2 M Bistris or Tris buffers in the presence of 5 mM 2,3-DPG. Protein concentrations (in g/dl) of hemoglobin S are given in parentheses near the experimental points. Values are rounded off to the nearest integer. (O) HBS, (Δ) HbA.

In Bistris and Tris buffers, the oxygen affinities of hemoglobins A and S at various pH values were very similar. The small amounts of fibers formed below pH 7.5 reduced very slightly the oxygen affinity of hemoglobin S.

The effect of IHP, which induced formation of large amounts of fibers, was very pronounced both on the oxygen affinity of hemoglobin S and on its pH dependence.

As a control, measurements of oxygen affinity of hemoglobin S at low (i.e., non-fiber-forming) concentrations showed that in the presence of equimolar amounts of IHP the oxygen affinity of hemoglobin S was higher than that of hemoglobin A at all pH values.

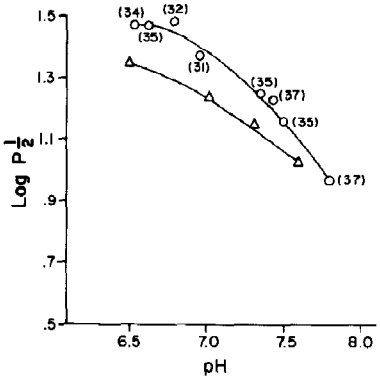

Fig. 4. Bohr effect of hemoglobins A and S in 0.2 M phosphate buffer at 30 °C. Protein concentrations (in g/dl) of hemoglobin S are given in parentheses near the experimental points. Values are rounded off to the nearest integer. (O) HbS, (Δ) HbA.

Fig. 3 shows the effect of 2,3-DPG on the oxygen affinity of fiber-forming solutions of hemoglobin S in comparison to hemoglobin A. As expected from the data in fig. 1 the effect was very similar to that of IHP, however, less pronounced, probably because of the lower amounts of fibers present in the samples.

Fig. 4 shows the Bohr effect of hemoglobin A and S in phosphate buffers. Consistent with the data shown in fig. 1, formation of fibers resulted in a lower oxygen affinity of hemoglobin S at all pH values investigated.

3.3. Oxygen binding cooperativity of hemoglobin S in the presence of fibers

When fibers were formed below pH 7.5, Hill plots of the oxygen saturation curves indicated the presence of a region of high slope, giving values of n between 5 and 6. This phenomenon was already noted in S/S erythrocytes and in concentrated solutions of hemoglobin S [3,17]. It should be noted that the high slopes of the curves were always in the region of 60–80% saturation of hemoglobin S with oxygen. In vivo, and under resting conditions, hemoglobin delivers about 40% of its oxygen to the tissues, therefore, this region of high cooperativity is relevant to the transport of oxygen by hemoglobin S.

4. Discussion

4.1. Buffer and effector conditions

The buffers chosen for measuring oxygen equilibria were the best compromise we could find between the necessities of: (a) using buffers of sufficiently high concentration to maintain a constant pH in highly concentrated solutions of hemoglobin during the oxygen binding experiment; (b) keeping the buffer concentrations low enough so that the negative ions necessarily introduced with the buffers were not competing with polyanionic effectors; and (c) keeping the anions of

the buffer at a constant concentration for all pH values. The chosen conditions produced a variation in ionic strength of the buffers between 0.15 and 0.3 at the various pH values, however, at all pH values there was a constant concentration of Cl^-, which is the relevant ion for the oxygen affinity of hemoglobin systems. $Tris^+$ and Na^+ were inert at the concentrations used here [18].

With regard to the concentrations of effectors, large amounts had to be used just for matching the equimolar ratio of effectors to hemoglobin. We did not want to exceed this ratio to prevent excessive increases of cations in our solutions. Also, as discussed below, to have equimolar quantities helped with the interpretation of the data.

4.2. C_{sat} measurements

The relevance of polyphosphates and polyanions to fiber formation, as shown by our data, is consistent with previous data of Briehl and Ewert [19], Briehl [20] and Poillon et al. [21]. We repeated some of those experiments to check on the amount of fibers which were formed under the identical conditions used for the measurements of oxygen absorption. A few considerations are here in order, which were not formulated by our predecessors.

The high value of C_{sat} for highly purified solutions of hemoglobin S in Tris and Bistris buffers suggests a specific role played by phosphates and polyphosphates in inducing fiber formation. Their effect was certainly linked to the electrostatic interaction of their negative charges with the positive groups present at the binding site for 2,3-DPG and IHP in deoxyhemoglobin. In fact, fiber formation was strongly inhibited above pH 7.5 where the α-amino groups and the histidines are mostly deprotonated [22]. Most probably this is a general phenomenon produced by anions and polyanions in the system. Experiments (not reported here) of oxygen binding by 5 mM solutions of hemoglobin S in the presence of 5 mM benzene hexakiscarboxylate gave results very similar to those obtained with IHP. It is even possible to speculate that the residual gelling power of hemoglobin S in Tris and Bistris buffers was due to the presence of 0.2 M Cl^- in those buffers.

Table 1

Proton Bohr effects computed from the regions of maximum slopes of the curves in figs. 2–4, using the equation $\Delta H^+/[\text{tetramer}] = -4\Delta \log P_{1/2}/\Delta\text{pH}$ [15]

Protein sample	Concentration (mM)	Buffer	pH range	$-\Delta \log P_{1/2}/\Delta$pH	$\Delta H^+/$[tetramers]
HbS	5	0.2 M Bistris or Tris	7.0–7.5	0.43	1.7
HbA	5	0.2 M Bistris or Tris	7.0–7.5	0.43	1.7
HbS	5	0.2 M phosphate	7.0–7.5	0.44	1.7
HbA	5	0.2 M phosphate	7.0–7.5	0.36	1.4
HbS	5	0.2 M Bistris or Tris +5.0 mM IHP	7.2–7.6	1.55	6.2
HbA	5	0.2 M Bistris or Tris +5.0 mM IHP	7.2–7.8	0.94	3.7
HbS	0.8	0.2 M Bistris or Tris +1.0 mM IHP	7.2–8.1	0.75	3.0
HbS	5	0.2 M Bistris or Tris +5.0 mM 2,3-DPG	7.2–7.6	1.18	4.4
HbA	5	0.2 M Bistris or Tris +5.0 mM 2,3-DPG	7.2–7.6	0.85	3.4

4.3. The additional Bohr effect of the fibers of hemoglobin S

Mere inspection of fig. 2 reveals that the pH dependence of the oxygen affinity of fiber-forming solutions of hemoglobin S is much more pronounced than that of the non-fiber-forming controls. This is particularly evident comparing the curves for 5 mM HbS in the presence of IHP (strongly fiber-forming) with those obtained with either 5 mM HbA or 1 mM HbS (non-fiber-forming) also in the presence of IHP.

It is a well-known phenomenon [11,23–25] that the interaction of hemoglobin with effectors increases the Bohr effect. The presence of both fibers and IHP had a much larger effect.

Table 1 lists the slopes of the various curves so as to give numerical values to the various Bohr effects. According to the theory of linked function of Wyman [15], the slopes should give the number of protons liberated by the various systems upon oxygenation. It should be borne in mind that these are only indicative quantities, due to the high concentration of hemoglobin which in the presence of stoichiometric amounts of effectors substantially altered the free effector concentration upon oxygenation. Nevertheless these data help in making comparisons with the appropriate controls.

Thus, it appears that fiber-forming solutions of hemoglobins had a larger Bohr effect than the non-fiber-forming controls.

It is possible that the fibers per se formed additional Bohr effect groups upon polymerization. The new groups could be either intramolecular, like the classic Bohr effect groups, or intermolecular in the new contacts in the fibers. Gill et al. [3] excluded the possibility of this kind of phenomenon. Scholberg et al. [10] proposed a possible pK shift of a histidyl residue in the system, upon fiber formation. However, the pH range in which this pK shift would be detectable (near pH 8) is too high for expressing itself as a Bohr effect in the pH range where fibers are formed.

More likely, in the presence of IHP, extra protons were absorbed by a different interaction with polyphosphates of the fibers of hemoglobin S. The use of stoichiometric amounts of IHP excluded the possibility that fibers formed additional binding sites for the effector, besides that present in the soluble hemoglobin tetramer. It follows that the different interaction is probably due to a conformational change in the protein upon fiber formation.

Much evidence supports the hypothesis of a specific conformational change of the hemoglobin S molecule upon fiber formation. Thermodynamic data [5,6] are unequivocal in demonstrating that fiber formation is an entropy-driven phenomenon, typical of hydrophobic interactions. Therefore, the pH dependence of their formation and the preponderant role played by the electrostatic interactions of the protein with polyphosphates can be explained only if they produce conformational changes in the system which in turn stabilize the hydrophobic interactions responsible for the formation of fibers.

This hypothesis is also supported by the findings of Arnone [26], who reports a slight modification of the position of the A helix of the β-subunits upon the interaction of hemoglobin with polyphosphates. It is also consistent with the proposition of Scholberg et al. [10] of a specific conformational change which produces a high pK for the $\beta 2$ histidine.

Padlan and Love [27,28] have recently described a displacement of the A helix of the β-subunits in the double-stranded quinary structure, in the crystals, of deoxyhemoglobin S. The displacement is similar to that described by Arnone [26] in normal hemoglobin upon binding of polyphosphates. This is consistent with the increased affinity of hemoglobin S fibers for polyphosphates and supports our hypothesis of a specific conformational change of the tetramers upon gelling, stabilized and possibly increased by polyphosphates.

Thus, it seems very likely that hemoglobin S undergoes a conformational change upon fiber formation, which increases the affinity of the tetramers for phosphates and polyphosphates, or more generally for anions and polyanions, thereby producing increased absorption of protons upon binding of the effectors.

4.4. Additional considerations

In contrast to its dramatic effect on fibers, the interaction of soluble hemoglobin S with polyphosphates was less effective in reducing the oxygen affinity of the system than in hemoglobin A. In fact, in the presence of stoichiometric amounts of IHP the affinity for oxygen of soluble hemoglobin S was higher than that of hemoglobin A, while in the absence of effectors the affinities of the two hemoglobins for the ligand were identical. This is shown in fig. 2 by lines 2, 3 and 5. A similar phenomenon has been reported for 2,3-DPG by Ueda et al. [29] and can be explained by a lower preferential affinity of deoxyhemoglobin S for the effector due to either the hydrophobicity of the β6 valine, or to a slight modification of the position of the β1 and β2 residues produced by the mutation, or both. This stresses the specificity of the conformational change proposed above for the fibers of hemoglobin S, which increased the affinity of the tetramers for polyphosphates.

Also, 2,3-DPG and phosphate increased both fiber formation and the Bohr effect of hemoglobin S. Qualitatively, the effect of 2,3-DPG on hemoglobin S was very similar to that of IHP and its lower efficiency was probably due to its lower net negative charge. It is interesting to note that, with regard to both fiber formation and Bohr effect, the interaction of phosphate with hemoglobin S was not only quantitatively different from that of the polyphosphates, but also differently distributed on the pH scale. This again stresses the predominant electrostatic nature of the interactions of hemoglobin with anions and polyanions, which in turn are dependent on the nature and configuration of the negative charges of the effectors.

An interesting characteristic of the oxygen affinity for the fibers of hemoglobin S is the high value of n in certain regions of the Hill plots. Besides the explanation provided by Gill et al. [3] and Monplaisir et al. [17], a value of n larger than 4 implies that the functional unit of the fibers within which binding cooperativity is established is larger than a single tetramer. This implies the formation of interfaces among the tetramers, capable of transmitting information with regard to the state of the heme. *

Furthermore, this high cooperativity was present at saturation levels above 50%, so as to be physiologically significant for the transport of oxygen. This makes hemoglobin S the best pump for oxygen delivery devised by nature for humans. In the homozygous individual fiber formation triggers the disease known as sickle cell anemia. In the heterozygous individual the situation is very different. In fact the aggregation of only two to three molecules of hemoglobin S may be sufficient to form a unit of high oxygen binding cooperativity as in the fibers. Further polymerization would be inhibited by the presence of the other hemoglobins in the red cells and by non-extreme deprivation of oxygen. These microscopic aggregates would not be of great significance for the biology and rheology of red cells, nevertheless, they would be able to provide an advantageous extra release of oxygen when they form. This would further justify the existence of hemoglobin S in the heterozygous individual as a favorable trait with respect to oxygen delivery, besides its possible antimalaric effects.

Acknowledgements

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* The objection can be raised that Sunshine et al. [2] found the disappearance of fibers, upon addition of oxygen, noncooperative up to a 14% average saturation of their samples. The noncooperativity of the fibers under these conditions only implies an explosion of their cooperativity at higher partial pressures of oxygen, which indeed prevented further monitoring of the phenomenon. It should be stressed that Sunshine et al. [2] clearly discuss in their paper the consistency of their findings with the MWC model of cooperative systems.

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