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The unusual behaviour of the putative terminal oxidases of *Bradyrhizobium japonicum* bacteroids revealed by low-temperature photodissociation studies

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In an attempt to identify the enigmatic terminal oxidase(s) of *Bradyrhizobium japonicum* bacteroids, cell suspensions were reduced and saturated with CO, then exposed to actinic light at temperatures down to −196 °C. A pigment was revealed with an absorption maximum in the unliganded (reduced) form at about 444 nm, but no characteristic absorbance in the α- and β-regions. Studies with the partially purified hydroperoxidase, haemoprotein *b*-590 (*Bradyrhizobium*) and with *B. japonicum* cytochromes *P*-450 and *c*-552 showed that none of these were responsible for the 444 nm band. In an effort to demonstrate ligand exchange (oxygen for carbon monoxide), photolysis of bacteroids was performed in the presence of oxygen at −143 °C. A band at 444 nm was again revealed, but of lower intensity. The diminution of signal height is attributable to the displacement of CO by O_2 during sample preparation or to the action of a CO-insensitive oxidase that allows partial cytochrome oxidation prior to photolysis. The photolysible component, with a band in the reduced form at 444 nm, also occurs in *Rhizobium leguminosarum* and *R. lupini* bacteroids. The nature of the 'effective' oxidase that mediates coupled respiration at low oxygen tensions is discussed.

Introduction

The nitrogen-fixing bacteria of the genera *Bradyrhizobium* and *Rhizobium* that are found in the root nodules of leguminous plants are called bacteroids. An apparent paradox [1] in the bacteroid-plant symbiosis is the incompatibility between the oxygen sensitivity of the Fe and MoFe protein components of bacteroid nitrogenase with the oxygen requirement of the aerobic respiratory chain that yields ATP for nitrogenase activity and bacterial metabolism. It is now known [2–4] that bacteroid respiration in the presence of part-oxygenated leghaemoglobin is most efficient, in terms of oxidative phosphorylation and consequent nitrogen fixation, at only 5–10 nM free O_2. At these low concentrations, probably the normal range for host symbiotic tissue [4], it is unlikely that nitrogenase in the bacteroid cytoplasm would suffer oxygen damage. At higher external oxygen concentrations, isolated bacteroids of *B. japonicum* enter into a phase of vigorous uncoupled respiration, which could conceivably offer extra protection against oxygen penetration to the bacteroid nitrogenase domain. Knowledge of the identity, localization and function of components of the oxygen-terminated respiratory chain will be central to the further understanding of this problem.

The most striking feature of the respiratory system of many strains of *Bradyrhizobium* and *Rhizobium* bacteroids [5–7] is the apparent absence of cytochrome aa_3 and *o* implicated as terminal oxidases in free-living (cultured) cells [8]. The nature of the terminal oxidase(s) of bacteroids has remained enigmatic. CO-reactive haemoproteins, and thus potential oxidases, in bacteroids are cytochromes *c*-552 and *c*-554, *P*-450, *P*-428 (haemoprotein *b*-590) and *P*-420 [5,9]. (Nomenclature of the last three is based on the absorption maxima of the respective CO compounds, which are observed at about 450, 428 and 420 nm in CO difference spectra.) Of these, *P*-428 (haemoprotein *b*-590, Appleby and Poole unpublished data) is special interest as a putative oxidase. It was seen in CO difference spectra with succinate as a reductant [5]. The peak at 428 nm and

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trough at about 441 nm suggested an *a*-type cytochrome, but the lack of discernable α- and β-bands prompted its tentative identification as 'cytochrome a_1' [3].

The triple-trapping procedure developed by Chance and his co-workers [10,11], for the study of the reactions of the mitochondrial cytochrome *c* oxidase with O_2 has proved very useful in identifying and elucidating the reactions of bacterial terminal oxidases with O_2 [12–14]. However, this experimental approach has not previously been applied to a study of the terminal oxidases of *Rhizobium* and *Bradyrhizobium* bacteroids. In this paper, we compare the spectral properties of CO-reactive haemoproteins in *B. japonicum*, *R. lupini* and *R. leguminosarum* bacteroids at sub-zero temperatures and comment on the possible involvement of the observed pigments and of a CO-insensitive oxidase in the bacteroid respiratory chains.

Materials and Methods

Organisms and growth conditions

Seeds of soybean (*Glycine max* cv. Lincoln) were inoculated with *Bradyrhizobium japonicum* strain CC 705 syn Wisconsin 505 as described by Appleby and Bergersen [15]. Nodules were picked from the roots of typically 4-week-old plants, grown at 25 °C (day) and 18 °C (night). The nodules were immersed in ice-cold 0.1 M potassium phosphate (pH 6.8) and either used immediately or stored frozen at −20 °C for short periods. Similarly, nodules were picked from broad bean (*Vicia faba* cv. Coles Dwarf prolific) and Russian yellow lupin (*Lupinus luteus* cv. Brystrorastuchii 4) which had been inoculated with *R. leguminosarum* strain SU391 and *R. lupini* strain USSR 359A, respectively.

Preparation of bacteroids

Freshly picked or thawed root nodules were placed in a Servall Omnimixer chamber at 0 °C, with 4 vols. of 0.1 M potassium phosphate (pH 6.8) and a trace of Dow-Corning antifoam A and ground at full speed for 2 min with the chamber immersed in an ice-water mixture. The homogenate was filtered through a 'Miracloth' (Calbiochem-Behring, La Jolla, CA) and cheesecloth sandwich into a 250 ml centrifuge tube cooled in ice, leaving a layer of cell debris on the 'Miracloth'. This debris was resuspended and washed three times in 50 ml of 0.1 M potassium phosphate (pH 6.8) to give a total filtered suspension volume of about 200 ml. This suspension had a pale pink, turbid appearance, due to oxyleghaemoglobin in solution. The suspension was centrifuged at 500 rpm for 5 min in the GSA head of a Sorvall centrifuge to remove cell debris and starch granules. The turbid pink supernatant was centrifuged at 8000 × *g* for 10 min to yield a clear red supernatant which was discarded, and a bacteroid pellet which was overlayed by a grey halo of plant mitochondria and membrane fragments. The pellet was swirled gently with about 25 ml of buffer, until most of the grey halo was removed, and then washed in 0.1 M potassium phosphate (pH 6.8) twice, resedimenting the bacteroids each time by centrifuging at 8000 × *g* for 10 min. Bacteroids were stored frozen at −20 °C until needed or resuspended to give a 25% (w/v) suspension in the same buffer which was diluted further as needed. *R. lupini* and *R. leguminosarum* bacteroids were prepared in a similar way.

Subcellular fractionation of B. japonicum bacteroids

To 120 ml of a 25% (w/v) bacteroid suspension were added a few grains of DNase and 1 mM $MgCl_2$. The bacteroid suspension was broken by two passages at 140 MPa through a 'French' pressure cell constructed in the workshops of CSIRO. The broken bacteroid suspension was centrifuged at 10000 × *g* for 10 min in a Beckman Ti 70 rotor to yield a supernatant designated S_0, which was recentrifuged at 200000 × *g* for 60 min to yield a supernatant S_1 and a membrane pellet P_1. The pellet P_1 was resuspended in 0.1 M potassium phosphate (pH 6.8), homogenised and again recentrifuged at 200000 × *g* for 60 min to give a washed membrane pellet P_2 and supernatant S_2.

Purification of B. japonicum bacteroid cytochromes P-450 and c-552 and of haemoprotein b-590

Cytochrome *P*-450 was purified according to Appleby [16]. Cytochrome *c*-552, obtained as a by-product of steps 2 and 3 of the *P*-450 purification procedure was further purified by anion-exchange chromatography and isoelectric focusing (C.A. Appleby, unpublished data). Haemoprotein *b*-590 was purified by a modification of the method developed for haemoprotein *b*-590 of *E. coli* [17]. Briefly, fresh bacteroids were disrupted in a French pressure cell, and the protein was precipitated from a cell-free extract by adding ammonium sulphate to a final saturation of 0.7. The redissolved, concentrated material was subjected to Sephacryl S-300 molecular exclusion chromatography and anion-exchange chromatography on DEAE-Sepharose CL-6B, followed by a second passage through Sephacryl S-300 (C.A. Appleby and R.K. Poole, unpublished data).

Spectrophotometry

B. japonicum bacteroid suspensions were diluted in 0.1 M potassium phosphate (pH 6.8). Thawed 25% (w/v) suspensions of *R. leguminosarum* bacteroids were homogenized (0 °C) to disperse clumps, then centrifuged at 5000 × *g* for 7 min, resuspended to 25% (w/v) and homogenized at 0 °C in the Omnimixer (50 ml chamber) for 1 min; this was repeated twice, finally giving 5 or 10% (w/v) suspensions in which the cells flocculated sufficiently slowly to be suitable for spectro-

photometric analysis. *R. lupini* bacteroid suspensions (25% w/v) were similarly homogenized and centrifuged to give a heterogenous pellet. The upper layer was decanted leaving a compact lower layer which was resuspended and centrifuged as above before suspending to 25% (w/v). Suspensions of all three organisms were diluted further to 10% (w/v) for room temperature spectra or to 5% (w/v) and supplemented with 30% (v/v) ethylene glycol for low temperature, ligand-binding experiments.

Low temperature experiments employed the 'triple trapping' principle [10] as described by Jones and Poole [11]. Bacteroid suspensions were reduced with 20 mM sodium succinate for 20 min in 2 mm pathlength cuvettes before bubbling with CO for 5 min, equilibrating at −23 °C in an ethanol/solid CO_2 bath and adding oxygen. This was achieved either by vigorous stirring with a close-fitting coiled steel wire for 30 s, or by adding air-saturated buffer containing 30% (v/v) ethylene glycol (2 mM O_2 at −20 °C [18]). In the latter method, argon-flushed pipettes were used and buffer addition and gentle mixing were performed under argon. The cuvette was thereafter frozen at −78 °C in an ethanol/solid CO_2 bath and transferred to the sample compartment of a Hitachi/Perkin Elmer 557 spectrophotometer, modified to allow control of sample temperature by a flow of cold nitrogen gas [11]. The instrument was connected by a serial interface to a Digital PDP-11 computer, enabling spectral data to be stored on floppy discs for manipulation. The sample was equilibrated for 10–20 min at the chosen temperature, monitored by a thermocouple at the side of the sample cuvette. The spectrum of the reduced, CO-liganded preparation was recorded in the dual wavelength mode (λ_1 fixed, λ_2 scanned) and stored in the computer memory before photolysis at a controlled low temperature (see Results) using the focussed beam from a 150 W projector lamp. A mechanically operated shutter prevented actinic light from reaching the photomultiplier.

Room temperature spectra were recorded in the PE557 spectrophotometer operating in its split (dual)-beam mode, using a pair of 1 cm pathlength cuvettes. Reduction in the sample cuvette was achieved with dithionite, succinate or NADH, whereas oxidation in the reference cuvette was achieved by adding ammonium persulphate or bubbling the cuvette contents with pure O_2 for ≈ 30 s. Carbon monoxide (Matheson, CP grade) was bubbled into reduced suspensions to form CO complexes.

Difference spectra at −196 °C were recorded using the cryogenic attachment of the same spectrophotometer. Portions (450 μl) of the ethylene glycol-supplemented cell suspension were reduced with succinate for 15 min in 2 mm pathlength cuvettes and bubbled with CO for 5 min. After freezing in liquid N_2, the reduced plus CO *minus* reduced plus CO baseline was recorded and the cuvette assembly removed from the instrument to a partly unsilvered Dewar vessel where it was immersed in liquid N_2. One cuvette was illuminated with the beam from a 150 W projector bulb, focussed by a condensing lens through the Dewar vessel and then the cuvette assembly was returned to the spectrophotometer for rescanning at −196 °C.

Results

Low-temperature photodissociation of reduced, CO-liganded B. japonicum bacteroids in the absence of oxygen

The CO difference spectrum of *B. japonicum* bacteroids with dithionite as reductant (Fig. 1A), recorded at room temperature, had maxima at 415, 537 and 569 nm and a trough at 552 nm indicating a CO-binding cytochrome *c*. A shoulder at 423 nm was probably due to the CO-complexes of pigments described as *P*-428 and *P*-420 [5]. The trough at 433 nm is thus attributable to the loss of absorbance at this wavelength of reduced *P*-420 (presumably a haem *b*-containing protein) on binding CO, while the binding of CO to *P*-428 [5] (haemoprotein *b*-590) contributes near 440 nm to this broad trough. The features between 600 and 620 nm suggest a low concentration of an *a*-type cytochrome. The prominent peak at 453 nm is due to cytochrome *P*-450.

The photodissociation spectrum (the inverse of the CO-difference spectrum) at −150 °C showed a maximum at 445 nm and a minimum 427 nm (Fig. 1B), but no signals in the α- or β-regions. Such a lack of absorbance changes in the 590 to 605 nm region of CO-difference spectra, and the signal at 428 nm, previously led Appleby [3] to describe this pigment as 'cytochrome a_1'. At −87 °C, the initial photodissociation spectrum (Fig. 1C) was similar to that recorded at −150 °C, but repetitive scanning revealed recombination of CO, as evidenced by the diminishing intensity of the Soret bands (Fig. 1D). Rephotolysis restored the original spectrum (not shown) confirming that the reacting ligand was CO. Small absorbance changes at 460, 545 and 588 nm during the experiment remain unattributed. Photolysis at −196 °C (Fig. 1E) revealed a spectrum similar to that obtained at −150 °C. This suggests that the additional features (414 nm peak, 420 nm trough) observed by Appleby [5] in a 'dark-*minus*-light' (i.e., inverted) photodissociation spectrum under continuous illumination, are due to components that rapidly combine with CO even at −196 °C unless exposed continuously to actinic light. Only one class of cytochrome oxidase has been reported so far to exhibit such behaviour, namely the cytochromes *d* of *E. coli* [19] and *Acetobacter pasteurianus* [12]. The 445 nm maximum and 427 nm minimum in the *B. japonicum* photodissociation spectrum recorded at −150 °C (Fig. 1B) could be

due to the presence of an *a*-type cytochrome or a high-spin *b*-type cytochrome [20,21], although the absence of significant absorption changes in the visible region makes an assignment difficult. For instance, although the CO-difference spectrum, at −142 °C, of dithionite-reduced, purified haemoprotein *b*-590 (*Bradyrhizobrum*), plotted as reduced *minus* CO-reduced, to allow direct comparison with signals in a photodissociation spectrum (Fig. 2), showed a peak at 444 nm and a trough at 424 nm, it also showed broad minima at 543 and 572 nm, the last feature being particularly prominent. Hence, it is unlikely that the bacteroid photodissociable pigment is haemoprotein *b*-590. Furthermore, when this sample of dithionite-reduced, CO-liganded haemoprotein *b*-590 was exposed to actinic light for 30 s at −142 °C, no stable photodissociated state was observed (data not shown).

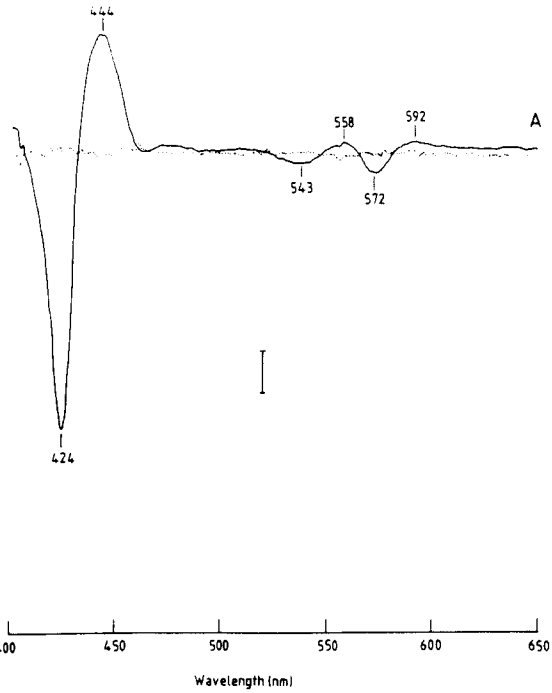

Fig. 2. Low temperature (−142 °C) reduced (with dithionite) *minus* reduced (with dithionite) plus CO difference spectrum of purified haemoprotein *b*-590 (*P*-428). The dotted line is a reduced *minus* reduced baseline. Spectra were recorded at a scan speed of 1 nm·s^{-1} and a slit-width of 2 nm. The reference wavelength was 500 nm. The vertical bar represents a ΔA of 0.02.

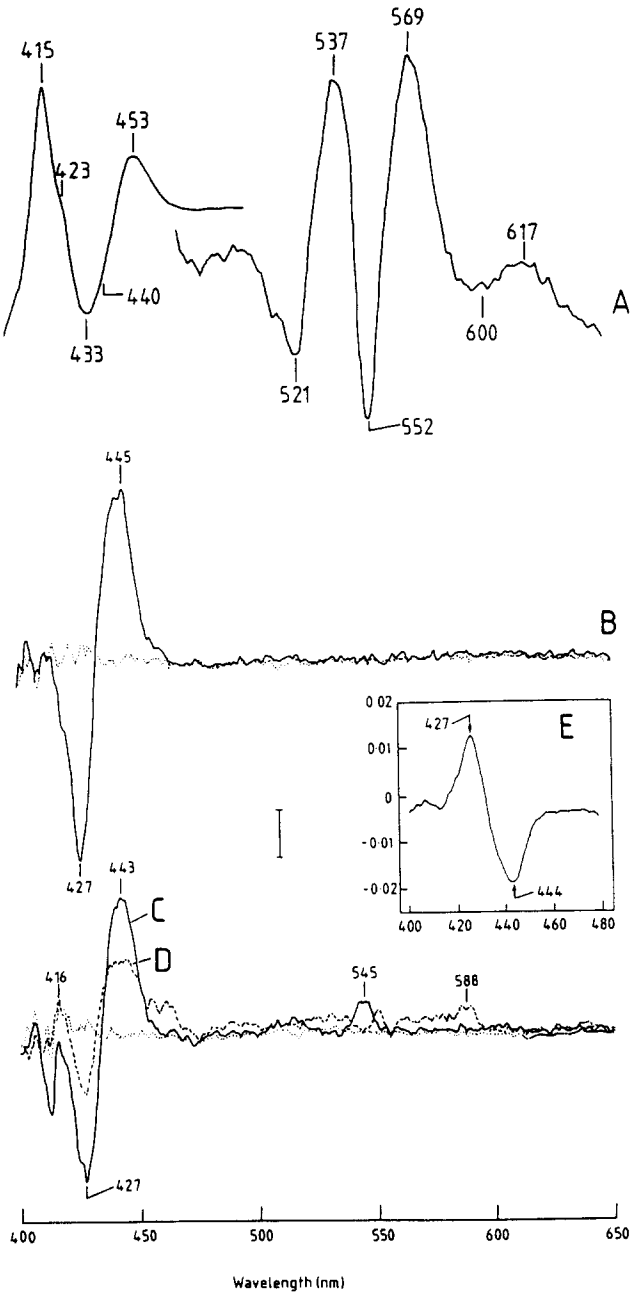

Fig. 1. Room temperature and photodissociation spectra of *B. japonicum* bacteroids. (A) shows a reduced (with dithionite) plus CO *minus* reduced (with dithionite) difference spectrum at room temperature. In (B) the spectrum of a suspension of CO-liganded, succinate-reduced anoxic bacteroids was scanned at −150 °C and stored in the memory of the dual-wavelength spectrophotometer. Subsequent scans were difference spectra, the stored pre-photolysis spectrum being subtracted. The first scan before photolysis yielded a reduced plus CO minus reduced plus CO baseline (dotted line). The solid line shows the first spectrum scanned following a 30 s exposure to white light. (C) and (D) show a similar experiment carried out at −87 °C. The dotted line is a reduced plus CO *minus* reduced plus CO baseline and (C) (solid line) shows the first spectrum scanned following 30 s exposure to white light, while (D) (dashed line) is a similar spectrum scanned after 28.5 min. Spectra were recorded at a scan speed of 1 nm s^{-1} and a slit-width of 2 nm. The reference wavelength was 500 nm. The vertical bar represents a ΔA of 0.003 in (B), (C) and (D), while in (A), up to 500 nm, it represents a ΔA of 0.022 and beyond 500 nm a ΔA of 0.0022. (E) is the photodissociation spectrum (CO reduced minus photolyzed) of a similar suspension recorded at −196 °C. In (E) the *A* scale is shown on the vertical axis and the wavelength in nm on the horizontal axis of the insert.

Photodissociation of reduced, CO-liganded B. japonicum bacteroids in the presence of O_2

At −143°C, photodissociation of an O_2-supplemented sample elicited a difference spectrum with similar spectral features to that observed in the absence of O_2 (Fig. 1B), with a distinct 442 nm peak but of much lower amplitude (Fig. 3A). At least two explanations may be offered for signal diminution in the presence of O_2. The first is the displacement of CO from the reduced oxidase at −23°C by oxygen during the triple-trapping procedure (see Materials and Methods), thereby lowering the amount of reduced, CO-liganded enzyme available for photodissociation and subsequent detection as the reduced form. A second is that a CO-insensitive oxidase reacted with O_2 at −23°C becoming oxidized itself and possible oxidizing other respiratory chain components. However, a cytochrome oxidase bound to CO, possibly the component revealed by our photodissociation spectra (Figs. 1, 2) would not be expected to become oxidized under these conditions.

Support for the first hypothesis comes from experiments in which absolute spectra of CO-saturated, reduced samples supplemented with 100 μM or 1 mM O_2 (Materials and Methods) were recorded at −141°C using frozen buffer as the reference sample (not shown). All cytochrome absorption bands in such spectra were

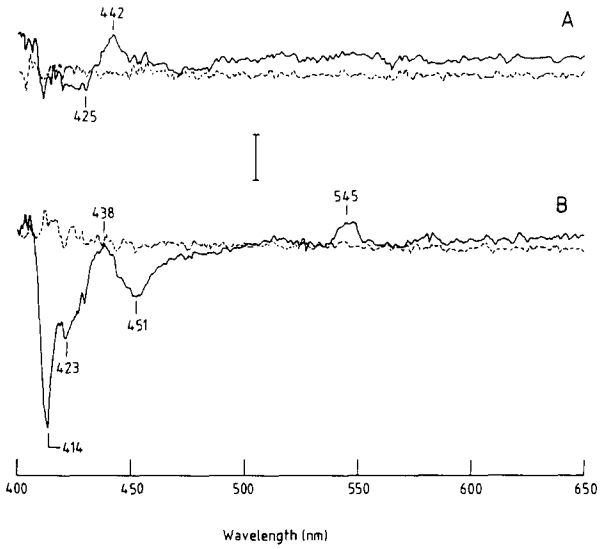

Fig. 3. Wavelength scanning following photolysis of CO-liganded *B. japonicum* bacteroids in the presence of O_2 at −143 and −88°C. (A) The spectrum of a suspension of CO-liganded, succinate-reduced bacteroids, to which O_2 had been added at −23°C by stirring, was scanned at −143°C and stored in the memory of the dual-wavelength spectrophotometer. Subtraction of the first spectrum from the subsequent scan of the unphotolysed sample gave the reduced plus CO *minus* reduced plus CO baseline (dashed line). The sample was then photolysed at −143°C by 30 s exposure to white light and a spectrum (solid line) recorded with the stored pre-photolysis spectrum as reference. In (B) the procedure was identical, but the spectra were recorded at −88°C. In each case the scan speed was 1 nm·s^{-1}, the slit-width 2 nm and the reference wavelength 500 nm. The vertical bar represents a ΔA of 0.005.

diminished (e.g., for the α-band of cytochrome *c*, by 65% at 100 μM O_2 and 78% at 1 mM O_2) with respect to the anoxic but CO-supplemented samples. Photodissociation of these samples at −141°C showed corresponding decreases in the intensity of the bands at approx. 442–445 nm and 425–428 nm (as in Fig. 3A). This argues against the possibility that, on photodissociation, oxygen immediately forms a compound with spectral properties similar to the CO-liganded form, thus being undetected in the photodissociation spectrum. At temperatures between −143 and −81°C no evidence for O_2 binding could be obtained. In the first spectrum obtained after photolysis at −88°C (Fig. 3B) troughs were at 414, 423 and 451 nm. At temperatures warmer than −88°C, similar troughs were observed but of greater magnitude (data not shown). Interpretation of such spectra was aided by recording difference spectra (not shown) in which the reference spectrum was of a bacteroid suspension (reduced plus CO), to which O_2 had been introduced at −23°C, mimicking the reference spectrum subtracted in Figs. 3A and B. Difference spectra of an O_2-oxidized sample *minus* the (reduced plus CO + O_2) sample were similar to Fig. 3B indicating that the troughs at 414, 424 and 450 nm were due to cytochrome oxidation. These data indicate that the oxidation of these cytochromes is as a consequence of photolysis at −80°C and warmer temperatures and, therefore, as a result of the reaction of a CO-sensitive terminal oxidase with O_2. There was no evidence for the reaction of cytochrome *P*-428 (haemoprotein *b*-590) with oxygen nor of its involvement in electron-transfer to oxygen via a CO-sensitive oxidase.

Evidence for the operation of a CO-insensitive oxidase

Appleby [3] has described a CO-insensitive oxidase, possibly a flavoprotein, that could be responsible for CO-insensitive electron flow to oxygen. He described this oxidase as being sensitive to EDTA and Atebrin. In the present investigation evidence for the operation of such an oxidase was sought as follows. EDTA (100 μM) was added to a CO-supplemented, succinate-reduced *B. japonicum* bacteroid suspension, to which O_2 was subsequently introduced at −23°C. Photolysis at −80°C (not shown) resulted in 20% greater oxidation of cytochromes with troughs at 414, 423 and 450 nm in the post-photolysis difference spectrum compared to similar samples without added EDTA. These signals are probably attributable to cytochromes *c*, *b* and *P*-450, respectively. This result is consistent with the inhibition by EDTA of a CO-insensitive oxidase which would otherwise react with oxygen prior to photolysis, causing partial oxidation of other lower-potential respiratory cytochromes which may be common electron-donors to more than one terminal oxidase, and thus diminish the proportion of reduced cytochrome(s) that are subsequently oxidised via the CO-sensitive route. Atebrin

could not be used in such experiments because of its strong masking fluorescence.

Low temperature photodissociation of reduced, CO-liganded R. lupini and R. leguminosarum bacteroids in the absence of O_2

To determine whether the CO-binding pigments observed in *B. japonicum* were common to the bacteroids of other species and whether the oxidases in such species could be more readily identified, we recorded low temperature photodissociation spectra of *R. lupini and R. leguminosarum*. The CO difference spectrum of an *R. lupini* bacteroid suspension (Fig. 4) indicated the presence of CO-reactive *b*-type cytochrome(s) (415 nm peak, 430 and 557 nm troughs) but no *a*-type, as previously noted by Kretovich et al. [6]. The photodissociation spectrum (Fig. 4B) was of low amplitude (and thus noisy) although otherwise similar to that of *B. japonicum* (Fig. 1B) with a maximum at 445 nm but no distinct absorbance in the α- and β-regions.

The CO-difference spectrum of a dithionite-reduced *R. leguminosarum* bacteroid suspension (Fig. 4C) revealed the presence of a large amount of CO-reactive *b*-type cytochrome, with a maximum at 418 nm and a minimum at 440 nm in the Soret-region, and maxima at 539 and 571 nm and minima at 553–557 and 592 nm in the α-region, as detected earlier by Kretovich et al. [7]. This CO-difference spectrum suggests to us that *R. leguminosarum* bacteroids might contain a relatively large amount of a haemoprotein *b*-590-like pigment.

The photodissociation spectrum of the dithionite-reduced, CO-equilibrated *R. leguminosarum* bacteroid suspension (Fig. 4D) was similar to those from *B. japonicum* (Fig. 1B) and *R. lupini* (Fig. 4B), showing that all these species contain a similar photodissociable pigment not exhibiting spectral changes in the visible region.

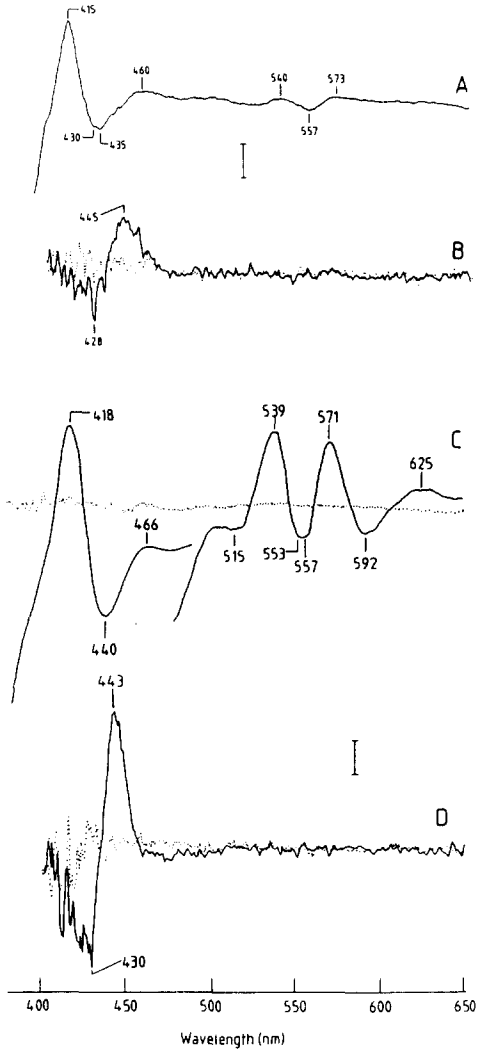

Fig. 4. Difference spectra of *Rhizobium lupini* and *R. leguminosarum* bacteroids. (A) and (C) are reduced (with dithionite) plus CO *minus* reduced (with dithionite), spectra of *R. lupini* and *R. leguminosarum* bacteroids, respectively. Spectra (A) and (C) were recorded at room temperature using the Hitachi-Perkin Elmer 557 spectrophotometer in dual (split)-beam mode at a scan speed of 1 $nm \cdot s^{-1}$ and a slit-width of 2 nm. In (B) the spectrum of a suspension of CO-liganded, dithionite-reduced *R. lupini* bacteroids was scanned at $-145\,^{\circ}C$ and stored in the memory of the spectrophotometer operating in the dual-wavelength mode. Subsequent scans were difference spectra, the stored spectrum being subtracted. The first scan before photolysis yielded a reduced plus CO *minus* reduced plus CO baseline (dotted line). The solid line shows the first spectrum scanned following a 30 s exposure to white light. Spectra were recorded at a scan speed of 1 $nm \cdot s^{-1}$ and a slit-width of 2 nm. The reference wavelength was 500 nm. Spectrum (D) of *R. leguminosarum* bacteroids, was recorded in the same way as spectrum (C) except that the temperature was $-146\,^{\circ}C$. The vertical bar represents a ΔA of 0.05 in (A) and (B) and a ΔA of 0.005 in (D). In (C) between 380 and 500 nm, it represents a ΔA of 0.04 and beyond 500 nm a ΔA of 0.005.

Discussion

In studies of the terminal oxidase systems of many microorganisms (e.g., *E. coli* [13,14]) and of yeast and animal mitochondrial [10,22] successful use has been made of the technique whereby CO-equilibrated suspensions of these microorganisms or mitochondria are examined by low temperature spectrophotometry before and after exposure to actinic light, to reveal moderately stable photodissociation spectra. But despite the presence of several CO-reactive pigments in the bacteroids of several *Rhizobium* and *Bradyrhizobium* species (and which have been seen as potential terminal oxidases) the present bacteroid photodissociation spectra showed only one photodissociable pigment that remained unliganded at temperatures down to $-196\,^{\circ}C$ in the dark. In contrast, Appleby [5] observed, under continuous illumination at this temperature, substantial photodissociation of *P*-428 (haemoprotein *b*-590), cytochrome *c*-552 and possibly *P*-420. In the dark-*minus*-light difference spectrum [5] there was a minimum at 445 nm, which presumably corresponded to the pigment described here and possibly to the 443 nm trough in CO

difference spectra reported by O'Brian and Maier [23]. The most likely explanation of the present failure to observe *P*-428, *c*-552 and *P*-420 photolysis is that their recombination with CO in the dark, i.e., after photolysis, was too rapid to be observable using conventional scanning spectrophotometry. This behaviour is typical of haemoglobin, myoglobin and cytochrome *d* [19].

The identify of the pigment absorbing at 445 nm in photodissociation spectra remains unclear. However, from photolysis of purified cytochromes *P*-450, *P*-428 (haemoprotein *b*-590) and *c*-552 these can be ruled out as candidates. We conclude that *P*-428, now identified as haemoprotein *b*-590 (*Bradyrhizobium*) (Appleby and Poole unpublished data, see Ref. 17] previously suggested to be an oxidase, does not contribute to the photodissociation spectrum obtained after transient illumination. Furthermore, the '445 nm pigment' is largely membrane-bound while haemoprotein *b*-590 is predominantly soluble (unpublished data). However, Appleby has postulated the role of a soluble oxidase in respiratory protection of nitrogenase and has demonstrated a 'soluble' cytochrome *c* oxidase activity in bacteroids [4]. Therefore, a possible respiratory role for *P*-428 still has to be considered. Following photodissociation in the presence of O_2, we were unable to demonstrate any oxygen-binding to *P*-428 and following photolysis at $-80\,^{\circ}C$ (Fig. 3B) and warmer temperatures in the presence of oxygen we did not observe oxidation of *P*-428. These data argue against an oxidase role for this pigment and demonstrates that it is not involved in electron-transfer to oxygen via a CO-sensitive terminal oxidase.

The low temperature 'triple-trapping' or ligand-exchange techniques, which have proved so powerful in the elucidation of the reactions of mitochondrial and bacterial terminal oxidases with oxygen and CO, have been less useful in attempting to clarify the enigma of terminal electron transfer to oxygen in *B. japonicum* and *Rhizobium* bacteroids. Thus the low temperature photodissociation spectra have failed to detect the readily recognisable signatures of potential oxidases and revealed instead a '445 nm pigment' of unknown structure and function. The inability to demonstrate oxygen-binding to a terminal oxidase component in these experiments has not allowed identification of a terminal oxidase component. Nevertheless, the photolysis-dependent oxidation of cytochromes indicates that a CO-sensitive terminal oxidase is functioning under the experimental conditions, which on the basis of its CO-sensitivity is probably a cytochrome. Our inability to detect oxygen-binding to these components, even at temperatures as low as $-150\,^{\circ}C$, may reflect their very high affinity for and very rapid reaction with oxygen. In fact, the terminal oxidase involved in 'effective' respiration of *B. japonicum* bacteroids is proposed to have a K_m for oxygen as low as 10 nM [4]. The terminal oxidase of *E. coli*, cytochrome *d*, has a relatively high affinity for oxygen ($K_m \approx 0.3\ \mu M$). Carbon monoxide and O_2 binding to this oxidase were only demonstrated at liquid helium temperatures and not at liquid nitrogen temperatures similar to those used in this study [19], and it may be that a similar experimental approach will be needed to identify the functional terminal oxidase(s) in *B. japonicum* bacteroids.

The results presented in this paper do suggest a role for *b*-type cytochromes, including *P*-450, and *c*-type cytochromes in electron-transfer to oxygen via a CO-sensitive terminal oxidase. The effect of EDTA on increasing the magnitude of photolysis-dependent oxidation of these components suggested a CO-insensitive oxidase (probably an EDTA-sensitive metalloflavoprotein) as previously described [3,23] to be operating, and suggests that *b*- and *c*-type cytochromes are involved in electron-transfer to this terminal oxidase.

The photodissociable 445 nm pigment, which resembles haemoprotein *b*-590 in the Soret absorbance region, might be an oxidase. Indeed we see it as especially significant that this '445 nm' pigment is a common component of *B. japonicum, R. lupini* and *R. leguminosarum* bacteroids, which otherwise have a diverse array of CO reactive haemoproteins. This pigment has photodissociation characteristics found for most other cytochrome-type oxidases and could possibly function as a bacteroid terminal oxidase in all three species. It may be equivalent to a cytochrome identified as an *a*-type in chemostat grown, N_2-fixing *Rhizobium* ORS 571 from *Sesbania* [24].

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