Identification of symbiosis-specific *c*-type cytochromes and a putative oxidase in bacteroids of *Rhizobium leguminosarum* biovar *viciae*

Carmen Vargas,¹† Guanghui Wu,² Maria-Jesus Delgado,¹ Robert K. Poole² and J. Allan Downie¹

Author for correspondence: J. Allan Downie. Tel: +44 1603 452571. Fax: +44 1603 456844.
e-mail: Downie@bbsrc.ac.uk

¹ John Innes Centre,
Norwich, NR4 7UH, UK

² Division of Life Sciences,
King's College London,
London W8 7AH, UK

Covalently bound haem proteins and cytochromes were analysed in *Rhizobium leguminosarum* biovar *viciae* free-living cells and nitrogen-fixing bacteroids isolated from pea nodules. Increased levels of spectroscopically detectable cytochrome *c* in bacteroids were correlated with the appearance of two proteins of *M*_r 30 000 and 28 000 that contained covalently bound haem. Conversely, bacteroids had undetectable levels of a periplasmic cytochrome *c* of *M*_r 14 000 that is normally present in free-living bacteria. Difference spectra confirmed that the terminal oxidases, cytochromes *aa*₃ and *d*, were absent, and photodissociation spectra revealed novel components that may be due to a bacteroid terminal oxidase.

Keywords: *Rhizobium*, cytochromes, nitrogen-fixation, bacteroids, haem

INTRODUCTION

When bacteria of the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (rhizobia) develop into nitrogen-fixing bacteroids in the root nodules of legumes, several genes involved in symbiotic nitrogen fixation are induced. Some of these genes are involved in the formation of the enzymes required for the reduction of nitrogen, while others are involved in the high level of bacteroid metabolism required to provide the energy to drive the nitrogen fixation process. The nitrogenase is very oxygen-labile and within legume nodules the oxygen concentration is maintained at a low level, estimated to be in the range 5–30 nM (Appleby, 1984). At such low oxygen concentrations, plant-made leghaemoglobin, which has a high affinity for oxygen and an appropriate oxygen 'off' rate, facilitates oxygen diffusion. To optimize respiration in this low-oxygen environment, a novel branch of the electron transport pathway encoded by the *fixNOQP* genes is induced in bacteroids of *Bradyrhizobium japonicum* (Preisig *et al.*, 1993), *Azorhizobium caulinodans* (Mandon *et al.*, 1994), *Rhizobium meliloti* (Kahn *et al.*, 1993) and *Rhizobium leguminosarum* (Schluter *et al.*, 1993). It is proposed that *fixO* and *fixP* encode two proteins with characteristics similar to *c*-type cytochromes and that *fixN*

encodes a haem-binding terminal oxidase that resembles subunit I of Cu-containing terminal oxidases (Preisig *et al.*, 1993).

When the *fixNOQP* genes are induced, some cytochromes decrease in amount in bacteroids. Thus, in *B. japonicum* and *R. leguminosarum*, cytochrome *aa*₃ is absent from bacteroids whereas it is a normal component of bacteria grown in aerobic culture (Appleby, 1969a, b). When cultured in the presence of normal or lowered levels of oxygen, *R. leguminosarum* cells were reported (Kretovich *et al.*, 1973) to show little alteration in the level of cytochrome *aa*₃ but showed significantly increased levels of cytochrome *d* when oxygen became limiting. Neither of these two oxidases was found in bacteroids (Kretovich *et al.*, 1973). We have extended the characterization of haem proteins present in *R. leguminosarum* bacteroids by identifying two symbiosis-specific *c*-type cytochromes. As well as confirming the lack of cytochromes *aa*₃ and *d*, we have used low-temperature photodissociation methods to reveal a ligand-reactive haem protein that is a candidate for the high-affinity symbiosis-specific terminal oxidase in *R. leguminosarum* biovar *viciae*.

METHODS

Microbiological techniques. *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI (Downie *et al.*, 1983) was used throughout. Bacteria were cultured in Y minimal medium (Sherwood, 1970)

† Present address: Departamento Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, 41012 Sevilla, Spain.

containing succinate (10 mM) and glutamate (10 mM) as carbon and nitrogen sources. Flasks (2 l) containing 500 ml medium were inoculated with 10 ml of a starter culture and shaken for 3 d at 30 °C in an orbital shaker. The cells were harvested by centrifugation, then washed once in 20 mM KH_2PO_4 buffer, pH 7.

For production of bacteroids, peas grown in sterilized Erlenmeyer flasks containing 200 ml Fahraeus medium with 0.5% agar (Beynon *et al.*, 1980) were inoculated with *R. leguminosarum* bv. *viciae* and grown for 24 d. The nodules were harvested and bacteroids were isolated by sucrose-density-gradient centrifugation as described by Brewin *et al.* (1985).

Cell fractionation, protein gel electrophoresis and haem staining. *R. leguminosarum* cells from a 300 ml culture grown in Y-succinate medium or bacteroids isolated from 5 g nodules were resuspended in 3 ml 100 mM potassium phosphate buffer (pH 7.0) containing 100 μM 4-amidinophenylmethanesulfonyl fluoride and DNase I (20 $\mu\text{g ml}^{-1}$). Cells were disrupted using a French pressure cell (SLM Aminco). The cell extract was centrifuged at 20 000 *g* for 20 min to remove unbroken cells and the supernatant was then centrifuged at 140 000 *g* for 2 h. The membrane pellet was resuspended in 2 ml of the same buffer, centrifuged at 140 000 *g* for 2 h and then resuspended in 100 μl buffer. The supernatant 'soluble fraction' from the first centrifugation at 140 000 *g* was concentrated using a Centricon-10 filter. Protein concentrations were estimated by the method of Bradford (1976) using BSA as standard. Membrane and soluble fractions were suspended in loading buffer (124 mM Tris, pH 7.0, 20%, v/v, glycerol, 4.6%, w/v, SDS), incubated at 20 °C for 10 min, separated by SDS-PAGE and transferred to a nitrocellulose filter following the procedures described by Bradley *et al.* (1988). Proteins containing covalently bound haem were stained by chemiluminescence as described by Vargas *et al.* (1993) using an Amersham enhanced chemiluminescence kit.

Potential contamination of the bacteroid extract by mitochondria was assayed using antibody to pea lipoamide dehydrogenase, a mitochondrial-specific marker (Turner *et al.*, 1992). Proteins separated by SDS-PAGE and transferred to a nitrocellulose filter were incubated with a 1:500 dilution of antiserum under the conditions described by Bradley *et al.* (1988). Binding of the antibody was visualized using rabbit anti-rat IgG conjugated to alkaline phosphatase (Sigma), used at a 1:2000 dilution, following the staining conditions described by Bradley *et al.* (1988). As a positive control, 1 g uninoculated pea root tips was homogenized in a mortar and pestle in 1 ml extraction buffer (50 mM Tris/HCl, pH 7.5, 10 mM DTT, 0.5 M sucrose, 5%, w/v, insoluble polyvinylpyrrolidone and 5 mM *p*-amino-benzamidine). The homogenate was filtered through Miracloth (Calbiochem) before solubilization in gel loading buffer. Relative M_r values of proteins were estimated using the 'Rainbow' M_r markers from Amersham.

Measurements of spectra. Spectra were measured with a Johnson Foundation SDB3 dual wavelength spectrophotometer (Williams & Poole, 1987) using a reference wavelength of 575 nm and a spectral band width of 4 nm. For reduced *minus* oxidized spectra, cells resuspended in buffer were reduced with a few grains of dithionite or oxidized with a few grains of ammonium persulphate. For the reduced-plus-CO *minus* reduced spectra, the dithionite-reduced cells were bubbled for 1 min with CO.

For the photodissociation spectra, dithionite-reduced cells or cells reduced in the presence of succinate (2 mM) and malate (2 mM) were bubbled with CO for 1 min, cooled to -20 °C for 5 min and then cooled to -78 °C for 10 min in the dark before

equilibration at -100 °C in the sample compartment (Poole *et al.*, 1983, 1994) using the cryostat illustrated by Jones & Poole (1985). The sample was scanned twice to generate a baseline (reduced-plus-CO spectrum *minus* reduced-plus-CO spectrum) and then photolysed using a focused 200 W light beam. The spectra shown are the differences between the CO-dissociated sample and the reduced-plus-CO sample.

RESULTS AND DISCUSSION

Novel covalently bound haem proteins in bacteroids

Cytochromes of the *c*-type have a covalently bound haem group that is retained during SDS-PAGE. These haem groups have an intrinsic peroxidase activity that can be used to identify the proteins after gel separation. *R. leguminosarum* bv. *viciae* cells lysed using a French pressure cell were fractionated into soluble and membrane fractions and the proteins separated by SDS-PAGE. Covalently bound haem proteins were visualized using a haem stain.

In the membrane fractions from free-living cells, two stained bands of M_r 31 000 and M_r 23 000 were detected (Fig. 1a, lane 1). The larger protein is similar in size to cytochrome c_1 from *B. japonicum* (Thöny-Meyer *et al.*, 1989), and a mutation in the *R. leguminosarum* bv. *viciae* cytochrome c_1 gene abolishes the formation of this protein (unpublished observations). The smaller band is similar in size to the membrane-bound *c*-type cytochrome CycM (M_r 20 000) described by Bott *et al.* (1991) in *B. japonicum*.

In the membrane fraction of bacteroids the total amount of haem-stained proteins was considerably higher than that seen with membranes from free-living cells (Fig. 1a, lanes 1 and 5). The increased level was principally due to the appearance of two strongly stained bands at estimated M_r values of 30 000 and 28 000. To resolve these components it was necessary to decrease the amount of protein loaded on the gel (Fig. 1a, lanes 2, 3 and 4). In addition to these two bands, there appears to be a more weakly stained band at about M_r 31 000 (arrowed), corresponding in size with the cytochrome c_1 component found in membranes from free-living cells (Fig. 1a, lanes 3 and 4). At about M_r 23 000 two bands were detected (Fig. 1a, lanes 4 and 5); presumably one of these corresponds to the component of M_r 23 000 found in membranes from free-living cells. It is not possible to distinguish if the other band in this range is a novel component or a degradation product of one of the other haem proteins; indeed other weakly staining bands that might be degradation products can be seen at the higher levels of protein loading (Fig. 1a, lane 5).

In the soluble fraction of free-living cells (Fig. 1b, lane 1) a stained protein of about M_r 14 000 is present and this was shown previously to be a periplasmic protein (Vargas *et al.*, 1994). This component was not detected in the soluble fraction from bacteroids (Fig. 1b, lane 2). Thus it appears that the expression of this cytochrome, like that of cytochrome aa_3 (see below), is greatly reduced in bacteroids. This contrasts with the significantly increased level of absorption of *c*-type cytochromes (see below), implying that the increase in absorption is due to the novel membrane-bound components.

Fig. 1. Covalently bound haem proteins in membrane (a) and soluble (b) fractions from cultured cells and bacteroids of *R. leguminosarum* strain A34. Proteins were separated by SDS-PAGE and stained for the presence of covalently bound haem as described previously by Vargas *et al.* (1993). In (a) lane 1 contains 50 μ g protein from the membrane fraction of free-living cells and lanes 2, 3, 4 and 5 contain, respectively, 2.5 μ g, 6 μ g, 12 μ g and 50 μ g proteins from the membrane fraction of bacteroids isolated from pea nodules. In (b) lanes 1 and 2 each contain 50 μ g protein from the soluble fraction of free-living cells and bacteroids, respectively. The positions of migration of M_r markers are indicated. The arrows indicate the band in bacteroids attributed to cytochrome c_1 .

Fig. 2. Immunostaining of bacteroids with antibody to pea lipoamide dehydrogenase. Protein extracts (10 μ g protein) from uninoculated pea roots (lane 1), bacteroids (lane 2) and cultured bacteria (lane 3) were separated by SDS-PAGE, transferred to nitrocellulose and stained with antibody to pea lipoamide dehydrogenase, which recognizes a pea mitochondrial protein of M_r 60000 (Turner *et al.*, 1992). The estimated sizes of stained bands are indicated.

We considered the possibility that the novel components in the bacteroid fraction might be due to contamination with plant mitochondria from nodule cells. However, when an immunoblot of bacteroid proteins and of root extract was probed with antibody to pea lipoamide dehydrogenase (a mitochondrial-specific marker) (Turner *et al.*, 1992), there was strong cross-reactivity with a protein of M_r 60000 in root extract (Fig. 2, lane 1) but no reaction at the corresponding size in the bacteroid fraction (Fig. 2, lane 2). Interestingly, a component of M_r 65000 was stained in the bacteroid extract and a protein of the same size was detected in an extract of cultured bacteria (Fig. 2, lanes 2 and 3), indicating that the antiserum to pea

lipoamide dehydrogenase recognizes a bacterial protein; this component may correspond to the *R. leguminosarum* lipoamide dehydrogenase. The absence of the pea lipoamide dehydrogenase in the bacteroid extract excludes significant mitochondrial contamination of the bacteroids, showing that the novel haem-staining bands are indeed bacteroid components.

Preisig *et al.* (1993) predicted that the *fixO* and *fixP* genes encode membrane-anchored c -type cytochromes. The *fixNOQP* operon is conserved in many (possibly all) rhizobia and is induced in bacteroids during nitrogen fixation. The predicted M_r values of the FixO and FixP apoproteins are 27352 and 31023, respectively, in *B. japonicum* (Preisig *et al.*, 1993) and very similar in *A. caulinodans* (Mandon *et al.*, 1994). The additional haem-staining proteins identified here at M_r 28000 and M_r 30000 in bacteroids correspond in size to those predicted for the *fixO* and *fixP* gene products. Thus, the additional haem-staining bands most probably correspond to FixO and FixP, but genetic characterization of *R. leguminosarum* bv. *viciae* *fixO* and *fixP* mutants will be required to confirm that the novel membrane-bound haem-stained proteins do indeed correspond to FixO and FixP.

Absorption spectra of culture cells and bacteroids

In an attempt to corroborate the haem stain and to identify a novel haem-containing terminal oxidase in bacteroids, we recorded absorbance difference spectra of intact cells and bacteroids, exploiting the photoreversible CO-binding properties of this class of cytochromes. The reduced minus oxidized difference spectrum of cultured cells of *R. leguminosarum* bv. *viciae* revealed an intense

Fig. 3. Visible absorption difference spectra of *R. leguminosarum* cells and bacteroids. Spectra (a) and (c) were recorded using intact cells suspended at a final protein concentration of 7.2 mg ml^{-1} . Spectra (b) and (d) were recorded using intact purified bacteroids suspended at a final protein concentration of 25 mg ml^{-1} . Spectra (a) and (b) are reduced (dithionite) *minus* oxidized (persulphate) difference spectra at room temperature, whereas (c) and (d) are reduced (dithionite)-plus-CO *minus* reduced difference spectra at room temperature. The pathlength was 10 mm.

Soret band at 427 nm (Fig. 3a). The α -peak at 551 nm is attributable to *c*-type cytochrome(s), the 558 nm shoulder to cytochrome(s) *b* (Kretovich *et al.*, 1973; Vargas *et al.*, 1994) and the peak at 600 nm to cytochrome *aa*₃. A reduced *minus* oxidized spectrum of bacteroids from pea nodules (Fig. 3b) showed a Soret (430 nm) band similar to

that of cultured cells, but the major α -band was highly asymmetric. The 552 nm peak attributable to *c*-type cytochromes is significantly increased in intensity relative to that of the 560 nm shoulder attributable to cytochromes *b*. The increased level of cytochrome *c* in bacteroids was also evident from the Soret and β (522 nm)-bands, which showed significant asymmetry at shorter wavelengths. As noted by others (Kretovich *et al.*, 1973), the absorption band at 600 nm due to cytochrome *aa*₃ was absent from bacteroids and the absence of a peak and trough at 630 nm and 650 nm indicates that cytochrome *d* is also absent from bacteroids.

CO difference spectra were used to reveal putative terminal oxidases. The CO-reduced *minus* reduced difference spectrum of cultured cells (Fig. 3c) showed a distinct peak at 419 nm due to absorbance of the CO-complex of a haem protein. This peak and the corresponding trough at 440 nm, due to loss of the ferrous form on CO binding, are at wavelengths too low to be attributed to cytochrome *a*₃ and arise from an additional component that is probably a ligand-binding, high-spin cytochrome *b*, or possibly *c* (Poole, 1994; Wood, 1984). The presence of cytochrome *d* in cultured cells (see below) suggests that this component is the high-spin cytochrome *b* (*b*₅₉₅) associated with cytochrome *d* in other bacteria (Poole, 1994). This component presumably masks the Soret absorbance band of cytochrome *a*₃ which is, however, detectable by the 590 nm peak. In contrast, the CO difference spectrum of intact bacteroids (Fig. 3d) showed a clear trough at 443 nm. Although this is the position anticipated for unligated cytochrome *a*₃, the absence of the α -band in Fig. 3(b) precludes this possibility. The trough is accompanied by a very broad Soret band centred at 420 nm; the band shape clearly suggests the presence of at least two CO-binding components, probably with maxima near 410 and 426 nm, respectively. At longer wavelengths, a broad trough centred at 560 nm and absorption maxima at 541 and 577 nm differed from the corresponding signals of cultured cells (Fig. 3c). The peak of absorbance at 640 nm in the cultured cells (Fig. 3c) corresponds with the expected signal due to carbon-monooxy-cytochrome *d*, but this was not seen with bacteroids (Fig. 3d).

Such CO difference spectra reveal all CO-binding haem proteins, whether or not they have oxidase function. The majority of terminal oxidases can be identified more specifically in photodissociation spectra at low temperature, where the CO dissociated from the photolabile carbon-monooxy form, does not immediately rebound to the haem, allowing recording of the photodissociated (i.e. unligated) *minus* pre-photolysis difference spectrum. Such spectra are shown in Fig. 4(a–c). In the photodissociation spectrum of cultured cells reduced with dithionite (Fig. 4a), the presence of cytochrome *a*₃ was confirmed by the appearance of the unligated reduced cytochrome at 446 nm. In the corresponding spectrum of bacteroids (Fig. 4b), the Soret band was at a slightly lower wavelength (443 nm) and the corresponding trough was at 428 nm. These spectral features closely resemble those of cytochrome *a*₃. However, the spectrum in Fig. 3(b)

Fig. 4. Photodissociation spectra, i.e. photolysed *minus* pre-photolysis, at -100°C . In each case, the spectrum is that obtained immediately after photolysis of the sample. Spectrum (a) shows cultured cells (protein concentration 5.0 mg ml^{-1}) reduced with dithionite before bubbling with CO, and (b) is the corresponding spectrum for bacteroids (protein concentration 25 mg ml^{-1}). In (c) the bacteroids were reduced with malate and succinate before bubbling with CO. The pathlength was 2 mm.

shows that cytochrome aa_3 is not present. The difference spectrum observed when bacteroids were reduced by endogenous respiration in the presence of succinate and malate (Fig. 4c) was clearly different from that obtained with dithionite in the absolute absence of oxygen (Fig. 4b). Fig. 4(c) shows that signals of narrow band width were observed in both Soret and β/α -regions after photolysis, the pre-photolysis baseline being featureless (not shown). Peaks were observed at 436, 497, 553 and 563 nm and troughs were observed at 410 and 546 nm. Re-scanning of the same sample after about 5 min revealed a shift of the Soret band to 431 nm (not shown). We consider that the most likely interpretation of these signals is that, in the absence of dithionite, residual oxygen in the sample reacts with the ligand-binding oxidase immediately after photolysis and before the scan shown in Fig. 4(c). This has been previously reported for the high oxygen affinity cytochrome d of *Escherichia coli* (Poole *et al.*, 1983). Thus, the difference spectrum recorded is

oxygenated *minus* CO-reduced and the spectral features indicate that the two forms are distinct. In particular, for example, the oxygenated form absorbs more intensely at 436 nm than does the CO-ligated sample, whereas the converse is true at 410 nm. As required by this interpretation the 410 nm trough attributed to loss of the CO-ligated oxidase (Fig. 4c) corresponds to the position of one of the components seen in the CO-plus-reduced *minus* reduced difference spectrum (Fig. 3d).

Further evidence that photolysis of the CO-ligated putative oxidase results in oxidation and turnover of respiratory chain components was obtained in an experiment in which oxygen was introduced into the substrate-reduced suspension at -23°C before freeze-trapping. This (Jones & Poole, 1985; Poole *et al.*, 1994) allows introduction of oxygen without displacement of CO until the sample is photolysed. The sample was scanned at -110°C , photolysed at -110°C , warmed to -40°C and then returned to -110°C for recording difference spectra (post-photolysis *minus* pre-photolysis). (This procedure overcomes potential artefacts in difference spectra that compare spectra recorded at different temperatures.) The difference spectrum (not shown) revealed clear α/β -troughs that indicated extensive cytochrome oxidation on raising the sample temperature. The α -trough was at 550 nm, with a shoulder at 560 nm, and the β -trough was at 520–530 nm, demonstrating the oxidation of both c - and b -type cytochromes. In contrast, cytochromes a_3 and o (Poole *et al.*, 1994) react relatively slowly with oxygen after photolysis of the CO compound to form intermediate oxy-compounds spectrally very similar to the CO-ligated forms.

These spectroscopic studies of bacteroids confirm the absence of cytochrome a_3 but confirm the presence of an unusual ligand-binding haemoprotein that appears to react with traces of oxygen at low temperatures. Such behaviour is consistent with the supposition that the bacteroid oxidase has a high affinity for oxygen.

There is clearly a strong induction of genes involved in c -type cytochrome formation in bacteroids and the unusual photodissociation spectrum may have revealed the new high affinity cytochrome oxidase in bacteroids. The *fixN* gene may encode such a high affinity oxidase, and photodissociation spectra measured using the purified oxidase will be required to confirm this. The bacteroid-specific components of the electron transfer chain appear to branch off from cytochrome c_1 since mutations in the gene encoding the cytochrome c_1 apoprotein completely block nitrogen fixation (Thöny-Meyer *et al.*, 1989; our unpublished observations).

In parallel with the appearance of the bacteroid-specific cytochromes, the data presented clearly show that there is a repression of synthesis of cytochrome aa_3 and of a periplasmic cytochrome c . It will be of particular interest to determine if the *fixL* and *fixK* genes involved in activation of *fixNOQP* expression play any role in the repression of expression of cytochrome aa_3 and the periplasmic cytochrome c .

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