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TEMPERATURE DEPENDENCE OF ESR SPECTRA OF SPIN-LABELLED MET-HEMOGLOBIN

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The maleimide spin-label, firmly fixed to the protein, was used to study conformation changes of various met-hemoglobin derivatives. The temperature dependence of the rotational correlation time shows a distinct conformation change in aquomet-hemoglobin at about 25°C. The other met-hemoglobin derivatives studied (fluoro-, cyano-, aquomet- complexed with inositol hexaphosphate) and carbonmonoxy-hemoglobin exhibit no conformation changes in the temperature range from 0–50°C.

1. Introduction

Spin labelling has proved to be very powerful in providing information on the protein conformation in the immediate vicinity of the bound label [1]. Because of the complexity of the paramagnetic resonance spectra of a nitroxide attached to a protein, studies have been mostly qualitative in nature and no measurements of temperature dependence have been performed.

The paramagnetic resonance spectrum of a nitroxide spin label depends on its rotational motion [1]. If one chooses the label that undergoes no rotational motion relative to the protein molecule, the resonance spectrum will describe the molecular tumbling of the protein itself, and its rotational correlation time can be deduced. Recently, McCalley and coworkers [2] have presented a theoretical analysis of the paramagnetic resonance line shapes for the slow-motion realm, and have described a method for measuring the protein rotational correlation time in solution. The rotational correlation time depends on the temperature and viscosity of the solution [3]. This fact makes it possible to study temperature-dependent changes in the protein conformation by using a spin label tightly bound to the protein. Such a spin label would reflect the rotational tumbling of the protein molecule and give information on the nature of its nearest neighbourhood.

Although the hemoglobin molecule has extensively been studied in terms of its structure and function, details relating these two aspects at the molecular level are not fully understood. The functional response of this molecule to the binding of effectors (allosteric) outside the active site is assumed to be based on the thermal equilibrium of different tertiary and quaternary structures (conformational states) [4]. Thus, temperature seems to be an important parameter. Atanasov has shown that temperature plays a considerable role in determining the conformation of myoglobin in solution. By using various methods, including a nonspecific binding of maleimide spin labels, Atanasov has demonstrated the existence of two myoglobin conformers in a temperature-dependent equilibrium, with different oxygen affinities [5]. It is well known that in some met-hemoglobins, components of high and low iron spin states coexist in thermal equilibrium and that this equilibrium depends on the ligand bound at the sixth coordination site of the heme iron [6,7]. The external factors, such as organic phosphates, inositol hexaphosphate (IHP), can change this equilibrium, changing at the same time the equi-

librium of the quaternary structures [6,7]. In order to study these quaternary effects by a spin label, its ESR spectra must be shown to reflect properly such structural changes. A great deal of effort has been put into this using nitroxide spin labels at β-93 cysteine [8]. This particular amino-acid is near the α_1/β_2-interface but it does not participate directly in the α_1/β_2-contact. Although it follows the proximal histidine (β-92) in the primary sequence, its R′ protrudes 100° away from histidine β-92 [9]. In the present work, the spin label was attached to the same site with the aim of checking its dependence on the quaternary and/or tertiary conformational characteristics of the hemoglobin molecule, as a function of temperature. The appropriate controls were various ligand forms of hemoglobin with different spin states "locked" in a given quaternary conformation.

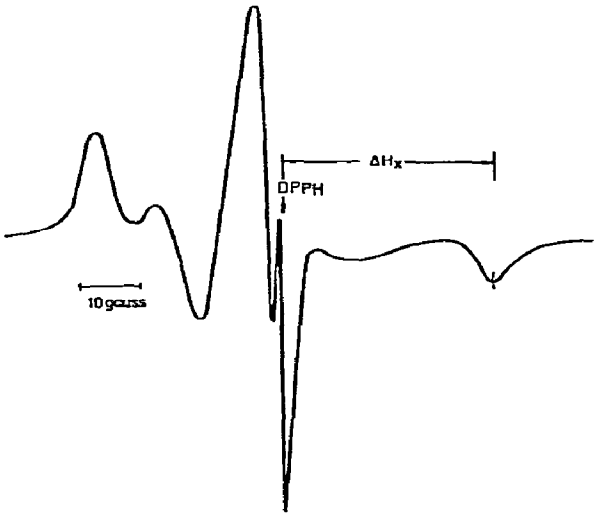

Fig. 1. ESR spectrum of maleimide spin-labelled aquomet-Hb, taken at 20°C. $\Delta H_x = H(T) - H(\text{DPPH})$ denotes the measured parameter.

2. Materials and methods

Hemoglobin samples were prepared from freshly drawn human blood following the procedure of Cameron and George [10]. No further purification of the major (> 98%) HbA component was made. Other liganded forms were obtained from oxy-Hb by (a) oxidation into met-Hb with $K_3Fe(CH)_6$ and subsequent dialysis, or (b) flushing with CO for carbonyl-hemoglobin; (c) cyanide- and (d) fluoridebound met-Hb were made by addition of a few grains of solid KCN and 1 M NaF to a final concentration of 0.1 M NaF. The reaction with inositol hexaphosphate (IHP) was performed following the description given by Perutz [6]. Hemoglobin was spin-labelled with maleimide (SYVA) at pH = 6.8 in a phosphate buffer at 10°C [2]. The reaction was terminated after 12 hours and followed by sephadex G-25 chromatography. Phosphates were removed in 0.1 M NaCl+Tris buffer at pH = 7.4 after spin labelling. The hemoglobin concentrations were about 5×10^{-4} M, as estimated spectrophotometrically [4]. ESR spectra were taken with a Varian E-3 spectrometer.

From the nitroxide ESR spectrum of the labelled Hb it is evident that the maleimide label undergoes little or no rotational motion relative to the tumbling protein molecule. In that case the label ESR spectrum reflects the motion of the whole molecule, which, in turn, is determined by the viscosity and temperature of the solution. From the label spectrum the rotational correlation time of the hemoglobin molecule can be derived from a quantitative relationship between the positions of the outer hyperfine extrema of the ESR pattern and the rotational correlation time [2]. The rotational correlation time for oxy-hemoglobin thus derived [2] was in agreement with the values obtained by other methods. The calculated shift of the high-field maximum relative to its position at a correlation time equal to infinity, $\Delta H(\tau_2)$, is proportional to $\tau_2^{-2/3}$ for a wide range of correlation times [11]. Bearing in mind that τ_2 is proportional to η/T and that the viscosity (η) of the water solution changes monotonically and only slightly in the temperature range from 0–50°C [12], the plot of the inward shift of the high-field hyperfine signal, ΔH, as a function of temperature, should be an exponential, continuous and monotonic function if the protein conformation does not change with temperature. Any change of conformation may result in changes of the shape and/or size of the protein and therefore may alter the rotational correlation time.

In our experiments, the temperature dependence of the high-field maximum in the ESR spectrum was measured relative to the standard DPPH line (ΔH_x in fig. 1). A sealed capillary tube with DPPH was fasten-

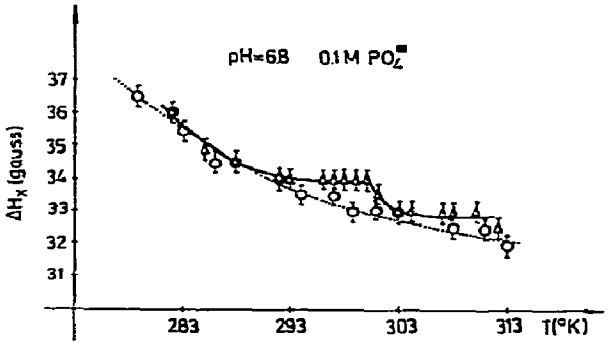

Fig. 2. The $\Delta H_x(T)$ plots for aquomet-Hb (triangles) and fluoromet-Hb (circles).

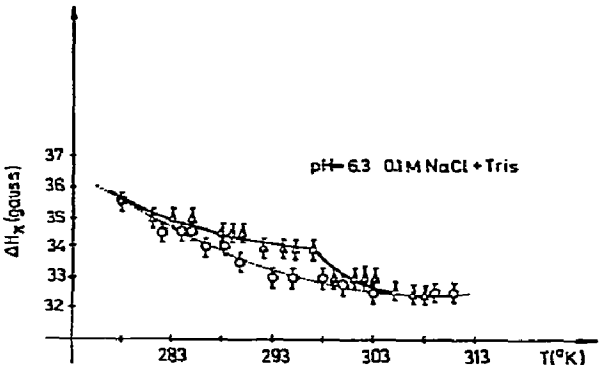

Fig. 4. The $\Delta H_x(T)$ plots for aquomet-Hb (triangles) and the same sample complexed with IHP (circles).

ed on the outer wall of the low-temperature aqueous-solution cell inserted in the ESR spectrometer. The temperature was maintained by a Varian variable-temperature controller and monitored by a thermocouple located above the sample.

3. Results

The $\Delta H_x(T)$ curves for aquomet-hemoglobin as compared with other met-hemoglobins are shown in figs. 2, 3 and 4. The three curves for aquomet-hemoglobin displayed in these figures come from three independent preparations. For cyanomet- and fluoromet-hemoglobin derivatives, $\Delta H_x(T)$ is a continuous and monotonic function, although differing in slope for the two hemoglobins. On the contrary, for aquomet-hemoglobin an apparent break point on the curve is observed at about 25–27°C. If, however, aquomet-hemoglobin is complexed with IHP (fig. 4), $\Delta H_x(T)$ is also a continuous and monotonic function of temperature and the curve is similar in shape to that of fluoromet-hemoglobin. For carbonmonoxy-hemoglobin, the curve $\Delta H_x(T)$ is similar in shape and behaviour to the curves for fluormomet and cyanomet derivatives; however, the numerical values are significantly different (fig. 5).

4. Discussion

We have observed a temperature-dependent change in the protein conformation of aquomet-hemoglobin; no similar change is present in other hemoglobin derivatives: fluoromet-, cyanomet- and carbonmonoxy-hemoglobin, or in the complex of aquomet-hemoglobin

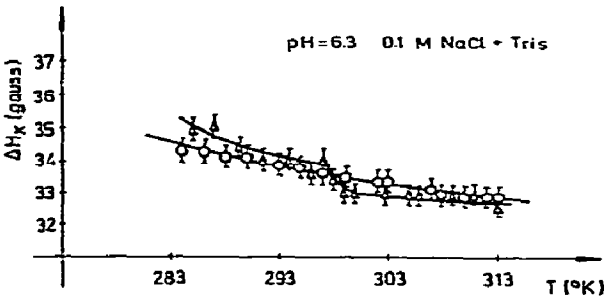

Fig. 3. The $\Delta H_x(T)$ plots for aquomet-Hb (triangles) and cyanomet-Hb (circles).

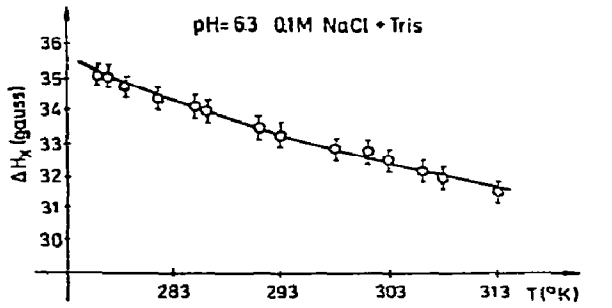

Fig. 5. The $\Delta H_x(T)$ for carbonmonoxy-Hb.

with inositol hexaphosphate. To evaluate the possible cause of the observed phenomenon, we shall consider the properties of the examined hemoglobin derivatives [4]. In met-hemoglobin derivatives, the ferric ion can exist in the low-spin state, like in the cyanomet form (pure low-spin state) or in the high-spin state, like in the fluoromet-Hb (nearly pure high-spin state). In aquomet-Hb, a mixture of spin states in thermal equilibrium is present. This derivative is also a mixture of two chemical species with a pH-dependent equilibrium. The species differ in the state of the water molecule bound at the sixth coordination site of the heme iron, H_2O(bound) + H_2O (solvent) $\rightleftarrows$ H_3O^+ (solvent) + OH^- (bound), the equilibrium being shifted to the left at pH 6–7. The spin-state equilibrium in aquomet-hemoglobin can be changed to the pure high-spin state by addition of organic phosphates (IHP), which is associated with the conversion into a tense (T) quaternary structure [7].

The observed temperature-dependent change of the protein conformation in aquomet-hemoglobin (see figs. 2, 3 and 4) appears to be related to the spin-state equilibrium, because it is absent when the quaternary structure is locked in the T-state, thereby forcing the iron ions into the high-spin state. The similarity of the curves for fluoromet-Hb (high spin) (fig. 2) and that of aquomet-Hb + IHP (fig. 4) corroborates our conclusion. As the spin state of aquomet-Hb–IHP complex is associated with the T-quaternary structure [7], the similarity of the curves $\Delta H_x(T)$ for aquomet-Hb–IHP complex and fluoromet-hemoglobin suggests that the fluoromet derivative is also in the T-quaternary state.

We conclude that the conformational change in the protein moiety observed in aquomet-hemoglobin should be related to the property of the ferric ion to form equilibrium between the low- and high-spin states. That makes this derivative so distinctly different from the others examined. This means that the temperature-induced shift in the spin-state equilibrium results eventually in a switch-over of the conformation of the protein moiety, or vice versa, the two effects being probably inseparable.

Recently, the study of heme-spin-labelled hemoglobin has also shown that the iron environment is different in fluoromet- and aquomet-hemoglobins [13], which is consistent with our findings. Our results show that carbonmonoxy-hemoglobin and aquomet-hemoglobin do not have identical quaternary structures. A similar distinction of aquomet-hemoglobin from oxy-hemoglobin [14] and from carbonmonoxy-hemoglobin [15] has been observed by heme-spin labelling and α_1/β_2-interface labelling, respectively. This point is discussed by McConnell, Deal and Ogata [15] in relation to X-ray studies of the two hemoglobin forms.

Two independent sets of measurements from two preparations at pH 6.3 in 0.1 M NaCl+Tris agree very well in the temperature of the break-point (297 K). In one of them (fig. 4), addition of IHP eliminated the break-point, while the set (fig. 2) obtained in the presence of a rather strong concentration of inorganic phosphates does show the break-point, but at a higher temperature (300 K). As the pH of the latter solution was 6.8, while that in the phosphate-free case (fig. 4) was 6.3, the shift of the break-point temperature is probably due to this difference, ΔpH = 0.5, and not to the different salts of the two solutions. Such a pH-effect would not be unexpected, but more experimental results are required for final elucidation.

Finally, some attention should be paid to the differences in the $\Delta H_x(T)$ curves (figs. 2, 3 and 4). The molecular weight and size of various met-hemoglobins are practically identical and no difference in τ_2 is expected [2]. Some differences, although not significant, may originate from the different protomer association–dissociation equilibrium at the concentrations used in our study [4]. We believe that the observed differences in the slopes come from the differences in the spin-label environment in different met-hemoglobins. Different local environments bring about different nitrogen couplings and hence different positions of the outer maxima in the nitroxide ESR spectra in the limit of no rotation. Consequently, the temperature-dependent shift $\Delta H_x(T) = H(T) - H_0$ will also depend on the local spin-label environment.

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