A POSSIBLE REDUCTION PATHWAY FOR LEGHEMOGLOBIN IN VIVO

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1. Introduction

Leghemoglobin (Lb), the myoglobin-like hemoprotein present at high concentration in legume root nodules [1] plays a role in facilitating oxygen diffusion to the bacteroids [2] and is involved in furnishing high ATP levels to support nitrogenase activity [3,4]. On the other hand, leghemoglobin has been shown to react with hydrogen peroxide [5,6] and to oxidize indole-3-acetic acid [7] and glycine [8], suggesting that this protein may act as an oxidase in old nodules [6,7].

Leghemoglobin in situ is present mainly in its ferrous form [9] and the absence of oxidized leghemoglobin during nodule development suggests that in vivo ferrileghemoglobin is reduced rapidly [10]. Until now, little information has been available about the mechanism involved in maintaining high levels of the ferrous form of Lb.

Here we report the reduction of ferrileghemoglobin during indole-3-acetic acid (IAA) oxidation and discuss the possible role of this process in reducing leghemoglobin within the nodule.

2. Material and methods

Soybean (*Glycine max* Merr. cv. Altona) nodules were obtained as in [11]. Leghemoglobin purification procedures have been described in [7]. The hemoprotein was freed from nicotinic acid, using the method in [12] and a ratio of A_{560}/A_{620} = 1.7 was regarded as characteristic of nicotinate-free ferrileghemoglobin [12]. Leghemoglobin concentrations were determined by the pyridine hemochromogen assay [13].

Spectra of dilute solutions of leghemoglobin, were recorded with a Varian Techtron spectrophotometer, using the 0–0.1 absorbance slidewire in the visible region and the 0–1 absorbance slidewire in the Soret region. The buffer used was 100 mM citrate–phosphate (pH 5). Experiments were performed at 20°C in 10 mm lightpath cuvettes in 3 ml total vol. In all cases the concentration of leghemoglobin was 6 μM and that of IAA 133 μM. Reactions were initiated by the addition of leghemoglobin to the cuvettes. The formation of ferrous Lb was determined from the ΔA_{574}, a well defined peak of this form, using ϵmM 15. The oxygenation level of leghemoglobin was estimated from A_{574}/A_{541} ratio [14]. In experiments with acetate and nicotinate, the initial reaction velocities were measured after the lag time.

3. Results and discussion

Figure 1a illustrates a typical spectrum of nicotinate-free ferric Lb in citrate–phosphate buffer (pH 5) (continuous line) exhibiting two characteristic peaks at 627 nm and 404 nm in the Soret band [9]. After addition of IAA (133 μM) the spectrum was modified (dashed line). In the visible wavelength range the A_{627} max of ferric Lb disappeared and new peaks, at 474 nm and 441 nm, were generated. The absorption of the Soret band decreased and the maximum shifted from 404 to 410 nm. These modifications are more apparent in the difference spectrum of ferric Lb after addition of IAA, minus ferric Lb (fig.1b), suggesting the formation of oxyleghemoglobin. The A_{574}/A_{541} ratio shows that the ferrous Lb was completely oxygenated (fig.1b).

The time course of the change in absorbance recorded at different wavelengths is shown in fig.2. The

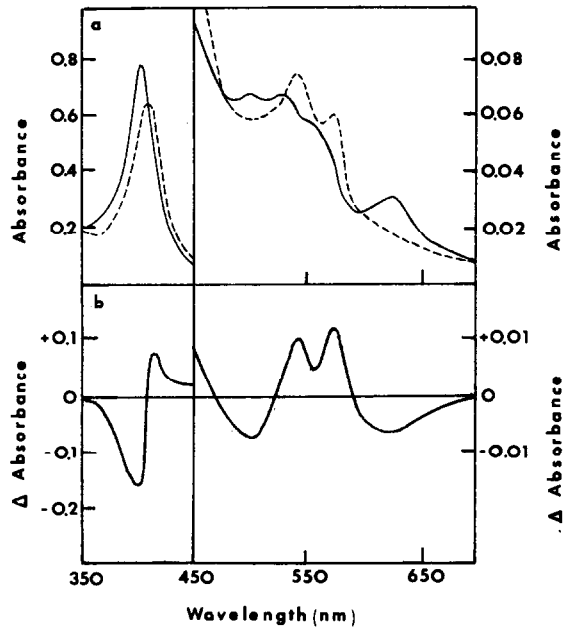

Fig.1. (a) Absorption spectra of ferric Lb before (———) and after (- - - - -) addition of IAA. (b) Difference absorption spectrum of ferric Lb in the presence of IAA minus ferric Lb.

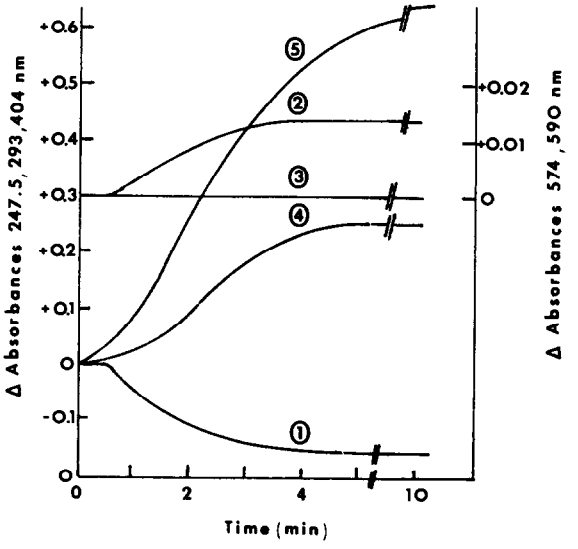

Fig.2. Time course of ferrous Lb formation and IAA oxidation. Absorbance changes were followed at 404 nm (1), 574 nm (2), 590 nm (3), 293 nm (4) and 247.5 nm (5).

reduction of ferric Lb, which is associated with a decrease at A_{404} (curve 1) and an increase at A_{574} (curve 2), showed a lag time of 30 s. The spectral change took place in 3 min. The level of oxyleghemoglobin remained constant for 7 min as confirmed by the absence of any change at A_{590} (curve 3); this wavelength corresponds to one of the isosbestic points for the spectral change observed during the conversion of ferric to ferrous Lb. After 7 min, the level of the ferrous form diminished slowly, suggesting the formation of a degradation product. The rate of disappearance of ferrous Lb was $\geqslant$ 50-fold lower than the rate of its formation. The oxidation of IAA resulted in the formation of two typical degradation products: indole-3-aldehyde and 3-methylene oxindole whose appearance was followed at 293 nm (fig.2, curve 4) and 247.5 nm (curve 5), respectively [15]. The reaction was favoured by acidic pH (4.9) close to the pK_a of IAA (4.75).

A well known characteristic of leghemoglobin is that it binds nicotinic acid [16]. The inhibitory effect of nicotinic acid upon the reduction of leghemoglobin was tested and on the basis of V_i values of the reaction, 83% inhibition was obtained at 17 μM nicotinate (fig.3, curve 2). On the other hand, acetic acid has

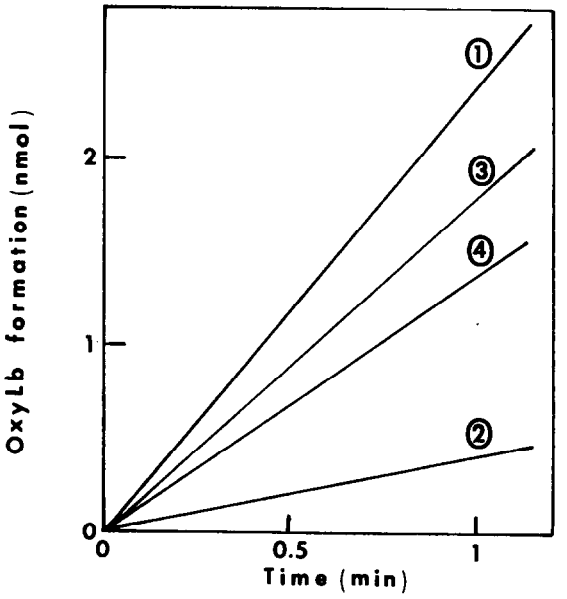

Fig.3. Initial velocities of ferrous Lb formation during IAA oxidation without (curve 1) and with addition of nicotinate 17 μM (curve 2) or acetate 1.33 mM (curve 3) or acetate 3.33 mM (curve 4), respectively.

been described as a ligand affecting the rate of leghemoglobin reduction [17]. Curves 3 and 4, corresponding to different concentrations of acetate (1.33 and 3.33 mM), gave 25% and 42% inhibition, respectively.

These results suggest, for the first time, a possible mechanism for leghemoglobin reduction which is able to keep this hemoprotein in its reduced, functional form. Oxyleghemoglobin was stable only for a few minutes in our experimental conditions but in vivo a protective system may increase its stability. The substantial amounts of IAA, detected in the nodules, [18–19] may act as substrate for the reaction described here. This type of reaction, in spite of its low rate, constitutes a model for similar systems involving other substrates. The reduction of leghemoglobin during oxidation processes could be connected with its peroxidatic activity, reported [6,7]. This mechanism does not exclude the operation of enzymatic systems, such as methemoglobin reductase [10], for maintaining leghemoglobin in the nodules in its ferrous state, the only form able to carry oxygen.

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