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Crystalline Leghemoglobin

XII. A Spectrophotometric Study of the Slow Component in the Acid pH Range

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A spectrophotometric study of the slow component of leghemoglobin was carried out in the acid pH range. The pH of 50 % cleavage is 4.26 at an ionic strength of 0.1 and the slope of the line is equal to 1.1. This implies one ionizing group, which was assumed to be the carboxyl group of a carboxyethyl side chain in the heme. The spectra of leghemoglobin were mainly of the low spin type in phosphate and phthalate buffers (μ=0.1) of pH 5.5 and a mixture of high- and low spin types at more neutral pH. The spectra at different pH values differed more in phosphate than in phthalate buffers. The spectra of leghemoglobin in cacodylate buffers indicated the existence of a complex similar to that formed by leghemoglobin and acetate.

The native form of leghemoglobin (Lhb) in root nodules has been shown to be ferrihemochrome.[1] The hemochrome was found to be labile and was transformed easily into a thermally balanced mixture of high- and low spin forms.[2] This has been assumed to indicate a conformational change in the apoprotein, probably one involving a shift of the heme binding groups to a greater distance from the heme iron.

In this investigation the spectral properties of ferriLhb in a mixture of high- and low spin forms were studied in the acid pH range. Specific effects of the various buffers used were also investigated spectrophotometrically.

MATERIALS AND METHODS

The slow component of Lhb was isolated as described previously [3] and stored as a suspension in 85 % ammonium sulphate at −16°C. Before use, the suspended Lhb was centrifuged and dissolved in 0.1 M phosphate buffer of pH 8.0 and the solution dialyzed against deionized water. No noticeable changes were observed to occur in the properties of Lhb during the storage at −16°C.

Chemicals. Sodium dihydrogen phosphate, disodium hydrogen phosphate, glycine (E. Merck AG), and potassium hydrogen phthalate (J. T. Baker Chemical Co.) were

of analytical grade, and cacodylic acid (Fluka AG) was of *purum* grade. Hydrochloric acid, glycine-HCl, and phthalate buffer solutions of $\mu=0.1$ were used when recording the stability curve in the pH range 1.0–6.12. The ionic strengths of the phthalate buffers were adjusted with sodium chloride.

Spectrophotometric measurements. A Beckman Model DK–1A recording spectrophotometer, a Perkin-Elmer Model 137–UV recording spectrophotometer, and a Beckman Model DU–2 spectrophotometer were used in the experiments.

In a typical experiment 0.05 ml of Lhb stock solution (5–10 mg/ml) was added to 2.95 ml of buffer. If not otherwise stated, the absorbances were measured at room temperature (23°C) one minute after mixing.

pH Measurements were performed with a Radiometer Titrator Type TTT 1c. The pH of 0.05 M potassium hydrogen phthalate was taken to be 4.01 at 23°C. The pH values of the Lhb solutions were measured immediately after the spectrophotometric measurements.

RESULTS

Fig. 1 shows the dependence of the absorbance at the Soret maximum on pH. The absorbances were measured at 404 nm one minute after adding the Lhb stock solution into the buffer solutions ($\mu=0.1$). A stability curve was constructed by plotting the absorbance at the Soret maximum against pH (Fig. 2). No further decrease of the Soret band intensity occurred when the pH fell below 2, which indicates that cleavage of the hemin-globin linkage

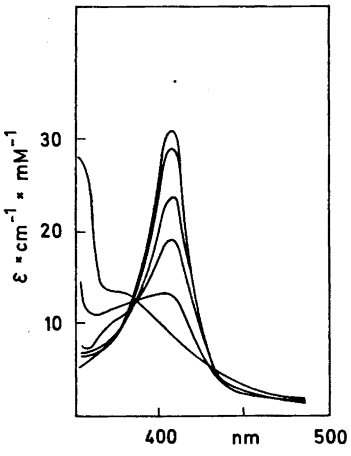

Fig. 1. The effect of pH on the absorption spectrum of ferriLhb in the Soret region (360–500 nm) 1 min after mixing a Lhb stock solution with buffers of varying pH value and $\mu=0.1$ at 23°C. The intensity of the absorption increases with increasing alkalinity. The buffers used were 0.1 N HCl, and phthalate buffers with pH values 3.91, 4.15, 4.39, 5.02, and 6.12. The Lhb concentration was 2.8 μM.

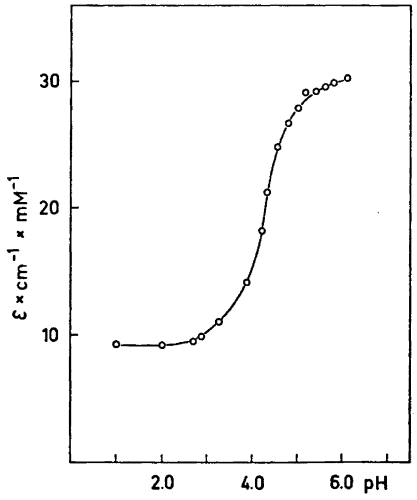

Fig. 2. The pH stability curve of ferriLhb in the acid pH range. The absorbance at 404 nm is plotted against pH. The buffers used were 0.1 N HCl, glycine–HCl, $\mu=0.1$ (pH 2.0–3.29), and phthalate buffers, $\mu=0.1$ (pH 3.91–6.12). The Lhb concentration was 1.4 μM.

had taken place. The pH value at which the hemin-globin linkage is intact without any observable influence of the alkaline form is estimated to be 6.12. (The spectrum was found to be stable at this pH for at least 20 h at 23°C). Assuming that Lhb is completely cleaved at pH 2 and intact at pH 6.12, the pH of 50 % cleavage can be calculated by plotting log $(\varepsilon_{pH\,2}-\varepsilon_{pH\,x})/(\varepsilon_{pH\,x}-\varepsilon_{pH\,6.1})$, where $\varepsilon_{pH\,2}$ is the absorbance of the Lhb solution of pH 2, $\varepsilon_{pH\,x}$ the absorbance of the solution at another pH value, and $\varepsilon_{pH\,6.1}$ the absorbance of the solution at pH 6.12, all at 404 nm, against pH. Two straight lines were obtained which intersected at pH 4.95. The line at pH <4.95 gives a $pH_{50\%}\sim$ 4.26 and a slope of 1.10. In this lower portion of the curve about 60 % of the hemin-protein linkage is cleaved. The slope of the line at pH >4.95 is equal to 0.75. After 20 h at room temperature, the $pH_{50\%}$ was equal to 4.70, and there was no break in the line.

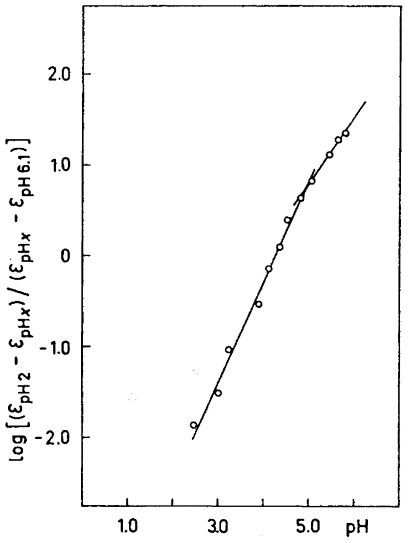

Fig. 3. Graphical determination of pH of 50 % cleavage of the hemin-globin linkage of ferriLhb. The experimental conditions were identical to those in Fig. 2. $\varepsilon_{pH\,2}$ is the absorbance at 404 nm of the Lhb solution at pH 2.0, where the linkage is totally cleaved, ε_{pHx} the absorbance of the Lhb solution at a different pH value, and $\varepsilon_{pH\,6.1}$ the absorbance at pH 6.12, where the linkage is intact. $pH_{50\%}$ is equal to 4.26. The slope of the line is equal to 1.10 at pH values below 4.95 and equal to 0.75 at pH values above 4.95.

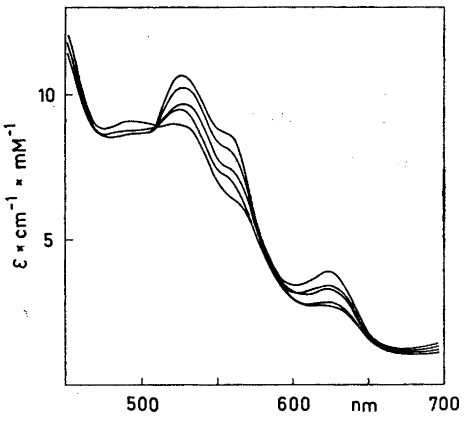

Fig. 4. The effect of pH on the absorption spectrum in the visible region (450—700 nm) of ferriLhb in phosphate buffers of varying pH value and $\mu=0.1$ at 23°C. The absorption spectra show diminishing band intensities with increasing alkalinity in the region 510—582 nm. The pH values were 5.51, 5.68, 5.91, 5.98, and 6.37. The Lhb concentration was 0.05 mM.

During the study, specific effects of different buffers were observed which were studied in detail. Visible spectra of Lhb in phosphate buffers ($\mu=0.1$) at pH 5.5—6.4 are shown in Fig. 4. The absorbances of the solutions increase

with decreasing pH between the isosbestic points at 510 and 582 nm with maxima at 525 and 561 nm. Between 582 and 655 nm, the absorbances decrease, and there is a maximum at 625 nm. No shifts in the positions of the maxima

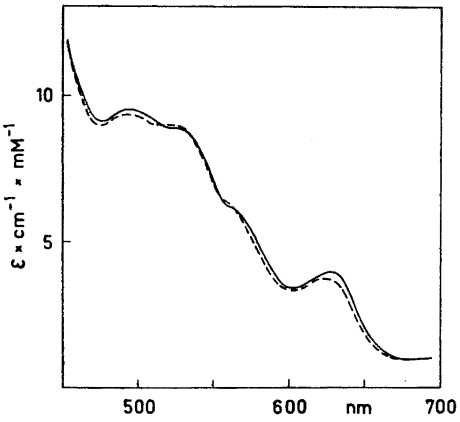

Fig. 5. The effect of pH on the absorption spectrum of ferriLhb in phthalate buffers (μ=0.1) at 23°C. ----- pH 5.30, ——— pH 6.03. The Lhb concentration was 0.05 mM.

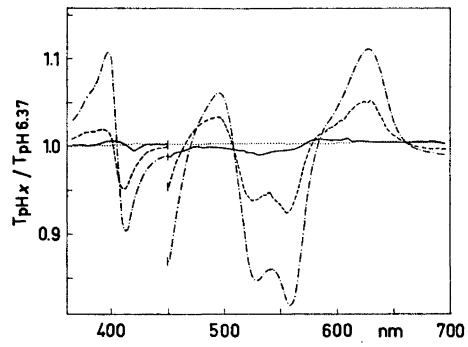

Fig. 6. Transmission ratio $T_{pHx}/T_{pH\ 6.37}$, where pH x denotes the pH value of the solution, as a function of wavelength for ferriLhb in phosphate buffers (μ=0.1) of varying pH value at 23°C. A solution of pH 6.37 was in the reference cuvette. pH x: ——— 6.12, ----- 5.98, ----- 5.68. The dotted line is the baseline obtained when both cuvettes contained the solution of pH 6.37. The Lhb concentrations were 0.05 mM (450—700 nm) and 0.005 mM (360—450 nm).

were observed. At pH 5.5 the spectrum is mainly of the low spin type with a maximum at 525 nm and a shoulder at 560—562 nm. At pH 6.4, the spectrum is that of "acid" Lhb, a mixture of high- and low spin forms.[2] Similar results were obtained in phthalate buffers of pH 5.30 and 6.03 that had the same ionic strength, but the differences were smaller (Fig. 5). Difference spectra of Lhb in phosphate buffers are shown in Fig. 6 and those in phthalate buffers in Fig. 7.

When cacodylate buffers in the same pH range were used, a different spectral pattern was obtained (Fig. 8). The absorbance decreased with decreasing pH at 480—560 nm, and increased with decreasing pH at 560—650 nm. In contrast to the above spectra, a hypsochromic shift of the absorption maximum from 625 to 615 nm with decreasing pH was observed.

DISCUSSION

The pH stability curve in the acid pH range has a pH of 50 % cleavage of 4.26. At pH 2 the heme group is completely displaced as indicated by the disappearance of the Soret maximum (Fig. 1). The curve of the logarithmic

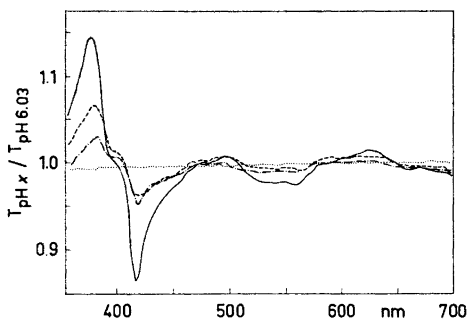

Fig. 7. Transmission ratio $T_{pH\,x}/T_{pH\,6.30}$, where pH x denotes the pH value of the solution, as a function of wavelength for ferriLhb in phthalate buffers ($\mu=0.1$) of varying pH value at 23°C. The solution of pH 6.03 was in the reference cuvette. pH x: ····· 5.95, ----- 5.72, ——— 5.31. The dotted line is the baseline obtained when both cuvettes contained solution of pH 6.03. The Lhb concentration was 0.05 mM.

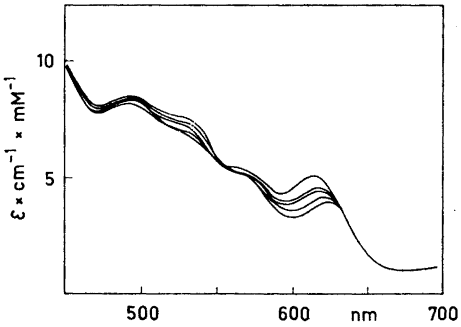

Fig. 8. The effect of pH on the spectrum of ferriLhb in cacodylate buffers ($\mu=0.075$) of different pH values at 23°C. The intensities of the maxima at 558—635 nm decrease with increasing alkalinity. The pH values are: 5.38, 5.59, 5.80, 5.96, 6.20, and 6.36. The last two spectra are superimposed. The Lhb concentration was 0.04 mM.

plot (Fig. 3) has a slope of 1.1, which suggests that one ionizing hydrogen ion is involved in the cleavage of the hemin from the protein. The curve has a break of the same type as found in the titration of myoglobin. This has been ascribed to a heterogeneity of the preparation.[4] The Lhb preparation used in this study was homogeneous, however. Hence a mixture of high- and low spin forms may be responsible for the break in the line. The fact that the break was not observed when measurements were made 20 h after mixing suggests that the Lhb was presumably transformed into the high-spin type.

It has previously been assumed that histidine imidazoles of the apoprotein interact with heme iron in Lhb [1] as in case of sperm whale myoglobin [5,6] and horse hemoglobin.[6,7] Myoglobins from different sources have an ionizing group with pK = 4.7 [8,9] and another with pK between 5.5 and 7.[8-11] Horse hemoglobin has ionizing groups with pK values equal to 5.1—5.3 and 6.3—6.7.[8,12] In both cases the values reported by different laboratories differ slightly depending on the method of measurement used. The ionizing groups in hemoglobin have been assumed to be two heme-binding histidines,[13] whose pK values are modified by the environment. The pK_2 of myoglobin is assumed to relate to ionization of the distal histidine (E 7), but the pK_1 may relate to ionization of a carboxyl group in the side chain of the heme with p$K \cong 4.8$.[14] This pK is in the same range as the $pH_{50\%}$ on the stability curve.[4,15] The $pH_{50\%}$ on the Lhb stability curve is also much lower than expected for the ionization of an imidazole group. The binding of hemin to apoprotein in Lhb is, however, less dependent on the linkage between heme iron and globin than on other groups such as the vinyl and carboxyethyl side chains of the porphyrin nucleus.[16] At least one carboxyl group is required to obtain a stable heme-

globin complex. In sperm whale myoglobin a heme carboxyl group is in contact with a histidine residue (FG 3)[17] forming a salt bridge. For the present, no such bonds are known to occur in Lhb. However, it is probable that protonation of one of the heme carboxyethyl side chains in Lhb may influence the cleavage of the heme group from Lhb.

The two histidine imidazoles binding the heme iron to globin greatly influence the spectral and magnetic properties of hemoglobins. In these hemoproteins a water molecule located between the distal histidine (E 7) and the heme iron [5,6] results in a high-spin complex.[11,18] Native Lhb is of the low-spin type.[1] In phosphate and phthalate buffers of low pH, the spectrum of Lhb is more of the low-spin type than at higher pH (Figs. 4 and 5). This indicates a tighter binding at low pH. A rough estimate of the pK value of the group involved is 5.8. The pK of the imino nitrogen atom in the glyoxaline ring of histidine is 5.97. The imidazole group is a positively charged acid below this pH, and a neutral base above this pH. It seems that in contrast to sperm whale myoglobin [19] the protonated form has a greater affinity for the heme group than the unprotonated form.

Different buffers do not have identical effects on the absorption spectra of Lhb. The effects of phosphate and phthalate buffers differ only in magnitude. However, Lhb spectra in cacodylate buffers are similar to the spectra of fatty acid complexes of Lhb.[20] The absorption maximum at 625 nm shifts to shorter wavelengths with decreasing pH. Undissociated acetic acid has been assumed to be bound to the heme iron.[20] The spectra of Lhb in cacodylate buffers indicate that a similar binding takes place also in these buffers.

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