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# Spectral Evidence for a Component Involved in Hydrogen Metabolism of Soybean Nodule Bacteroids[1]

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## ABSTRACT

A component with a difference spectrum similar to that of *b*-type cytochromes which becomes reduced upon the addition of $H_2$ has been demonstrated in soybean nodule bacteroids. This electron carrier, referred to as component 559-$H_2$, is present in hydrogenase-positive strains of *Rhizobium japonicum* but has not been detected in mutants that lack hydrogenase activity or in hydrogenase-negative wild-type strains. A positive correlation between concentrations of component 559-$H_2$ and hydrogenase activities has been established. These results provide further evidence that component 559-$H_2$ is involved in $H_2$ metabolism in *R. japonicum*.

While investigating $H_2$-dependent Cyt reduction in *Rhizobium japonicum* (8, 9), we have detected a component in chemolithotrophically grown cells with a difference spectrum similar to that of *b*-type Cyt. Chemolithotrophically grown *R. japonicum* cells contain high amounts of this component (hereafter referred to as component 559-$H_2$) and, as a consequence, an alteration in the difference spectrum of reduced *versus* oxidized cells was evident when compared to difference spectra of nodule bacteroids or heterotrophically grown cells (9). The ratio of *b* to *c*-type Cyt was drastically increased in chemolithotrophically grown cells. The behavior of component 559-$H_2$ in the presence of KCN with and without $H_2$ indicated an involvement in $H_2$ metabolism (9). If this is the case, component 559-$H_2$ should also be detected in $H_2$ uptake-positive nodule bacteroids and in bacteria grown under conditions where $H_2$ uptake capability in $H_2$ uptake-positive strains of *R. japonicum* (6) is derepressed (12). In this communication, we present results of our investigation with cells grown under these conditions.

## MATERIALS AND METHODS

**Materials and Growth Conditions.** *Rhizobium japonicum* strains SR, PJ17, PJ18 (11), PJ17-1, and PJ18-1 were grown heterotrophically in 1-liter Erlenmeyer flasks in a yeast-arabinose-mannitol (YAM) medium as described previously (9) or in hydrogen uptake medium (HUM) under hydrogenase derepressing conditions as described by Maier *et al.* (12) with the modification that $KH_2PO_4 \cdot H_2O$ and $NaH_2PO_4 \cdot H_2O$ were added at 100 and 15 mg/l, respectively. The $Hup^+$ strains PJ17-1 and PJ18-1 are spontaneous revertants of the $Hup^-$ mutant strains PJ17 and PJ18 (11), respectively, and were isolated by Dr. M. A. Cantrell and Mr. F. J. Hanus in this laboratory. Chemolithotrophically grown *R. japonicum* cells were provided by Dr. K. Purohit from this laboratory (13). All preparations were stored frozen at −80°C until used.

**Methods.** Nodule bacteroids were grown, isolated, and resuspended in a buffer adjusted to pH 7.0 and containing 12.8 mM $K_2HPO_4$, 13.6 mM $KH_2PO_4$, and 2.5 mM $MgCl_2$ (hereafter referred to as K-phosphate-$MgCl_2$ buffer). $H_2$ and $O_2$ uptake rates, protein concentrations, Cyt difference spectra, and Cyt concentrations were determined as described previously (8, 9). The rates of $H_2$ and $O_2$ uptake were in the same range as described previously (8, 9).

**Concentration of Component 559-$H_2$.** To determine the concentration of component 559-$H_2$, we first established a 'baseline' difference spectrum, where both 3 mM KCN and about 300 $\mu$M $O_2$ were present in the sample and reference suspensions. After this, we added 1 ml $H_2$ into the air-containing gas phase of the sealed sample cuvette and shook it 10 times. The initial concentrations of dissolved $H_2$ and $O_2$ were about 500 and 300 $\mu$M as determined with a duplicate reaction cuvette described previously (8). After the addition of $H_2$, the difference spectrum was recorded with wavelength changes at 1 nm/s and chart speed of 20 nm/cm. We used the mM extinction coefficient of 17.9 (559–580 nm) (2) to calculate the concentration of *b*-type Cyt and the concentration of component 559-$H_2$.

**Determining the Extent of Cyt Reduction.** After establishing a baseline difference spectrum where both sample and reference suspensions contained about 300 $\mu$M $O_2$, 100% Cyt reduction was determined by recording a difference spectrum where the anaerobic sample suspension containing about 500 $\mu$M $H_2$ was measured *versus* an air-oxidized reference suspension.

## RESULTS

**Appearance of Component 559-$H_2$ in Bacteroids.** In chemolithotrophically grown *R. japonicum*, Eisbrenner *et al.* (9) found that about 37% of the *A* difference at 559 nm (Cyt *b*) in a difference spectrum of reduced *versus* oxidized cells was accounted for by a component (559-$H_2$) that was concluded to be involved in $H_2$ metabolism. Using the technique described in "Materials and Methods" in an investigation of nodule bacteroids, we have obtained evidence for the occurrence of component 559-$H_2$ in those strains that are $Hup^+$ (Fig. 1A, trace b). The addition of Ar to the sample suspension revealed a nonspecific decrease in the absorbance in the Soret region (trace c). This may be due to slight differences in cell concentrations in thick suspensions which occur when one of the cuvettes is shaken after adding a gas. We observed no specific response to the addition of an inert gas. When the same type of experiment was done with nodule bacteroids of the $Hup^-$ mutant PJ18, no specific influence of the addition of $H_2$

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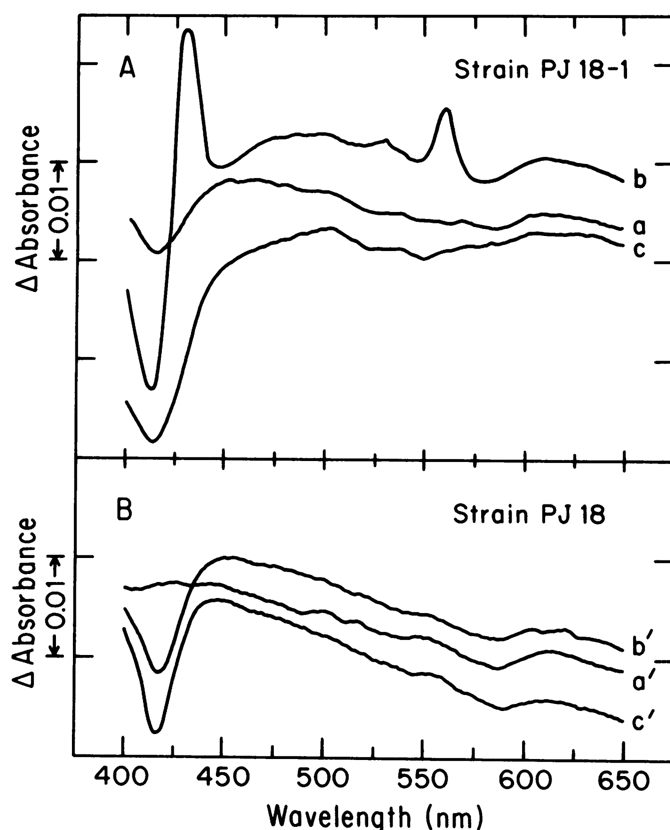


FIG. 1. Difference spectra in the presence of cyanide in the range 400 to 650 nm of *R. japonicum* nodule bacteroids. The experiments were performed in sealed cuvettes as described previously (8, 9). A, Strain PJ18-1 was examined at a concentration of 4.7 mg protein/ml. B, Strain PJ18 was examined at a concentration of 4.5 mg protein/ml. Traces a and a′ are baseline spectra of an air-oxidized suspension where 3 mM KCN was present in both sample and reference cuvettes. Traces b and b′ are difference spectra of sample suspensions containing 3 mM KCN, about 500 nmol/ml $H_2$, and about 300 nmol/ml $O_2$ *versus* reference suspensions containing the same components, except $H_2$ was omitted. Traces c and c′ are difference spectra of sample suspensions containing 3 mM KCN, about 300 nmol/ml $O_2$ and Ar *versus* reference suspensions containing the same components, except Ar was omitted. Ar was added to the sample suspension by injecting 1 ml into the gas phase of the sealed sample cuvette and then the cuvette was shaken 10 times.

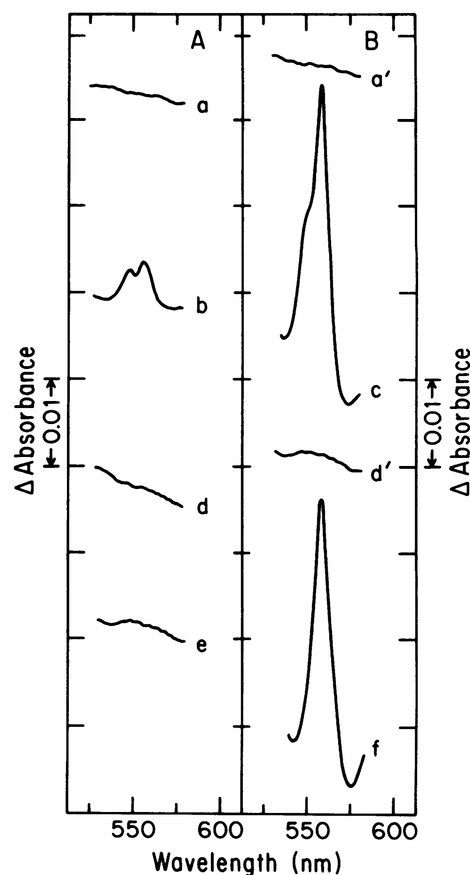


FIG. 2. Difference spectra in the presence of $O_2$ in the range 530 to 580 nm of chemolithotrophically grown *R. japonicum* strain SR. The experiments were performed with cell suspension of 5.1 mg protein/ml in sealed cuvettes as described previously (8, 9). The effects caused by adding succinate (A) and $H_2$ (B) are shown. Traces a and a′ are baseline spectra of an air-oxidized sample suspension *versus* air-oxidized reference suspensions. Trace b is a difference spectrum recorded about 20 s after 25 mM disodium succinate (50 µl volume) was added to the sample cuvette which contained about 300 nmol/ml $O_2$ *versus* a reference suspension containing about 300 nmol/ml $O_2$ to which 50 µl K-phosphate-$MgCl_2$ buffer was added. Trace c is a difference spectrum of a sample suspension containing about 200 nmol/ml $H_2$ and about 300 nmol/ml $O_2$ *versus* an air-oxidized reference suspension. Traces d and d′ are baseline spectra of an air-oxidized sample suspension where 3 mM KCN is present *versus* a reference suspension under the same conditions. Trace e is a difference spectrum recorded about 20 s after 25 mM disodium succinate (50 µl volume) was added to the sample suspension containing 3 mM KCN and about 300 nmol/ml $O_2$ *versus* a reference suspension containing the same components, except 50 µl buffer was added instead of succinate. Trace f is a difference spectrum of a sample suspension containing 3 mM KCN, about 500 nmol/ml $H_2$, and about 300 nmol/ml $O_2$ *versus* a reference suspension containing the same components, except $H_2$ was omitted.

could be detected (Fig. 1B). Similar results were obtained when bacteroids of the $Hup^-$ mutant PJ17 and its $Hup^+$ revertant PJ17-1 were examined.

**Difference Spectra Resulting from the Addition of Substrates Other than $H_2$.** The additional *A* at 559 nm, when $H_2$ is present in $Hup^+$ strains of *R. japonicum*, cannot be explained on the basis of $H_2$ functioning as a nonspecific additional substrate and, therefore, resulting in a shift in the steady-state level of Cyt toward a more reduced state. When chemolithotrophically grown cells were provided with 25 mM succinate, a substrate readily and immediately utilized by *R. japonicum* bacteroids (14) and chemolithotrophically grown cells (this investigation), the Cyt became only slightly reduced while $O_2$ was still present (Fig. 2A, trace b). This increased reduction level resulting from the addition of succinate accounted for only 8% of the *b*-type and 5.2% of the *c*-type Cyt (see "Materials and Methods"). The addition of higher concentrations (50 mM) of succinate failed to change these percentages significantly. When the cells were provided with $O_2$ and treated with KCN, the addition of succinate had no effect (Fig. 2A, trace e). When a similar experiment was performed, in which $H_2$ and $O_2$ were provided but neither KCN nor succinate was added (Fig. 2B, trace c), 52% of the *b*-type Cyt and 12.5% of the *c*-type Cyt were reduced. With KCN-treated cells, the addition of $H_2$ resulted in an appearance of 45.9% of all absorbance difference that was observed at 559 nm (Fig. 2B, trace f; see also Ref. 9). In these experiments and those presented in Figure 3, we recorded only the critical wavelength range between 530 and 580 nm. It was difficult to monitor the spectrum over a longer period of time because the untreated cells used up $H_2$ very quickly. That $H_2$ and $O_2$ were still present after each spectrum was recorded was proven by measuring the kinetics of *A* changes at 559 nm. When $H_2$ was depleted and $O_2$ was still present, an *A* decrease at 559 nm was

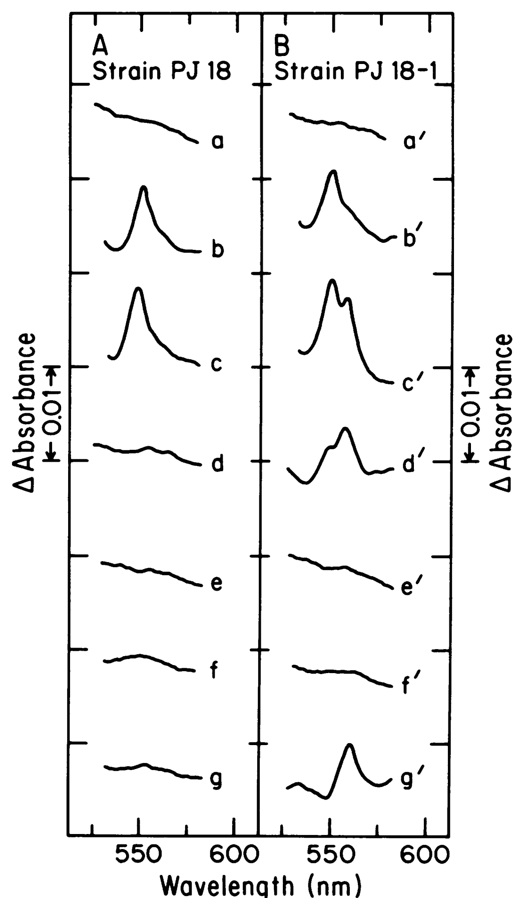


FIG. 3. Difference spectra in the presence of $O_2$ in the range of 530 to 580 nm of *R. japonicum* nodule bacteroids. The experiments were performed in sealed cuvettes as described previously (8, 9). Strain PJ18 (A) and strain PJ18-1 (B) were examined at a concentration of 5.7 mg/ml each. Traces a and a′ are baseline spectra of air-oxidized sample *versus* air-oxidized reference suspensions. Traces b and b′ are difference spectra recorded about 20 s after 25 mM disodium succinate (50 μl volume) was added to the sample cuvette which contained about 300 nmol/ml $O_2$ *versus* references containing the same but instead of succinate, 50 μl K-phosphate-$MgCl_2$ buffer was added. Traces c and c′ are difference spectra where the sample suspension contained about 500 nmol/ml $H_2$ in addition to all other conditions described for traces b and b′. Traces d and d′ are difference spectra where the sample suspension contains about 100 nmol/ml $H_2$ and about 300 nmol/ml $O_2$ *versus* air-oxidized reference suspensions. Traces e and e′ are baseline spectra of an air-oxidized sample suspension where 3 mM KCN is present *versus* reference suspensions under the same conditions. Traces f and f′ are difference spectra recorded about 20 s after 25 mM disodium succinate (50 μl volume) was added to the 3 mM KCN and about 300 nmol/ml $O_2$ containing sample suspension *versus* reference suspensions containing the same, but instead of succinate, 50 μl buffer was added. Traces g and g′ are difference spectra where, in addition to the conditions present where traces f and f′ were recorded, about 500 nmol/ml $H_2$ were added to the sample suspensions.

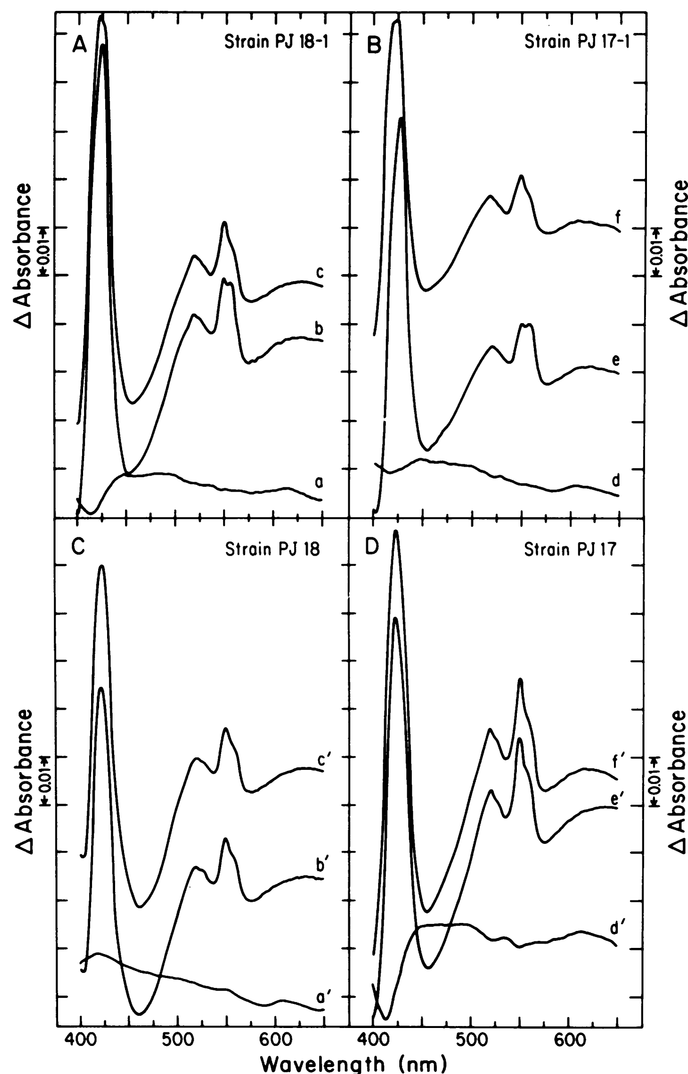


FIG. 4. Effect of endogenous respiration on difference spectra in the presence of cyanide in the range 400 to 650 nm of different Hup⁻ mutants and their revertants of *R. japonicum* nodule bacteroids. The experiments were performed in sealed cuvettes as described previously (8, 9). Strains PJ18-1 (A), PJ17-1 (B), PJ18 (C), and PJ17 (D) were examined at protein concentrations of 6.5, 4.6, 5.2, and 9.0 mg/ml, respectively. Traces a, a′, d, and d′ are baseline spectra of air-oxidized sample suspensions in the presence of 3 mM KCN *versus* reference suspensions under the same conditions. Traces b, b′, e, and e′ are difference spectra recorded 30 min after $O_2$ became depleted in the sample suspensions *versus* air-oxidized reference suspensions. Both sample and reference suspensions contained 3 mM KCN. Traces c, c′, f, and f′ are difference spectra recorded 35 min after $O_2$ became depleted in the sample suspensions *versus* air-oxidized reference suspensions where about 500 nmol/ml $H_2$ was added. Both sample and reference suspensions contained 3 mM KCN.

observed.

Similar experiments also were conducted using nodule bacteroids of the Hup⁻ strain PJ18 (Fig. 3A) and its Hup⁺ revertant PJ18-1 (Fig. 3B). When strain PJ18 was examined, $H_2$ could not interfere because it contains no hydrogenase (Fig. 3A, trace d), the addition of 25 mM succinate resulted in a 16.5 and 9.8% reduction of Cyt of the *b* and *c*-types, respectively (Fig. 3A, trace b). The addition of $H_2$ (Fig. 3A, trace c) or 50 mM succinate caused no further changes. The addition of succinate (Fig. 3A, trace f) or $H_2$ (Fig. 3A, trace g) to KCN-treated Hup⁻ mutant cells did not change the absorbance difference.

When a similar experiment was conducted with Hup⁺ nodule bacteroids of strain PJ18-1, the response to the addition of succinate (Fig. 3B, trace b′) was not measurably different from that observed with the mutant (Fig. 3A, trace b). The addition of $H_2$ to the cuvette containing succinate, however, resulted in a significant increase in the *A* difference at 559 nm (Fig. 3B, trace c′). Furthermore, the extent of *b*-type Cyt reduction in presence of $H_2$ and $O_2$ (Fig. 3B, trace d′) was greater than in the presence of succinate and $O_2$. Also, the addition of succinate to KCN-treated

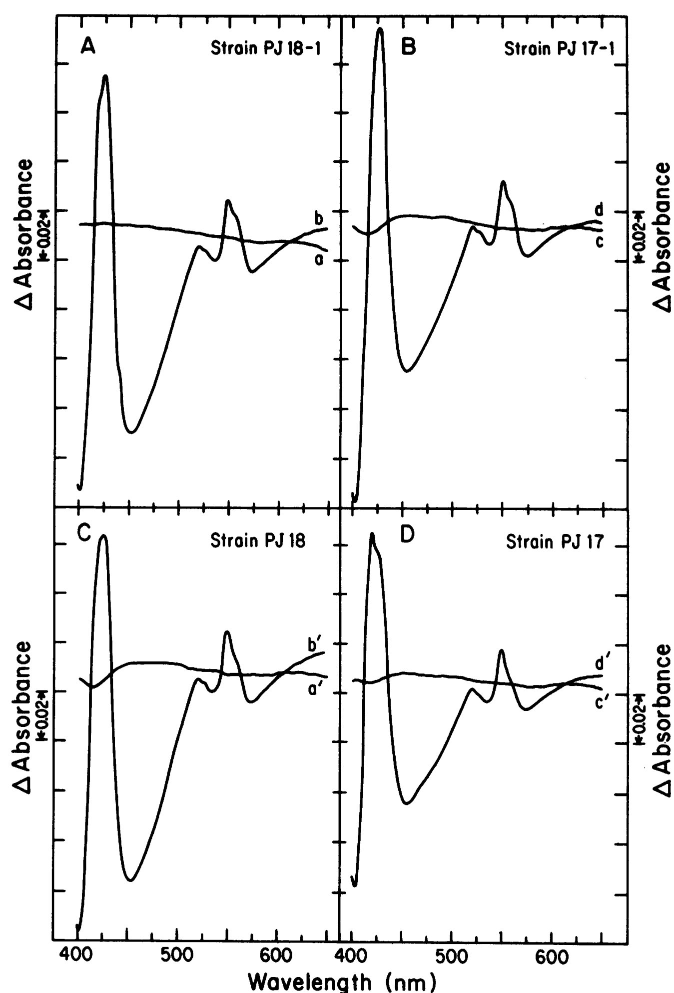


FIG. 5. Effect of dithionite on difference spectra in the presence of cyanide in the range of 400 to 650 nm of different Hup⁻ mutants and their revertants of *R. japonicum* nodule bacteroids. The experiments were performed in sealed cuvettes as described previously (8, 9). Strains PJ18-1 (A), PJ17-1 (B), PJ18 (C), and PJ17 (D) were examined at protein concentrations of 5.1, 5.4, 5.2, and 4.5 mg/ml, respectively. Traces a, a′, c, and c′ are baseline spectra of air-oxidized sample suspensions in the presence of 3 mM KCN *versus* references under the same conditions. Traces b, b′, d, and d′ are difference spectra after 0.5 mg $Na_2S_2O_4$ as a powder (2) was added to the sample suspensions against air-oxidized references. Both sample and reference suspensions contained 3 mM KCN.

$Hup^+$ PJ18-1 bacteroids caused no appreciable changes in the absorbance difference (Fig. 3B, trace f′), while the addition of $H_2$ caused a marked change (Fig. 3B, trace g′; see also Fig. 1A, trace b).

**Component 559-$H_2$ Detected Only in $Hup^+$ Strains of *R. japonicum*.** To find out whether component 559-$H_2$ was also present in the $Hup^-$ strains of *R. japonicum*, we investigated several $Hup^-$ wild type and $Hup^-$ mutant strains. By use of the observation that component 559-$H_2$ becomes reduced by endogenous substrates after a 30-min incubation in the absence of $H_2$, we designed an experiment that enabled us to determine qualitatively whether component 559-$H_2$ was present or not. In the experiment utilizing $Hup^-$ mutants (Fig. 4, C and D) and $Hup^+$ revertants (Fig. 4, A and B), we first recorded a baseline where sample and reference suspensions contained 3 mM KCN and $O_2$ (traces a, a′, d, and d′). The suspension in the reference cuvette was maintained in an oxidized state all the time. The depletion of $O_2$ in the sample cuvette was detected by an increase in *A* difference at 550 nm (Cyt of the *c*-type that are involved in the cyanide-insensitive pathway). In the experiment, cuvettes were incubated for 30 min which allowed endogenous substrates to reduce the electron carriers (Fig. 4, A–D, traces b, b′, e, and e′). It is clearly apparent that the *A* difference at 559 nm in suspensions containing $Hup^+$ revertants was greater than that of the $Hup^-$ mutants. This increased absorbance was eliminated by adding $H_2$ to the reference cuvette. The resulting difference spectrum then became similar to that found

Table I. *Measurements of b-Type Cyt, Component 559-$H_2$ Contents, and Hydrogenase Activity under Different Growth Conditions*

The total concentration of *b*-type Cyt was determined as *A* difference between 559 and 580 nm of an anaerobic sample suspension in the presence of $H_2$ *versus* an air-oxidized reference. The concentration of component 559-$H_2$ was measured as *A* difference between 559 and 580 nm of an aerobic sample suspension that contained 3 mM KCN and about 500 nmol/ml $H_2$ *versus* an aerobic reference suspension that contained 3 mM KCN. The total Cyt of the *b*-type (column 3) contain Cyt *o* and *Rhizobium* hemoglobin where present (3). Hydrogenase activity was determined with $O_2$ as electron acceptor. Cells from two different harvests were used to examine chemolithotrophic growth conditions. Strains PJ17-1-20 and PJ17-1-52 are two independently isolated revertants.

| Growth Conditions | Strains | Total Cyt of the *b*-Type | Component 559-$H_2$ | Hydrogenase |
|---|---|---|---|---|
| | | *nmol/g protein* | | *μmol $H_2$ consumed/h·mg protein* |
| Chemolithotrophic | SR ($Hup^+$) | 713 | 319 | 11.55 |
| | SR ($Hup^+$) | 726 | 356 | 13.63 |
| Symbiotic (as soybean nodule bacteroids) | SR ($Hup^+$) | 412 | 41 | 1.35 |
| | PJ18-1 ($Hup^+$) | 414 | 79 | 2.66 |
| | PJ17-1-20 ($Hup^+$) | 361 | 73 | 3.21 |
| | PJ17-1-52 ($Hup^+$) | 405 | 61 | 2.25 |
| | 110 ($Hup^+$) | 420 | 66 | 2.03 |
| | 143 ($Hup^+$) | 425 | 51 | 2.34 |
| | PJ18 ($Hup^-$) | 333 | <10 | <0.01 |
| | PJ17 ($Hup^-$) | 339 | <10 | <0.01 |
| | 16 ($Hup^-$) | 385 | <10 | <0.01 |
| | 54 ($Hup^-$) | 364 | <10 | <0.01 |
| Heterotrophic (YAM medium) | SR ($Hup^+$) | 251 | <10 | <0.01 |
| | PJ18 ($Hup^-$) | 221 | <10 | <0.01 |
| Heterotrophic (depressed for hydrogenase; Ref. 12) | SR ($Hup^+$) | 228 | <10 | 0.04 |
| | PJ18 ($Hup^-$) | 220 | <10 | <0.01 |

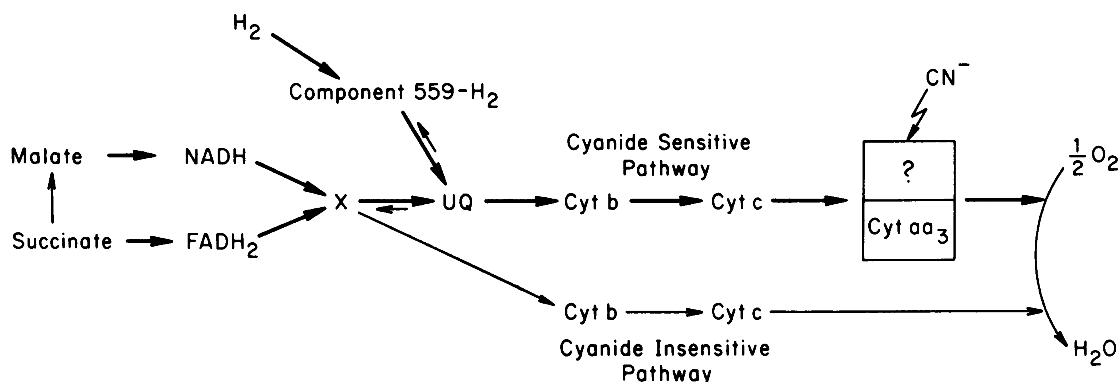


FIG. 6. Tentative scheme for electron transport in Hup$^+$ *R. japonicum*. Thick arrows indicate main electron transport pathways. Component 559-$H_2$ is an electron carrier that is involved in the $H_2$-metabolism (see text). An unknown electron carrier is assumed to function at the branching point between cyanide-sensitive and cyanide-insensitive $O_2$ uptake. The terminal oxidase (?) that is involved in the cyanide-sensitive $O_2$ uptake in bacteroids has so far not been characterized. The assumed location of cyanide inhibition is indicated in this figure. Ubiquinone has been established to be present in *R. japonicum* (7). Possible further branched electron transport involving Cyt like Cyt *o*, when present (2, 3), are not considered in this scheme. Also, not considered are the likely involvement of several Cyt of the *b* and *c* types in either the cyanide-sensitive or cyanide-insensitive electron transport pathways.

in Hup$^-$ strains because under these conditions, component 559-$H_2$ also was reduced in the reference cuvette (Fig. 4, A and B, traces c and f). The addition of $H_2$ to the reference cuvette containing Hup$^-$ strains had no effect on the difference spectrum whatsoever (Fig. 4, C and D, traces c′ and f′).

In another approach to this problem, after establishing a baseline in the presence of 3 mM KCN and $O_2$ (Fig. 5, traces a, a′, c, and c′), we added 0.5 mg/ml solid $Na_2S_2O_4$ to the sample cuvette. This resulted in the reduction of all electron carriers that were not already reduced in the presence of 3 mM KCN and $O_2$ (Fig. 5, trace b, b′, d, and d′). Calculation of the ratios of *b* to *c*-type Cyt always showed higher values in suspensions of Hup$^+$ revertants than in suspensions of Hup$^-$ mutants. Similar experiments with the Hup$^+$ wild-type strains 3I1b 143 and 3I1b 110 and the Hup$^-$ wild-type strains USDA 16 and R54a produced similar results. All of our results can be explained by assuming that an electron transport scheme as shown in Figure 6 is functional.

**Hydrogenase Activities and Concentrations of Component 559-$H_2$.** We have summarized in Table I data concerning the relationship of contents of *b*-type Cyt, component 559-$H_2$, and hydrogenase activities of *R. japonicum* strains grown under different conditions. In chemolithotrophically grown cells which need the $H_2$ uptake pathway for their energy demands, the concentration of component 559-$H_2$ is about 7 times higher than in hydrogenase-containing *R. japonicum* bacteroids from soybean nodules. It is known that hydrogenase activity in chemolithotrophically grown cells is several-fold higher than that of soybean nodule bacteroids (9, 10). Since the concentration of component 559-$H_2$ is relatively low compared to the total Cyt *b* background concentration, component 559-$H_2$ was not detected in our earlier work (8). When we calculated the hydrogenase activities on a series of *R. japonicum* strains grown under different growth conditions, against estimated concentrations of component 559-$H_2$, a positive correlation coefficient of $r = 0.98$ was obtained. The correlation coefficient for these same parameters for the nodule bacteroids listed in Table I was $r = 0.89$.

We were not successful in detecting component 559-$H_2$ in *R. japonicum* strain SR grown on HUM medium under derepressing conditions (12). The hydrogenase activity of strain SR under derepressing conditions was 40 nmol $H_2$ consumed/h·mg protein which was in the same range as the 39 ± 6 nmol $H_2$ consumed/h·mg protein reported for strain 122 DES (12). It appears that our ability to detect component 559-$H_2$ is limited to *R. japonicum* preparations that show $H_2$ uptake rates measured as $\mu$mol $H_2$ consumed/h·mg protein of 0.5 or greater.

## DISCUSSION

We have shown that Hup$^+$ strains of *R. japonicum* contain a component with a *b*-type Cyt spectrum which rapidly becomes reduced upon the addition of $H_2$. It can be argued that the addition of $H_2$ as a substrate to Hup$^+$ strains of *R. japonicum* shifts to a more reduced level the steady-state oxidation-reduction status of those Cyt that are involved in cyanide-sensitive electron transport and that no additional component is associated with the increased absorbance. Against this argument are the findings of Eisbrenner *et al.* (9) showing that the addition of readily oxidizable substrates such as succinate to chemolithotrophically cultured cells does not cause a marked increase in *A* at 559 nm. Furthermore, the experiments reported in Figure 3 provide evidence that the results obtained with chemolithotrophically cultured cells may be extended to *R. japonicum* bacteroids. Also, against the argument that the appearance of additional *A* at 559 nm is a nonspecific response to the addition of $H_2$, are the data of Table I demonstrating a positive correlation between amounts of component 559-$H_2$ and hydrogenase activities of cells grown under different conditions. Chemolithotrophically cultured cells contain activities of hydrogenase and concentrations of component 559-$H_2$ that are about 7 times higher than values observed in *R. japonicum* bacteroids, while the *c*-type Cyt content of chemolithotrophically cultured cells was only 1.6-fold higher than that observed with nodule bacteroids.

Furthermore, the results that we have obtained using $Na_2S_2O_4$ as a reductant are inconsistent with the argument that the response to $H_2$ is nonspecific. The data in Figure 5 show that the ratio of *b* to *c*-type Cyt determined after adding $Na_2S_2O_4$ was consistently higher in Hup$^+$ than in Hup$^-$ nodule bacteroids. Such differences in the *b*-type Cyt content were not expected because the bacteroids were grown under comparable conditions that exist in nodules. This supports the view that Hup$^+$ strains contain an additional component that absorbs at 559 nm.

We cannot rule out the possibility that some of the *A* difference at 559 nm observed after adding $H_2$ in the presence of KCN and $O_2$ to Hup$^+$ cell suspensions is due to an increased reduction level of *b*-type Cyt but the evidence indicates that the majority of the *A* difference at 559 nm is a result of the reduction of a carrier which is involved in $H_2$ metabolism and referred to as component 559-$H_2$.

We have observed that only Hup$^+$ strains of *R. japonicum* that are cultured under conditions where hydrogenase activity is measurable contain component 559-$H_2$. This suggests that component 559-$H_2$ is very closely related to hydrogenase function or

perhaps associated with the enzyme *per se*. The fact that component 559-$H_2$ was not detected in two non-sibling revertible mutants PJ17 and PJ18 (11) which are considered to have point mutations in the hydrogenase or regulatory genes for hydrogenase provides support for an assumed close interaction between component 559-$H_2$ and hydrogenase. Furthermore, our data show that all of a whole series of wild-type $Hup^-$ strains of *R. japonicum* are devoid of component 559-$H_2$. Inasmuch as these strains show no $H_2$ uptake capability in the presence of $O_2$ or methylene blue, the latter of which is known to accept electrons directly from hydrogenase (4), again we are forced to conclude that component 559-$H_2$ and hydrogenase may be closely related.

Obviously, it is necessary to purify hydrogenase and component 559-$H_2$ in order to obtain definitive information regarding their relationship. The hydrogenase from the $Hup^+$ *R. japonicum* strains USDA 110 has been highly purified by Arp and Burris (4). According to Dr. D. J. Arp (personal communication, 1982) the most purified fraction showed evidence of contamination by a Cyt component. This information together with our results (9, this paper) might be interpreted by assuming that *R. japonicum* contains a *b*-type Cyt with an oxidation reduction potential more negative than ubiquinone (Fig. 6). The possibility needs to be considered that component 559-$H_2$ is a *b*-type Cyt such as that found in the electron transport from ubiquinone to $O_2$. A more negative oxidation reduction potential could be caused by its association with the membrane-bound hydrogenase. Cyt of the *b*-type with an oxidation reduction potential more negative than ubiquinone have been observed in the anaerobic electron transport from formate to nitrate in *Escherischia coli* (5; see 1, 15) and in electron transfer from formate to fumarate in *Vibrio succinogenes* (see 15).

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