Biochimica et Biophysica Acta, 387 (1975) 461–474

BBA 46937

INVOLVEMENT OF OXYLEGHAEMOGLOBIN AND CYTOCHROME *P*-450 IN AN EFFICIENT OXIDATIVE PHOSPHORYLATION PATHWAY WHICH SUPPORTS NITROGEN FIXATION IN *RHIZOBIUM*

C. A. APPLEBY, G. L. TURNER and P. K. MACNICOL

Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra (Australia)

(Received December 6th, 1974)

SUMMARY

Cellular ATP level, ATP/ADP ratio and nitrogenase activity rise when oxyleghaemoglobin is added to respiring suspensions of *Rhizobium japonicum* bacteroids from soybean root nodules. Increased gaseous O_2 tension is much less efficient than oxyleghaemoglobin in stimulation of bacteroid ATP production. Studies with the inhibitor carbonyl cyanide *m*-chlorophenylhydrazone show this ATP to be generated as a consequence of oxidative phosphorylation. *N*-Phenylimidazole, a specific cytochrome *P*-450 inhibitor, also lowers the efficiency of bacteroid oxidative phosphorylation. An approximately linear relationship is observed between ATP/ADP ratio and nitrogenase activity as *N*-phenylimidazole concentration is lowered. It is suggested that cytochrome *P*-450 is a component of the leghaemoglobin-facilitated respiration pathway and that it may act as intracellular O_2 carrier rather than terminal oxidase. A less efficient oxidase appears to function when cytochrome *P*-450 is inhibited.

INTRODUCTION

The addition of oxyleghaemoglobin to respiring bacteroids isolated from soybean root nodules considerably increases their nitrogen fixing efficiency [1–3]. The function of oxyleghaemoglobin appears to be the delivery of free, dissolved O_2 to the bacteroids at a stable, low O_2 tension [1–4], and it was proposed [2, 3] that bacteroids might contain two or more oxidase systems of differing O_2 affinity and phosphorylating efficiency. One of these systems, in responding specifically to leghaemoglobin-facilitated O_2 flux, was proposed to raise bacteroid ATP/ADP ratio to a level required for high nitrogenase activity (cf. refs 5 and 6). Other oxidase(s), possibly responsible for cellular maintenance respiration [3] or protective respiration [7], were proposed to have lower phosphorylating efficiencies. Bacteroids contain a number of autoxidizable cytochromes, including *P*-428 (possibly cytochrome a_1) and

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; K_1, ligand equilibrium dissociation constant; K_i, inhibition constant, measured during enzymic reactions.

cytochrome *P*-450, which are not found in air-grown rhizobia [8], and these bacteroid cytochromes have been proposed as alternative oxidases [8, 9]. Very recently [3, 10], studies with inhibitors of cytochrome *P*-450 have directly implicated this cytochrome in bacteroid respiration and nitrogenase activity.

In the present work we offer experimental evidence that bacteroid ATP level, ATP/ADP ratio and nitrogenase (acetylene-reducing) activity increase more in response to O_2 supplied via oxyleghaemoglobin than to O_2 supplied by free diffusion. By using the inhibitors *N*-phenylimidazole and CCCP at concentrations which have negligible effect on cell-free anaerobic nitrogenase activity or on leghaemoglobin, we also demonstrate that cytochrome *P*-450 appears to be part of an efficient oxidative phosphorylation pathway which functions when oxyleghaemoglobin is present. We argue that cytochrome *P*-450 may be acting as an intracellular O_2 carrier, rather than an oxidase.

MATERIALS AND METHODS

Rhizobium bacteroid and Azotobacter suspensions. Nodules were produced on roots of soybeans (*Glycine max* cv. Lincoln) by inoculation with *Rhizobium japonicum* strain CC705 (syn. Wisconsin 505), and bacteroids prepared anaerobically from fresh nodules as previously described [1, 2], except that they were finally suspended in anaerobic 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM $MgSO_4$ but no sucrose.

Azotobacter vinelandii strain O was grown with vigorous aeration at 25 °C in a nitrogen-deficient, inorganic salts medium plus sucrose [11], and the unwashed cells in culture medium used for measurements of nitrogenase activity.

Soybean oxyleghaemoglobin. This was prepared as a 2 mM stock solution in 50 mM phosphate buffer (pH 7.4) as previously [2], and was divided into small samples and stored in liquid N_2.

Reagents. N-Phenylimidazole was kindly synthesized by Dr J. L. Huppatz, CSIRO, and CCCP was a gift from Dr P. G. Heytler, Du Pont and Co., Wilmington, Delaware. Reagents were analytical grade, and gas mixtures were made and stored in a metal reservoir with O-ring seals [1].

Gas analysis. Oxygen uptake and nitrogenase (acetylene reduction to ethylene) activities were calculated following analysis bymass spectrometry and gas chromatography of zero time gas samples and gas samples removed from reaction vessels at the end of 30-min incubations [1, 2]. Specific activities are expressed as nmol/min per mg bacteroid dry weight.

Bacteroid adenine nucleotide analysis. Incubations (2 ml) were terminated at 30 min by injecting 1.0 ml of 1.2 M $HClO_4$/3 mM EDTA, with rapid mixing and cooling to 0 °C; the suspensions were then held for 45 min at 0 °C with occasional shaking. After centrifuging (20 000 × *g*, 10 min) the clear supernatants were brought to pH 7.4 with one-fourth volume of a triethanolamine/KOH buffer such that final triethanolamine concentration was 50 mM, and then recentrifuged at 0 °C. Levels of ATP, ADP and AMP in the final supernatant were determined by a fluorimetric procedure [12] and are recorded as nmol/mg bacteroid dry weight.

Effect of inhibitors on anaerobic nitrogenase activity, and on leghaemoglobin. Partially purified soybean bacteroid nitrogenase was used, and assayed for activity

by acetylene reduction as previously described [13], with and without inhibitors present. In these anaerobic assays, electrons were supplied from dithionite and ATP from a generating system (creatine phosphate, creatine phosphokinase and ATP).

To study the effect of inhibitors on pure oxyleghaemoglobin, this protein was diluted to 0.5 mM in 50 mM phosphate (pH 7.4) and absorption spectra (700–450 nm) recorded in 1-mm optical path cuvettes in the absence and presence of inhibitor. Each spectrum was repeated after deoxygenation of oxyleghaemoglobin to ferrous leghaemoglobin with dithionite. The wavelength pair, 556 minus 475 nm, was used to measure the extent of haemochrome formation from ferrous leghaemoglobin, and ligand dissociation constants (K_I) measured from Lineweaver and Burk plots (cf. pp. 68–69 of ref. 14) of $1/\Delta A_{(556-475nm)}$ versus 1/(inhibitor concentration).

Bacteroid respiration, phosphorylation and aerobic nitrogenase activity; effects of oxyleghaemoglobin and inhibitors. Incubations were carried out at 25 °C as previously [1,2] in 34-ml capacity vials closed with serum stoppers and shaken at 150 cycles/min. The reaction mixtures (2 ml) contained 20–24 mg bacteroid dry weight, 50 mM potassium phosphate (pH 7.4), 2 mM $MgCl_2$, 50 mM sodium succinate and (when present) 0.5 mM oxyleghaemoglobin. Inhibitors were added from freshly prepared, concentrated solutions (pH 7.4). The gas phase (32 ml) contained O_2 (20 mm Hg pressure), acetylene (173 mm) and argon to 700 mm. In some experiments (Table II) the O_2 pressure was increased and argon pressure correspondingly decreased. Gas samples were withdrawn for assay from triplicate reaction vessels at the end of 30-min incubations, and average values reported. For adenine nucleotide assays, reactions were terminated by acid injection (see above) at the end of 30 min incubations, and the results reported are the averages from duplicate or triplicate assays.

N-Phenylimidazole reactivity with bacteroid cytochrome P-450; comparisons with N-phenylimidazole effect on aerobic nitrogenase activity. *N*-Phenylimidazole is a known ligand for other microbial cytochromes *P*-450 [15, 16] and the objective of the following assays was to see if it reacted with bacteroid *P*-450 at the levels which inhibited the aerobic nitrogenase activity of whole bacteroids. Cytochrome *P*-450 difference spectra were measured on anaerobically prepared whole bacteroid suspensions, on the anaerobically prepared, dithionite-reduced 144 000 $\times g$ supernatant from ruptured bacteroids [8, 13] and on dithionite-reduced solutions of purified *Rhizobium* bacteroid cytochromes *P*-450 [17]. Preliminary assay of *P*-450 concentration was made by CO difference spectra on dithionite-reduced reaction pairs in 2-mm optical path cuvettes, using the scattered transmission accessory of a Cary 14 spectrophotometer [8]; all samples were then diluted to 1.4 μM cytochrome P-450 in 50 mM phosphate (pH 7.4). Increments of concentrated *N*-phenylimidazole solution (pH 7.4) were then added to a sample cuvette and equal volumes of buffer to a reference cuvette. Succeeding difference spectra were recorded and values of ligand dissociation constant (K_I) measured as above from plots of $1/\Delta A$ versus 1/free *N*-phenylimidazole concentration, where ΔA represents absorbance change at a convenient wavelength pair (cf. Table V).

Approximate values of K_i for the inhibition of bacteroid nitrogenase activity were measured according to Dixon and Webb [14] (pp. 328–329) from plots of $1/v$ versus inhibitor concentration. In the absence of meaningful information about competing ligand or substrate concentrations we assumed the condition of non-competitive inhibition.

RESULTS

Choice of inhibitors, and permissible concentrations

In this study of leghaemoglobin-stimulated bacteroid nitrogenase activity, any inhibitor directed against oxidative phosphorylation or the suspected oxidase component, cytochrome *P*-450 [3, 8–10] had to be shown to have minimal or no effect on leghaemoglobin or anaerobic (ATP-supported) nitrogenase activity. After preliminary experiments with a range of reputed inhibitors of oxidative phosphorylation, bacterial oxidases and of cytochrome *P*-450 (Appleby, C. A. and Turner, G. L., unpublished) we selected CCCP, a phosphorylation inhibitor [18] and *N*-phenylimidazole, a cytochrome *P*-450 inhibitor [15, 16] as least likely to have non-specific effects. Table I shows that up to 100 μM CCCP or 10 mM *N*-phenylimidazole caused variable, but slight inhibition of purified nitrogenase. These effects are within the precision limits of the assay. Furthermore, 100 μM CCCP had no effect on the spectra of ferrous or oxyleghaemoglobin (cf. Materials and Methods) so that the 50 μM limiting concentration of CCCP actually used was unlikely to have any effect on the O_2-carrying function of the 0.5 mM oxyleghaemoglobin added to relevant assays. But 10 mM *N*-phenylimidazole had a considerable effect on the spectrum of ferrous leghaemoglobin, and K_I for ferrous haemochrome formation was 25 mM. At 1 mM N-phenylimidazole, the limiting concentrations finally chosen, this inhibitor had no discernible effect on the spectrum of oxyleghaemoglobin and caused only 3 % formation of ferrous haemochrome from dithionite-reduced leghaemoglobin. In anticipation of later experimental results (cf. Fig. 2) we also point out that leghaemoglobin when present, is always added at a concentration (0.5 mM) which is much higher than the concentration of *N*-phenylimidazole ($\cong$ 140 μM) found to half inhibit bacteroid nitrogenase activity. Hence, the principal effect of this inhibitor cannot be cryptic binding to and inactivation of leghaemoglobin.

TABLE I

EFFECT OF CCCP AND *N*-PHENYLIMIDAZOLE ON ANAEROBIC NITROGENASE ACTIVITY OF THE PARTIALLY PURIFIED ENZYME FROM BACTEROIDS

Ethylene evolution, from acetylene, shown as percentage of uninhibited control reactions is the mean of duplicate analyses. All reactions included an ATP generating system, and dithionite as reductant. Experimental conditions are given in Materials and Methods.

CCCP (μM)	Activity (%)	*N*-Phenylimidazole	Activity (%)
5	95	10 μM	103
10	103	100 μM	109
20	88	1 mM	104
100	90	10 mM	97

A comparison of increased gaseous O_2 tension versus oxyleghaemoglobin in the stimulation of bacteroid activities

Ratio of O_2 uptake to acetylene reduction. A useful way to detect changes in the efficiency of bacteroid nitrogenase (acetylene-reducing) activity is to calculate the ratio, ΔO_2 consumed/Δ C_2H_4 evolved following different experimental treat-

TABLE II

STIMULATION OF BACTEROID OXIDATIVE PHOSPHORYLATION AND NITROGENASE ACTIVITY BY INCREASING GASEOUS O_2 TENSION OR BY OXYLEGHAEMOGLOBIN-FACILITATED O_2 SUPPLY

The standard assay, with free gas phase, is described in Materials and Methods. Nitrogenase activity is measured by acetylene reduction to ethylene, and specific activities are expressed/mg bacteroid dry weight. Use of the ratio $\Delta O_2/\Delta C_2H_4$ is explained in the text. Nucleotide levels are measured at the end of standard 30-min assays and expressed/mg bacteroid dry weight.

Treatment (mm O_2)	nmol/min per mg		ΔO_2	nmol/mg				ATP
	O_2 uptake	C_2H_4 evolution	ΔC_2H_4	AMP	ADP	ATP	Total adenine nucleotide	ADP
20	3.85	1.33	—	0.13	0.55	0.67	1.35	1.2
30	8.26	1.95	7.11	0.10	0.47	0.81	1.39	1.7
40	10.6	2.22	7.58	0.09	0.45	0.93	1.47	2.1
20 plus 0.5 mM oxyleghaemoglobin	9.04	4.93	1.44	0.05	0.30	1.08	1.42	3.6

ments [2]. Thus, Table II shows that adding 0.5 mM oxyleghaemoglobin to bacteroid suspensions shaken under 20 mm O_2 increases their O_2 uptake from 3.85 to 9.04 nmol/min per mg ($\Delta O_2 = 5.19$) and at the same time stimulates ethylene production from 1.33 to 4.93 nmol/min per mg ($\Delta C_2H_4 = 3.60$). The ratio $\Delta O_2/\Delta C_2H_4$ of 1.44 in this particular experiment is somewhat lower than the overall mean value of 2.13 obtained in the present series (Table III) although close to the mean (1.54) obtained in earlier experiments [2] using a different strain of *R. japonicum*. When bacteroid respiration is raised to just below or just above the leghaemoglobin-simulated level by simply increasing gaseous O_2 tension to 30 or 40 mm (Table II), the ratio $\Delta O_2/\Delta C_2H_4$ rises to 7.11 and 7.58, respectively; i.e. the efficiency of bacteroid O_2 utilization is low in the absence of oxyleghaemoglobin. This is in agreement with earlier work (cf. footnote 2 of ref. 2) and with recent results of Bergersen and Turner [19]

TABLE III

EFFECT OF OXYLEGHAEMOGLOBIN ON BACTEROID O_2 UPTAKE, NITROGENASE ACTIVITY AND OXIDATIVE PHOSPHORYLATION

The values given are the means and standard errors (S.E.) of nine separate experiments made under standard conditions (20 mm O_2 tension, with or without 0.5 mM oxyleghaemoglobin; cf. Table II) on bacteroids prepared from separate batches of soybean nodules. Oxygen uptake and ethylene evolution are expressed as nmol/min per mg bacteroid dry weight; ATP concentrations are expressed as nmol/mg at the end of 30-min incubations.

	Minus oxyleghaemoglobin				Plus oxyleghaemoglobin				
	O_2 uptake	C_2H_4 evolution	ATP	$\frac{ATP}{ADP}$	O_2 uptake	C_2H_4 evolution	$\frac{\Delta O_2}{\Delta C_2H_4}$	ATP	$\frac{ATP}{ADP}$
Mean	4.63	1.41	0.94	1.34	12.91	5.25	2.13	1.74	3.44
S.E.	0.76	0.10	0.07	0.12	1.67	0.38	0.25	0.11	0.31

where O_2 consumption and nitrogenase (acetylene-reducing) activity of bacteroid suspensions were measured in the absence of a gas phase. This latter technique had removed the possibility of anomalies due to restrictions on gas exchange across the gas-liquid interface and through the stirred liquid. In our experiments, observations with a hand spectroscope suggested that leghaemoglobin remained 50–75% oxygenated in shaken, uninhibited, reactions to which oxyleghaemoglobin was initially added. This is close to the optimum found for nitrogenase activity by Bergersen and Turner [19].

Efficiency of oxidative phosphorylation. In standard incubations (Table II) the total nucleotide level remained almost constant, but ATP level and ATP/ADP ratio both rose when oxyleghaemoglobin was added under 20 mm gaseous O_2 pressure (Table II). In nine similar experiments (Table III), the mean ATP concentration rose from 0.94 to 1.74 nmol/mg bacteroid dry weight and the mean ATP/ADP ratio rose from 1.34 to 3.44 when oxyleghaemoglobin was added to incubations under 20 mm O_2. These observations allow the important conclusion that leghaemoglobin-facilitated O_2 supply significantly increases the level of bacteroid ATP and hence the ATP/ADP ratio. Table II also shows that the increased O_2 consumption which followed the rise in gaseous O_2 tension to 40 mm is much less efficient than leghaemoglobin-stimulated respiration in support of bacteroid oxidative phosphorylation; the ratio ATP/ADP rose to only 2.1, compared with 3.6 for leghaemoglobin-stimulated respiration.

Effect of CCCP and N-phenylimidazole on bacteroid respiration, phosphorylation and nitrogenase activity, with and without oxyleghaemoglobin

The activities reported in Tables II and III are consistent with the hypothesis [1–3] that bacteroids may possess two or more separate oxidase systems, of differing phosphorylating efficiency, but say nothing about their nature. The following experiments establish that the ATP produced during leghaemoglobin-stimulated respiration

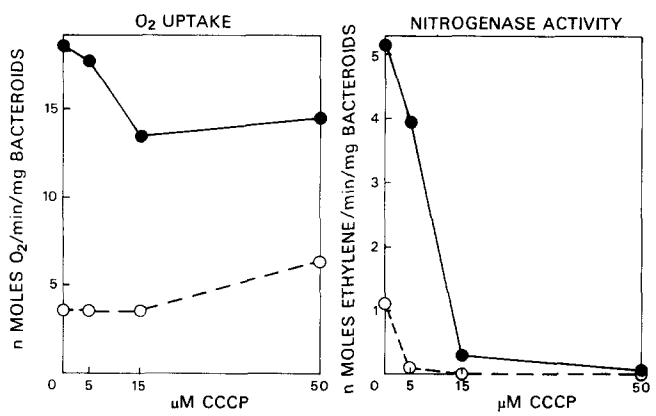

Fig. 1. Inhibition of bacteroid O_2 uptake and nitrogenase activity by CCCP, in the absence (○- - -○) and presence (● — ●) of 0.5 mM oxyleghaemoglobin. Each shaken assay (2 ml) contained approx. 20 mg bacteroids (equivalent dry wt) and the gas phase contained 20 mm O_2 pressure. General experimental conditions are given in Materials and Methods. The concentrations of ATP, and ATP/ADP ratios measured at the end of this experiment are recorded in Table IV.

TABLE IV

EFFECT OF INHIBITORS ON BACTEROID OXIDATIVE PHOSPHORYLATION

These values are directly related to the experimental points shown on Figs 1 and 2 at increasing inhibitor concentration, being obtained from the same two experimental series. ATP concentrations are expressed as nmol/mg bacteroid dry weight at the end of 30-min incubations.

Inhibitor (μM)	Minus oxyleghaemoglobin		Plus oxyleghaemoglobin	
	ATP	$\frac{ATP}{ADP}$	ATP	$\frac{ATP}{ADP}$
(A) CCCP				
0	0.73	1.0	1.83	5.2
5	0.66	0.8	1.42	2.4
15	0.48	0.6	0.84	1.5
50	0.53	0.6	0.78	1.1
(B) *N*-Phenylimidazole				
0	0.91	1.4	1.42	2.7
100	0.48	0.6	1.17	2.1
300	0.56	0.65	0.62	0.75
1000	0.16	0.2	0.52	0.7

is a consequence of oxidative, rather than substrate-level phosphorylation, and that cytochrome *P*-450 may be a component of this respiration pathway.

CCCP effects. The results obtained with this inhibitor were as expected for an uncoupler of oxidative phosphorylation [18]. Bacteroid O_2 uptake did not show much change (Fig. 1A), but there was a considerable decrease in bacteroid ATP level and ATP/ADP ratio (Table IVA) especially in the presence of oxyleghaemoglobin, as the CCCP level was raised to 50 μM. This was accompanied (Fig. 1B) by a dramatic decline of bacteroid nitrogenase activity, including almost complete prevention of the leghaemoglobin-stimulated activity. We note, however, that the correlation between ATP/ADP ratio and nitrogenase activity was not complete. For instance, in the absence of leghaemoglobin, and of CCCP, the ATP/ADP ratio was 1.0 (Table IVA) and nitrogenase activity was 1.1 nmol C_2H_4 evolved/min per mg bacteroid dry weight (Fig. 1B). Yet in the presence of oxyleghaemoglobin, with 50 μM CCCP, the ATP/ADP ratio was 1.1 and nitrogenase activity had fallen to 0.02 nmol C_2H_4/min per mg. Since 50 μM CCCP has negligible effect on purified nitrogenase (Table I) we consider, as one of several possible interpretations, that there may be separate domains of ATP formation and accumulation within the bacteroid, and that one of these is more sensitive to uncoupling by CCCP.

Effects of N-phenylimidazole, a cytochrome P-450 inhibitor. As found for CCCP, *N*-phenylimidazole had little effect on bacteroid O_2 consumption, in the presence or absence of oxyleghaemoglobin. In the experiment recorded as Fig. 2A, a slight decrease occurred in total respiration, but the mean values from five separate experiments (Turner, G. L. and Appleby, C. A., unpublished) showed this to be insignificant. There was, however, a considerable decrease in bacteroid aerobic nitrogenase activity (Fig. 2B) caused by *N*-phenylimidazole at concentrations which did not inhibit the anaerobic, ATP-supported nitrogenase activity of the purified

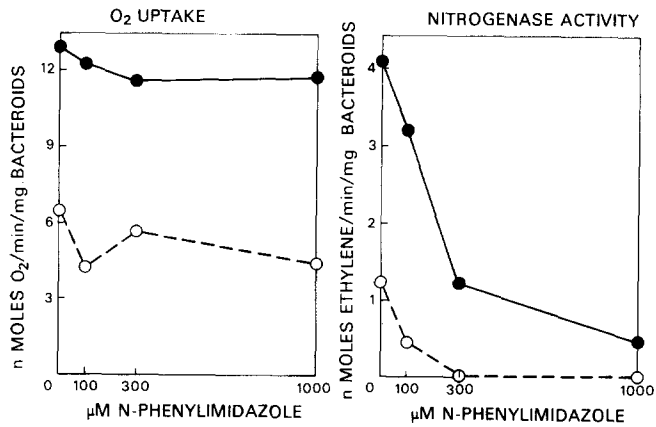

Fig. 2. Inhibition of bacteroid O_2 uptake and nitrogenase activity by *N*-phenylimidazole, in the absence (○- - -○) and presence (●—●) of 0.5 mM oxyleghaemoglobin. Conditions as for Fig. 1. The concentrations of ATP, and ATP/ADP ratios measured at the end of this experiment are recorded in Table IV and Fig. 4.

enzyme (Table I). Estimates of inhibition constant (cf. Materials and Methods) from the nitrogenase activities recorded in Fig. 2B gave $K_i \cong 20\ \mu M$ *N*-phenylimidazole in the absence and $K_i \cong 140\ \mu M$ in the presence of oxyleghaemoglobin.

Bacteroid ATP level and ATP/ADP ratio both decline when *N*-phenylimidazole is added to respiring bacteroid suspensions (Table IV) and when the relevant results from this table, and from Fig. 2B are combined to give Fig. 3, an approximately linear relationship is observed between the ATP/ADP ratio and nitrogenase activity.

Although the pattern of inhibition of bacteroid processes by *N*-phenylimidazole has a superficial resemblance to that obtained with CCCP, the following evidence

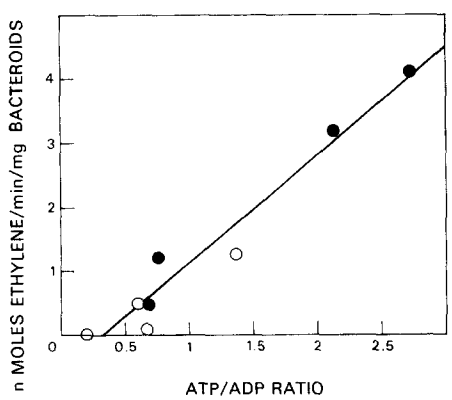

Fig. 3. The relationship between bacteroid nitrogenase activity and ATP/ADP ratio in the presence (solid circles) and absence (open circles) of 0.5 mM oxyleghaemoglobin, during inhibition with increasing amounts of *N* phenylimidazole. These experimental points are plotted from the data recorded in Fig. 2 and Table IV. The straight line of best fit was determined by least squares analysis, and the product moment correlation coefficient, $r = +0.972$.

suggests that *N*-phenylimidazole does not resemble CCCP in acting primarily as an uncoupler of oxidative phosphorylation [18]. Dr I. J. Ryrie, Australian National University (personal communication), could not demonstrate any effect of *N*-phenylimidazole on overall oxidative phosphorylation or phosphate exchange processes in yeast mitochondria. Furthermore, *A. vinelandii* resembles *R. japonicum* in relying on oxidative phosphorylation to support nitrogenase activity [6, 7] yet we found (Fig. 4) only a slight effect of *N*-phenylimidazole on aerobic nitrogenase activity in this organism. The apparent value of K_i, 3 mM *N*-phenylimidazole, calculated from Fig. 4 for inhibition of *Azotobacter* nitrogenase is 150-times higher than the corresponding value for inhibition of bacteroid nitrogenase in the absence of oxyleghaemoglobin. Bacteroids contain cytochrome *P*-450, *A. vinelandii* apparently does not [20] and we show below that a high affinity interaction occurs between bacteroid cytochrome *P*-450 and *N*-phenylimidazole.

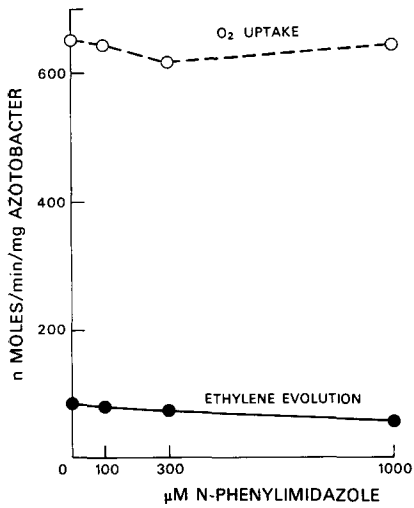

Fig. 4. Inhibition of *A. vinelandii* O_2 uptake and nitrogenase activity by *N*-phenylimidazole. A small volume of unwashed nitrogen-fixing liquid culture of *A. vinelandii* was assayed under the standard conditions described for bacteroids, except that oxyleghaemoglobin was omitted. Each assay contained 3.2 mg (dry wt) of bacterial cells.

Reactions of bacteroid cytochrome P-450 with N-phenylimidazole

Much of the cytochrome *P*-450 present in bacteroids is released into solution when they are ruptured [8], and Fig. 5A, trace 1 shows the *N*-phenylimidazole difference spectrum of a dithionite-reduced $144000 \times g$ supernatant from anaerobically prepared and broken bacteroids. It has a major absorption peak at 425 nm and a lesser peak at 445 nm, so the reaction of bacteroid *P*-450 with *N*-phenylimidazole has at least a superficial resemblance to that which occurs between ethyl isocyanide and the reduced cytochrome *P*-450 from bacteroids [21] or from animal microsomes [22]. In this reaction with isocyanide the twin absorption peaks, at 430 and 455 nm, are thought to represent the reactions of ligand with two conformational states of cytochrome *P*-450 in equilibrium with each other [22]. When purified, high-spin

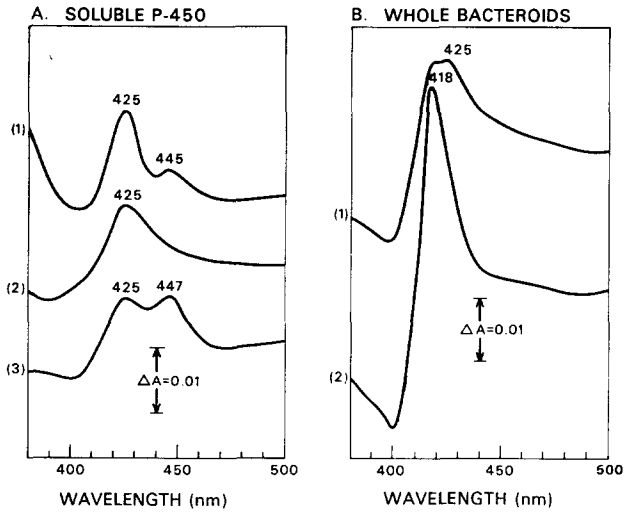

Fig. 5. Difference spectra of the reactions between *N*-phenylimidazole and bacteroid cytochrome *P*-450. In each experiment, *N*-phenylimidazole solution was added to the sample cuvette and an equivalent volume of buffer to the reference cuvette. (A) Dithionite-reduced soluble preparations, at saturating ligand concentrations, measured in 2-mm light path cuvettes. Trace 1 is the 144 000 × *g* supernatant from ruptured bacteroids, trace 2 is purified high-spin cytochrome *P*-450*b* and trace 3 is purified low-spin cytochrome *P*-450*c* (ref. 15). (B) Anaerobic whole bacteroid suspensions, no dithionite present, in 2-mm light path cuvettes. Trace 1 was recorded approx. 1 min after addition of 1 mM *N*-phenylimidazole to the sample cuvette and trace 2 recorded after 15 min. All preparations contain approx. 1.4 μM total cytochrome *P*-450, at pH 7.4 (cf. Materials and Methods).

TABLE V

REACTIVITY OF BACTEROID CYTOCHROME *P*-450 SPECIES WITH *N*-PHENYLIMIDAZOLE

These values were determined from *N*-phenylimidazole difference spectra of an anaerobic suspension of whole bacteroids, of a dithionite-reduced 144 000 × *g* supernatant from ruptured bacteroids and of dithionite-reduced solutions of the purified components P-450*b* and *P*-450*c*, as recorded in Fig. 5. Values of K_1 were measured as described in Materials and Methods. Values of $\Delta\varepsilon$ (mM) were measured from difference spectra, at saturating ligand concentration, at the wavelength pair λ_{max}-470 nm and are based on total cytochrome *P*-450 in each preparation as calculated from CO difference spectra [8].

Fraction	λ max (nm)	$\Delta\varepsilon$ (mM)	K_1 (μM)
144 000 × *g* supernatant	425	48	130
Cytochrome *P*-450*b* (high spin)	425	31	140
Cytochrome *P*-450*c* (low spin)	447	29	670
Whole bacteroids	418	(111)	($\simeq$3)

Rhizobium cytochrome *P*-450*b* [17] reacts with *N*-phenylimidazole the 425-nm-absorbing species predominates (Fig. 5A, trace 2); when purified, low-spin *Rhizobium* cytochrome *P*-450*c* [17] reacts, the 447-nm-absorbing species is also observed, with slightly greater peak height than the 425-nm-absorbing species (Fig. 5A, trace 3).

From this evidence, we make the tentative assumption that the predominant cytochrome *P*-450 species in the bacteroid 144 000 $\times g$ supernatant (Fig. 5A, trace 1) is high spin (cf. Discussion).

The ligand dissociation constant, K_I, for the reaction with reduced cytochrome *P*-450 in the bacteroid supernatnt is 130 μM *N*-phenylimidazole (Table V). This happens to be almost identical with the value of $K_i \cong 140\ \mu$M *N*-phenylimidazole calculated above (cf. Fig. 2B) for the inhibition of bacteroid nitrogenase activity in the presence of oxyleghaemoglobin. This value is also very close to $K_i = 140\ \mu$M *N*-phenylimidazole found for the reaction with purified high-spin cytochrome *P*-450*b*, compared with $K_I = 670\ \mu$M for the reaction with purified, low-spin cytochrome *P*-450*c* (Table V).

It was not possible to discern the formation of a cytochrome *P*-450-*N*-phenylimidazole complex in respiring bacteroids (i.e. with dissolved O_2 in solution) since such difference spectra were dominated by changes in cytochrome redox state. However, if *N*-phenylimidazole was added to an anaerobic suspension of whole bacteroids, and the difference spectrum recorded immediately (Fig. 5B, trace 1) a 425-nm peak was apparent (cf. the 425-nm peak due to cytochrome *P*-450-*N*-phenylimidazole complexes in Fig. 5A) together with a shoulder at 418–419 nm. This 425-nm peak rapidly faded, as the 418-nm peak became more intense (Fig. 5B, trace 2). On the tentative assumption (cf. Discussion) that this 418-nm-absorbing species represents a third type of cytochrome *P*-450-*N*-phenylimidazole complex we present (Table V) measurements of $\Delta\varepsilon$(mM) and K_I ($\cong 3\mu$M) made from the 418-nm peak in stabilized (15 min) difference spectra.

DISCUSSION

This investigation was undertaken primarily to test the hypothesis [1–3] that leghaemoglobin-facilitated bacteroid respiration is more efficient than unfacilitated respiration in producing the ATP required for nitrogenase activity [5] and to provide evidence for the nature of this respiration pathway. Wittenberg et al. [2] has assumed that a bacteroid oxidase which operated best at 'leghaemoglobin-delivered' O_2 tension was more efficient in ATP production than was a principal oxidase which functioned in the absence of leghaemoglobin. An alternative explanation, offered by Stokes [4] is that delivered O_2 tension might vary greatly in the absence of leghaemoglobin and that the extreme concentration of O_2 could directly inhibit or inactivate nitrogenase.

We show (Table II and III) that bacteroid ATP production is more efficient during leghaemoglobin-facilitated respiration and that an approximately linear relationship exists between the bacteroid ATP/ADP ratio and nitrogenase activity, at least during *N*-phenylimidazole inhibition (Fig. 3).

We also show that the leghaemoglobin-stimulated production of ATP occurs through oxidative rather than substrate-level phosphorylation, since the effect of adding oxyleghaemoglobin is reversed (Table IV, cf. Fig. 1) at levels of CCCP which have no effect on isolated nitrogenase (Table I) nor on ferrous or oxyleghaemoglobin. This inhibitor is known as a specific uncoupler of oxidative phosphorylation [18].

ATP is required for activity of purified nitrogenase, and ADP is a competitive inhibitor of ATP binding [6, 23], so it has been proposed (e.g. ref. 5) that nitrogenase activity in the bacterial cell is regulated by ATP/ADP ratio. Experimental proof of

this hypothesis has been obtained for Azotobacter (e.g. ref. 6), and our Fig. 3 suggests that similar regulation occurs in *Rhizobium* with a threshold ATP/ADP ratio below which nitrogenase activity is negligible. Nevertheless, the scatter of experimental points (Fig. 3) and our inability to discern a linear relationship between ATP/ADP and nitrogenase activity during CCCP inhibition (cf. Results), mean that a more rigid proof is required.

In our standard shaken incubation, under 20 mm gaseous O_2 tension, the average oxygenation of leghaemoglobin (when present) was 50–75 %. Hence it can be calculated (cf. ref. 24) that in the presence of leghaemoglobin the free O_2 tension in the bulk of the stirred solution would be only 0.03–0.05 mm, and would have to be somewhat lower at the bacteroid surface [2, 4]. However, our experiments say nothing about average O_2 tension at the bacteroid surface in the absence of oxyleghaemoglobin, when a less efficient (Table II) oxidative phosphorylation system is operating. Wittenberg et al. [2] had argued that O_2 tension at the bacteroid surface might be extremely low in the absence of oxyleghaemoglobin; this could mean that a high O_2 affinity, low phosphorylating efficiency oxidase such as cytochrome a_1 (cf. ref. 4) might be predominant. On the other hand, recent O_2 electrode measurements by Bergersen and Turner [19] (also personal communication) in the newer, 'no gas phase' system suggest that O_2 consumption occurring in the range of low free O_2 concentration associated with the discharge of O_2 from oxyleghaemoglobin is more efficient than similar rates of O_2 consumption occurring at higher O_2 concentration. This could imply the operation of a low phosphorylating efficiency 'protective' respiration pathway such as occurs in *Azotobacter* [5, 25] at high O_2 concentration.

Whatever the nature of inefficient, unfacilitated respiration, our results with *N*-phenylimidazole suggest that cytochrome *P*-450 is a component of the efficient oxidative phosphorylation pathway which is stimulated in the presence of oxyleghaemoglobin. This ligand reacts with reduced cytochrome *P*-450 in crude bacteroid extracts (Fig. 5A, trace 1) to give a recognizable *N*-phenylimidazole complex with $K_1 = 130\ \mu M$, almost identical with the value of $K_1 = 140\ \mu M$ found for the interaction with purified, high-spin cytochrome *P*-450*b* (Table V), and the value of $K_i = 140$ mM for the inhibition of nitrogenase activity by *N*-phenylimidazole during leghaemoglobin-facilitated bacteroid respiration.

In the absence of leghaemoglobin, *N*-phenylimidazole is even more effective in inhbiting the residual nitrogenase activity (Fig. 2B) and K_i was estimated as $\cong 20\ \mu M$. This could mean that O_2 and *N*-phenylimidazole compete for the same binding site on cytochrome *P*-450 (cf. ref. 26) and that, in the experiment reported as Fig. 2, there was a very low O_2 concentration in the cytochrome *P*-450 domain in the absence of leghaemoglobin. On the other hand, we note that under anaerobic conditions a slow, but very high affinity reaction ($K_1 \cong 3\ \mu M$) occurred between *N*-phenylimidazole and modified cytochrome *P*-450 or between *N*-phenylimidazole and some other cytochromes to give the 418-nm-absorbing species recorded as Fig. 5B, trace 2 and in Table V. Perhaps this means that two separate, but *N*-phenylimidazole-inhibited oxidative phosphorylation pathways are operating in unfacilitated and leghaemoglobin-facilitated bacteroid respiration, respectively. It should be possible, by means of photochemical action spectra (cf. ref. 27) for the reversal of CO-inhibited bacteroid processes, to decide whether cytochrome *P*-450 is a constituent of one or both of these proposed respiration pathways. We have undertaken a study of this problem.

Our present hypothesis is that, at least for the leghemoglobin-facilitated respiration pathway, *N*-phenylimidazole acts by binding to cytochrome *P*-450 and is not itself a direct inhibitor of leghaemoglobin or nitrogenase (Table I) or uncoupler of oxidative phosphorylation (cf. Fig. 4). It follows, then, that uninhibited bacteroid cytochrome *P*-450 may be concerned in some way with electron transport, O_2 activation or O_2 transport within the bacteroid. By the use of a non-hydroxylatable steroid substrate [28] the cytochrome *P*-450 of animal microsomes can be diverted from its normal function of hydroxylation, and apparently made to reduce O_2 to water. But there is so far no experimental evidence that this sort of artificial oxidase, nor indeed any electron flow associated with the reduction of oxidized *P*-450, is linked with oxidative phosphorylation. For the present, then, we offer an alternative explation of bacteroid cytochrome *P*-450 function.

When the soluble, low-spin cytochrome *P*-450 of *Pseudomonas putida* is reacted with its substrate, camphor, to make a high-spin complex, it can be readily reduced and shown to form an O_2 complex [26, 29]. If further electron flow and camphor hydroxylation are prevented this O_2 can be rapidly dissociated by challenge with CO [26, 29] or released as a part-reduced product during autoxidation [30]. Since bacteroid cytochrome *P*-450*b* (Table V; Fig. 5A) can be isolated as a high-spin structure in the apparent absence of dissociable substrate [17] we suggest the possibility that it may act as an intracellular O_2 carrier, with final delivery of O_2, or O_2^{2-} to a specific, high efficiency oxidase or peroxidase (cf. ref. 31) concerned with ATP production in the bacteroid domain.

ACKNOWLEDGEMENTS

Drs F. J. Bergersen, A. N. Stokes, B. A. Wittenberg and J. B. Wittenberg are thanked for their stimulating discussions which preceded and followed this work. Dr J. L. Huppatz is thanked for his synthesis of *N*-phenylimidazole. Mrs L. Grinvalds, Mrs L. Stipnieks and Miss R. M. Totterdell are thanked for their assistance with the experiments.

REFERENCES

1 Bergersen, F. J., Turner, G. L. and Appleby, C. A. (1973) Biochim. Biophys. Acta 292, 271–282
2 Wittenberg, J. B., Bergersen, F. J., Appleby, C. A. and Turner, G. L. (1974) J. Biol. Chem. 249, 4057–4066
3 Appleby, C. A., Bergersen, F. J., Macnicol, P. K., Turner, G. L., Wittenberg, B. A. and Wittenberg, B. A. and Wittenberg, J. B. (1974) in International Symposium on Nitrogen Fixation: Inter-disciplinary Discussions (Newton, W. E. and Nyman, C. J., eds), Pullman, Washington, in the press
4 Stokes, A. N. (1975) J. Theoret. Biol., in the press
5 Dalton, H. and Mortenson, L. E. (1972) Bacteriol. Rev. 36, 231–260
6 Haaker, H., DeKok, A. and Veeger, C. (1974) Biochim. Biophys. Acta 357, 344–357
7 Postgate, J. R. (1971) in The Chemistry and Biochemistry of Nitrogen Fixation (Postgate, J. R, ed.), pp. 170–172, Plenum Press, London
8 Appleby, C. A. (1969) Biochim. Biophys. Acta 172, 71–87
9 Kretovich, L., Matus, V. K. and Melik-Sarkissian, S. S. (1972) Fiziol. Rast. 19, 1060–1065
10 Kretovich, L., Melik-Sarkissian, S. S., Raikschinstein, M. V. and Archakov, A. I. (1974) FEBS Lett. 44, 305–308
11 Strandberg, G. W. and Wilson, P. W. (1968) Can. J. Microbiol. 14, 25–31

12 Macnicol, P. K. (1972) Anal. Biochem. 45, 624–633
13 Bergersen, F. J. and Turner, G. L. (1973) Biochem. J. 131, 61–75
14 Dixon, M. and Webb, E. C. (1964) Enzymes, 2nd edn, Longmans, London
15 Hollenberg, P. F. and Hager, L. P. (1973) J. Biol. Chem. 248, 2630–2633
16 Yu, C. A., Gunsalus, I. C., Katagiri, M., Suhara, K. and Takemori, S. (1974) J. Biol. Chem. 249, 94–101
17 Appleby, C. A. and Daniel, R. M. (1973) in Oxidases and Related Redox Systems (Proc. 2nd Int. Symp.), (King, T. E., Mason, H. S. and Morrison, M., eds), pp. 515–528, University Park Press, Baltimore
18 Heytler, P. G. (1963) Biochemistry 2, 357–361
19 Bergersen, F. J. and Turner, G. L. (1975) J. Gen. Microbiol. in the press
20 Jones, C. W. and Redfearn, E. R. (1967) Biochim. Biophys. Acta 143, 340–353
21 Appleby, C. A. (1967) Biochim. Biophys. Acta 147, 399–402
22 Imai, Y. and Mason, H. S. (1971) J. Biol. Chem. 246, 5970–5977
23 Tso, M. W. and Burris, R. H. (1973) Biochim. Biophys. Acta 309, 263–270
24 Wittenberg, J. B., Appleby, C. A. and Wittenberg, B. A. (1972) J. Biol. Chem. 247, 527–531
25 Jones, C. W., Brice, J. M., Wright, V. and Ackrell, B. A. C. (1973) FEBS Lett. 29, 77–81
26 Lipscomb, J. D. and Gunsalus, I. C. (1973) Drug Metab. Disp. 1, 1–5
27 Estabrook, R. W., Cooper, D. Y. and Rosenthal, O. (1963) Biochem. Z. 338, 741–755
28 Narasimhulu, S. (1971) Arch. Biochem. Biophys. 147, 384–390
29 Peterson, J. A., Ishimura, Y. and Griffin, B. W. (1972) Arch. Biochem. Biophys. 149, 197–208
30 Sligar, S. G., Lipscomb, J. D., Debrunner, P. G. and Gunsalus, I. C. (1974) Biochem. Biophys Res. Commun. 61, 290–296
31 Erecińska, M., Oshino, N., Loh, P. and Brocklehurst, E. (1973) Biochim. Biophys. Acta 292, 1–12