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TEMPERATURE-DEPENDENT CONFORMATION CHANGE IN SPIN-LABELED HEMOGLOBIN

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Hemoglobin was spin labeled at β-93(F9)-cysteine with *N*-oxyl-2,2,6,6-tetramethylpiperidinylmaleimide. The inward shift of the high-field hyperfine line (ΔH_+) position in the ESR spectra of the spin label was measured as a function of temperature. One can expect that an abrupt change in the microenvironment around the tightly bound spin label will be reflected in the function $\Delta H_+(T)$ as a discontinuity (break point). This was shown for aquo-, azido-, nitro- and oxyhemoglobin derivatives. The presented results suggest that the microenvironment around the tightly bound spin label in those methemoglobin derivatives that exhibit the mixed-spin state of the heme iron is prone to an abrupt change above a certain ligand-specific temperature. The change in microenvironment of the spin label is probably due to a temperature-dependent change in the tertiary structure of the protein.

1. Introduction

The essential part of the mechanistic model proposed by Perutz [1–3] for cooperative oxygenation is the interrelation of heme-iron spin states and the tertiary and quaternary hemoglobin structure. Most of the research related to this point has been performed at room temperature using different techniques. There is, however, ample evidence for the temperature dependence of iron spin states in some hemoglobin derivatives [4–8]. The question arises as to whether temperature-induced spin-state alterations are interrelated with hemoglobin structural readjustments, and, if so, at what structural level.

In this paper we use the method developed originally by McConnell and co-workers [9,10] for determining the rotational correlation time of macromolecules with a tightly bound spin label. In this method, the inward shift of the high-field hyperfine line position in the ESR spectrum is a function of temperature (T) and viscosity (η), i.e., $\Delta H \propto (T/\eta)^{2/3}$ [10]. If an abrupt change in the microenvironment around the tightly bound spin label occurs at some temperature, it will be reflected as a discontinuity (break point) in the $\Delta H(T)$ function. This was shown for maleimide spin-labeled aquohemoglobin [11].

In this paper we extend the measurements to a number of hemoglobin derivatives at various pH values of the hemoglobin solutions. The change in the microenvironment around the tightly bound spin label at a particular temperature was found in methemoglobin derivatives: H_2OHb, NO_2Hb, N_3Hb and O_2Hb. The temperature required for the change to occur was ligand specific. Oxyhemoglobin, although being a diamagnetic molecule with the iron in the ferro state, underwent a similar temperature-dependent conformational change at high pH of the hemoglobin solution.

Abbreviations: Hb, hemoglobin; DPPH, α,α'-diphenyl-β-picrylhydrazyl.

2. Materials and methods

Hemoglobin samples were prepared from freshly drawn human blood following the procedure of Cameron and George [12]. The major (98%) HbA

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component was not purified any further. Methemoglobin was obtained from oxyhemoglobin by oxidation with $K_3Fe(CN)_6$ and subsequent extensive dialysis. Azide and cyanide derivatives were prepared by the addition of solid NaN_3 and KCN to aquohemoglobin at the appropriate pH in 0.1 M NaCl, 0.05 M Tris. Nitrohemoglobin was prepared from oxyhemoglobin with 1 M $NaNO_2$ and subsequent purification on a Sephadex G-25 column.

Hemoglobin was spin labeled with maleimide (Syva, Palo Alto, CA) in 0.1 M NaCl, 0.05 M Tris at 10°C [9]. The reaction was terminated after 36 h. The sample was chromatographed on the Sephadex G-25 column at pH 7.6 to remove phosphates as well as unreacted spin label. The samples were then dialyzed to various pH values. The hemoglobin concentrations were determined spectrophotometrically [13] to be about 0.25×10^{-5} M.

ESR spectra were taken with a Varian E-3 spectrometer. The temperature dependence of the high-field hyperfine line ($M_I = -1$) position in the ESR spectrum was measured relative to the standard DPPH line, $H_{-1} - H_{DPPH} = \Delta H_x$ (fig. 1). A sealed capillary tube containing DPPH was fastened 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 at the same position in all measurements.

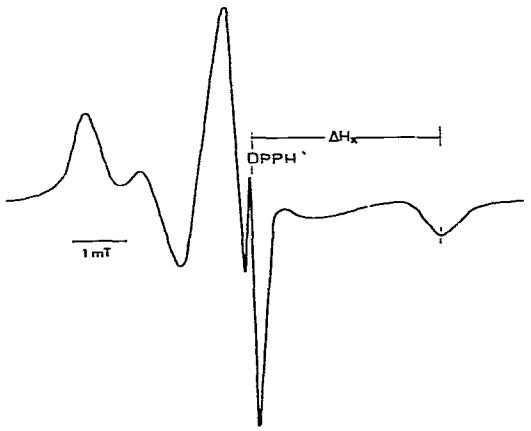

Fig. 1. ESR spectrum of maleimide spin-labeled aquohemoglobin. The spectrum was taken at 20°C.

3. Results

Fig. 2 shows $\Delta H_x(T)$ curves for aquohemoglobin, cyanohemoglobin, azidohemoglobin and nitrohemoglobin in 0.1 M NaCl, 0.05 M Tris. The curve for cyanohemoglobin is a continuous and monotonic function while the curve for aquohemoglobin shows a break point at about 25°C. The $\Delta H_x(T)$ curves taken for aquohemoglobin at pH 6.3 [11], 6.8 (fig. 2a), 7.3 (fig. 2b) and 7.6 (fig. 2c) coincide within experimental error. The break point observed at 25°C does not depend on the solution pH between pH 6.3 and 7.6. At pH 8.2 break points were observed at 25 and 35°C (fig. 2d), while at pH 9.0 a break point was observed only at 35°C (fig. 2c, open circles). At the latter pH value,

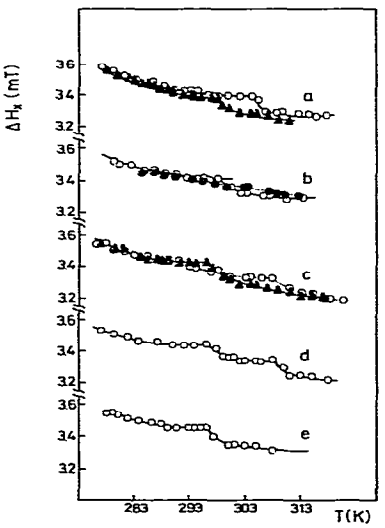

Fig. 2. (a) Plot of $\Delta H_x(T)$ for aquohemoglobin (▲) and for azidohemoglobin (○) at pH 6.8. (b) Plot of $\Delta H_x(T)$ for aquohemoglobin (○) and for cyanohemoglobin (●) at pH 7.3. (c) Plot of $\Delta H_x(T)$ for aquohemoglobin at pH 7.6 (▲) and for hydroxyhemoglobin at pH 9.0 (○). (d) Plot of $\Delta H_x(T)$ for aquohemoglobin at pH 8.2. Two break points can be observed: one at 25°C as in the acid H_2O form and the other at 35°C as in the alkaline OH^- form. (e) Plot of $\Delta H_x(T)$ for nitrohemoglobin at pH 7.6.

the heme iron ligand is predominantly OH^-.

When aquohemoglobin was converted into azidohemoglobin, the break point was observed at 30°C (fig. 2a) and was independent of pH between pH 6.3 and 9.0.

Fig. 2e presents the $\Delta H_x(T)$ curve for nitrohemoglobin with a break point at 23°C.

The break points of $\Delta H_x(T)$ curves were observed in H_2O, OH^-, N_3 and NO_2 methemoglobin derivatives. The curves for fluorohemoglobin, aquohemoglobin with inositol hexaphosphate [11], and for cyanohemoglobin (fig. 2) were all monotonic functions of the temperature. A similar temperature dependence was observed for carbonmonoxyhemoglobin with a zero-spin divalent heme iron (fig. 3c).

Fig. 3a and b shows the curves for the other Fe^{2+} zero-spin form, oxyhemoglobin, at different pH values in 0.1 M NaCl, 0.05 M Tris. The break point of the $\Delta H_x(T)$ curve at 23°C can be observed at pH 9.0 (fig. 3a). At lower pH, i.e., at pH 6.8 and 7.1, the break points are uncertain, but the curves differ in slope.

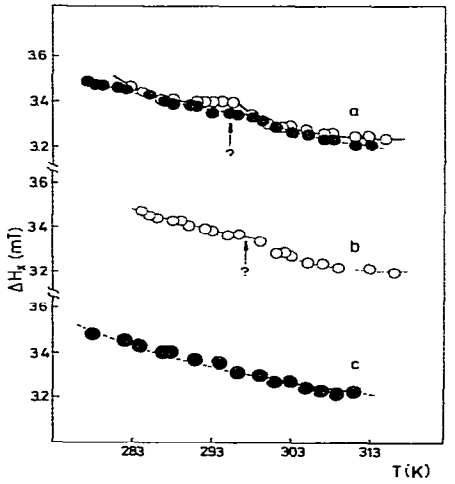

Fig. 3. (a) Plots of $\Delta H_x(T)$ for oxyhemoglobin at two different pH values of hemoglobin solutions: at pH 6.3 (●) and at pH 9.0 (○), where the break point at 23°C was determined. (b) Plot of $\Delta H_x(T)$ for oxyhemoglobin at pH 7.1. The break point is uncertain. (c) Plot of $\Delta H_x(T)$ for carbonmonoxyhemoglobin at pH 9.0.

4. Discussion

The maleimide spin label is bound to hemoglobin at the β-93(F9)-cysteine position, i.e., near the α_1/β_2 interface along which the movements of the subunits take place in a quaternary conformation change [1,2]. However, the aquohemoglobin structure is intermediate at this position between the structure of oxy- and deoxyhemoglobin [2,14], so that β-93-cysteine fluctuates with a high degree of freedom of movement between the position in the solvent and another position within the 'pocket'.

Ohnishi et al. [15] and Moffat [16] have shown, and our spectra (fig. 1) are in agreement with their observation, that in oxyhemoglobin, carbonmonoxyhemoglobin and methemoglobin the maleimide spin label is actually immobilized within the protein pocket. In other words, the maleimide-labeled aquohemoglobin is frozen in a relaxed (R) quaternary conformation state [16]. On the other hand, Moffat has shown that in maleimide-labeled carbonmonoxyhemoglobin the protein structure around the hemes in both types of subunits (α and β) is perturbed by the label [16]. It can, therefore, be expected that structural alterations in the vicinity of the hemes induced by a change in temperature may affect the ESR spectra of the spin label.

Using the saturation-transfer technique, Johnson [17] showed that maleimide spin label immobilized in carbonmonoxyhemoglobin exhibits motional fluctuations (librational motion) whose amplitude and frequency depend on the solution conditions. In precipitated hemoglobin, these motional fluctuations were not detectable even at 40°C. Fluctuations in the spin-label motion were ascribed to fluctuations in the protein conformation that affect the spin-label-binding site. Such fluctuations are obviously too slow to be detected in the linear response in our measurements.

The observed monotonic dependence of the position of the high-field maximum on temperature, i.e., the slope of the function $\Delta H_x(T)$ in fig. 3c, for COHb, is due to the temperature dependence of the rigid-limit spectral parameters [11,18]. Hence, the observed temperature breaks of the hyperfine splitting in some hemoglobin forms may be due only to the protein conformation change in

the microenvironment around the spin label (fig. 2).

The blocked position of the spin label within the β-subunit of hemoglobin and rather small changes in the $\Delta H_x(T)$ curves at the critical temperature probably reflect a conformational readjustment within the β-chain itself. (Although much less probable, the influence of alterations in the quaternary structure at the gliding surface between α_1- and β_2-chains cannot be excluded in the native unlabeled molecules.)

The transition temperature (t_{tr}) is ligand specific (table 1). The magnetic moment of Fe^{3+} in aquohemoglobin is constant up to pH 7.6 but is lowered on further titration with $pK_{H_2O \rightarrow OH^-} = 8.05$, being interrelated with the change of the iron ligand from H_2O to OH^- [4,13]. In the alkaline region the equilibrium is shifted towards hydroxyhemoglobin, to which the break point at the higher temperature (35°C) at pH 9 (fig. 2d) is ascribed. While this discontinuity appears at different temperatures for the acid (25°C) and alkaline (35°C) forms of methemoglobin, the slopes of the $\Delta H_x(T)$ curves for the two forms are alike.

Oxyhemoglobin is a diamagnetic species. For this derivative a transition temperature of 23°C was established at pH 9. At pH 6.3 (fig. 3a) and pH 7.1 (fig. 3b) the break points are not certain. However, as the slopes of the $\Delta H_x(T)$ curves differ for each pH value, it is likely that the microenvironment of oxyhemoglobin around the spin label reflects the structural alterations induced by the surface charge rearrangement on varying pH. In contrast, the curves for carbonmonoxyhemoglobin, another diamagnetic hemoglobin derivative, are a pH-insensitive and monotonic functions of temperature at pH 6.4 [11] and 9.0 (fig. 3c). This finding suggests that the CO derivative is structurally different from oxyhemoglobin, which corroborates a number of observations [19–21].

Also, no pH dependence either of $\Delta H_x(T)$ slopes or of t_{tr} was observed for mixed-spin-state methemoglobin derivatives. The present observations point out again (for an earlier survey see ref. 22) that particular care should be exercised in delineating the interdependence of various contributions in the hemoglobin cooperative mechanism. There seems to be no doubt that the protein inlet towards the met-heme in hemoglobin is under control of the protein-surface charge distribution in the physiological Bohr-effect pH region [23]. The lack of pH dependence in the present measurements indicates that such effects are not transferred to the microenvironment of the spin label.

No break points for spin-state 'frozen' derivatives (cyanohemoglobin and fluorohemoglobin [11]) were observed. As t_{tr} is attributed to a 'structural transition' of the spin-label microenvironment, the fact that it is observed only with mixed-spin-state methemoglobin derivatives points strongly towards the interrelationship between the temperature-controlled spin state of the heme and the protein structural dynamics. Thus, it seems that the thermal equilibrium of the iron spin states

Table 1

Transition temperature for various heme-ligand forms of hemoglobin

The slope of the $\Delta H_x(T)$ curve depends on the pH of the hemoglobin solution.

Ligand	Iron spin	pH	t_{tr} (°C)	$\Delta H_x(T)$
H_2O	5/2 (spin mixed)	6.3–7.6	25	–
OH^-	5/2 (spin mixed)	9.0	35	–
NO_2	5/2 (spin mixed)	6.3–7.6	23	–
N_3	1/2 (spin mixed)	6.3–7.6	30	–
F^-	5/2 (spin only)	6.3–9.0	–	–
CN	1/2 (spin only)	6.3–9.0	–	–
CO	0	6.3–9.0	–	–
O_2	0	9.0	23	yes

is, in some as yet unknown way, coupled with the protein structure.

We have reported here temperature-dependent changes in the globin structure in hemoglobin derivatives with the mixed-spin states and with thermal equilibrium of the iron spin states. One may envisage that the observed ligand-specific temperature discontinuities are caused by the inability of the low-temperature conformation to accommodate the changes in the heme-protein interaction induced by the enhanced high-spin state of the heme iron. As the spin label attached at β-93-cysteine feels the change in the protein structure, this can be the pathway along which the iron spin-state changes are coupled to the subunit surface.

To conclude, from an experimental point of view, the present observations show that the structural information furnished by a variety of methods should be obtained below and/or above the temperature of transition for those hemoglobin derivatives with mixed iron spin states, for which a temperature-dependent protein conformation change was found.

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