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THE SEPARATION AND PROPERTIES OF LOW-SPIN (HAEMOCHROME) AND NATIVE, HIGH-SPIN FORMS OF LEGHAEMOGLOBIN FROM SOYBEAN NODULE EXTRACTS

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SUMMARY

1. About 95% of the ferroleghaemoglobin (Lb^{2+}) in N_2-fixing soybean root nodules is extracted and purified as autoxidized ferrileghaemoglobin (Lb^{3+}) at pH 5.5. As the pH of extraction is raised less autoxidation occurs, and at pH 7.9, 70% more of the leghaemoglobin (Lb) is isolated as ferrous oxyleghaemoglobin ($Lb^{2+}O_2$).

2. Although this $Lb^{2+}O_2$, when oxidized or deoxygenated, has the properties of a high-spin haemoprotein, autoxidized Lb^{3+}, as present in crude extracts, is separable by chromatography on 'DEAE-Sephadex' in 13 mM sodium acetate (pH 5.2) into high-spin and low-spin (ferrihaemochrome)forms.

3. This low-spin (ferrihaemochrome) form of Lb^{3+} probably arises by combination of autoxidized, high-spin Lb^{3+} with a low-molecular-weight ligand during extraction or initial purification. High-spin Lb^{2+} (or $Lb^{2+}O_2$) appears to be the natural, functional form of Lb.

INTRODUCTION

To understand the function of Lb in symbiotic N_2 fixation[3] its native form must be known, and recent work from this laboratory[4] shows that Lb^{2+} is present in soybean root nodules as a Mb-like (high-spin) structure able to undergo reversible oxygenation. When extracted as high-spin Lb^{2+} under very mild conditions (pH 6.4, low salt strength) and gently oxidized, the Lb^{3+} formed also has the spectroscopic properties of a predominantly high-spin haemoprotein (ref. 4, *cf.* refs. 5 and 6). These results are in apparent conflict with those of ELLFOLK AND SIEVERS[7] who established that Lb^{3+} was extracted at pH 5.6 as a ferrihaemochrome (low-spin) structure and suggested that the native form of Lb in root nodules might be a ferrohaemochrome. It is im-

Abbreviations and definitions: abbreviations for myoglobin (Mb) or haemoglobin (Hb) forms follow those for Lb given in the SUMMARY. "Lb*a*, Lb*c* and Lb*d*" refer, respectively, to ELLFOLK's[1] major components of Lb, separable by chromatography on DEAE-cellulose. "Low-spin" refers to ferro- or ferrihaemoproteins having a covalent (haemochrome) structure with, respectively, 0 and 1 unpaired iron electrons, and "high-spin" refers to ferro- or ferrihaemoproteins with Mb-like structure and 4 or 5 unpaired iron electrons; in this paper these spin states are identified from the type-spectra assigned to them by BRILL AND WILLIAMS[2]. HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; MES, 2(*N*-morpholino)ethane sulphonic acid.

portant to resolve this problem, as haemochomes (*e.g.* cytochromes) generally function as electron rather than O_2 carriers.

This paper shows that during extraction from the nodule, native Lb^{2+} can be converted to a mixture of high-spin and low-spin ferrihaemoproteins, somewhat analogous to the high- and low-spin forms of peroxidase, which are readily separable by ordinary physical procedures[8]. These moderately stable and separable high- and low-spin structures are not identical with the high- and low-spin states which constitute a thermal-equilibrium mixture in conventionally isolated Lb^{3+} (ref. 6) and in Mb^{3+} (refs. 2 and 9).

A comparison of properties of the separated Lb^{3+} forms suggests that the high-spin form is the one directly related to native Lb^{2+}, although the ability of this Lb^{3+} to make a low-spin ligand complex during extraction could perhaps be of physiological significance.

MATERIALS AND METHODS

Leghaemoglobin extraction

All extraction and purification procedures were performed at 0–4°. Soybean root nodules[10] were homogenized in 0.1 M buffer in a Waring 'Blendor', or Servall 'Omnimixer' fitted with a gas-tight chamber and tubes allowing equilibration with CO or other gas. Lb in the 10000 × *g* supernatants from these breis was precipitated between 0.55 and 0.8 $(NH_4)_2SO_4$ saturation. This Lb was desalted by brief dialysis, then passage through columns of Sephadex equilibrated with the desired buffer.

Chromatography

Molecular-weight separations were achieved on columns of Sephadex gel types G-10, G-15 or G-25 (Pharmacia, Uppsala). K_{av} (partition coefficient between liquid phase and gel phase) was calculated according to the manufacturer's instructions[11]. Ion-exchange chromatography was performed on DEAE-Sephadex, Type A-50 (Pharmacia) or DEAE-cellulose 'microgranular type DE-52' (Whatman). In calculating R_F, the volume occupied by the equivalent dry weight of swollen Sephadex A-50 was ignored. After chromatography, dilute fractions were reconcentrated in a Diaflo Model 50 ultrafiltration cell fitted with a type UM10 (10000 mol. wt. exclusion) membrane, both supplied by the Amicon Corp., Lexington, Mass., U.S.A.

Spectrophotometry

General procedures are described elsewhere[10]. Total Lb was measured as pyridine haemochrome[4,12]. The per cent oxidation of Lb to Lb^{3+} was calculated from the relative heights of the 562-nm absorption peak (due to $Lb^{2+}CO$) in CO-equilibrated solutions of Lb measured in three conditions: as prepared; after reduction by $Na_2S_2O_4$ (0%); after oxidation by $K_3Fe(CN)_6$ (100%). Oxidation of $Lb^{2+}CO$ to Lb^{3+} was achieved by simultaneous illumination (2 × 500-W Photoflood lamps) and titration with $K_3Fe(CN)_6$ of a thin film of Lb solution in an evacuated (< 1 mm Hg) Buchner flask at 0°. In this way < 3 equiv of $K_3Fe(CN)_6$ were needed, compared with > 100 equiv for a similar titration in the dark at 1 atm.

Reagents

Phosphate buffers were prepared by mixing Na_2HPO_4 and KH_2PO_4 to stated phosphate molarity and pH; sodium acetate or sodium succinate buffers are also referred to as total anion molarity. Low ionic strength, zwitterion buffers were prepared using *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) and 2(*N*-morpholino)ethanesulphonic acid (MES) obtained from Calbiochem, Los Angeles. All buffer pH values (glass electrode) and conductivities were measured at 20°. *N*-Ethylmaleimide was obtained from Calbiochem, Los Angeles and iodoacetamide from the Sigma Chemical Co., St. Louis.

RESULTS

Predominance of low- or high-spin forms of Lb according to extraction conditions

During chromatographic separation of the major Lb components, Lba^{3+} and Lbc^{3+}, on columns of DEAE-cellulose equilibrated with 13 mM acetate (I = 0.01, pH 5.2), ELLFOLK[1] noted that the appearance of these Lb bands was not uniform, but showed a colour change from red-brown to green; the green colouration was due to the selective ability of the 'tail' of each band to combine with undissociated acetic acid in the buffer[1,5]. APPLEBY[13] confirmed this observation, and since the red 'front' fractions showed ferrohaemochrome spectra when reduced (C. A. APPLEBY, unpublished observations) they were discarded as 'modified Lb' from preparations used for determination of $Lb^{2+}O_2$ equilibria[13].

In the present work, attempts were made to separate these red 'front' and green 'tail' forms of both Lba^{3+} and Lbc^{3+} on 'Microgranular' DEAE-cellulose (Whatman, DE-52) in 0.013 M acetate (pH 5.2) to see if they represent respectively the ferrihaemochrome reported by ELLFOLK AND SIEVERS[7] and the Mb-like Lb^{3+} described by APPLEBY[4]. Only partial resolution was achieved, but it was sufficient to allow a semi-quantitative estimation of the proportions of red and green Lb^{3+}, and of $Lb^{2+}O_2$, extracted by various procedures.

Nodule extraction (see MATERIALS AND METHODS) in 0.1 M acetate or phosphate (pH 5.5) and $(NH_4)_2SO_4$ fractionation at pH 5.5 in air, with final dialysis against H_2O or pH 5.5 buffer yielded about 5 % $Lb^{2+}O_2$ and 95 % Lb^{3+}. About two-thirds of this Lb^{3+} were in the red, low-spin ferrihaemochrome form and one third in the green, high-spin form (*cf.* Fig. 3B). The small amount of $Lb^{2+}O_2$ which survived autoxidation did not separate into analogous high- and low-spin forms. However, nodule extraction and $(NH_4)_2SO_4$ fractionation in 0.1 M Tris (pH 7.9) confirmed THOROGOOD'S[14] observation that most Lb is extracted as $Lb^{2+}O_2$ at alkaline pH; less than 30 % oxidation to Lb^{2+} occurred. When chromatographed at pH 5.2 this Lb^{2+} ran only as the green, high-spin form.

Extraction and $(NH_4)_2SO_4$ fractionation in air in the presence of 0.1 M phosphate at physiological[4] pH (6.4–6.8) gave approximately equal amounts of $Lb^{2+}O_2$, red 'front' Lb^{3+} and green 'tail' Lb^{3+}. If the extraction was made in 0.01 M phosphate (pH 6.8) and $(NH_4)_2SO_4$ fractionation omitted, $Lb^{2+}O_2$ accounted for more than 50 % of the total Lb (ref. 4). If this extraction and $(NH_4)_2SO_4$ fractionation at pH 6.8 was carried out under pure CO then autoxidation was even less; the final solution, in 0.01 M phosphate (pH 6.8), contained mainly $Lb^{2+}CO$. 50 μmoles of this $Lb^{2+}CO$ (1.25 mM) when converted to Lb^{3+} with $K_3Fe(CN)_6$ (see MATERIALS AND

METHODS) and chromatographed (pH 5.2), contained only the green, high-spin forms of Lba^{3+}, Lbc^{3+} and Lbd^{3+}.

In summary, these experiments suggest that Lb^{2+} autoxidation is more rapid as pH is lowered during extraction and salt fractionation procedures. Furthermore, it is only when Lb^{3+} is formed by autoxidation during extraction (especially at acid pH) that the red, low-spin, ferrihaemochrome can be detected. In support of this conclusion it is noted that $Lb^{2+}O_2$ (and $Lb^{2+}CO$) which survived autoxidation at any pH (and which ran just behind the green form of Lb^{3+} during chromatography) showed no trace of low-spin spectra when reduced with $Na_2S_2O_4$ (*cf.* ref. 4).

Separation of the low- and high-spin forms of Lb^{3+}

A satisfactory separation of the red and green forms of Lba^{3+}, Lbc^{3+} and Lbd^{3+} was eventually achieved on DEAE-Sephadex. Fresh or thawed nodules (300 g) were ground in 0.1 M phosphate, 0.1 mM EDTA (pH 6.8) and the $(NH_4)_2SO_4$-precipitated Lb (see MATERIALS AND METHODS) redissolved and equilibrated with 0.1 mM EDTA (pH 6.8) by passage through Sephadex G-25. 20 ml (33 μmoles) of this Lb were applied to a column of DEAE-Sephadex type A-50 (details in Fig. 1) and after 1000 ml of buffer (13 mM acetate, pH 5.2) had passed through the Lba^{3+} appeared on the column as discrete red-pink 'front' and green 'tail' bands followed by a bright red band of $Lba^{2+}O_2$. Similar but compressed patterns appeared for the slower running Lb*c* and Lb*d* components.

Immediate spectrophotometry of the peak fractions of these Lba^{3+} subcomponents after elution (Fig. 1) showed a considerable divergence of properties, and this was best seen by examining the $Na_2S_2O_4$-reduced fractions. At pH 5.2 the spectrum of Lba^{2+} derived from red, 'front' Lba^{3+} (Fig. 1) showed ferrohaemochrome

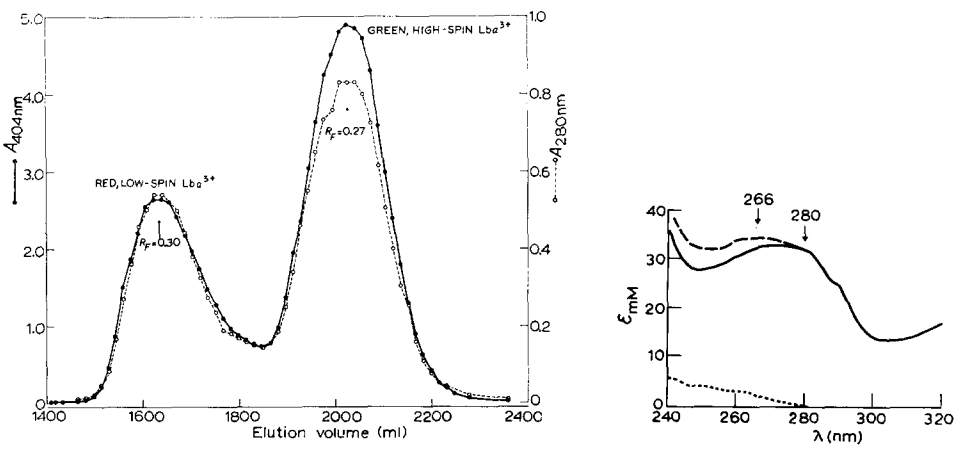

Fig. 1. Chromatographic resolution of low-spin (red) and high-spin (green) forms of Lba^{3+}. The 8-ml fractions were eluted at 4° by upward flow from a 105 cm × 2.6 cm column of Sephadex A-50, equilibrated and developed with 13 mM acetate (pH 5.2). Absorbance (*A*) was measured on undiluted fractions, immediately after elution.

Fig. 2. Ultraviolet absorption of low- and high-spin Lba^{3+}. These are the peak fractions from chromatography (Fig. 1) diluted 1:4 with 13 mM acetate (pH 5.2); – – – –, low-spin Lba^{3+}; ———, high-spin Lba^{3+}; ·····, the computed absorption difference between low- and high-spin Lba^{3+}.

characteristics, with a sharp α peak at 554 nm ($\varepsilon_{mM} = 21.8$) and β peak at 526 nm ($\varepsilon_{mM} = 12.7$) (Table I; *cf.* Fig. 3A). In contrast, Lba^{2+} derived by reduction of green, 'tail' Lba^{3+} had a Mb^{2+}-like spectrum with a broad absorption band centered at 555 nm ($\varepsilon_{mM} = 13.9$) (Table I; *cf.* Fig. 3A) and no suggestion of ferrohaemochrome double peaks.

The decreased ratio, $A_{404\,nm}/A_{280\,nm}$, of 4.95 for red, low-spin Lba^{3+} compared with 5.9 for green, high-spin Lba^{3+} (Fig. 1), suggested that the low-spin form contained extra protein, but haem analysis showed this ratio to be low because of a decreased Soret (404 nm) absorption peak in low-spin Lba^{3+} (*cf.* Fig. 3B). Further, Fig. 2 shows that low- and high-spin Lba^{3+} have identical absorption at 280 nm ($\varepsilon_{mM} = 34$) although the low-spin form has its ultraviolet maximum at 266 nm. This does not mean that low-spin Lba^{3+} is characterized by the presence of bound nucleotide; the computed difference spectrum (Fig. 2) between the low- and high-spin subcomponents shows no well-defined peak near 260 nm, and $\delta\varepsilon_{mM} = 3.7$ (at 260 nm) is much less than an average $\delta\varepsilon_{mM} = 14$ (ref. 15) required for each nucleotide residue bound per haem.

Optimal conditions for recording the spin state of Lb fractions by spectrophotometry; loss of low-spin properties during manipulations

The spin state of Lb^{2+} is apparently unaffected by the presence or absence of acetate buffer (*cf.* Fig. 3A) so wherever possible diagnostic spectra were recorded on $Na_2S_2O_4$-reduced Lb fractions. However, the tendency to form a Lb^{3+}-acetic acid complex[5], with exaggerated high-spin properties[6], made it desirable to remove acetate before recording diagnostic spectra of Lb^{3+}.

TABLE I

LOSS OF HAEMOCHROME PROPERTIES DURING CONCENTRATION AND BUFFER EXCHANGE OF LOW-SPIN Lba^{3+}

The concentration and buffer-exchange manipulations were performed on Lba^{3+} components isolated as shown in Fig. 1, and absorption peaks determined after dilution to 50 μM and reduction to Lba^{2+} with $Na_2S_2O_4$. Data on reduced cytochrome b_1 (ref. 16) are included to show the spectral characteristics of a native 100% ferroprotohaemochrome. The peak wavelength of each absorption band is shown in nm, and corresponding ε_{mM} value in parentheses.

Haemoprotein and treatment	*Absorption peaks*					
	α	(ε_{mM})	$\alpha\beta$ *fused*	(ε_{mM})	β	(ε_{mM})
Reduced cytochrome b_1	559	(24.4)	—		530	(14.2)
Low-spin Lba^{2+}						
As isolated, pH 5.2	554	(21.8)	—		526	(12.7)
Reconc. from DEAE-cellulose with 0.1 M phosphate (pH 6.8); read at pH 5.2	555	(15.2)	—		528	(9.6)
Reconc. by ultrafiltration; read at pH 5.2	554	(17.9)	—		526	(10.6)
Reconc. by ultrafiltration; read at pH 6.8	—		555	(13.9)	—	
High-spin Lba^{2+} as isolated or reconc.; pH 5.2 or 6.8	—		555	(13.9)	—	

The standard procedure[1] for acetate removal and reconcentration of dilute Lb^{3+} fractions (from chromatography) involves readsorption onto DEAE-cellulose at very low ionic strength and elution as a sharp band with 0.1 M phosphate (pH 6.8). However, as shown in Table I, this treatment caused a considerable loss of haemochrome properties (conveniently measured after conversion to Lb^{2+}) when applied to the low-spin form of $Lb\alpha^{3+}$ obtained as in Fig. 1. It was therefore necessary to devise a gentler reconcentration and buffer-exchange procedure.

When 15-ml vol. (0.8 μmole) of the peak fractions of red and green $Lb\alpha^{3+}$ forms (Fig. 1) were freed of acetate by passage through Sephadex G-15 equilibrated with unbuffered NaCl (50 mM) and reconcentrated by ultrafiltration in an AMICON Model 50 cell, less reversion occurred. In 0.1 M MES (pH 5.2), after $Na_2S_2O_4$ reduction, the reconcentrated low-spin $Lb\alpha^{2+}$ retained much of its ferrohaemochrome character (Table I; Fig. 3A) with $\varepsilon_{mM} = 17.9$ (554 nm). Such fractions, concentrated by ultrafiltration, were used for recording standard spectra of low- and high-spin Lb.

Spectra of low- and high-spin forms of $Lb\alpha^{2+}$

Fig. 3A and Table I show the striking contrast between spectra of the 'front' and 'tail' forms of $Na_2S_2O_4$-reduced Lbα at pH 5.2. The 'tail' form had a Mb^{2+}-like, high-spin spectrum, with fused $\alpha\beta$ band at 555 nm and Soret (γ) band at 427 nm; the 'front' form had a ferrohaemochrome, low-spin spectrum, with absorption peaks at 554, 526 and 420 nm. However, this 'front' fraction did not contain 100% haemochrome; a shoulder at 427 nm in the spectrum (Fig. 3A, dashed trace) was attributed to the presence of some high-spin $Lb\alpha^{2+}$, and the absorption coefficients of the α and β bands were less than in 'fresh' low-spin $Lb\alpha^{2+}$ or in reduced cytochrome b_1 (Table I).

Effect of pH. The spectrum of high-spin $Lb\alpha^{2+}$ was stable between pH 5.2 and 6.8, but the ferrohaemochrome spectrum of low-spin $Lb\alpha^{2+}$ was transformed to a Mb^{2+}-like (high-spin) spectrum when pH of the diluent buffer was raised from 5.2 to 6.8 (Fig. 3A; Table I).

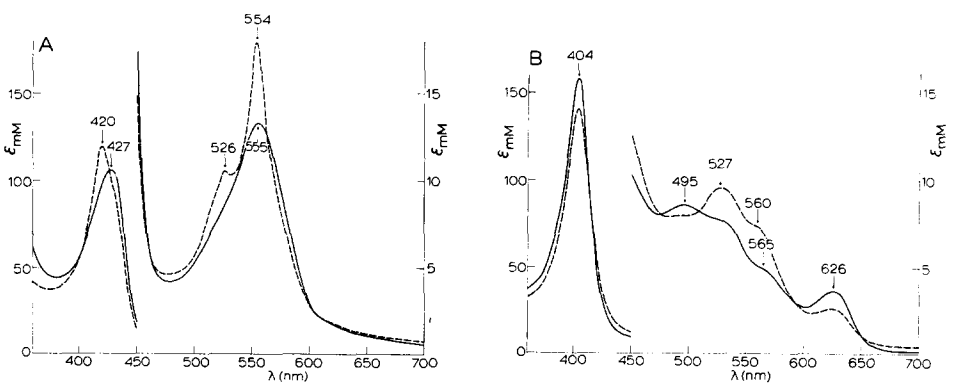

Fig. 3. Spectra of low- and high-spin forms of Lbα. The peak fractions from ion-exchange chromatography (Fig. 1), freed from acetate by Sephadex G-15 chromatography and ultrafiltration, were diluted to 50 μM in the appropriate buffer. A. $Lb\alpha^{2+}$ ($Na_2S_2O_4$-reduced); – – – –, low-spin form at pH 5.2 (0.1 M acetate or 0.1 M MES); ———, high-spin form at pH 5.2 (0.1 M acetate or 0.1 M MES) or pH 6.8 (0.1 M phosphate), also the low-spin form at pH 6.8 (0.1 M phosphate). B. $Lb\alpha^{3+}$; – – – –, low-spin form at pH 5.2 (0.1 M MES) or pH 6.8 (0.1 M phosphate); ———, high-spin form at pH 5.2 or 6.8.

An apparent pK near 5.4 for this change (Fig. 4) suggested that an undissociated acidic group participated in formation of the covalent, ferrohaemochrome structure. It is unlikely that this transformation was due to an acid-sensitive conformational change of the whole Lb molecule; titrations of Lb^{2+} or Lb^{3+} solutions with reagents which rupture H bonds (urea or guanidine) showed no tendency towards increased or decreased haemochrome formation. A possible model of the relationship between low-spin and high-spin Lb is presented in DISCUSSION (Fig. 8).

Spectra of the low- and high-spin forms of Lba^{3+}

Fig. 3B shows the low-spin spectral characteristics of the 'front' component and high-spin characteristics of the 'tail' component of Lba^{3+}, although the tendency of the ferrihaemoprotein (contrasted to ferrohaemoprotein) to form a thermal-equilibrium mixture of high- and low-spin states (see INTRODUCTION) lessens the contrast. Thus, the solid trace of Fig. 3B with 'charge-transfer' bands[2] at 495 and 626 nm (characteristic of high-spin ferrihaemoproteins), dominating the ferrihaemochrome bands[2] at 527 and 560–565 nm, is identical with the published spectrum[5] of the thermal-equilibrium mixture (predominantly high-spin[6]) of a classical preparation of Lba^{3+}. For convenience this is referred to as a 'high-spin' spectrum, as it is this material, which gives (when reduced) the 100 % high-spin spectrum of Lba^{2+} (Fig. 3A). The dashed trace of Fig. 3B (low-spin Lba^{3+}) shows dominating low-spin, ferrihaemochrome bands at 527 and 560 nm; the weak band at 626 nm and shoulder at 495 nm are due to the presence of a small amount of high-spin Lba^{3+}.

Effect of pH. Although ferrohaemochrome properties, apparent at pH 5.2, are

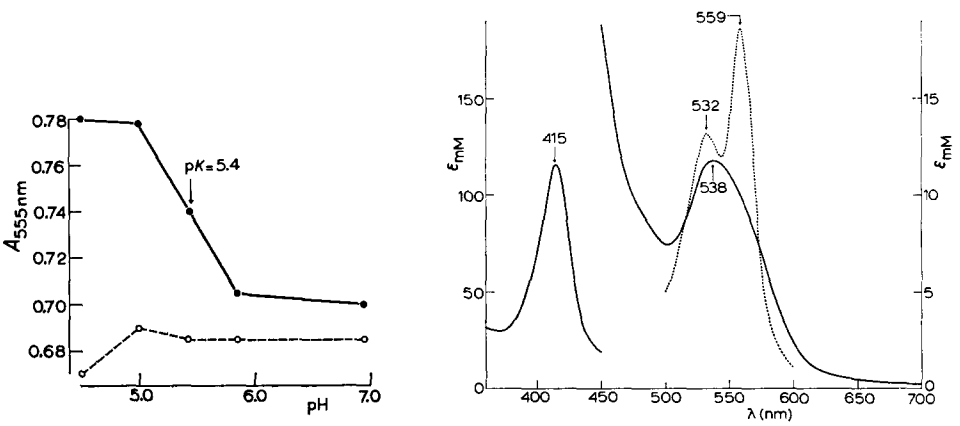

Fig. 4. pH sensitivity of low- and high-spin forms of Lba^{2+}. The peak fractions of Lba^{3+} (Fig. 1) after reconcentration on DEAE-cellulose (see RESULTS), were diluted in 0.1 M succinate (pH 4.5–6.0) or 0.1 M phosphate to 50 μM, reduced with $Na_2S_2O_4$ and spectra recorded immediately. The high-spin form (O---O) showed insignificant change between pH 5 and 7. The low reading at pH 4.5 is due to instability and consequent slow precipitation; the low-spin form (●—●) shows an acid protonation with apparent pK near 5.4.

Fig. 5. Cyanide complexes of Lba. The preparations of Fig. 3 (50 μM) were used to record these spectra in the presence of 1 mM KCN. The same Lba^{3+}CN spectrum (———) was obtained from low- or high-spin Lba^{3+} forms at pH 5.2 or 6.8. The unstable Lba^{2+}CN spectrum (·····) was recorded 1–2 min after $Na_2S_2O_4$ addition to low-spin Lba^{3+}CN in 0.1 M MES (pH 5.2). Similar, rapidly decaying, 559- and 532-nm peaks were seen in all the other preparations, following $Na_2S_2O_4$ addition.

lost at neutral pH (Figs. 3A and 4) the ferrihaemochrome spectrum is stable between pH 5.2 and 6.8 (Figs. 3B and 4). If the same ligand X is responsible for ferro- and ferrihaemochrome formation in low-spin Lb*a*, then dissociation of a weakly acidic group (see below) must cause expulsion of the ligand from Lba^{2+} but not from Lba^{3+}.

Possible nature of the haemochrome ligand X

The spectra of Fig. 3B show that Lb^{3+}, like peroxidases[8] may be extracted from plant tissues as a separable mixture of low- and high-spin ferrihaemoproteins. It is now known[8] that the ligand which confers low-spin properties on paraperoxidase is cyanide, but Fig. 5 shows that the corresponding ligand X in low-spin Lb is not cyanide. At pH 5.2 or 6.8, titration of either low- or high-spin Lba^{3+} (*cf.* Fig. 3B) with neutralized KCN caused considerable spectral change, giving finally the identical 100 % low-spin Lba^{3+}CN complex, with absorption bands at 415 and 538 nm (Fig. 5, solid trace). At pH 5.2, K_m (KCN) was 47 μM for low-spin Lba^{3+} and 69 μM for high-spin Lba^{3+}. This compares with K_m (KCN) of 0.03 μM for low-spin and 2.0 μM for high-spin horseradish peroxidases[8]. The most convincing evidence that X is not cyanide was obtained following $Na_2S_2O_4$ reduction of the Lba^{3+}CN complexes which had been formed at either pH 5.2 or 6.8. A transitory formation of Lba^{2+}CN, with peaks at 532 and 559 nm (Fig. 5, dotted trace) compared with 526 and 554 nm for the stable Lba^{2+}X complex (Fig. 3A, dashed trace) was followed by a rapid decay ($<$ 10 min) to the respective low- or high-spin forms of Lba^{2+} previously found in the absence of cyanide. Like Hb^{2+} (ref. 17), but unlike ferroperoxidase[8], Lb^{2+} appears unable to make a stable cyanide complex.

The foregoing experiments do not necessarily mean that X is a separable ligand; it could be an acidic sidechain, possibly carboxyl, of the Lb protein. It is not even certain that the group with apparent pK of 5.4 and involved in ferrohaemochrome formation (Fig. 4) is part of the ligand X or is a necessary secondary binding site on the protein. Identification of X as a low-molecular-weight ligand was finally achieved by its quantitative displacement from low-spin Lba^{2+} or Lba^{3+} and separation from Lb by molecular-weight chromatography.

Displacement of X from low-spin Lba^{2+}. 5 ml (0.4 μmole) of low-spin Lba^{3+} (*cf.* Fig. 3B) were reduced to Lba^{2+} with $Na_2S_2O_4$, and run through a 30 cm $\times$ 2.61 cm column of Sephadex G-15 equilibrated with 10 mM HEPES (pH 7.3). Reduction of the Lba^{2+} was maintained by addition of $Na_2S_2O_4$ (0.5 mg/ml) to this buffer, which was continuously purged with pure N_2, and by collecting effluent fractions under N_2. After chromatography, this Lba^{2+} had an identical high-spin (Mb^{2+}-like) spectrum at both 7.3 and 5.2. In a control experiment, an aliquot of the same Lba^{3+} preparation was passed through the same column of Sephadex G-15, equilibrated with 0.01 M HEPES (pH 7.3), but in the absence of $Na_2S_2O_4$. The effluent Lba^{3+}, after reduction to Lba^{2+} with $Na_2S_2O_4$, showed a high-spin spectrum at pH 7.3 and low-spin spectrum at pH 5.2, as in Fig. 3A.

These experiments were interpreted to mean that X, normally dissociated from Lba^{2+} (but not from low-spin Lba^{3+}) at pH 7.3, had been physically separated from Lba^{2+} on the Sephadex G-15 column. The Lba^{2+} (mol. wt. 15400)[18] was completely excluded from the column ($K_{av} = 0$) so it is assumed that X ran more slowly ($K_{av} > 0$) and that it had a lower molecular weight than the Lb.

Displacement of X from low-spin Lba^{3+}. YAMAZAKI *et al.*[8] were able to displace

the ligand, cyanide, from low-spin ferriperoxidase I by titration with $HgCl_2$. Although the ligand X, which confers low-spin properties on Lb*a* is not cyanide (Fig. 5), titration with $HgCl_2$ was nevertheless able to cause the displacement of X from Lba^{3+}.

The solid trace of Fig. 6 is the spectrum of a 50 μM solution of low-spin Lba^{3+} in 13 mM acetate (pH 5.2); at 70 μM $HgCl_2$, complete conversion to high-spin Lba^{3+} had occurred (Fig. 6, dashed trace), and almost complete reconversion to low-spin Lba^{3+} (Fig. 6, dotted trace) was achieved following a reverse titration with cysteine to 60 μM. This result cannot by interpreted as showing the reversible inactivation

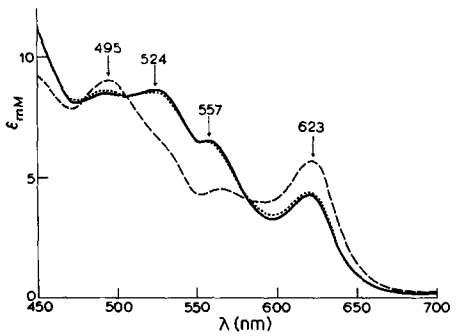

Fig. 6. Displacement of the ligand X from low-spin Lba^{3+}. ———, spectrum of low-spin Lba^{3+} (*cf.* Fig. 1) diluted to 50 μM in 13 mM acetate (pH 5.2). After $HgCl_2$ titration which produced maximal spectral change (– – –) half the preparation was titrated with cysteine, causing reversion to a low-spin spectrum (·····). The other half of the $HgCl_2$-titrated material was passed through a column of Sephadex G-10. The spectrum of high-spin Lba^{3+} (– – –) remained unchanged after titration with cysteine. Experimental details are given in the text.

of a protein ligand by mercury mercaptide formation, because Lb*a* is known to be devoid of sulphur amino acids[19]. Neither could it indicate mercury mercaptide formation with a non-protein ligand, as the specific thiol reagents, *N*-ethylmaleimide (100 μM) or iodoacetamide (100 μM) were quite unable to cause a transformation of low-spin to high-spin Lba^{3+} in similar experiments. A simple explanation is that Hg^{2+}, or more likely $CH_3 \cdot COOHg^+$, combines with a weakly acidic group of X or a secondary binding site on the protein, thereby allowing dissociation of X from the 6th coordination position of the haem (*cf.* Fig. 8), with consequent loss of ferrihaemochrome properties. In the reverse titration, cysteine, by forming a mercury mercaptide, would unmask the essential acidic group and allow X to recombine.

When 5 ml of this Hg^{2+}-titrated material, showing the spectral properties of high-spin Lba^{2+} (Fig. 6, dashed trace) were passed through a 40 cm × 1.5 cm column of Sephadex G-10 equilibrated with 13 mM acetate (pH 5.2) the eluted Lba^{3+} (K_{av} = 0) retained its high-spin spectrum (Fig. 6, dashed trace) even after titration with cysteine to 100 μM. As in the experiments on Lba^{2+} reported above, it is assumed that the dissociated X had separated from Lb on this Sephadex G-10 column and so must be of lower molecular weight than Lb.

The nature of X is still unknown, but it is unlikely to be an aliphatic mono- or dicarboxylic acid; undissociated acetic, propionic, butyric or valeric acids[5] or succinic acid (C. A. APPLEBY, unpublished experiments) all form 100% high-spin complexes with Lb^{3+} *in vitro*. In similar studies (C. A. APPLEBY, unpublished experi-

ments) citric, malonic or maleic acids formed no Lba^{3+} or Lba^{2+} complexes at all, nor did a variety of mononucleotides.

Possible distinction between high-spin Lb^{2+} and "ligand-free" low-spin Lb^{2+}

In their studies on low-spin horseradish peroxidase I (paraperoxidase) YAMAZAKI *et al.*[8] showed that removal of a ligand (cyanide) gave a haemoprotein having spectral properties identical with those of high-spin peroxidase II, but which was not chemically identical. Their 'ligand-free' ferric peroxidase I had a much higher affinity (K_m = 0.03 μM) for cyanide than did peroxidase II (K_m = 2.0 μM).

Experiments reported above, and in a previous paper[4] show that Lb^{2+}, which survived autoxidation during extraction at pH 6.4–6.8 and was purified as Lb$^{2+}O_2$ or Lb^{2+}CO, did not show ferri- or ferrohaemochrome spectra when subsequently oxidized, reduced or deoxygenated at either pH 5.2 or 6.8. If low-spin Lb is formed by artifactual association of Lb^{3+} (but not Lb^{2+}) with X during isolation (see DISCUSSION, Fig. 8) then this 'protected' Lb^{2+} might show a dichotomy of properties, suggesting that the portion of total Lb able to combine with X is different from Lb unable to combine with X. Some occasional and non-reproducible observations suggest that this may be so. In a preparation similar to that recorded in Fig. 1, but using DEAE-cellulose (Whatman DE-52) instead of DEAE-Sephadex, low- and high-spin Lba^{3+} were eluted with 13 mM acetate (I = 0.01, pH 5.3) and the closely following band of Lb$a^{2+}O_2$ was eluted with 13 mM acetate, 20 mM NaCl (I = 0.03, pH 5.3). As isolated, this Lb$a^{2+}O_2$ had its α absorption peak (574 nm) higher than its β peak (540 nm) (*cf.* ref. 3) and after oxidation with $K_3Fe(CN)_6$ it showed the 100% high-spin spectrum of Lba^{3+}–acetic acid complex (*cf.* Fig. 6, dashed trace). However, deoxygenation of this Lb$^{2+}O_2$ solution (pH 5.2) with $Na_2S_2O_4$ did not produce the expected high-spin Lb^{2+} with 427- and 555-nm absorption peaks (*cf.* Fig. 3A); instead there appeared (Fig. 7) a complex spectrum with 560-nm peak and 545-nm shoulder, 417-nm peak and 430-nm shoulder. The 560-nm peak and 430-nm shoulder possibly represent 'classical' high-spin Lba^{2+}; the 418- and 545-nm absorption, not characteristic of

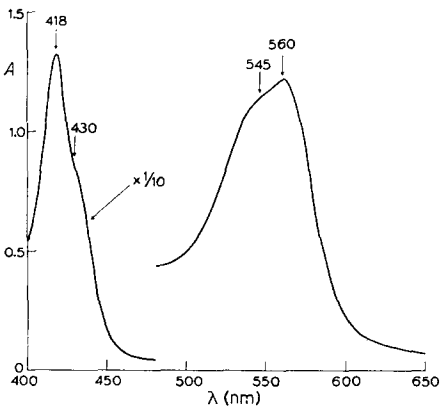

Fig. 7. Appearance of an unstable form of high-spin Lba^{2+}. A fresh preparation of Lb$a^{2+}O_2$, in 13 mM NaCl (pH 5.2) had this spectrum after deoxygenation with $Na_2S_2O_4$; oxidation with $K_3Fe(CN)_6$ gave the spectrum of high-spin Lba^{3+} identical with the dashed trace of Fig. 6. After storage for 5 days at 0°, deoxygenation of the same Lb$a^{2+}O_2$ preparation gave Lba^{2+} with a conventional high-spin spectrum (*cf.* Fig. 3A, solid trace).

low-spin Lba^{2+} (Fig. 3A), might conceivably represent a form of Lb^{2+} able to receive the ligand X and thence assume a haemochrome structure. After this same Lb$a^{2+}O_2$ preparation had stood at 0° for 5 days, deoxygenation with $Na_2S_2O_4$ caused the appearance of 'classical' high-spin Lba^{2+} only. This phenomenon was also seen in one preparation of Lb$a^{2+}O_2$ isolated on a column of DEAE-Sephadex A-50 at 10 mM phosphate, 15 mM NaCl (I = 0.035, pH 6.8), so it is unlikely that whatever is responsible for the appearance of 418- and 545-nm absorption bands has the acidic group (pK 5.4) essential for ferrohaemochrome formation from Lb^{2+} (Fig. 4).

DISCUSSION

These observations on Lba, and similar, unreported, observations on Lbc and Lbd, clearly show that the red 'front' and green 'tail' subcomponents of Lba^{3+} as separated on DEAE-Sephadex (Fig. 1) have the respective spectral properties of Lb^{3+} ferrihaemochrome extracted from nodules by ELLFOLK AND SIEVERS[7] at pH 5.6, and the oxidized form of high-spin Lb^{2+} isolated by APPLEBY[4] at pH 6.4–6.8.

If it is accepted that the natural form of Lb in the nodule is Lb^{2+}, and that the natural pH is near 6.4 (ref. 4), then the properties of Lba^{2+} and Lba^{3+} revealed by Figs. 3 and 4 allow the conclusion that high-spin Lb^{2+}, not low-spin Lb^{2+} is the natural form. At pH 6.4 or above (Figs. 3A and 4) the low-spin form of Lb^{2+} reverts to a high-spin form; the ligand X has apparently dissociated. A $Lb^{2+}X$ complex is therefore unlikely to exist *in vivo* at pH 6.4, or unlikely to be formed by justaposition of native Lb^{2+} and X during extraction at pH 6.4. Any Lb^{2+} autoxidized to Lb^{3+} during extraction (pH 6.4) might be able to combine with X, since Fig. 3B shows the ferrihaemochrome (Lba^{3+}X) to be stable at neutral as well as acid pH values.

In pH 5.6 extracts, formation of Lba haemochrome would be greatly favoured. Most Lba^{2+} is autoxidized to Lba^{3+} at this pH, and therefore able to form a Lba^{3+} complex. Of the surviving Lba^{2+}, almost half should be able to form the Lba^{2+}X complex, since Fig. 4 shows the pK for Lba^{2+}X dissociation to be about pH 5.4. Autoxidation of this Lba^{2+}X (but not of uncomplexed Lba^{2+}), during later stages of purification when X may no longer be present, would result in further appearance of Lba^{3+}X.

The experiments relating to molecular-weight chromatography of (originally) low-spin Lba^{2+} at pH 7.3 or of $HgCl_2$-titrated low-spin Lba^{3+} at pH 5.2 show that X is a low-molecular-weight ligand, but one which must have a very high affinity for Lba^{3+}. Complete reformation of Lba^{3+}X can occur when X is released from its association with Hg^{2+} (or Hg^{2+} is removed from a secondary binding site on the protein) during a reverse titration of unchromatographed material with cysteine.

It is not possible to decide whether X, the protein, or both, have the (presumably) acidic binding site for Hg^{2+}. Some observations (C. A. APPLEBY, unpublished experiments), show that when the $HgCl_2$-titrated, low-spin Lba^{3+}, which has assumed high-spin properties (Fig. 6) is passed through Sephadex G-10, traces of Hg^{2+} can be detected in both the high-molecular-weight (protein) and low-molecular-weight (salt) fractions. For this reason, a tentative formulation (Fig. 8) of the reactions of Lb^{2+} and Lb^{3+} with X at pH 5.2 and 6.8 show both X and the Lb protein (in a region distal to haem iron) to possess weakly acidic groups. For convenience these groups are presented as carboxyls, although no chemical evidence can yet be offered. At pH 5.2 the sup-

pressed ionization of these acidic groups would permit the insertion of ligand X in both low-spin Lba^{3+} (Fig. 8, Structure B) and low-spin Lba^{2+} (Fig. 8, Structure D). At pH 6.8 repulsion between the two ionized groups might be sufficient to cause expulsion of X from its haem-binding site (Fig. 8, Structure C). An obvious weakness of this formulation is the ability of X to remain tightly bound to Lba^{3+} at pH 6.8 (Fig. 8, Structure A, *cf.* Fig. 3B). It is possible that loss of an iron electron during Lb oxidation could in turn cause sufficient weakening of the acidic properties of bound X to permit it to remain close to the acidic region on the protein. Alternatively it is possible that X has an electron-dense region rather than a dissociable acidic group.

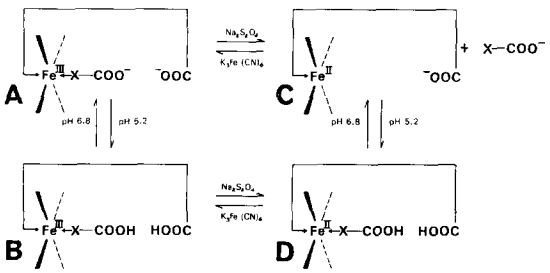

Fig. 8. A formulation of the relationships between low- and high-spin forms of Lb. Structure A represents the low-spin, covalent $Lb^{3+}X$ complex at pH 6.8; B, the spectroscopically identical $Lb^{3+}X$ complex at pH 5.2. Structure D represents the low-spin $Lb^{2+}X$ complex at pH 5.2 and C, the expulsion of ligand X at pH 6.8 to form a high-spin Lb^{2+} molecule. In all structures, four coordination positions of the haem iron are occupied by the tetrapyrrole nitrogens and the fifth by a Lb-protein ligand. In Structures A, B, and D the sixth coordination position is occupied by the ligand X, giving a covalent, low-spin, haemochrome structure. High-spin Lb^{3+} is not depicted, but high-spin Lb^{2+} (pH 5.2 only) is represented by Structure C.

The origin of X is unknown. If it is released from a remote site during nodule crushing, and this release is favoured at acid pH, then at pH 6.4 or higher there may be insufficient X available to bind all Lb^{3+} molecules. This would explain the failure to achieve complete formation of low-spin Lba^{3+}X at neutral or alkaline pH. Alternatively, there may be two forms of natural high-spin Lba^{2+}, only one of which (when oxidized) has a high affinity for X. The rare appearance (Fig. 7) of an unstable variant form of high-spin Lba^{2+} is the only indication that this may be so. The new absorption peak at 418 nm and shoulder at 545 nm suggest some change (other than ferrohaemochrome formation) in the haem environment[22]. Such a change might be related to the extraordinary affinity of Lb^{2+} for O_2 (ref. 13) or to a still unproven (*cf.* ref. 4) ability of Lb to bind N_2.

In summary, these experiments support the recent conclusion[4] that high-spin Lb^{2+} is the natural form of Lb in N_2-fixing soybean nodules, and offer an explanation for the appearance of a low-spin, ferrihaemochrome form of Lb^{3+} when nodules are crushed at pH 5.6 (ref. 7). The observed interconversion of high- and low-spin forms of Lba^{3+} (*e.g.* Fig. 6) may explain the earlier observations of change in the electrophoretic[20] and titration[21] properties of ageing Lb preparations.

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REFERENCES

1 N. ELLFOLK, *Acta Chem. Scand.*, 14 (1960) 609.
2 A. S. BRILL AND R. J. P. WILLIAMS, *Biochem. J.*, 78 (1961) 246.
3 R. W. F. HARDY AND R. C. BURNS, *Ann. Rev. Biochem.*, 37 (1968) 331.
4 C. A. APPLEBY, *Biochim. Biophys. Acta*, 188 (1969) 222.
5 N. ELLFOLK, *Acta Chem. Scand.*, 15 (1961) 975.
6 A. EHRENBERG AND N. ELLFOLK, *Acta Chem. Scand.*, 17 (1963) S343.
7 N. ELLFOLK AND G. SIEVERS, *Acta Chem. Scand.*, 21 (1967) 1457.
8 I. YAMAZAKI, R. NAKAJIMA, H. HONMA AND M. TAMURA, *Structure and Function of Cytochromes, Osaka, 1967*, Univ. Park Press, Baltimore, Md., 1968, p. 552.
9 I. YAMAZAKI, K. YOKOTA AND K. SHIKAMA, *J. Biol. Chem.*, 239 (1964) 4151.
10 C. A. APPLEBY, *Biochim. Biophys. Acta*, 172 (1969) 71.
11 *Sephadex-Gel Filtration in Theory and Practice*, Pharmacia, Uppsala, 1966, p. 49.
12 C. A. APPLEBY, *Biochim. Biophys. Acta*, 172 (1969) 88.
13 C. A. APPLEBY, *Biochim. Biophys. Acta*, 60 (1962) 226.
14 E. THOROGOOD, *Science*, 126 (1957) 1011.
15 H. M. RAUEN, *Biochemisches Taschenbuch*, Vol 1, Springer, Berlin, 1964, p. 643.
16 T. FUJITA, E. ITAGAKI AND R. SATO, *J. Biochem. Tokyo*, 53 (1963) 282.
17 R. LEMBERG AND J. W. LEGGE, *Hematin Compounds and Bile Pigments*, Interscience, New York, 1949, p. 226.
18 N. ELLFOLK, *Acta Chem. Scand.*, 14 (1960) 1819.
19 N. ELLFOLK, *Acta Chem. Scand.*, 15 (1961) 545.
20 K. ABEL AND N. BAUER, *Arch. Biochem. Biophys.*, 99 (1962) 8.
21 E. THOROGOOD, *Biochem. J.*, 87 (1963) 114.
22 K. DUS, H. DE KLERK, R. G. BARTSCH, T. HORID AND M. D. KAMEN, *Proc. Natl. Acad. Sci. U.S.*, 57 (1967) 367.