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COLLECTIVE BEHAVIOUR OF HEMOGLOBIN IN DENSE SOLUTIONS

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The formation of radical pairs of spin-labelled hemoglobin molecules (β-93 cysteine) in a mixture with unlabelled hemoglobin of concentrations higher than the critical one [$(5.7 < c < 6.3)$ mM/ℓ per heme] is due to some kind of long-range order characterizing these solutions. Labelled molecules induce perturbation in an unlabelled hemoglobin solution of concentration higher than the critical one. The collective behaviour of unlabelled molecules gives rise to the formation of radical pairs of spin-labelled hemoglobin molecules. Such behaviour is dependent on the ligand bound at the heme iron (carbonmonoxide, oxygen, or water).

Introduction

Spin labels have been successfully used to probe conformational changes occurring within the hemoglobin molecule during its transition from the deoxygenated to the oxygenated state [1]. These studies have shown that spin-labelled hemoglobin molecules are slightly different from unlabelled molecules with respect to oxygen binding, i.e., labelled molecules differ physically from unlabelled ones [2].

It has been shown recently that in an unlabelled hemoglobin solution of high concentration, spin-labelled molecules added in small portion are arranged in such a way that two nitroxide labels form a radical pair. The formation of radical pairs with a distance of about 10 Å between unpaired electrons gives rise to broadening of ESR lines of the pair which are practically impossible to detect [3]. The rearrangement of spin-labelled hemoglobin molecules in a dense unlabelled hemoglobin solution can thus be studied by following the decrease with time of the ESR signal of the mono-radical.

The formation of radical pairs of labelled CO-hemoglobin molecules in a mixture with unlabelled CO-hemoglobin depends on the concentration of unlabelled hemoglobin. The critical concentration (≈ 6 mM/ℓ per heme) at which the ESR-signal decrease with time started to appear was observed [4].

It will be shown in this paper that the formation of radical pairs of labelled molecules in a mixture with dense unlabelled hemoglobin is due to the property of unlabelled hemoglobin solutions that some kind of long-range order characterizes dense oxy- and carbonmonoxyhemoglobin solutions.

2. Materials and methods

Hemoglobin samples were prepared from freshly drawn human blood following the procedure of Cameron and George [5]. Purification of the samples was performed on DEAE-Sephadex with NaCl gradient at pH = 7.6. Aquomet-hemoglobin was obtained from oxy-form by oxidation with $K_3Fe(CN)_6$ and subsequent dialysis. Carbonmonoxyhemoglobin was prepared by flushing erythrocites with carbonmonoxide. The same procedure was applied to hemolysate and to hemoglobin after dialysis to a desired buffer. NaCl+TRIS buffers were used at pH of nearly physiological value (7.4). Solutions of various concentrations up to 24 mM/ℓ per heme were prepared by dialysis under nitrogen pressure.

Hemoglobin was spin-labelled with maleimide (4-maleimido-2,2,6,6,-tetramethylpiperidinooxyl) or with iodoacetamide (4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinooxyl) [2]. Hemoglobin concentrations were determined spectrophotometrically.

A small amount of ($\approx 0.6\%$) of spin-labelled hemo-

globin (≈ 1 mM/ℓ per heme) was added to dense unlabelled hemoglobin solutions of given concentrations. The decrease of the ESR monoradical signal was measured as a function of time. The ratio of the amplitude of the spectrum central line to the amplitude of one manganese (Mn^{2+}) marker was chosen as a measuring parameter (I). All functions representing the dependence of parameter I on time were normalized with respect to the value of I at time zero. The manganese marker was fixed on the inner wall of a Dewar flask (produced by SCANCO) inserted in the ESR cavity. A temperature of 20°C was maintained constant by a VARIAN variable-temperature controller and monitored by a thermocouple located above the sample. ESR spectra were taken on a VARIAN E-3 spectrometer.

3. Results

Gradual decrease in intensity of the ESR signal without changes in line-shapes of the spectra was observed when a small portion of spin-labelled hemoglobin (about 1 mM/ℓ per heme) was added to dense unlabelled-hemoglobin solutions of high concentrations.

Fig. 1a shows the ESR spectrum of acetamide spin-labelled carbonmonoxyhemoglobin (0.9 mM/ℓ per heme) added to a dense carbonmonoxyhemoglobin solution (19 mM/ℓ per heme) in 1 to 9 volume ratio immediately after addition of labelled molecules. The ESR signal decreased in time and disappeared completely within one hour (fig. 1b). If the sample represented in fig. 1b was agitated in a vortex, the signal gradually reappeared, remained constant for about 20 minutes and then began to decrease again (fig. 1c).

The signal could also be recovered by slow freezing of the sample [4]. When the sample was frozen in liquid nitrogen, the signal in the frozen sample did not reappear; however, it gradually reappeared when the sample was rewarmed at temperature above 0°C.

Nitroxide 2,2,6,6,-tetramethyl-4-oxopiperidinooxyl (TANO) was introduced to a dense unlabelled CO-hemoglobin solution in an adequate proportion as spin-labelled hemoglobin. This nitroxide molecule cannot react with hemoglobin, and shows an ESR spectrum characteristic of the nitroxide molecule freely tumbling into the hemoglobin solution. The amplitude of the spectrum remains constant in dense unlabelled

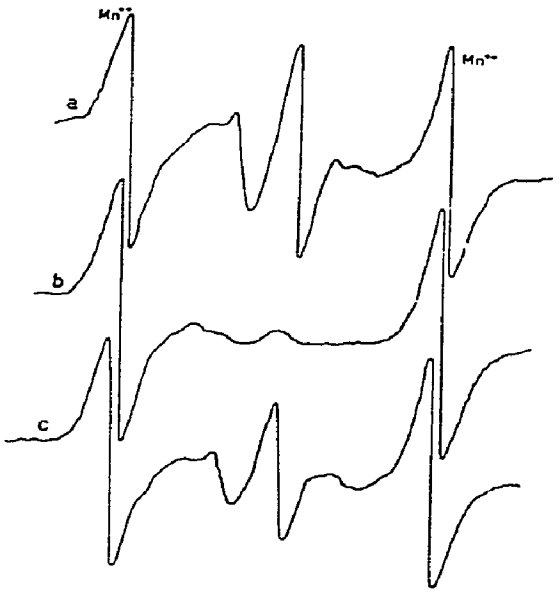

Fig. 1. ESR spectra of spin-labelled CO hemoglobin (0.9 mM/ℓ per heme) added to an unlabelled CO hemoglobin solution (19.1 mM/ℓ per heme) in 1:9 volume ratio, in 0.1 M NaCl+ TRIS buffer, pH = 7.2; (a) immediately after addition of labelled molecules, (b) 60 min after addition of labelled molecules, (c) sample (b) was agitated in a vortex for 5s.

hemoglobin solutions.

It is obvious from the results described above that the disappearance of the ESR signal is not brought about by reduction of the nitroxide radical. The disappearance of the signal could, therefore, be associated with a process resulting in a special arrangement of labelled molecules, i.e., in the formation of radical pairs of spin-labelled hemoglobin molecules [3].

The following experiments were performed in order to elucidate the possible cause of the phenomenon of radical-pair formation. Dense aquomet-, oxy-, and carbonmonoxyhemoglobin solutions of similar concentrations were prepared. Adequate amounts of spin-labelled hemoglobins were added to these solutions: labelled aquomethemoglobin (1 mM/ℓ per heme) to dense methemoglobin (18 mM/ℓ per heme), labelled oxyhemoglobin (0.8 mM/ℓ per heme) to dense oxyhemoglobin (20 mM/ℓ per heme), and labelled carbonmonoxyhemoglobin (0.9 mM/ℓ per heme) to dense carbonmonoxyhemoglobin (19 mM/ℓ per heme). In all solutions, labelled molecules were added in 1 to 9 volume ratio.

The results are shown in fig. 2. The slopes of the lines representing the ESR-signal decrease with time

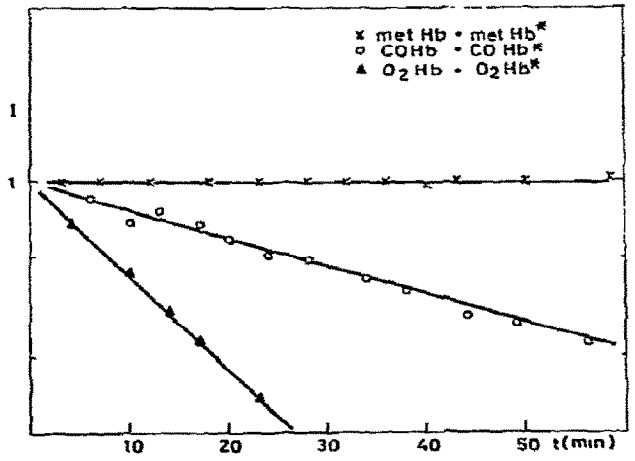

Fig. 2. ESR-signal decrease with time of acetamide spin-labelled hemoglobins (≈ 1 mM/ℓ per heme) added to dense unlabelled hemoglobins of high concentrations in 1:9 volume ratio in 0.1 M NaCl+TRIS buffer, pH = 7.2. (Star denotes spin label attached at β-93 cysteine.)

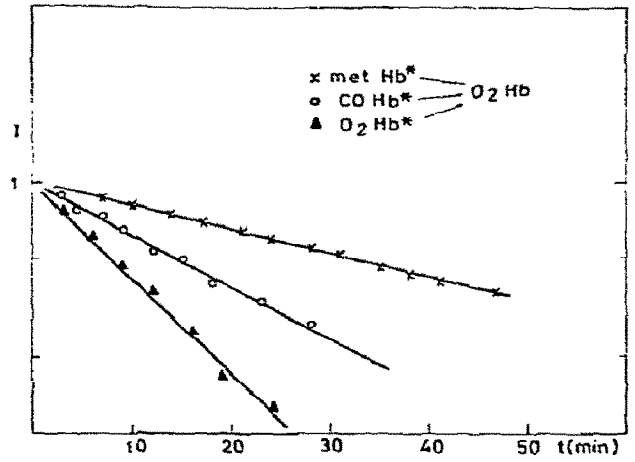

Fig. 4. ESR-signal decrease with time of acetamide spin-labelled hemoglobin (≈ 1 mM/ℓ per heme) added to dense unlabelled oxy-hemoglobin (20 mM/ℓ per heme) in 1:9 volume ratio, in 0.1 M NaCl+TRIS buffer, pH = 7.2. (Star denotes spin label attached at β-93 cysteine.)

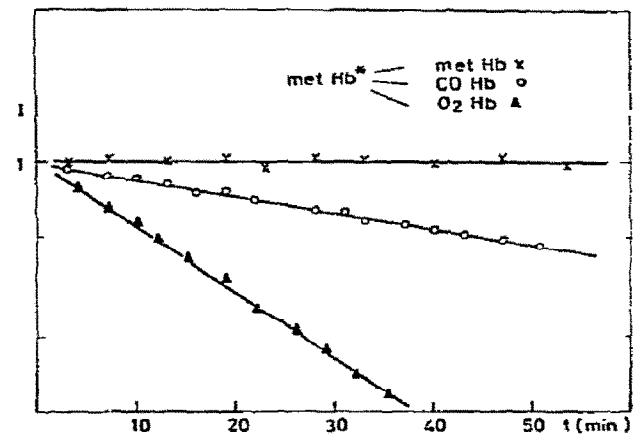

Fig. 3. ESR-signal decrease with time of acetamide spin-labelled methemoglobin (1 mM/ℓ per heme) added to dense unlabelled hemoglobin solutions of high concentration in 1:9 volume ratio in 0.1 M NaCl+TRIS buffer, ph = 7.2. (Star denotes spin label attached at β-93 cysteine.)

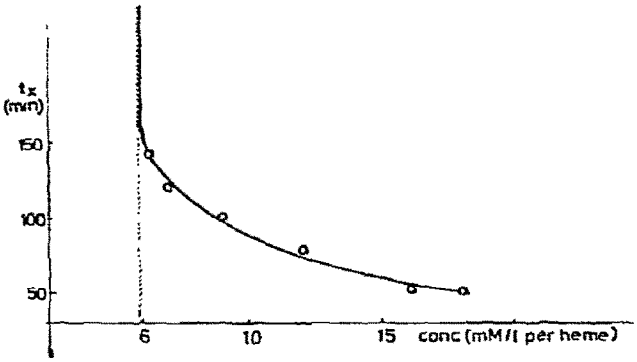

Fig. 5. Dependence of time (t_x) required for complete disappearance of the ESR signal on the concentrations of unlabelled hemoglobin. Spin-labelled CO hemoglobin (0.9 mM/ℓ per heme) was added to unlabelled CO-hemoglobin solutions of various concentrations in 1:9 volume ratio in 0.1 M NaCl+TRIS buffer at pH = 7.2.

are different in different ligand forms of hemoglobin. The ESR-signal decrease with time was not observed in dense methemoglobin solutions.

Fig. 3 shows the effect of labelled methemoglobin molecules added in adequate amounts to dense hemoglobin solutions of different ligand forms. In dense oxy- or carbonmonoxyhemoglobin solutions, labelled methemoglobin molecules form radical pairs, while in dense methemoglobin solutions they remain distributed through the bulk of unlabelled solution. The slopes of the lines representing the ESR-signal decrease with time depend on the ligand form of dense solutions to which labelled methemoglobin molecules were added.

Fig. 4 shows the effect of various ligand forms of spin-labelled hemoglobins on dense unlabelled oxyhemoglobin. The slopes of the lines representing the ESR-signal decrease with time depend on the labelled-hemoglobin ligand form (oxy-, carbonmonoxy-, or aquomet-hemoglobin).

Fig. 5 shows the time required for complete disappearance of the ESR signal (t_x) as a function of concentration of unlabelled hemoglobin. Adequate amounts of spin-labelled carbonmonoxyhemoglobin (0.9 mM/ℓ per heme) were added in the same volume ratio (1 to 9) to unlabelled carbonmonoxyhemoglobin of various concentrations. In unlabelled hemoglobin solutions of very high hemoglobin concentrations, the ESR monoradical signal disappeared in shorter time (t_x) than in lower concentrations. t_x attained an infinite value at the concentration of $5.7 < c < 6.3$ mM/ℓ per heme, which is the critical concentration for the phenomenon of radical-pair formation.

4. Discussion

Spin-labelled hemoglobin molecules present in small amount in dense unlabelled hemoglobin solutions are arranged in such a way that two nitroxide labels of two hemoglobin molecules form a radical pair [3]. The consequence of such an arrangement is the disappearance of the monoradical signal. For the ESR signal to disappear, the majority of labelled molecules must be arranged in radical pairs. However, since labelled molecules represent only a small amount (about 0.6%) of the total hemoglobin, it is apparent that the association of labelled hemoglobin molecules must be very specific; otherwise, they would remain distributed throughout the bulk of hemoglobin solution.

The association of labelled hemoglobin molecules in radical pairs might be due to a certain property of labelled molecules. Contrary to this assumption, neither the ESR-signal decrease in dense spin-labelled hemoglobin solutions (up to 24 mM/l per heme) nor the relative ESR-signal increase in such solutions upon dilution, was observed. Pairs of nitroxides were not found in solutions where the concentration of unlabelled hemoglobin was below 6 mM/ℓ per heme [4].

The results shown in fig. 3 suggest that the association of labelled hemoglobin molecules in radical pairs is due to a certain property of unlabelled hemoglobin matrix. In fact, spin-labelled methemoglobin molecules in a dense unlabelled methemoglobin solution remain distributed as monoradicals. In another matrix, i.e., in dense oxy- or carbonmonoxyhemoglobin solutions, the same molecules rearrange in such a way as to form radical pairs (fig. 3). Shaking these samples in a vortex, after the ESR signal has disappeared, gives rise to the reappearance of the monoradical spectrum (fig. 1). Hence, the external disturbance induced by the mechanical force or freezing caused disruption of radical pairs.

The formation of radical pairs of spin-labelled hemoglobin molecules might be a consequence of the long-range order among hemoglobin molecules in dense solutions of high concentrations (above 6 mM/ℓ per heme) [4]. Labelled molecules induce perturbation of the long-range order, i.e., they behave as impurities: these are arranged in radical pairs by the collective behaviour of unlabelled hemoglobin molecules. Such an arrangement diminishes the perturbation induced by spin-labelled molecules.

Two arrangements of radical pairs can be visualized as a pair of mixed hemoglobin tetramers consisting of two spin-labelled dimers in contact, or as a ring of labelled tetramers. Both arrangements lead to complete disappearance of the ESR signal.

The existence of rapid equilibrium between tetramers and dimers ($\alpha_2\beta_2 \rightleftarrows 2\alpha\beta$) in hemoglobin solutions is well established. The dissociation of a tetramer at neutral pH occurs via cleavage along the same contact region to form a symmetrical $\alpha\beta$ dimer pair [6].

Although the tetramer $\rightleftarrows$ dimer dissociation constant of labelled molecules is not known, hybrid tetramers of type $\alpha\beta^*$ (star denotes the spin label attached covalently at β-93 cysteine) may be assumed to exist in solutions in which some fractions of hemoglobin molecules are spin-labelled. It has been recently shown that hybrid tetramers of hemoglobin A and hemoglobin S are formed in hemoglobin mixtures [7]. As spin-labelled hemoglobin A differs much less from unlabelled hemoglobin A than hemoglobin S from hemoglobin A, it is reasonable to assume that hybrid tetramers of type $\alpha\beta^*/\alpha\beta$ are present in all hemoglobin solutions examined in this article. In the process of radical-pair formation, hybrid tetramers of this type should play an important role, i.e., they behave as impurities in an ordered unlabelled solution.

It has been shown recently that the concentration of labelled molecules has no influence on the forma-

tion of radical pairs [4]. This suggests that the final arrangement of labelled hemoglobin molecules in an unlabelled matrix can be visualized as rings of labelled molecules.

Differences in slopes of the lines representing the ESR-signal decrease with time (figs. 2 and 3) indicate that the behaviour of the dense hemoglobin solution depends on the ligand bound at the sixth coordination place of the heme iron. Moreover, the ligand form of labelled molecules also affects the slopes of the lines representing the ESR-signal decrease with time (fig. 4). It may be concluded that the process of radical pair formation should be governed by the tetramer $\rightleftarrows$ dimer dissociation constant of labelled and unlabelled molecules, by the formation of mixed tetramers $\alpha\beta^*$, and by the long-range order present in dense hemoglobin solutions. Fig. 5 corroborates this conclusion, suggesting that some sort of phase transition occurs at a concentration of 6 mM/ℓ per heme of unlabelled hemoglobin.

Acknowledgement

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References

[1] S. Maričić, in: Magnetic resonance in chemistry and biology, eds. J.N. Herak and K.J. Adamič (Dekker, New York, 1975) p. 445.
[2] S. Ogawa and H.M. McConnell, Proc. Natl. Acad. Sci. US 58 (1967) 19.
[3] J.N. Herak and V. Nöthig-Laslo, Manuscript in preparation.
[4] V. Nöthig-Laslo, Biochem. Biophys. Res. Comm. (1977), in press.
[5] B.F. Cameron and P. George, Biochim. Biophys. Acta 194 (1969) 16.
[6] G.K. Ackers, H.R. Halvorson, Proc. Natl. Acad. Sci. US 71 (1974) 4312.
[7] K. Moffat, Science 185 (1974) 274.