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The redox potential of leghaemoglobin

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SUMMARY

1. Soybean leghaemoglobin was prepared in two stages of purity (a) "non-fractionated" and (b) fractionated by known chromatographic procedures into three components.

2. Redox potential determinations of these preparations were carried out by potentiometric titration. At pH 7.0 all preparations gave a mid-point potential close to +0.22 V and with $n = 1$. "Non-fractionated" material was examined over the pH range 5–7.5; dE/dpH was found to be 0 and −0.05 with an inflexion at pH 6.1. There was indication of a change to a less negative slope between pH 7 and 7.5.

3. These results contradict an earlier reported suggestion that leghaemoglobin functions at low pH values as a vigorous electron donor capable of producing atomic hydrogen.

Although leghaemoglobin (Lb) is invariably present in active, nitrogen-fixing root nodules, there remains doubt as to its role in the nitrogen-fixing process. It has been postulated[1] that leghaemoglobin is involved in redox reactions during nitrogen reduction, but the demonstration by Bergersen and Turner[2] of nitrogen reduction by washed, Lb-free *Rhizobium* bacteroids from nitrogen-fixing root nodules indicates that direct participation is unlikely. Alternatively, Appleby[3], and also Wittenberg *et al.*[4], have shown that ferro-leghaemoglobin (Lb^{2+}) has properties which could allow it to function in the facilitated diffusion of oxygen to the nitrogen-fixing bacteroids.

The iron atom of Lb is capable of reversible oxidation and reduction, so there is the theoretical possibility of Lb participating in oxidation–reduction reactions. The only

Abbreviations: Lb, leghaemoglobin; Mb, myoglobin; Hb, haemoglobin; see also ref. 7.

report to our knowledge of the redox properties of Lb is the unsubstantiated report by Bauer[5] that Lb has the value +0.03 V at pH 5.7. It was also claimed that the redox potential of the pigment showed an unlikely pH dependence (dE/dpH) of +0.126 V/pH unit. It seemed desirable therefore to substantiate or otherwise the results of Bauer[5] and thus to provide reliable values for comparison with other haem–protein systems in any consideration of the role of Lb. Redox potential determinations were accordingly carried out at several pH values on Lb from soybean root nodules. Comparisons were made between the non-fractionated Lb obtained by ammonium sulphate precipitation and several homogeneous fractions which may be separated by ion-exchange chromatographic procedures[6,7].

Leghaemoglobin preparation. Young (4 weeks) root nodules from Lincoln strain soybeans infected with *Rhizobium japonicum* Strain 505 (Wisc.) were ground in 0.1 M phosphate (pH 6.8) at 0 °C and the turbid extract centrifuged at 6000 x *g* for 30 min. Lb was precipitated from the resulting clear supernatant between 0.55 and 0.8 ammonium sulphate saturation, redissolved in 1.0 mM EDTA, pH 6.8, and dialysed against glass-distilled water. Complete conversion to ferrileghaemoglobin (Lb^{3+}) and some purification was achieved by passing this solution through a column of Sephadex G-25 equilibrated with 1.0 mM phosphate (pH 7.0) and pre-loaded with a 10-fold molar excess of potassium ferricyanide as compared with Lb haem. This "non-fractionated" Lb was then chromatographed on DEAE-cellulose at pH 5.2 according to Ellfolk[6] and Appleby[7]; the haemoproteins Lb*a* (= slow electrophoretic component), Lb*c* (= fast electrophoretic component) and Lb*d* were eluted as green acetate complexes[6,8]. No attempt was made to crystallize these leghaemoglobins which all appeared homogeneous by electrophoresis on cellulose acetate strips at pH 6.0 or 8.0. When re-concentrated and equilibrated with 1.0 mM phosphate buffer (pH 6.5), all fractions had spectra typical of normal Lb^{3+} which is a thermal equilibrium mixture of high-spin and low-spin species[9–11]. On reduction with dithionite at pH 6.5 or 5.5 these fractions all gave spectra with a single broad absorption peak at 556–558 nm.

Redox potential measurement. Potentiometric titrations were carried out on solutions of Lb^{3+} (usually 0.06 mM) in 0.05–0.1 M phosphate buffer in an atmosphere of oxygen-free nitrogen at 25 °C. The titrant was an aqueous solution of the sodium salt of reduced anthraquinone 2-sulphonate. The apparatus and conditions used were essentially as described by Henderson and Rawlinson[12]. By means of a hand spectroscope and illumination of the sample with a small tungsten lamp mounted behind the titration vessel, the spectrum could be observed at any time during the titration. The pH values of the solutions were checked before and after titration at 25 °C using a Radiometer Model 22 pH meter.

Spectrophotometry. Spectra of Lb fractions were recorded on a Cary 14 spectrophotometer and concentrations calculated following pyridine haemochrome estimations[3].

The most satisfactory redox titrations of Lb were obtained within the pH range 6–7. Within this range all samples tested were found to be electromotively active without

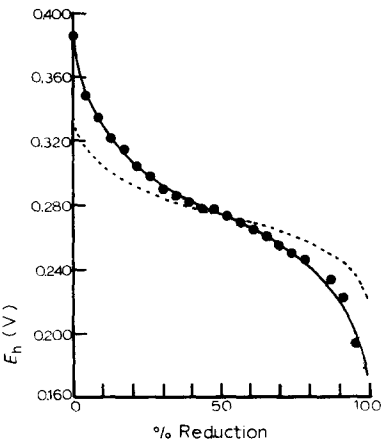
the necessity of adding a mediator. Stable potentials were attained in 3–5 min following each addition of titrant and smooth titration curves were obtained. At the start of such titrations the spectrum of Lb^{3+} was apparent with its characteristic absorption band in the red region (623 nm). As titrant was added, this band gradually became weaker while the absorption band in the green region (556 nm) of Lb^{2+} became stronger. Finally and coincident with the end point of the titration, the red absorption band of Lb^{3+} completely disappeared and the only band apparent with the hand spectroscope was the one in the green due to Lb^{2+}. Immediately air was allowed into any of the fully reduced solutions, the typical spectrum of ferrous oxyleghaemoglobin $Lb^{2+}O_2$ with absorption bands at 574 and 540 nm became apparent.

"Non-fractionated" leghaemoglobin. A typical result for this type of preparation, titrated in 0.05 M phosphate buffer at pH 6.1 and 25 °C, is shown in Fig. 1. Also included in this figure are theoretical curves for the cases where $n = 1$ and $n = 2$. These were obtained by substituting the experimentally obtained E_m value of +0.27 V along with the particular n value in the standard electrode equation. It will be seen that the experimental curve lies close to $n = 1$. On the acid side of neutrality it was found possible with this preparation to determine redox potentials in the pH range 7–5. Some difficulty due to instability of potentials was experienced at pH values lower than 6. This was not usually apparent, however, until after about 50% reduction and points of inflexion could be ascertained graphically. A titration was attempted at pH 4.0 in 0.2 M acetate buffer. However, no spectral change or alteration of potential took place on addition of titrant; an opacity which developed in the solution indicated that some precipitation had occurred. At pH 8.0 the preparation was found to be electromotively inactive; even at pH 7.5 some instability was apparent. Addition of 2,4-dichlorophenolindophenol (to a final concentration of 2 μM) did not improve the result in these cases.

Fig. 2 shows a plot of E_m *versus* pH in the pH range 5.1–7.5. Two slopes are apparent with values (dE/dpH) of 0 and −0.05; the point of intersection of the slopes occurs at pH 6.1. There is some indication of a change of slope from −0.05 to a lower value in the vicinity of pH 6.8–7.0.

Separated fractions of leghaemoglobin. Redox titrations of the various fractions separated chromatographically were carried out at pH 7.0 in 0.05 M phosphate buffer. The values obtained are shown in Table I.

The titration curves of Lb in the pH range 5–7.5 gave n values (of the electrode equation) which were close to 1. In this regard Lb is like myoglobin (Mb) rather than haemoglobin (Hb) which gives n values intermediate between 1 and 2[13]. In view of the electromotive inactivity experienced with Hb and Mb in the absence of a mediator[14,15], it was surprising that no such difficulty was experienced with Lb. It is well known that haemochromes are usually electromotively active and it may be that a small amount of the haemochrome, $Lb^{3+}X$ [7,11] was present in all of the preparations and that this compound was acting as a mediator. The value E_m (pH 5.7) = +0.27 V obtained for Lb from Fig. 2 is considerably higher than the earlier claimed value of E_m (pH 5.7) = +0.03 V[5]. Lb with average E_m (pH 7.0) = +0.22 V compares with E_m (pH 7.0) = +0.15 V for Hb from several



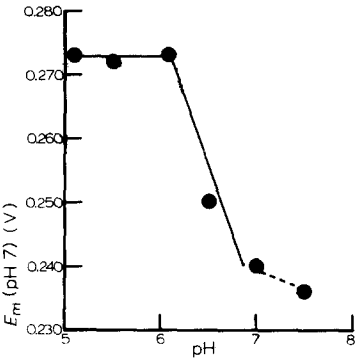


Fig. 1. E_h *versus* % reduction curve for "non-fractionated" Lb. •, experimental points obtained by reductive titration of 10^{-4} M Lb in 0.05 M phosphate buffer (pH 6.1) with reduced sodium anthraquinone 2-sulphonate at 25 °C. Solid line is theoretical curve where: E_m = +0.27 V and n = 1, dotted line where n = 2.

Fig. 2. E_m *versus* pH curve for "non-fractionated" Lb. •, E_m values obtained from reductive titrations of 10^{-4} M Lb in 0.05 M phosphate buffer at 25 °C.

TABLE I

E_m VALUES OBTAINED FOR VARIOUS PREPARATIONS OF Lb; (SEE TEXT) BY POTENTIOMETRIC TITRATION AT 25 °C

Sample	E_m *(pH 7.0) (V)*
"Non-fractionated" Lb	+0.24
Lb*a*	+0.19
Lb*c*	+0.23
Lb*d*	+0.23

sources (see, *e.g.* ref. 16) and E_m (pH 7.0) = +0.06 V for horse Mb[13] and thus is one of the most positive in redox potential of the haemoglobin types capable of carrying oxygen. We wonder if the structural features responsible for this high redox potential are also responsible for Lb having the fastest oxygen "ON" reaction ($K^1 = 118 \cdot 10^6\ M^{-1} \cdot s^{-1}$) yet recorded for any haemoglobin[4]. The redox potentials of the soybean Lb components are fairly close together (Table I), suggesting that the known differences in amino acid composition[17] do not greatly affect the essential structure of the active centre.

The dE/dpH curve of "non-fractionated" Lb (Fig. 2) is invariant with pH in the range 5–6 after which it becomes −0.05 which is close to the classical −0.06 slope for a haem-linked dissociation of the oxidized form[18]. As the pH value is increased from 7 to 7.5 there is evidence for a decrease in slope which could be due to a haem-linked dissociation of the reduced form in this region[18]. Such dissociations have been reported in horse Mb ($pK' = 7.9$)[13] and in human Hb ($pK' = 7.7$) when the latter was in 4 M urea[19]. It is interesting that

there is an overall similarity of pattern of the E_m *versus* pH relationships between Lb, Mb and the dissociated form of Hb in 4 M urea. It can thus be seen that these results are in contradiction to those reported earlier[5] and make unlikely the claim[5] that Lb functions at low pH values as a vigorous electron donor capable of producing atomic hydrogen.

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