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Crystalline Leghemoglobin

VII. Magnetic and Spectrophotometric Properties of Leghemoglobin and its Derivatives

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The paramagnetic susceptibilities of soya bean leghemoglobin (Lhb) and some of its derivatives have been determined. Acidic and alkaline ferriLhb have molar paramagnetic susceptibilities of 9 500 and 5 200$\times 10^{-6}$ cgs emu at 20°C, respectively. The susceptibilities of the complexes of ferriLhb formed upon addition of cyanide, fluoride and acetate and of ferroLhb show no large deviations from values for other hemoglobins. Difference optical absorption spectra at 8 and 38°C show for both acidic and alkaline ferriLhb characteristic maxima and minima. This indicates that both forms of ferriLhb are temperature dependent equilibria between high- and low-spin states. No such temperature dependent difference spectrum was obtained for the acetate complex of ferriLhb, showing that this complex is a pure high spin form, which is also inferred from its magnetic moment. The electron spin resonance at low temperatures verify that acidic ferriLhb is a mixed spin compound.

Leghemoglobin (Lhb), the hemoglobin(Hb) of the root nodules of leguminous plants, has been found to differ from ordinary Hb in several respects. The molecular weight of Lhb is similar to that of myoglobin (Mb), *i. e.* only one fourth of that of Hb[1]. The affinity for oxygen of ferroLhb is even higher than that of Mb. In fact it is the highest measured for any hemoprotein[2]. This might be due to a partially exposed heme group on the surface of the globin molecule of Lhb[3], whereas the heme groups of Hb and Mb are more shielded by the globin helices. It has been shown that a positive correlation exists between the amount of nitrogen fixed and the concentration of Lhb in the nodules[4,5]. More recently it has been demonstrated by gas chromatography that one molecule of Lhb is able to fix one molecule of nitrogen[6]. This interesting observation might help to explain the biological function of Lhb.

In order to understand the reactions of Lhb it seemed desirable to obtain further information about the mode of binding of the heme iron of Lhb to the globin and to other coordinated ligands. In this paper the magnetic properties of Lhb and some of its derivatives are reported and are correlated to spectroscopic data.

EXPERIMENTAL

Leghemoglobin. The *fast* and *slow* components of crystallized Lhb were prepared chromatographically as described previously[7]. The preparations were checked by electrophoresis as well as by light absorption spectroscopy. The concentration of a preparation was evaluated on the basis of determination of the pyridine hemochrome[8]. For the magnetic measurements it was desirable to use concentrated solutions. However, with the technique applied, dialysis of ammonium sulphate precipitate, it was not possible to obtain higher concentrations than about 1 mM.

Magnetic susceptibility measurements. The apparatus described by Theorell and Ehrenberg[9,10] was used. The instrument was calibrated with a dilute solution of nickel chloride for which the molar susceptibility was assumed to be $4\,434 \times 10^{-6}$ cgs emu at 20°C. The calibration constant was 2.200×10^{-11} volume susceptibility units per scale division (μ). The samples were made anaerobic by rapid evacuation in Thunberg tubes and twice flushing with nitrogen or argon. The pH of each sample was adjusted by addition of small amounts of 1 N NaOH for the alkaline pH values and 1 N HCl and/or buffer for other values. Reduction was obtained by addition of 20 μl 15 % $Na_2S_2O_4$ per ml of sample. The reaction with CO was performed by flushing Thunberg tubes with this gas.

ESR (electron spin resonance) measurements were performed with a Varian V-4500 X-band spectrometer with 100 kc/s magnetic field modulation. Liquid nitrogen and variable temperature accessories were used.

pH measurements were made at 20°C with a Radiometer PHM3 pH-meter which was standardized against phtalate and borate buffers.

Spectrophotometric measurements. A Beckman spectrophotometer model DK2 was used. A special cell holder was constructed in order to study the effect of temperature on the spectra of the different Lhb derivatives. Each cuvette in the holder was provided with a separate well insulated thermospacer. By circulating thermostated water from two thermostates through the thermospacers each cuvette could separately be kept at a desired temperature. The temperature was checked in the cuvettes before the spectra were recorded by means of a thermistor in a bridge device. Phosphate and borate buffers with known temperature dependence were used. Care was taken to adjust the high temperature buffer to the same pH at the high temperature as the pH of the low temperature buffer at the low temperature.

RESULTS AND DISCUSSION

The CO compound of ferroLhb was found to be diamagnetic. The diamagnetic susceptibility per g of protein was calculated to be $-0.597\ (\pm 0.015) \times 10^{-6}$ cgs emu, when the value 0.740 ml/g was used for the partial specific volume[1]. The paramagnetic susceptibilities of other Lhb derivatives have been referred to the diamagnetic level of the CO compound, taking into due consideration the residual orbital paramagnetism of the iron of a compound of this type[11].

The paramagnetic data of the derivatives investigated are shown in Table 1. No significant difference could be detected between the fast and slow components. When calculating the mean value of susceptibility from several determinations with different samples the values were given weights in proportion to the concentrations, because the relative errors within a series are roughly inversely proportional to the concentrations. The effective magnetic moments, μ_{eff}, were calculated under the assumption that Curie-Weiss constant was zero.

A comparison of the data of Table 1 and the predicted μ_{eff}-values for different spin forms[12] shows the following. The cyanide complex of ferriLhb is a pure low-spin form with 1 unpaired electron. The fluoride and acetate complexes of ferriLhb are, or very nearly are, pure high-spin forms with 5 unpaired electrons. The ferroLhb at pH 10.5 is a pure high-spin form with 4 unpaired electrons. All

Table 1. Paramagnetic data of some derivatives of leghemoglobin (Lhb).

Derivative	Valence of iron	Component	Conc. μM	pH		$\chi_{Fe} \times 10^6$ cgs emu	μ_{eff} Bohr magnetons
Acidic form	Fe^{3+}	fast	265	6.10		8 900	
		„	265	6.10		7 600	
		„	1 000	5.70		10 100	
		slow	710	5.80		9 700	
					mean	9 500	4.74
Alkaline form	Fe^{3+}	fast	251	10.75		4 500	
		„	251	10.80		4 500	
		„	960	10.80		5 200	
		slow	701	10.80		5 800	
		„	612	10.80		5 000	
					mean	5 200	3.50
Cyanide complex	Fe^{3+}	slow	601	10.80		2 500	
		„	601	10.80		2 200	
					mean	2 350	2.36
Fluoride complex	Fe^{3+}	fast	333	–		13 600	5.66
Acetate complex	Fe^{3+}	slow	640	5.5		14 000	5.75
Ferro Lhb	Fe^{2+}	„	395	10.5		12 300	5.39

these data are similar to what has been found for the corresponding derivatives of other hemoglobins.

The magnetic moments 4.17 B. m. (Bohr magnetons) of the acidic form and 3.50 B. m. of the alkaline form, however, are both intermediate between the expected moments of the high- and low-spin forms, 5.92 and 1.73–2.8 B. m., respectively. On several occassions it has been discussed that intermediate magnetic properties of Hb derivatives were due to mixtures of forms with different numbers of unpaired electrons[13–16]. Experimental support for this hypothesis was obtained by George *et al.*[16], who studied the temperature dependence of the light absorption and the paramagnetic susceptibility of alkaline ferriMb, and further evidence was obtained by Ehrenberg[10] by means of the ESR technique. The temperature dependence of the susceptibility of alkaline ferriHb indicates the presence of a thermally balanced mixture of two spin forms in this case also[12]. Experiments of the mentioned types were thus indicated for both alkaline and acidic Lhb.

The temperature dependence of the light absorption was investigated by recording the difference spectra of samples kept at two different temperatures (Fig. 1). Curves A show that alkaline ferriLhb at low temperatures has increased absorption in the regions 405–475 mμ and 515–580 mμ with maxima of the increase at 425, 540 and 565 mμ. At high temperatures the absorption is increased in the complementary regions with maxima of the increase at 385, 495 and 595 mμ. This is essentially the same as observed by George *et al.*[16] for alkaline ferriMb and would suggest an increase in the portion of low-spin form as the temperature is decreased.

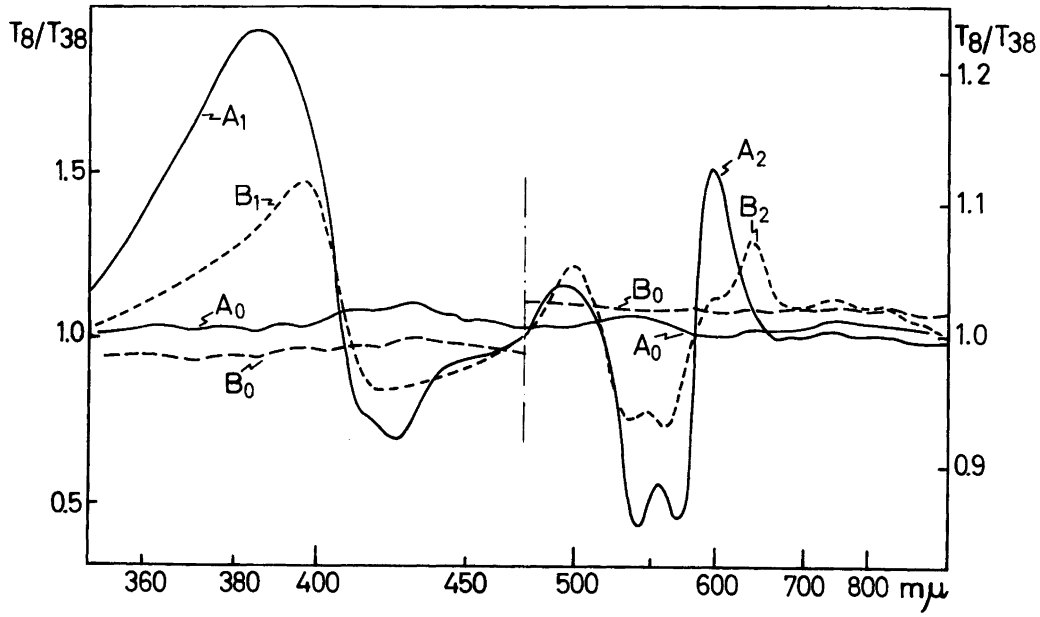

Fig. 1. Difference spectra of ferrileghemoglobin at 8 and 38°C recorded as the transmission ratio T_8/T_{38}. Curve A_1 with ordinate scale to the left, other curves with ordinate scale to the right. ———: glycine buffer pH 10.8, 52.2 μM Lhb, 350–475 mμ (A_1), 475–800 mμ (A_2) and baseline with both samples at room temperature (A_0). – – – –: phosphate buffer pH 5.8, 6.65 μM Lhb at 350—475 mμ (B_1), 66.6 μM Lhb at 475–800 mμ (B_2) and baselines with both samples at room temperature (B_0).

Curves B of Fig. 1 show that the light absorption of acidic Lhb changes in much the same way with temperature as that of alkaline Lhb. The positions of the peaks are, however, slightly shifted. The maximum increase of light absorption at low and high temperatures are at 420, 533 and 558 mμ and at 397, 500 and 635 mμ, respectively. It should be noted that the difference spectra of Fig. 1 are in most respects quite different from those between acidic and alkaline ferriLhb. The size of the changes of the light absorption with temperature suggests that in case of acidic ferriLhb there is a relatively smaller increase in the portion of low-spin form as the temperature is decreased than in case of alkaline ferriLhb.

The light absorption of the acetate complex was investigated in the same way. No temperature effect of the type demonstrated in Fig. 1 was detected. If there was any effect at all it was in the opposite direction. This confirms our conclusion that the acetate complex is a pure high-spin form, which, however, could be dissociated at elevated temperatures.

Frozen solutions of the acetate complex of ferriLhb show the ESR absorption with $g \approx 6$, typical for high-spin heme compounds. This absorption was identical in size and shape to the absorption of acidic ferriMb[10], and both compounds are

classified as essentially pure high-spin forms. Acidic ferriLhb also gives an ESR absorption of the same shape but with reduced intensity. The susceptibility data (Table 1) indicate a high-spin portion of about 60 % for acidic ferriLhb at room temperature. A comparison of the intensities of the ESR absorptions shows that the high-spin portion is only slightly reduced with decreasing temperature and is in the range of 35–50 % at 77°K.

Alkaline ferriLhb has a high-spin portion of about 25 % at room temperature as calculated from the susceptibility (Table 1). The ESR recordings at 77°K revealed only the anisotropic g-values around $g = 2$ typical of low-spin compounds. The concentration of the sample was not high enough to allow a definite conclusion whether any high-spin character remained at 77°K.

The magnetic and spectral data demonstrate unanimously that both acidic and alkaline ferriLhb are thermally balanced mixtures of high- and low-spin forms. The assumption by George *et al.*[16] that alkaline ferriLhb should represent a pure low-spin compound at room temperature has not been confirmed. Acidic ferriLhb has a smaller magnetic moment and more low-spin character than most other "acidic" forms of the hemoproteins investigated so far. In this respect it resembles most closely the intermediate form of the peptic hemopeptide from cytochrome *c*[12,17] and some of the "cytochromoids"[18] which have recently been investigated magnetically[19]. Another common feature of these compounds is that their heme groups are more exposed to the solvent medium than in other related compounds. At the present state of our knowledge it might be permitted to speculate about this feature as a possible common denominator of the increased low-spin character of these compounds.

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