STRUCTURAL CHARACTERIZATION OF HEMOGLOBIN OF LIOPHIS MILIARIS

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INTRODUCTION

Hemoproteins have been attracting the attention of many investigators from different areas for many years. Since the determination of the three-dimensional structure by X-ray methods for sperm whale myoglobin, for human and horse hemoglobins, the possibility of applying different biophysical techniques for the studies of hemoproteins was considerably opened. More recently the research related to these macromolecules has been directed to the understanding of the fundamental process of electron transfer in biological systems on the one hand and to the study of the relation structure-function on the other. In the case of structure-function relation comparative studies with hemoproteins play an important role. Using homologous proteins it is possible, in principle, to see what are the relevant aminoacid residues in the protein structure and function. In this respect the existence of a great variety of hemoglobins has given a considerable relevance to this protein. One of the techniques that have been used successfully to study hemoglobin structure is the spin labeling.

In the present study an attempt was made to characterize the hemoglobin of an aquatic snake, Liophis Miliaris. It has been recently shown to be a tetrameric hemoglobin(1) and the aminoacid analysis showed the presence of 10 SH-groups per tetramer(1). In recent years also some consideration was given on the possible role of SH-groups in the hemoglobin oxygenation so a characterization of the SH-groups is made both through their titration and also through ESR spectroscopy with a maleimide spin label.

EXPERIMENTAL

- Preparation of the solution of Hemoglobin of Liophis Miliaris (Hb L.M.):

The blood is colected from the heart of the snakes in NaCl 1.8% (W/V) and 1mM EDTA, pH 7.0. This high concentration of NaCl is used since the erythrocyte membranes of L.M. are very fragile and hemolize easily. After centrifugation in 5000 rpm for 5 min, the erythrocytes are separated from plasma and washed for 3 times with the same solution as above. The hemolysis is done through exposure of erythrocytes to 3 volumes of 1mM EDTA, pH 9.4, followed by centrifugation in 10.000 rpm during 5 minutes.

The Hb solution is cromatographed in a column of Sephadex G-25 followed by one of Dowex 1-X_8 with acetate buffer.

The concentration of the Hb solution was determined at 541 nm in a Shimadzu UV-VIS spectrophotometer and was generally 0.1-0.5 mM.

- Spin labeling of the Hb L.M.:

The reaction of the Hb with 2,2,5,5-tetramethyl-pirrolidine-1--oxyl (MAL) was performed generally overnight in three different ratios [MAL]/Hb: 4:1, 6:1 and 8:1 at T=4°C.

The unreacted label was removed by filtration in small Sephadex G-25 columns.

- Spectra of the spin labeled Hb L.M.:

X-band ESR measurements were performed on a E-9 Varian spectrometer. The field scan was 100 G, the microwave power 10mW, the modulation amplitude 1G and the spectra were recorded at room temperature (25°C).

- Determination of the number of labels bound to Hb L.M.:

This estimation was done through a

comparison of the ESR spectrum of denatured protein in a solution of 2M NaOH (the ESR spectrum becomes characteristic of free label) with a standard calibration curve for a nitroxide (TANOL) in solution under the same instrumental conditions.

- Titration of the -SH groups of Hb L.M.:

It was performed by the method of Boyer(2) measuring the absorbance at 250 nm upon addition of p--chloromercuribenzoate to Hb solutions, using a Shimadzu spectrophotometer.

- Kinetics of maleimide spin labeling of Hb L.M.:

Experiments were made monitoring the decrease of the free signal in solution as a function of time at a constant temperature (25°C) at a fixed magnetic field (M_I = -1 component) as well as recording the whole spectrum at fixed time intervals (5 min).

- Effect of copper(II) on the ESR signal of the label in the spin labeled Hb L.M.:

Typical hemoglobin concentrations were 0.1-0,2 mM. Copper was added directly to the spin-labeled hemoglobin solution in an ESR flat cell as small aliquots from $CuCl_2$ solution.

RESULTS AND DISCUSSION

Spin labeling with maleimide shows a composite spectrum with a restricted and mobile components and a hyperfine splitting of 32 G for the immobilized label.

Control experiments with sodium cyanate to block NH_2-terminal and with N-ethylmaleimide to block the SH-groups showed that the maleimide spin label is bound effectively to SH-groups. Fig.1 shows the ESR spectra for Hb L.M. labeled under different conditions. From the estimate of the number of bound spin labels, it is observed that for most of the experiments three-four maleimide labels are bound to Hb L.M. This number increases with the ratio of label to protein used in the labeling reaction. A maximum of six bound labels was observed for 8 MAL: 1 Hb L.M. reaction ratio. The proportion of immobilized and mobile components seems to be dependent upon the temperature of the reaction. At lower temperature (4°C) greater amount of immobilized label is obtained.

In order to compare the amount of

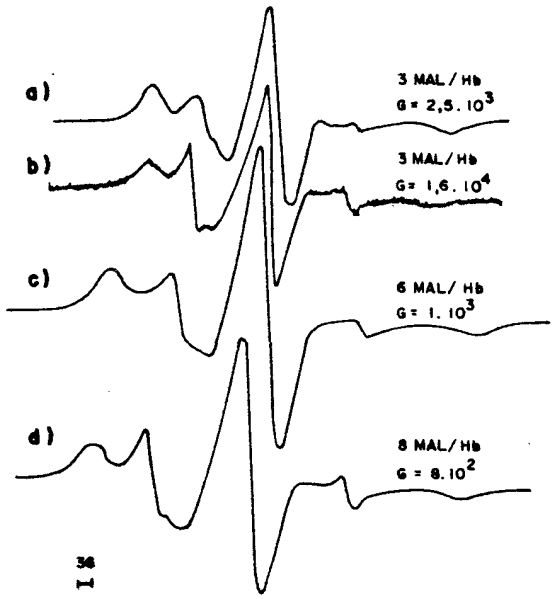

Fig.1 - ESR spectra for Hb L.M. spin labeled with maleimide: a) native Hb L.M. and ratio of MAL/Hb 3.0, b) Hb L.M. pretreated with N-ethyl-maleimide and labeled with MAL/Hb ratio of 3.0, c) native Hb L.M. labeled with MAL/Hb ratio of 6.0, d) native Hb L.M. labeled with MAL/Hb ratio of 8.0. Spectra measured at 9.15 GHz, 10 mW, modulation amplitude 1.0 gauss, 25°C.

bound label with the available titratable SH-groups titrations were performed both for native and spin labeled Hb L.M. Fig.2 shows typical results for this titration. It is seen that the native Hb L.M. has a greater number of titratable SH-groups than the spin labeled protein again indicative of binding of maleimide spin label to SH-groups. It has also been noticed that the number of titratable SH-groups is reduced upon aging. It is possible that aging promotes the oxidation of SH-groups even in the presence of EDTA. The maximum number of SH-groups titratable for fresh Hb is six; the modification with maleimide spin label in a ratio 4:1 lead to a reduction of this number to half its value which agrees with the number of MAL bound to Hb L.M. estimated as described above. In order to obtain more information about the binding of maleimide spin label to Hb L.M. some kinetic

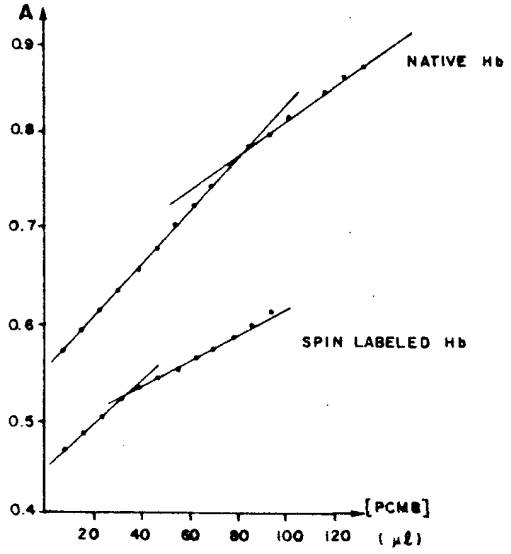

Fig.2 - Titration of SH-groups of Hb L.M. with PCMB: native Hb L.M. and Hb L.M. spin labeled with maleimide at a ratio MAL/Hb equal 4.0. Addition of PCMB aliquots were done to a Hb solution and measurements done at 250 nm.

experiments were performed monitoring the binding through the decrease of free signal as a function of time. Fig.3 shows the spectra obtained in such experiment where the ratio of MAL to Hb L.M. was 2.5. It is clearly demonstrated that most of the spin label is bound within a relatively short time (around one hour). This suggests the existence of 2-3 very reactive SH-groups. Increasing the ratio of MAL to Hb L.M. two-three fold shows the presence of slowly reacting SH-groups since in this case a considerable amount of free label remains in solution. Fig.4 shows the results of a typical kinetic experiment for a ratio of MAL/Hb L.M. of five where the decrease in intensity of the $M_I = -1$ component of free label is monitored as a function of time. The kinetics is biphasic with characteristic rates of $1.8 \times 10^{-3} min^{-1}$ for the slow phase and $10.8 \times 10^{-2} min^{-1}$ for the rapid phase. Besides that from the intensities extrapolated for time $t = 0$ it is obtained that 30% of the signal corresponds to the rapid phase which is then correlated with 1.5 bound spin labels. This is again indicative that 2 SH-groups are very reactive towards the maleimide spin label. It is also interesting that for the lower MAL/Hb ratio of 2.5 a biphasic kinetics is observed. In this case, however, the kinetic constants are different, $k_{rapid} = 2.10^{-1} min^{-1}$ and $k_{slow} = 2.10^{-2} min^{-1}$ and the bound labels are practically equally distributed among the rapid and slow process. This seems to indicate that for Hb L.M. there are in fact two different classes of reactive SH-groups.

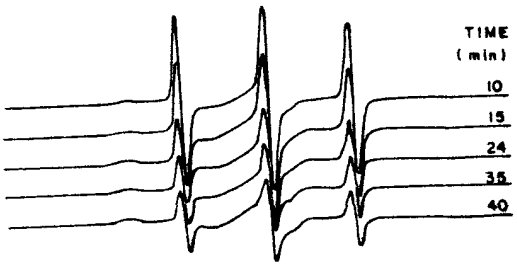

Fig.3 - ESR spectra for maleimide spin label reacting with Hb L.M. as a function of time from the start of the reaction. Microwave frequency 9.15 GHz, 10 mW, modulation amplitude 0.5 gauss, ratio of MAL/Hb 2.5 and Hb concentration 0.5 mM.

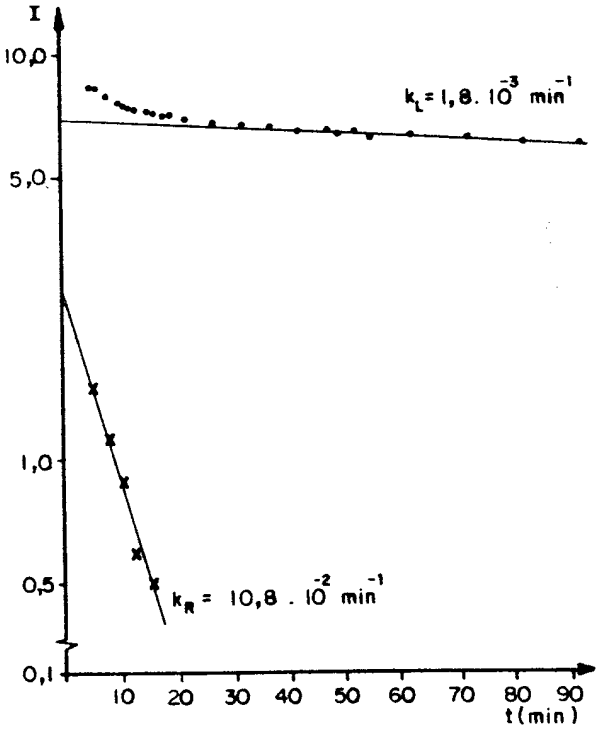

Fig.4 - Decrease of intensity of the $M_I = -1$ component for the free spin label as a function of time for a ratio of MAL/Hb equal 5.

In order to further characterize the SH-groups of Hb L.M. and to try to classify these groups experiments were

performed to monitor the effect of Cu(II) ions on the ESR spectra of spin labeled Hb L.M. It has been shown previously for human hemoglobin that a strong magnetic interaction takes place between Cu(II) and a maleimide spin label bound to β-93 SH-group (3,4). This is due to the fact that copper ion binds to a high affinity site in human Hb which is in close proximity to the SH-group. In human Hb the addition of 2 moles of Cu(II) per mole Hb reduces the intensity of the signal of the maleimide spin label to near 10% of its original value. Fig.5 shows the results of addition of Cu(II) to spin labeled Hb L.M.

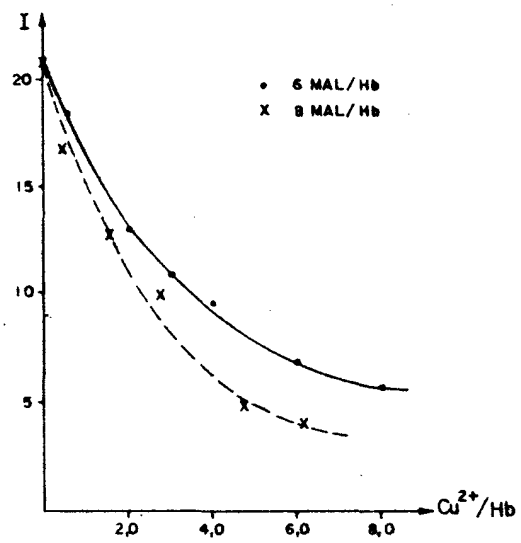

Fig.5 - Titration of a spin labeled solution of Hb L.M. with copper ions. The intensity of the ESR signal of spin labeled Hb L.M. (M_I = +1 low field component) is plotted as a function of Cu^{2+}/Hb ratio. Two different ratios of MAL/Hb were used and are indicated in the figure.

As can be seen from this figure the addition of two moles of Cu(II) per mole Hb L.M. a reduction of around 50% of the signal amplitude takes place independently of the fact that there are four or six maleimide molecules bound to Hb L.M. The addition of another 2 moles of Cu(II) reveals a different behaviour as a function of the number of MAL bound to the protein: when there are four MAL bound the second pair of Cu(II) induces only a small change in the amplitude of the spin label signal, while in the case of six MAL bound a significant decrease in amplitude is observed for the spin label signal. This result suggests that it is quite probable that the first pair of maleimide spin labels bound to Hb L.M. is bound to β-93 SH-groups which are also present in this Hb and that the copper ions also bind to a high affinity copper site (as in human Hb) interacting strongly with the spin label. This interaction is responsible for 50% reduction of the spin label signal intensity. The second pair of SH-groups is relatively insensitive to copper ion addition to Hb L.M.

Finally the third pair of SH-groups titratable do interact with copper ions leading to further reduction of the spin label signal intensity.

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