Crystalline Leghemoglobin

VII. Magnetic and Spectrophotometric Properties of Leghemoglobin and its Derivatives

ANDERS EHRENBERG and NILS ELLFOLK

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

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 9500 and 5200 × 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. The affinity for oxygen of ferroLhb is even higher than that of Mb. In fact it is the highest measured for any hemoprotein. This might be due to a partially exposed heme group on the surface of the globin molecule of Lhb, 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. More recently it has been demonstrated by gas chromatography that one molecule of Lhb is able to fix one molecule of nitrogen. 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.

Acta Chem. Scand. 17 (1963) Suppl. 1
EXPERIMENTAL

Hemoglobin. The fast and slow components of crystallized Lbb were prepared chromato-
graphically as described previously. 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. 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 Ehren-
berg was used. The instrument was calibrated with a dilute solution of nickel chloride for
which the molar susceptibility was assumed to be 4.394 × 10−4 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 ob-
tained by addition of 20 µl 15% Na₂S₂O₄ per ml of sample. The reaction with CO was per-
formed 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 tempera-
ture accessories were used.

pH measurements were made at 20°C with a Radiometer PHM3 pH-meter which was
standardized against phthalate 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 Lbb derivatives. Each cuvette in the holder was provided with a separate well
insulated thermospace. By circulating thermostated water from two thermostats through the
thermospacer 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 thermometer 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 ferroLbb was found to be diamagnetic. The diamagnetic
susceptibility per g of protein was calculated to be −0.597 (±0.015) × 10−6 cgs
emu, when the value 0.740 ml/g was used for the partial specific volume. The
paramagnetic susceptibilities of other Lbb 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.

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 determina-
tions 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 shows the following. The cyanide complex of ferriLbb is a pure
low-spin form with 1 unpaired electron. The fluoride and acetate complexes of
ferriLbb are, or very nearly are, pure high-spin forms with 5 unpaired electrons.
The ferroLbb at pH 10.5 is a pure high-spin form with 4 unpaired electrons.

Acta Chem. Scand. 17 (1963) Suppl. 1
Table 1. Paramagnetic data of some derivatives of leghemoglobin (Lhb).

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Valence of iron</th>
<th>Component</th>
<th>Conc. μM</th>
<th>pH</th>
<th>$\chi_{Fe} \times 10^6$ cgs emu</th>
<th>$\mu_{eff}$ Bohr magnetons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic form</td>
<td>Fe³⁺</td>
<td>fast</td>
<td>265</td>
<td>6.10</td>
<td>8 900</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>„</td>
<td>265</td>
<td>6.10</td>
<td>7 600</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>„</td>
<td>1 000</td>
<td>5.70</td>
<td>10 100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>slow</td>
<td>710</td>
<td>5.80</td>
<td>9 700</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mean 9 500</td>
<td>4.74</td>
</tr>
<tr>
<td>Alkaline form</td>
<td>Fe³⁺</td>
<td>fast</td>
<td>251</td>
<td>10.75</td>
<td>4 500</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>„</td>
<td>251</td>
<td>10.80</td>
<td>4 500</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>„</td>
<td>960</td>
<td>10.80</td>
<td>5 200</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>slow</td>
<td>701</td>
<td>10.80</td>
<td>5 800</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>„</td>
<td>612</td>
<td>10.80</td>
<td>5 000</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mean 5 200</td>
<td>3.50</td>
</tr>
<tr>
<td>Cyanide complex</td>
<td>Fe⁴⁺</td>
<td>slow</td>
<td>601</td>
<td>10.80</td>
<td>2 500</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>„</td>
<td>601</td>
<td>10.80</td>
<td>2 200</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mean 2 350</td>
<td>2.36</td>
</tr>
<tr>
<td>Fluoride complex</td>
<td>Fe³⁺</td>
<td>fast</td>
<td>333</td>
<td>–</td>
<td>13 600</td>
<td>5.66</td>
</tr>
<tr>
<td>Acetate complex</td>
<td>Fe³⁺</td>
<td>slow</td>
<td>640</td>
<td>5.5</td>
<td>14 000</td>
<td>5.75</td>
</tr>
<tr>
<td>Ferro Lhb</td>
<td>Fe³⁺</td>
<td>„</td>
<td>395</td>
<td>10.5</td>
<td>12 300</td>
<td>5.39</td>
</tr>
</tbody>
</table>

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 occasions it has been discussed that intermediate magnetic properties of Hb derivatives were due to mixtures of forms with different numbers of unpaired electrons\textsuperscript{13–16}. Experimental support for this hypothesis was obtained by George et al.\textsuperscript{16}, who studied the temperature dependence of the light absorption and the paramagnetic susceptibility of alkaline ferriMb, and further evidence was obtained by Ehrenberg\textsuperscript{16} 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\textsuperscript{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.\textsuperscript{16} for alkaline ferriMb and would suggest an increase in the portion of low-spin form as the temperature is decreased.

*Acta Chem. Scand. 17 (1963) Suppl. 1*
Fig. 1. Difference spectra of ferrileglobin 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 8.8, 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

*Acta Chem. Scand. 17 (1963) Suppl. 1*
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. 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 c12,17 and some of the "cytochromoids"18 which have recently been investigated magnetically16. 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.

Acknowledgements: The investigations have been supported by grants from Statens Medicinska Forskningsråd, Statens Naturvetenskapliga Forskningsråd and by PHS Research Grant A-5895 from the National Institute of Arthritis and Metabolic Diseases, Public Health Service.

REFERENCES


Received April 1, 1963.

Acta Chem. Scand. 17 (1963) Suppl. 1