SPIN-LABELING STUDY OF SOLUTIONS OF CO-HEMOGLOBIN IN HIGH CONCENTRATIONS

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SUMMARY - In an unlabeled hemoglobin solution of a concentration higher than the critical one ((5.7<c<6.3)mM/l per heme), spin-labeled hemoglobin molecules behave as impurities; these are brought together by the collective behavior of unlabeled hemoglobin molecules, thus giving rise to the formation of radical pairs of spin-labeled hemoglobin.

INTRODUCTION - It has been shown recently that in an unlabeled hemoglobin solution of high concentration, spin-labeled hemoglobin molecules added in small amount 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 (1). The rearrangement of spin-labeled hemoglobin molecules in an unlabeled hemoglobin solution can thus be studied by observing the decrease with time of the ESR signal of the monoradical.

It will be shown in this communication that the formation of radical pairs of spin-labeled hemoglobin is due to a certain property of the unlabeled hemoglobin solution. A model for a superstructure of hemoglobin in erythrocites has recently been proposed (2). In this model the existence of a long-range order among hemoglobin molecules is assumed. This model fits well to our observation.

MATERIALS AND METHODS - Hemoglobin samples were prepared from freshly drawn human blood following the procedure of Cameron and George (3). Hemoglobin was purified on DEAE-Sephadex-A-50 column with 0.3M NaCl gradient. Carbonmonoxyhemoglobin was prepared by flushing erythrocites with carbonmonoxide. Solutions of various concentrations up to 24mM/l per heme were prepared by dialysis under the pressure of nitrogen. Carbonmonoxyhemoglobin was spin-labeled with 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (SYVA) or with 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinooxyl at pH=6.8 and 4°C (4). The reaction was terminated after 36 hours and followed by Sephadex G-25 chromatography. Hemoglobin concentrations were determined spectrophotometrically.

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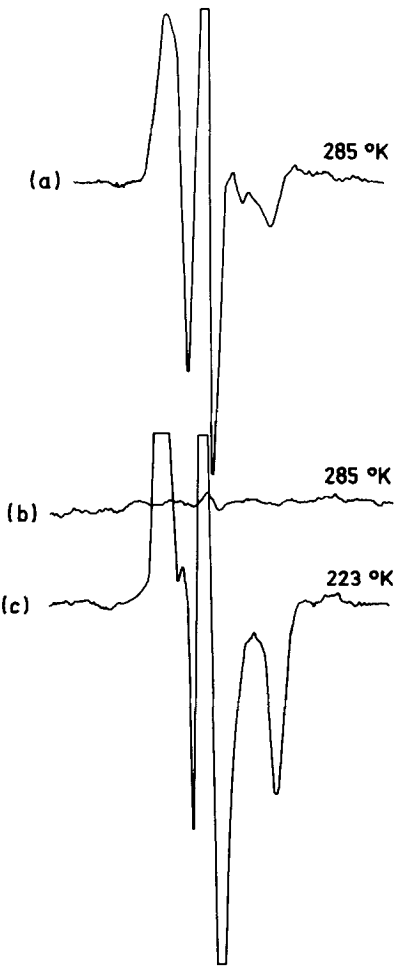

FIGURE 1. ESR spectra of acetamide spin-labeled CO-hemoglobin (concentration 0.6mM/l per heme) added to an unlabeled CO-hemoglobin solution (21.0mM/l per heme) in 1:3 volume ratio, in 0.1 M phosphate buffer at pH=7.0.
(a) 10 min. after addition of spin-labeled CO-hemoglobin,
(b) 60 min after addition of spin-labeled CO-hemoglobin,
(c) temperature of the sample (b) was slowly lowered to 223°K.

The decrease in intensity of the ESR 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 line 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 inserted in the ESR cavity. The temperature 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.

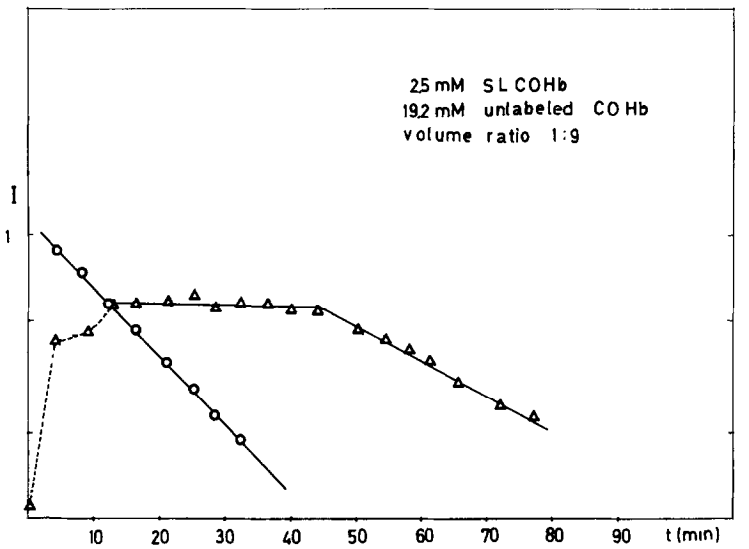

FIGURE 2. ESR-signal decrease with time of acetamide spin-labeled CO-hemoglobin added to an unlabeled CO-hemoglobin solution in 0.1 M NaCl+TRIS buffer at pH=7.0 (open circles). Reappearance of the ESR signal due to vortexing (15 seconds) (triangles).

RESULTS - Gradual decrease in intensity of the ESR signal without changes in line-shapes was observed when a small amount of spin-labeled carbonmonoxyhemoglobin was added to an unlabeled hemoglobin solution of high concentration. The signal disappeared completely within one hour or two, depending on the concentration of the unlabeled carbonmonoxyhemoglobin solution. The signal was recovered by diluting the sample below the concentration of 6mM/l per heme (1), or by slowly lowering the temperature below $0^{o}C$ (Fig. 1). When a mechanical stress (vortex) was applied to the sample after the signal had disappeared, the signal reappeared, remained constant for some time, and then started to decrease again (Fig. 2). The reappearance of the signal was also observed after several successive agitations of the sample in the vortex. When nitroxide 4-oxo-2,2,6,6-tetramethylpiperidinooxyl was added to unlabeled hemoglobin solutions of various concentrations, the amplitudes of the ESR spectra remained constant during several days in all the samples. This nitroxide cannot react with hemoglobin, and the ESR

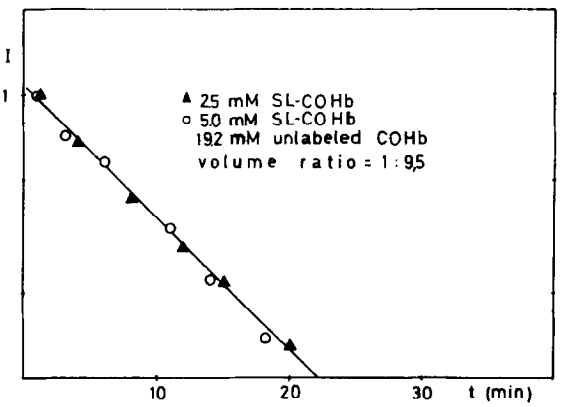

FIGURE 3. The effect of two different spin-labeled CO-hemoglobin concentrations of equal volumes added to equal amounts of unlabeled CO-hemoglobin in 0.1 M NaCl+TRIS buffer at pH=7.2.

spectra reflect microviscosity of the unlabeled hemoglobin solution to which nitroxide was added. Nitroxide was added to unlabeled hemoglobin solutions in similar amounts as spin-labeled hemoglobin (with respect to spin-labels attached at β-93 cysteine).

From the results described above it is obvious that the disappearance of the ESR signal (Figs. 1 and 2) is not brought about by the reduction of the nitroxide radical. If this phenomenom were due to the reduction of nitroxide by some contaminating enzymes, it would also be observed in the samples to which nitroxide 4-oxo-2,2,6,6-tetramethylpiperidinooxyl was added.

The disappearance of the ESR signal might be caused by the formation of aggregates of hemoglobin molecules sedimenting out of the ESR cavity during experiment. In that case, the ESR signal intensity of the vortexed sample would be reduced after the low-speed centrifugation. Mechanical instability has already been established in liganded hemoglobin A solutions (5). In order to answer this question, we performed the following experiment. The temperature of the sample was lowered to 10°C after the ESR signal had disappeared, and the sample was vortexed for a few seconds. The amplitude of the recovered signal remained constant in time at this temperature. The sample was

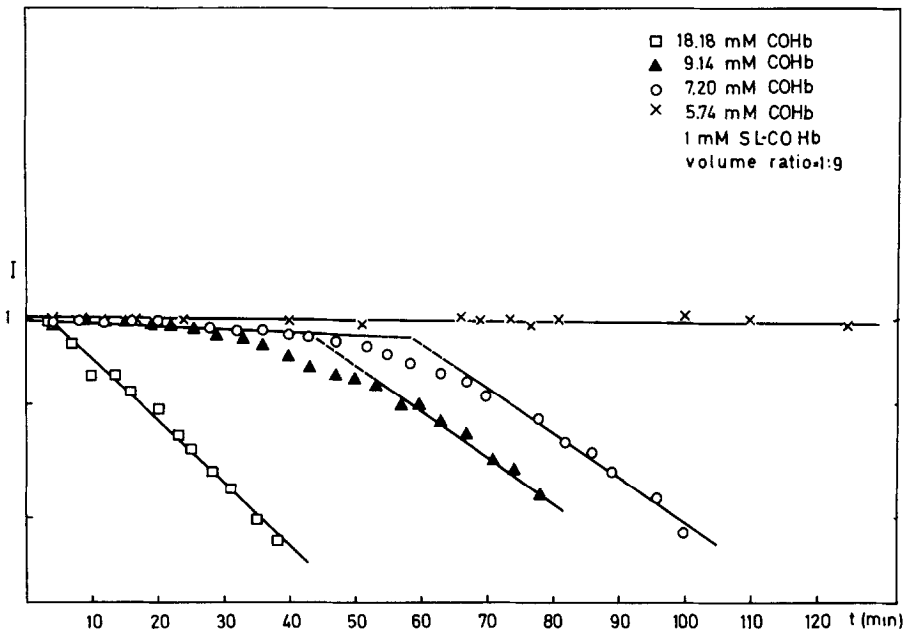

FIGURE 4. Dependence of the ESR-signal decrease with time of maleimide spin-labeled CO-hemoglobin upon unlabeled CO-hemoglobin concentrations in 0.1 M phosphate buffer at pH=7.0.

then centrifuged at low speed (1000 RPM). Centrifugation of the sample did not reduce the ESR signal intensity.

The disappearance of the ESR signal can therefore be associated with the process resulting in a special arrangement of labeled molecules, characterized by different paramagnetic behavior. The formation of radical pairs seems to be a possible explanation (1).

The association of labeled hamoglobin molecules might be due to a certain property of labeled hemoglobin molecules. Fig. 3 shows the effect of the number of spin-labeled hemoglobin molecules added in equal volumes to solutions of unlabeled hemoglobin. The volumes of labeled and unlabeled hemoglobin solutions were 0.02 Ml and 0.2 ml, respectively, and the concentrations of labeled hemoglobin were 2.5mM/l per heme and 5mM/l per heme. There is practically no difference in functions describing the ESR-signal decrease with time. The phenomenon of ESR-signal decrease was not observed in solu-

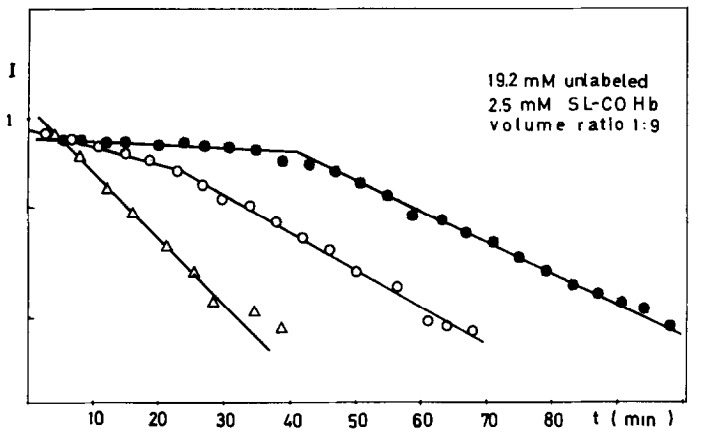

FIGURE 5. The effect of the mechanical force (vortex) applied to the sample at the moment when spin-labeled CO-hemoglobin was added to an unlabeled CO-hemoglobin solution. ESR-signal decrease with time of acetamide spin-labeled CO-hemoglobin in 0.1 M NaCl+TRIS buffer at pH=7.4: without vortexing (triangles), vortexing was applied for 5 seconds (open circles), vortexing was applied for 40 seconds (full circles).

tions of spin-labeled hemoglobin up to concentrations of 24mM/l per heme, in which all hemoglobin molecules were spin-labeled. Fig. 4 represents the ESR-signal decrease as a function of time for various concentrations of unlabeled hemoglobin solutions. Equal amounts of spin-labeled hemoglobin (concentration 1mM/l per heme) in equal volumes (10% of the total volume) were added to equal volumes of unlabeled hemoglobin of various concentrations. Addition of the spin-labeled hemoglobin to a concentration of 5.7mM/l per heme of unlabeled hemoglobin had no effect on the ESR spectra. The signal remained constant in time. Addition of an equal amount of spin-labeled hemoglobin to a concentration higher than that mentioned above resulted in a gradual decrease of the ESR signal. The time required for complete disappearance of the ESR signal depends on the concentration of unlabeled hemoglobin. It decreases with increasing concentration of unlabeled hemoglobin. The decrease of the ESR signal may be represented by two intersecting straight lines with different slopes. The results described indicate that the phenomenom of ESR-signal decrease is

a function of unlabeled-hemoglobin and not of labeled-hemoglobin concentrations. This fact indicates that the "bulk" of unlabeled hemoglobin has some influence on the formation of radical pairs of spin-labeled hemoglobin. Fig. 5 shows the effect of the mechanical force. Vortexing was applied to the sample at the moment when spin-labeled hemoglobin was added to a dense unlabeled hemoglobin solution. The speed of vortexing was kept constant and the time was controlled. The decrease of the ESR signal depends on the duration of vortexing.

DISCUSSION - The association of spin-labeled hemoglobin molecules in radical pairs, i.e., the phenomenon of ESR-signal decrease, might be due to a certain property of spin-labeled molecules. Contrary to this assumption, the ESR-signal decrease was not dependent on the concentration of labeled hemoglobin (Fig. 3). This phenomenon was not observed in the hemoglobin solutions of high concentrations in which all hemoglobin molecules were spin-labeled. These results suggest that the phenomenon of radical-pair formation of spin-labeled hemoglobin molecules is not due to a certain property of spin-labeled hemoglobin.

There is a critical concentration ((5.7 c 6.3)mM/l per heme) of unlabeled hemoglobin at which the ESR-signal decrease starts to appear (Fig. 4). The external disturbance induced by a mechanical force (vortex) causes disruption of radical pairs (Fig. 2) and also influences the formation of radical pairs when applied before addition of spin-labeled hemoglobin to an unlabeled hemoglobin solution of high concentration (Fig. 5). The results presented in Figs. 4 and 5 suggest that the association of spin-labeled hemoglobin molecules in radical pairs is due to the property of the unlabeled hemoglobin solution that some kind of long-range order characterizes unlabeled hemoglobin solutions of concentrations higher than the critical one. Spin-labeled hemoglobin molecules induce perturbation in dense unlabeled hemoglobin solutions, i.e., they behave as impurities. They are brought together by the collective behavior of unlabeled hemoglobin molecules. Arrangement in radical pairs diminishes the perturbation induced by spin-labeled molecules.

Spin-labeled hemoglobin is present in small amount in a

mixture with unlabeled hemoglobin. For the ESR signal to disappear, the majority of labeled molecules must be associated in radical pairs. Such association must, therefore, be very specific; otherwise, labeled molecules would remain distributed as monoradicals throughout the bulk of unlabeled hemoglobin solution. Two arrangements of spin-labeled molecules can be visualized: a pair of mixed tetramers consisting of two spin-labeled dimers in contact or a ring of spin-labeled tetramers in contact. Both arrangements lead to complete disappearance of the ESR signal.

We conclude that the arrangement of spin-labeled hemoglobin molecules in radical pairs (1) reflects a certain property of the unlabeled hemoglobin solution to which spin-labeled hemoglobin was added. The presence of the critical concentration suggests that a phase transition between dense and normal solutions may be assumed. Dense solutions may be characterized by a long-range order among hemoglobin molecules.

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