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The nature of a supposed N_2 complex of ferroleghaemoglobin

ABEL AND BAUER[1] prepared leghaemoglobin (Lb) by grinding soybean root nodules in 3 M $(NH_4)_2SO_4$ at pH 9 under H_2 followed by fractionation of the crude extract with excess $(NH_4)_2SO_4$. This ferrous leghaemoglobin (Lb^{2+}) was claimed to be isolated as a $Lb^{2+}N_2$ complex[2] since addition of $Na_2S_2O_4$ did not cause the disappearance of specific absorption peaks thought to be due to a Lb^{2+}–gas complex. If the gas had been O_2 the spectrum should have reverted to that of Lb^{2+} (*cf.* Fig. 1, dashed trace). The addition of $K_3Fe(CN)_6$ to their preparation resulted in the liberation of gas following oxidation of the haem iron; the gas was claimed to be identified as N_2 by a gas-chromatographic procedure[2].

When their preparative procedure was repeated in our laboratory using nodules picked from "Lincoln" strain soybean roots inoculated with *Rhizobium japonicum* strain 505 (Wisconsin)[3] the final precipitate, redissolved in 0.1 M potassium phosphate (pH 6.8) or 0.1 M Tris (pH 7.9) showed absorption peaks at 538 and 574 nm, characteristic of $Lb^{2+}O_2$, and a small shoulder at 626–628 nm, characteristic of oxidized leghaemoglobin (Lb^{3+}). Titration of such preparations with $Na_2S_2O_4$ caused the collapse of these peaks and shoulder and in the final spectrum (Fig. 1, solid trace) the broad peak at 558 nm was consistent with the formation of Lb^{2+} and the 550-nm peak and 520-nm shoulder were assumed to be due to the "$Lb^{2+}N_2$ complex" for which no spectrum had previously been presented[2].

1.06 μmoles of Lb^{2+} containing this supposed "$Lb^{2+}N_2$ complex", diluted to 3 ml in 0.1 M phosphate (pH 6.8), were placed in a 10-mm light-path optical cuvette

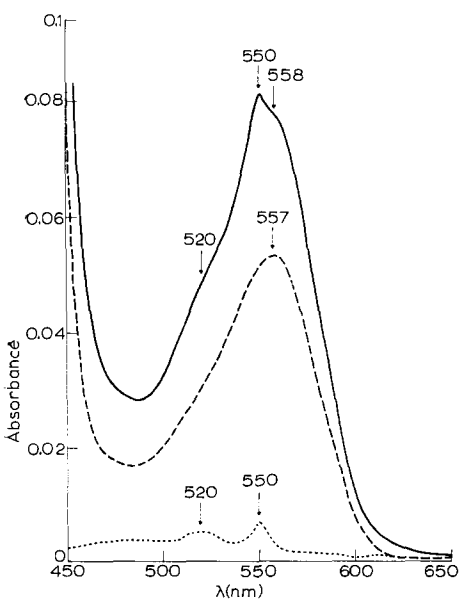

Fig. 1. Spectra of the supposed "$Lb^{2+}N_2$ complex" and its separated components, measured in a Cary 14 spectrophotometer using 10-mm light-path cuvettes. All samples reduced with $Na_2S_2O_4$ (1 mg/ml). Experimental details are given in the text. ———, "$Lb^{2+}N_2$", before chromatography; — — —, Lb^{2+}, after chromatography; -------, reduced cytochrome *c* after chromatography.

fused to the bottom of a Thunberg tube, and gassed with pure N_2 to allow complete formation of any N_2 complex, followed by evacuation and refilling with pure argon to 0.1 atm. Spectrophotometric analyses[4] showed that the Lb^{2+} in this solution had become about 50% oxygenated (0.5 μmole $Lb^{2+}O_2$) during these manipulations, and that the solution contained about 5% of Lb^{3+}. This Thunberg tube was connected through an attached tap and B12 "Quickfit" glass socket to the sample inlet system of an Atlas M-86 mass spectrometer. Analysis of the gas phase (17 ml) before and after conversion of all Lb to Lb^{3+} by 6 μmoles of $K_3Fe(CN)_6$ added from the Thunberg tube sidearm showed that 0.63 μmole O_2, but less than 0.08 μmole N_2 were liberated.

A similar preparation of this supposed "$Lb^{2+}N_2$ complex" was dialysed against 1 mM phosphate (pH 6.8) and passed through a small column of Whatman cellulose phosphate powder, equilibrated with 1 mM phosphate (pH 6.8). The first, major effluent fraction had an absorption spectrum characteristic of conventional Lb^{2+}, with 557-nm peak (Fig. 1, dashed trace). A second, minor band of coloured material, eluted with 1 mM phosphate–100 mM NaCl (pH 6.8), had the absorption spectrum characteristic of reduced cytochrome *c* (Fig. 1, dotted trace) with 550- and 520-nm absorption peaks.

Crude cytochrome *c* (550, Rhizobium) was liberated from conventionally isolated nodule bacteria (Rhizobium bacteroids)[3] following re-extraction with 3.0 M $(NH_4)_2SO_4$ at pH 9. After purification on ion-exchange columns[5] a mixture of this homogeneous reduced cytochrome *c* (1-2%) with 98-99% of Lb^{2+} had the spectral properties of the "$Lb^{2+}N_2$ complex" (Fig. 1, solid trace).

It is suggested that the "$Lb^{2+}N_2$ complex" apparently detected[2] by spectrophotometry of $Na_2S_2O_4$-reduced, alkaline-extracted Lb is in fact a simple physical mixture of reduced cytochrome *c* and Lb^{2+}. The gas liberated by oxidizing this "complex" in the absence of excess $Na_2S_2O_4$ appears to be O_2 rather than N_2. These results, and observations to be reported elsewhere on the state of Lb *in vivo*, do not support a role for Lb in N_2 transport or activation during symbiotic N_2 fixation.

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