EFFECTS OF MODIFICATIONS OF LEGHEMOGLOBIN STRUCTURE ON ITS ABILITY TO OXIDIZE INDOLE-3-ACETIC ACID

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1. Introduction

Leghemoglobin (Lb) is a monomeric hemoprotein that facilitates oxygen diffusion into the symbiotic nitrogen-fixing bacteria in leguminous root nodules [1,2]. In addition to oxygen transport, leghemoglobin may have certain other functions, which become important in aging root nodules. Soybean ferric leghemoglobin has been shown to oxidize indole-3-acetic acid [3,4], which occurs abundantly in infected root nodules, and to have pseudoperoxidatic activity [5,6].

The heme iron in the oxygen-binding form of leghemoglobin is in the ferrous state. However, the iron atom has a tendency to be oxidized by molecular oxygen. Auto-oxidation studies on leghemoglobin and other oxygen carriers imply a correlation between oxygen activation and the strength of the iron-dioxygen bond [7]. A reductase has been detected in root nodules which returns iron into the ferrous state [8]. Another mechanism for the reduction of ferric leghemoglobin to ferrous leghemoglobin in vivo has been suggested [4], in which indole-3-acetic acid (IAA) acts as an electron donor. The oxygenated form of leghemoglobin, stable only for a few minutes in the experimental conditions, is involved in the IAA oxidation process [4].

The aim of this study was to characterize the structural features in the leghemoglobin molecule which makes it capable to oxidize IAA. This was done by measuring the IAA oxidation catalyzed by artificial leghemoglobins, in which unnatural hemes, modified in the 2,4-substituents of the porphyrin ring, were combined with soybean apoleghemoglobin. Secondly, the IAA oxidation catalyzed by leghemoglobins of 3 plants, namely soybean, kidney bean and pea, was measured and evaluated in view of their different amino acid sequences. Of these leghemoglobins, diacetyldeuteroleghemoglobin and pea leghemoglobin did not cause IAA oxidation. Myoglobin, which closely resembles leghemoglobin in its structure, was tested in comparison and found unable to oxidize IAA.

2. Materials and methods

Leghemoglobin a from soybean *(Glycine max)*, leghemoglobin a from kidney bean *(Phaseolus vulgaris)* and leghemoglobin I from pea *(Pisum sativum)* root nodules were isolated and purified essentially as in [9–11]. Nicotinic acid, which occurs in root nodules and binds tightly to leghemoglobin, was removed from leghemoglobin preparations by filtration at alkaline pH [12]. This was necessary because nicotinic acid has been observed to affect IAA oxidation by leghemoglobin [3,4]. Leghemoglobin concentrations were calculated from the heme content obtained by the pyridine hemochrome method [13]. Horse heart myoglobin was a lyophilized preparation purchased from Sigma. Procedures for the preparation of artificial leghemoglobins from soybean apoleghemoglobin and meso-, deutero- and diacetyldeuteroheme have been described [14].

The initial rate of the oxidation of indole-3-acetic acid by leghemoglobin was determined essentially as in [3,4]. About 3 μM leghemoglobin solutions were used in the oxidase assays, while the IAA was 0.2–0.7 mM. The buffer was 25 mM phosphate (pH 6.0). The oxidation rate was followed by measuring colorimetrically the concentration of IAA in samples taken from the reaction mixture [15]. The simultaneous

formation of an oxidation product, 3-methylene oxindole, was evaluated by measuring the absorbance of the reaction mixture at 247.5 nm [3,4]. Catalytic activity was expressed as mol indole-3-acetic acid oxidized . mol leghemoglobin^{-1} . min^{-1}.

3. Results and discussion

In fig.1 are shown the Lineweaver-Burk plots of IAA oxidation by different leghemoglobins. Table 1 summarizes the kinetic constants calculated from the Lineweaver-Burk plots. The 2,4-substituents of the porphyrin ring are ethyls in mesoheme, hydrogens in a deuteroheme, acetyls in diacetyldeuteroheme and vinyls in protoheme, the natural prosthetic group of hemoglobins.

The closely similar K_m-values for proto-, meso- and deuteroleghemoglobin indicate the same degree of stability in the leghemoglobin–indole-3-acetic acid interaction. In control experiments protoheme or apoleghemoglobin separately did not catalyze oxidation of IAA. Therefore it is concluded that only the protein-bound heme group is provided with the IAA oxidation capacity. The proper binding of heme to apoprotein, in view of the IAA oxidation, seems to be preserved in meso- and deuteroleghemoglobin, although the maximum velocity of the oxidation is considerably lower than by protoleghemoglobin. Only when protoheme is replaced with diacetyldeuteroheme, does the leghemoglobin molecule lose its capacity to oxidize IAA. This could be due to steric reasons, since the acetyl groups are bulkier than the other 2,4-substituent groups, and may cause considerable disturbance in the heme–protein interactions.

The electron-withdrawing effect of the 2,4-substituents, which is considered as a significant factor in determining reactivities of hemoproteins [16,17], increases in the order ethyl, hydrogen, vinyl, acetyl, and does not seem to correlate with the catalytic con-

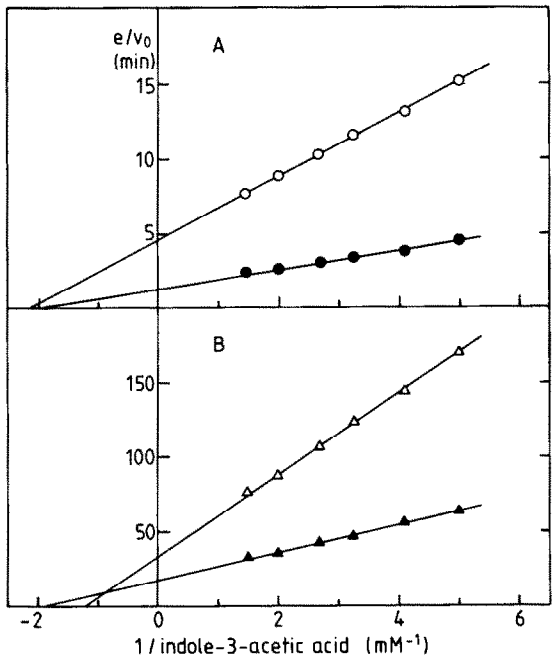

Fig.1. Lineweaver-Burk plots of oxidation of indole-3-acetic acid by leghemoglobin: (A) oxidation by kidney bean leghemoglobin (○) and by soybean leghemoglobin (●); (B) oxidation by soybean leghemoglobin reconstituted from deuteroheme (△) and mesoheme (▲); e is the concentration of leghemoglobin; V is expressed as mol IAA oxidized/min, and IAA (s) 0.2–0.7 mM. The values for K_m and e/V calculated from the plots are given in table 1.

Table 1
Kinetic constants for the oxidation of indole-3-acetic acid by leghemoglobin

Protein	Heme	K_m (mM)	V/e (min^{-1})
Soybean leghemoglobin	Proto	0.50	0.83
	Meso	0.55	0.06
	Deutero	0.83	0.03
	Diacetyldeutero		a
Kidney bean leghemoglobin	Proto	0.48	0.22
Pea leghemoglobin	Proto		a
Horse heart myoglobin	Proto		a

[a] No detectable IAA oxidation

Kinetic studies were performed under conditions in section 2. Michaelis-constants and turnover numbers were calculated from the plots in fig.1

stants obtained here. The natural prosthetic group, with the unique property of the vinyl side chains to act as either electron donors or acceptors, being involved in a conjugated system, seems to be the best suited of the four hemes for the IAA oxidation.

According to the kinetic constants in table 1, soybean leghemoglobin and kidney bean leghemoglobin are about equally capable to effect IAA oxidation, whereas pea leghemoglobin, as well as myoglobin, fail to react. These results correlate with the high degree of homology between the primary structures of soybean leghemoglobin [18,19] and kidney bean leghemoglobin [20] (21% of the amino acid residues are different) with pea leghemoglobin [21] differing by 40% from soybean leghemoglobin and by 36% from kidney bean leghemoglobin.

The parts of the polypeptide chain of leghemoglobins which make up the heme pocket on the ligand-binding site have been best preserved during evolution. However, some residues are different, like residue E 11, which is valine in pea leghemoglobin (and myoglobin) and leucine in soybean leghemoglobin and kidney bean leghemoglobin, and residue G 4, which is histidine in pea leghemoglobin, glutamic acid in soybean leghemoglobin and glutamine in kidney bean leghemoglobin (tyrosine in myoglobin). The results obtained from heme degradation studies on different leghemoglobins [11] implied more restricted rotational freedom of heme-bound oxygen in pea leghemoglobin and myoglobin than in soybean leghemoglobin and kidney bean leghemoglobin. This was suggested to be due to steric hindrance by adjacent amino acid residues, especially by Val E 11 and His G 4, in pea leghemoglobin. These residues most probably also affect the leghemoglobin–indole-3-acetic acid interaction.

Assuming that this kind of mechanism for leghemoglobin reduction is in operation in leguminous root nodules (an unresolved question) then it is likely that not only indole-3-acetic acid but also other suitable molecules in root nodules can be electron donors in the reaction. It may be expected that subtle variations in the spatial arrangement of the polypeptide chain in the vicinity of the ligand-binding site determine the substrate specificity of leghemoglobin as an oxidase.

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