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Crystalline Leghemoglobin

X. The Ferrihemochrome of Leghemoglobin

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The absorption spectrum of anhydro-ferrileghemoglobin (ferriLhb) in anhydrous glycerol has been measured and found to be that of a ferrihemochrome. A faint absorption observed at 623 nm is assumed to indicate complex formation between the ferric iron of the ferrihemochrome and the alcohol groups of glycerol. The ferrihemochrome was rapidly converted to acidic ferriLhb on addition of water. Acidic ferriLhb could be transformed into ferrihemochrome by exposure to high concentrations of neutral salts like $NaClO_4$ and $MgCl_2$. On dilution, a re-transformation into ferriLhb occurred. The spectral curves representing different concentrations of salt intersect at five isosbestic points, 664, 592, 514, 483, and 412 nm, respectively. Rapidly prepared centrifugates of crushed soya bean nodules showed a ferrihemochrome type of spectrum, which on reduction was transformed into that of ferrohemochrome. Only a low percentage of ferriLhb could be demonstrated in the extract, the predominant form of Lhb being that of ferrihemochrome. Precipitation with ammonium sulfate caused a partial transformation of the ferrihemochrome to acidic ferriLhb. Ferri-(ferro-)hemochrome is concluded to be the native form of Lhb present in the root nodules.

The acidic ferrileghemoglobin (ferriLhb) has recently been shown to be a thermally balanced mixture of high- and low-spin forms, a property rather unusual among the heme proteins.[1] Acidic ferrihemoglobin (ferriHb) and acidic ferrimyoglobin (ferriMb), *e.g.*, are both high-spin compounds. However, dehydration of these two hemin proteins has been found to cause a characteristic change in their spectra to that of a typical low-spin ferrihemochrome.[2–5]

In this investigation the ferrihemochrome of Lhb has been prepared by different dehydration procedures and compared spectroscopically with the Lhb obtained by rapidly extracting crushed soya bean root nodules.

EXPERIMENTAL

Leghemoglobin. The *slow* component of crystalline Lhb was prepared chromatographically as described previously.[6] The preparation was checked by electrophoresis

and spectroscopy. Before lyophilization it was carefully dialyzed against distilled water in order to remove all ammonium sulfate.

Myoglobin. A lyophilized preparation of crystalline myoglobin (Nutritional Biochemicals Co.) was used.

Pyridine hemochrome was determined according to Paul, Theorell, and Åkeson.[7]

Lyophilization. Acidic ferriLhb of pH 5.6 was lyophilized, the final dehydration being performed with a Hg diffusion pump for 6—8 h at a pressure of about 10^{-6} mm Hg at room temperature. The preparation obtained was immediately used for spectroscopic studies.

Anhydrous glycerol. Bidistilled glycerol (Merck) was distilled at high vacuum to remove the bulk of the water. The product was then treated for 30 min with magnesium methylate prepared by activation of magnesium with iodine.[8] The mixture was finally redistilled in a high vacuum with exclusion of moisture. The anhydrous ferriLhb dissolved reasonable well in this solvent. Undissolved material was removed by centrifugation at 45 000 rpm for 30 min in a Spinco L 50 preparative ultracentrifuge.

Neutral salt solutions for preparation of ferrihemochrome of Lhb were made up with $NaClO_4 \cdot H_2O$ (Fluka) and $MgCl_2 \cdot 6H_2O$ (Merck). The solutions were usually prepared immediately before use.

pH Measurements. The pH values of the solutions were measured with a Radiometer PHM 3 pH meter, which was standardized against phthalate and borate buffers.

Spectrophotometric measurements. A Beckman Recording spectrophotometer model DK-1 was used in most experiments.

RESULTS

The absorption spectrum of anhydrous acidic ferriLhb dissolved in anhydrous glycerol is given in Fig. 1, which shows the molecular extinction coefficients from 370 to 700 nm. Anhydrous ferriLhb exhibited a spectrum typical of a ferrihemochrome, with two absorption bands, the α-band at 563 and the β-band at 530 nm, the β-band being much stronger than the α-band.

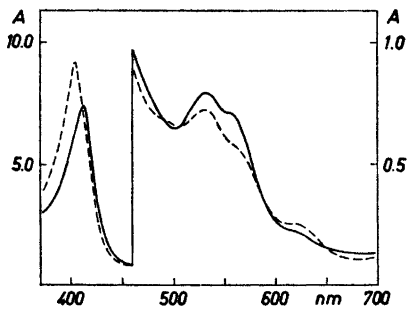

Fig. 1. Absorption spectrum in the visible and ultraviolet (370—700 nm) of anhydro-ferriLhb, pH 5.6, in anhydrous glycerol before (———) and after addition of water (- - - -) at 23°C.

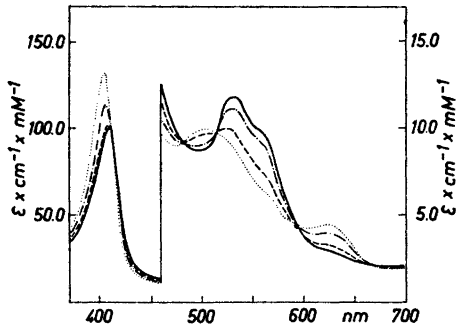

Fig. 2. The effect of concentrated solutions of $NaClO_4$ upon the absorption spectrum of ferriLhb in the visible and ultraviolet (370—700 nm) at pH 5.0 and 23°C. The absorption curves corresponding to the different concentrations of $NaClO_4$ may be distinguished in the spectral region between 514 and 592 nm, where absorption increases, or at the Soret band, where absorption decreases with increasing salt concentration equal to 0, 2.0, 3.0, and 4.0 M $NaClO_4$.

The Soret band was situated at 409 nm. A small band was still present at 623 nm, which was assumed to indicate complex formation between the alcohol groups of glycerol and the ferric iron of Lhb. On addition of water, the spectrum of ferrihemochrome was replaced by that of ferriLhb, which included a characteristic increase of light absorption at 625 and 500 and at the Soret band, with a shift of the maximum to 403 nm. The two absorption curves intersected at 664, 590, 512, 486, and 412 nm, respectively. In general, the ferrihemochrome spectrum of Lhb showed great similarity to that of Mb obtained under identical experimental conditions.

Addition of neutral salts like $NaClO_4$ or $MgCl_2$ to an acidic ferriLhb solution caused a change of the spectrum to that of ferrihemochrome. Fig. 2 shows the molar extinction coefficients from 370 to 700 nm in increasing concentrations of $NaClO_4$ at pH 5.0. The maximum of the α-band was situated at 563 nm, that of the β-band at 530 nm and that of the Soret band at 410 nm. The intensity of the Soret band was gradually decreased with increasing concentration of the neutral salt, with simultaneous increase in the formation of ferrihemochrome. No maximum at 625 nm could be detected in the spectrum of quantitatively transformed salt ferrihemochrome of Lhb. In the visible region, the absorption curves seem to intersect at five isosbestic points, 664, 592, 514, 483, and 412 nm. The salt effect was found to be reversible. By dilution or by dialysis the original acid ferriLhb spectrum was restored followed by an increase of light absorption at the Soret band and a shift of the maximum to 403 nm.

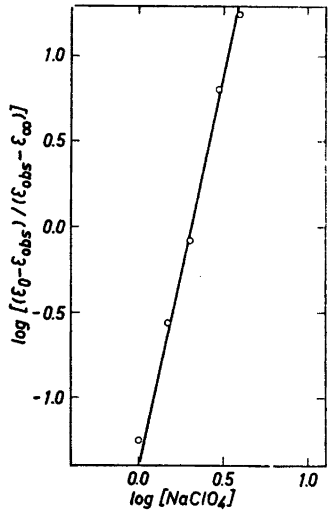

Fig. 3. Graphical determination of $[NaClO_4]_{50\ \%}$ of the transition of acidic ferriLhb into ferrihemochrome of Lhb in the presence of varying concentrations of $NaClO_4$. All solutions had a pH of about 5.0 and temperature of 23°C, $[NaClO_4]_{50\ \%} =$ 2.1 M. The slope of the line equalled 4.6.

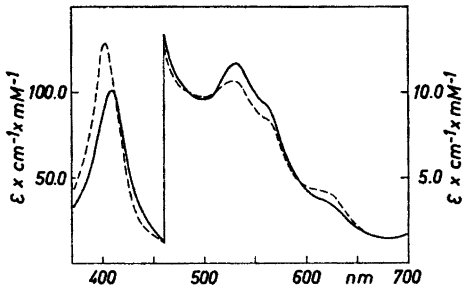

Fig. 4. Qualitative absorption spectrum in the visible and ultraviolet (370—700 nm) of a crude extract of crushed soya bean root nodules in phophate buffer of pH 5.6 (———) and that of the ammonium sulfate precipitate dissolved in the same buffer (- - - -) at 23°C.

Since the change in light absorption was maximal at 405 nm, a series of determinations was made at this wave length at different concentrations of $NaClO_4$ and pH 5.0 On plotting log $[(\varepsilon_0-\varepsilon_{obs})/(\varepsilon_{obs}-\varepsilon_\infty)]$ against log $[NaClO_4]$, a straight line was obtained with $n_{NaClO_4} = 4.6$ and $[NaClO_4]_{50\,\%}$ calculated to be 2.1 M (Fig. 3). This shows that removal of the water molecule bound to the iron in acidic ferriLhb and conversion to ferrihemochrome is a complicated process.

A crude extract of Lhb was prepared from 1 g of freshly picked soya bean nodules by grinding in a Potter-Elvehjem homogenizer in 7 ml of 0.02 M phosphate buffer of pH 5.6 and centrifuging for 15 min at 12 000 rpm at 4°C. The spectrum of the centrifugate shows an α-band at 563, a β-band at 530 nm, and a Soret band at 412 nm (Fig. 4). The identity of the spectrum with that of the ferrihemochrome of Lhb obtained by two different dehydration procedures is obvious. The faint absorption at 625 nm is assumed to indicate traces of acidic ferriLhb, the preparation as a whole consisting to about 95 % of ferrihemochrome. On reduction with dithionite a ferrohemochrome spectrum was obtained. In order to elucidate the extent to which the ferrihemochrome might be converted to ferriLhb during purification procedures like fractionation with ammonium sulfate, the crude extract was dialyzed against a 75 % saturated solution of ammonium sulfate and the precipitate formed dissolved in phosphate buffer of pH 5.6. The absorption spectrum obtained showed an increase of intensity at 625 and 500 nm and at the Soret band, with a shift of the spectral maximum from 412 to 403 nm. Similar spectral changes were found to take place with anhydro-Lhb as well as salt ferrihemochrome of Lhb on addition of water and the formation of the aquo compound. As a whole, the preparation still exhibited a spectrum of mainly ferrihemochrome character.

DISCUSSION

The spectrum of anhydro-ferriLhb in anhydrous glycerol was found to be that of a ferrihemochrome. An identical ferrihemochrome spectrum was obtained by adding neutral salts to a solution of acidic ferriLhb. The reversibility of the transformation of both these ferrihemochromes was shown by their retransformation to acidic ferriLhb upon addition of water. This indicates that the anhydrous complex structure of the iron in the two ferrihemochrome compounds is identical.

It has previously been demonstrated that acidic ferriLhb exists in a thermally balanced mixture of high- and low-spin forms.[1] An increase of temperature was found to increase the high-spin form of the compound with a corresponding increase of light absorption at 397, 500, and 635 nm. A decrease of temperature increased the low-spin character of the compound with a mutual increase in the light absorption at 420, 533, and 558 nm. A temperature decrease evidently caused a change in the conformation of the molecule of Lhb that resulted in a decrease of the high-spin form. A similar type of conformational change was found to take place by subjecting ferriLhb to dehydration. The removal of water from the protein as well as the prosthetic group evidently caused shrinkage of the molecule, which resulted in a decrease

in the distance between the ferric iron of the hemin ring and the protein causing the formation of the ferrihemochrome compound.

The ferrihemochrome formation of Lhb can not be correlated with an exact picture of the Lhb molecule. However, the spectral similarity between the ferrihemochromes of Lhb and Mb justifies the assumption that the binding of hemin to the apoprotein in the two proteins is identical. Crystallographic data on ferriMb show that the fifth of the six coordination positions of the iron is occupied by His-93 and that the sixth position on the far side of the hemin is left for a water molecule connected to the distal His-64 by hydrogen bonds.[9-10] In ferrihemochrome there is no water molecule attached to the hemin iron and a bond is probably established between the iron and the distal histidine. It is assumed that in the ferrihemochrome of Lhb both the fifth and sixth coordination positions are also occupied by the ring nitrogen of histidine, in particular as both the main components of Lhb have been found to contain two histidine residues per molecule.[11]

The ferrihemochrome spectrum of Lhb obtained by different procedures of dehydration was found to be identical with that of a crude Lhb preparation obtained by rapid centrifugation of crushed root nodules of soya bean. After precipitation with ammonium sulfate, partial transformation into acidic ferriLhb was observed. In no case could more than traces of ferriLhb be found in the soya bean nodules. It is evident that the native form of Lhb which can be demonstrated in the root nodules is ferri-(or ferro-)hemochrome of Lhb. The ferrihemochrome was found to be very unstable and readily transformed into ferriLhb, a fact which suggests that some of the discrepant results obtained on Lhb [12-14] may be attributable to differences in the purification procedures used which might have yielded preparations representing different stages of transformation of ferrihemochrome into ferriLhb.

Some additional properties of ferrihemochrome of Lhb will be published in a separate paper.

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