THE BINDING OF MICROSOMAL HYDROXYLATION SUBSTRATES TO CYTOCHROME $P\text{-}450_{Rh}$ AND ITS EFFECT ON THE NITROGEN FIXATION BY LUPIN BACTEROIDS

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1. Introduction

The bacteroids, a symbiotic form of the nodule bacteria of *Rhizobium* genus, are known to contain a soluble auto-oxidizable hemoprotein which is similar to cytochrome *P*-450 from other organisms in its spectral characteristics [1,2].

Recently it has been shown that cytochrome $P\text{-}450_{Rh}$* content in lupin nodule bacteroids undergoes considerable changes during plant development [2]. Its content was greatest at the period of active nitrogen fixation and maximal respiration intensity. Cytochrome $P\text{-}450_{Rh}$ was not found either in cells cultured under aerobic conditions [3] or in bacteroids from *Lupinus luteus* nodules inoculated by ineffective *Rhizobium lupini* strain (400) which is known to be incapable of fixing molecular nitrogen [4].

Liver microsomal cytochrome *P*-450 is not specific with respect to hydroxylation substrates giving two types of spectral changes with various compounds [5] in contrast to cytochromes *P*-450 from other sources.

The soluble cytochrome $P\text{-}450_{cam}$ isolated from *Pseudomonas putida* binds and hydroxylates only D(+)-camphor out of all the compounds causing type I spectral changes [6], and the cytochrome *P*-450 from adrenal cortex mitochondria displays such spectra changes only with steroids [7].

The present work compares the spectral properties of cytochrome $P\text{-}450_{Rh}$, isolated from lupin bacteroids, to those of cytochromes *P*-450 from other sources. The effect on nitrogen fixation of some substances forming complexes with cytochrome *P*-450 has also been investigated in this work.

2. Methods

Bacteroids were isolated under aerobic conditions from 7-week-old *Lupinus luteus* L. plants grown in soil. The isolation medium consisted of 300 mM sucrose, 1% polyvinylpyrrolidone, 200 mM sodium ascorbate and 1 mM Mg^{2+} in 100 mM K-phosphate buffer, pH 7.0.

Cytochrome $P\text{-}450_{Rh}$ was prepared following essentially the method described previously [2]. Bacteroid suspension was disrupted in an Iaton press, and centrifuged (144 000 *g*, 60 min). The supernatant was then treated with ammonium sulphate. The fraction

* We suggest that bacteroid cytochrome *P*-450 be named cytochrome $P\text{-}450_{Rh}$ (*Rhizobium*) to distinguish it from cytochromes *P*-450 from other organisms.

Table 1
Difference spectra cytochrome *P*-450 bound minus cytochrome *P*-450 free

Substance	Type	Absorption maximum, (nm)		Absorption minimum (nm)		K_s, mM		A_{max}*, cm^{-1} mM^{-1}	
		Bacteroids	Microsomes	Bacteroids	Microsomes	Bacteroids	Microsomes	Bacteroids	Microsomes
Dimethylaniline	I	388	385	422	425	5.9	0.44	6.5	4.0
Hexobarbital	I	384	389	430	423	1.1	0.039	19	3.8
D(+)-Camphor	I	385	390	425	420	1.6	0.044	8.5	5.9
SKF-525 A	I	382	388	428	420	0.31	0.001	13	8.9
Aniline	II	427	430	402	395	18	1.0	18	7.3
Metyrapone	II	429	425	400	395	3.7	0.079	66	14

Both cuvettes contained 6.6 nmoles of cytochrome $P\text{-}450_{Rh}$ or 5.4 nmoles of cytochrome *P*-450 from liver microsomes in 3.0 ml 0.1 M phosphate buffer, pH 7.0. The concentrations of substances were $10^{-4}-10^{-2}$ M in the case of cytochrome $P\text{-}450_{Rh}$ and $10^{-5}-10^{-3}$ M in the case of microsomal cytochrome *P*-450.

* A_{max} values were determined as the maximal amplitudes of the absorbance changes at the wavelengths of peak and trough.

precipitated between 30% and 60% saturation was, after passage through a Biogel P-30 (Bio-Rad) column, adsorbed on to a DE-52 (Whatman) cellulose column. Cytochrome $P\text{-}450_{Rh}$ (0.410 nmoles/mg protein) with trace amounts of cytochromes *P*-420 and C-552 was eluted by means of a linear 10 mM phosphate/10 mM phosphate + 500 mM NaCl gradient (pH 7.0).

Nitrogen fixation was determined by the acetylene reduction method [8]. The vessels (7 ml) contained 1 ml of the bacteroid suspension (50 mg protein) in 10 mM phosphate buffer (pH 7.0) with 12 mM sodium succinate and 1 mM Mg^{2+}. The gas mixture consisted of 78% argon, 12% oxygen and 10% acetylene. Incubation time was 60 min, temperature 28°C. The samples under such conditions formed 160 nmoles of ethylene, i.e. 3.2 nmole $hr^{-1} \cdot mg^{-1} \cdot$ protein.

The oxygen consumption rate was measured in a LP-7 polarograph (ČSSR) with a Clark electrode.

The preparation of rat liver microsomal fraction was described previously [9].

Protein was determined by the method of Lowry et al. [10].

All spectra were recorded in a Hitachi-356 spectrophotometer assuming molar extinction coefficients to be 87 cm^{-1} mM^{-1} for cytochrome $P\text{-}450_{Rh}$ [1] and 91 cm^{-1} mM^{-1} for microsomal cytochrome *P*-450 [11]. The K_s (concentrations of substances required for half-maximal spectral changes) and ΔA_{max} (see legend to table 1) values were calculated according to Lineweaver–Burk as described by Remmer et al. [5].

3. Results and discussion

Cytochrome $P\text{-}450_{Rh}$ from lupin bacteroids was able to bind some substrates and inhibitors of microsomal hydroxylation (table 1). The complexes formed gave the same types of spectral changes as complexes of rat liver microsomal cytochrome *P*-450.

All the peaks and troughs in the difference spectra of the cytochrome $P\text{-}450_{Rh}$ complexes resemble those of the microsomal cytochrome *P*-450 complexes. The K_s values of complexes formed by cytochrome $P\text{-}450_{Rh}$ considerably exceed the K_s of the corresponding complexes with microsomal cytochrome *P*-450. ΔA_{max} was higher in the case of cytochrome $P\text{-}450_{Rh}$.

The ethyl isocyanide complex of reduced cytochrome *P*-450 from liver microsomes is characterized by two absorption maxima in the Soret region, at 430 nm and 455 nm; their relative heights depend on the pH and the ionic strength of the solution [11]. The reduced cytochrome $P\text{-}450_{cam}$ gives only one peak at 453 nm with ethyl isocyanide [12]. Cytochrome $P\text{-}450_{Rh}$ isolated from soybean bacteroids by Appleby also combines reversibly with ethyl isocyanide

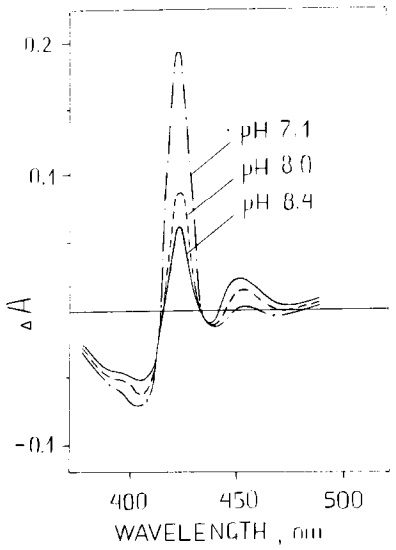

Fig. 1. Difference spectra (dithionite reduced + 240 μM ethyl isocyanide minus dithionite reduced) of 1.10 μM cytochrome $P\text{-}450_{Rh}$ in 0.1 M phosphate buffer.

giving two peaks in the Soret region (428 nm and 453 nm); their heights depend on pH [1].

Fig. 1 shows the respective spectra of cytochrome $P\text{-}450_{Rh}$ from lupin bacteroids. The location of the absorption maxima is close to those of microsomal cytochrome and cytochrome $P\text{-}450_{Rh}$ from soybean bacteroids. The peak at 456 nm was intensified progressively when the pH was raised, and the peak at 427 nm decreased at the same time. The K_s value for ethyl isocyanide complex was 19.6 μM at pH 7.0.

Thus, according to the spectra presented, cytochrome $P\text{-}450_{Rh}$ seems to be similar to membrane-bound cytochrome *P*-450 from liver microsomes rather than to soluble bacterial cytochrome $P\text{-}450_{cam}$.

According to the data in table 2, the substrates and inhibitors of the microsomal hydroxylation, which interact with cytochrome $P\text{-}450_{Rh}$, inhibited bacteroid N_2-fixation. 50% Inhibition of ethylene formation occured at concentrations close to the K_s values (see table 1); that indirectly points to the participation of cytochrome $P\text{-}450_{Rh}$ in the process of nitrogen fixation.

Oxygen consumption also decreased under the action of these substances, but somewhat less than the reduction of acetylene to ethylene (except the case of SKF-525 A inhibition).

The fact that respiration of ineffective bacteroids, neither fixing nitrogen nor containing cytochrome $P\text{-}450_{Rh}$ [4], was not inhibited by the substances studied, favours the suggestion that cytochrome $P\text{-}450_{Rh}$ participates in processes tightly connected with nitrogen fixation in lupin bacteroids.

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Table 2
The effect of substances giving complexes with cytochrome $P\text{-}450_{Rh}$ on ethylene formation and oxygen consumption by lupin nodule bacteroids

Substance	Concentration (mM)	Inhibition, %		
		Ethylene formation	Oxygen consumption by	
			Effective bacteroids	Ineffective bacteroids
Dimethylaniline	7.0	50	22	0
Hexobarbital	1.9	50	20	0
D(+)-Camphor	2.6	50	34	0
SKF – 525 A	0.35	50	66	22
Aniline	10.1	50	24	0
Metyrapone	3.5	50	26	0

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