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Hydrogen-Oxidizing Electron Transport Components in Nitrogen-Fixing *Azotobacter vinelandii*†

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Membranes from N_2-fixing *Azotobacter vinelandii* were isolated to identify electron transport components involved in H_2 oxidation. We found direct evidence for the involvement of cytochromes *b*, *c*, and *d* in H_2 oxidation by the use of H_2-reduced minus O_2-oxidized absorption difference spectra. Carbon monoxide spectra showed that H_2 reduced cytochrome *d* but not cytochrome *o*. Inhibition of H_2 oxidation by cyanide was monophasic with a high K_i (135 μM); this was attributed to cytochrome *d*. Cyanide inhibition of malate oxidation showed the presence of an additional, low K_i (0.1 μM cyanide) component in the membranes; this was attributed to cytochrome *o*. However, H_2 oxidation was not sensitive to this cyanide concentration. Chlorpromazine (at 160 μM) markedly inhibited malate oxidation, but it did not greatly inhibit H_2 oxidation. Irradiation of membranes with UV light inhibited H_2 oxidation. Adding *A. vinelandii* Q8 to the UV-damaged membranes partially restored H_2 oxidation activity, whereas addition of UV-treated Q8 did not increase the activity. 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide inhibited both H_2 and malate oxidation.

The redox carriers in the membrane fraction of *Azotobacter vinelandii* include several flavin-dependent dehydrogenases, ubiquinone Q8, and at least six detectable cytochromes, which form a complex branched electron transport pathway (6, 9, 10). One branch, from cytochromes *b* through *c* to $a_1 + o$, is involved in energy conservation, and the other branch, from cytochromes *b* to *d*, is thought to be the major pathway of electrons to O_2, but does not appear to produce ATP. The respiratory system of *A. vinelandii* produces ATP via oxidative phosphorylation and also seems to protect nitrogenase by consuming O_2 (21, 22). Additionally, when H_2 is provided in carbon substrate-limited cultures, O_2 consumption is more rapid, due to an H_2 oxidation electron transport system (24). This H_2 oxidation system uses electrons from the H_2 that is evolved by the nitrogenase reaction and carries them through an unidentified electron transport chain to O_2, yielding water as the final product (16, 26). Wilson and colleagues (5, 16) concluded that the hydrogenase and nitrogenase systems involved in H_2 metabolism in *A. vinelandii* were somehow related; the activity of hydrogenase increased four-fold when nitrogenase was derepressed (5). In *Azotobacter chroococcum*, H_2 oxidation allows nitrogenase to function optimally at high O_2 levels (24, 27).

Early studies (7) have suggested the involvement of hemoproteins in H_2 oxidation in *A. vinelandii*; however, these components have not been identified. In this report, we demonstrate the reduction of specific electron transport components in *A. vinelandii* membranes by H_2.

MATERIALS AND METHODS

Chemicals. 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), sodium ascorbate, sodium succinate, sodium malate, *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine (TMPD), and sodium dithionite were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were purchased from J. T. Baker Chemical Co., Phillipsburg, N.J.

Cell growth and membrane preparation. *A. vinelandii* CA (obtained from P. E. Bishop, Raleigh, N.C.) was grown under N_2-fixing conditions as described previously (12). Whole cells were suspended in 50 mM phosphate buffer (pH 7.5), and the suspension was stirred overnight at 4°C to remove endogenous cellular substrates. The cells were harvested by centrifugation and disrupted by passing them twice through a French pressure cell at 10,000 lbs/in^2. The membrane fraction was isolated by differential centrifugation as described previously (12). Membranes were stored at −20°C until used. The O_2-dependent H_2 oxidation activity in these membranes remained stable for 3 weeks.

Spectroscopic analysis. All absorption spectra were determined with a Perkin-Elmer model 557 dual-beam spectrophotometer. Difference spectra were recorded by subtracting the memorized oxidized spectrum from the reduced sample spectrum as described previously (18, 19). First, an oxidized spectrum was obtained by scanning 0.8 ml of sample (3 mg of protein per ml) in a semimicro quartz cuvette (10-mm pathlength; Savant Instruments, Inc., Hicksville, N.Y.). The cuvette was then tightly stoppered with a sleeve type rubber stopper and flushed with Ar gas for 2 min to remove O_2. An Ar minus O_2 difference spectrum was recorded to be sure no endogenous substrate was present. The sample was then flushed with H_2 for 2 min and then equilibrated at room temperature for 20 to 30 min. Absorption difference spectra were recorded (to obtain an H_2 minus O_2 spectrum) after the cuvette was again flushed with H_2 for 2 min to ensure H_2 saturation. The spectral properties used to identify specific cytochrome types have been described by Smith (23). H_2-reduced difference spectra with CO_2 was performed as described previously (18).

H_2 and O_2 uptake assay. H_2 and O_2 uptake rates were simultaneously monitored amperometrically as described previously (25). A potassium phosphate buffer solution (50 mM, pH 7.5) was flushed thoroughly with N_2 gas and used to fill the electrode chamber. H_2 was added to the chamber by injecting 500-μl samples of H_2-saturated phosphate buffer. Membrane samples (10 to 20 μl of ca. 30 mg of protein per ml) were injected into the chamber, and O_2 consumption was monitored until the chamber was completely anaerobic. The H_2 concentration was adjusted to 40 μM. Reactions were then started by injecting 50 μl of O_2-saturated buffer into the chamber. There was no H_2 uptake once the O_2 was depleted,

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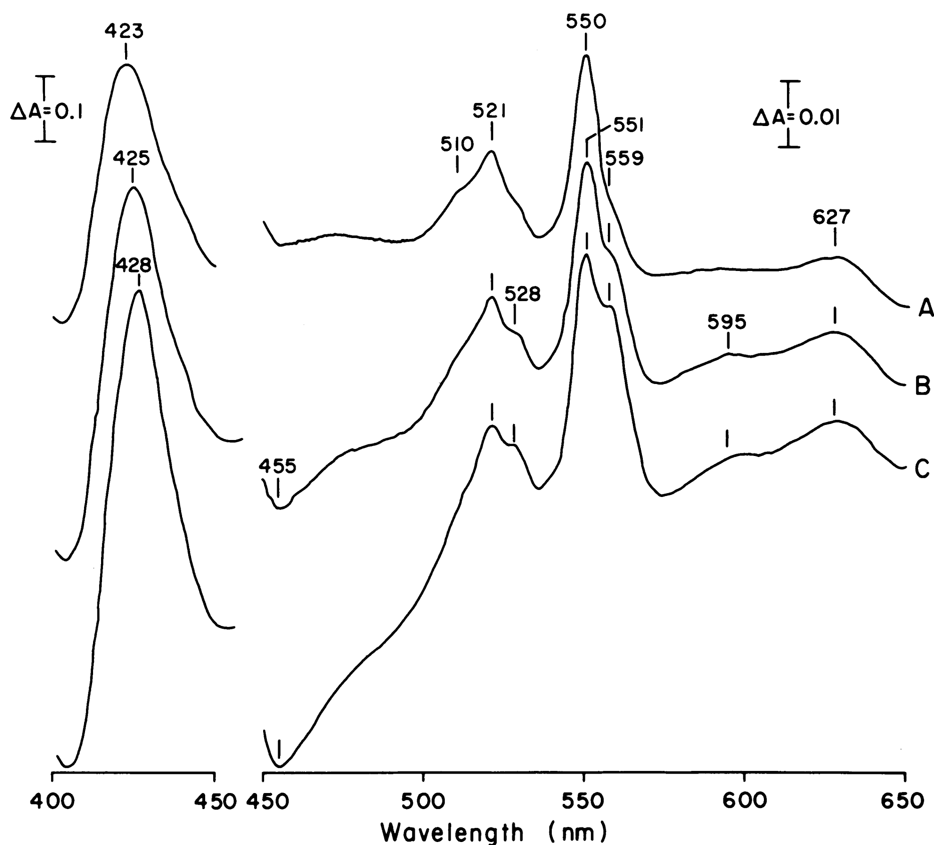

FIG. 1. Substrate-reduced minus oxidized absorption difference spectra in membranes. H_2 absorption difference spectra were recorded as described in the text. Malate (1 mM) and dithionite (1 mM) absorbance spectra were recorded by direct injection into the oxidized membrane sample in the stoppered cuvette. The sample was equilibrated for 10 min with substrate before the absorption spectrum was recorded. (A) H_2; (B) malate; (C) dithionite.

and the ratio of H_2 consumed to O_2 was ca. two (data not shown). Injecting more than 50 μl of O_2-saturated buffer did not increase the rate of H_2 uptake. Both the O_2 and H_2 uptake rates were linear during the assay periods. When performing inhibitor studies, the inhibitor was injected into the anaerobic electrode chamber and equilibrated for 1 min before the addition of O_2 to begin the assay. The oxidation of malate and of ascorbate plus TMPD were monitored similarly (O_2 uptake in the presence of the substrates). The final concentrations were as follows: malate, 5 mM; ascorbate, 5 mM; and TMPD, 10 mM. All experiments were performed at room temperature. Portions of H_2- and O_2-saturated buffer were used as standards before and after each experiment (50-μl volume containing 37.7 nmoles of H_2 or 63.3 nmoles of O_2). Dixon plots ($1/v$ versus [cyanide]) were used to calculate the K_i values. The correlation of all lines constructed with linear regression was better than 0.9.

UV light treatments. A sample of membranes (10 mg of protein per ml) was irradiated with UV light (254-nm maximum wavelength) for 30 min at 4°C in a 3-ml cuvette. The light source (27-cm length; General Electric Co., Cleveland, Ohio) was held 5 cm from the cuvette. The ubiquinone extract was treated identically.

H_2 uptake rates in UV-treated and untreated membranes were assayed as described above. H_2-sparged ubiquinone extract was added directly to the electrode chamber as an ethanolic solution in 100-μl volume and then allowed to equilibrate for 1 min before the addition of 50 μl of O_2-saturated buffer.

Total lipids were extracted from 2 g (wet weight) of *A. vinelandii* by the method of Bligh and Dyer (2). Phospholipids were then precipitated from the total lipid extract with cold acetone (15). The supernatant was dried under dry N_2. This ubiquinone extract was suspended in ethyl alcohol and used subsequently for reconstitution studies. This extraction procedure gave a preparation consisting of a single spot that ran with ubiquinone on thin-layer chromatograms when stained with I_2.

RESULTS

We observed no O_2 uptake by membranes without the addition of oxidizable substrates; in the presence of H_2, the H_2/O_2 uptake rate ratio was ca. 2. These membranes were therefore useful for studying the electron transport system that oxidizes H_2. After rupturing *A. vinelandii* cells and fractionating the crude extract, ca. 20% of the whole-cell H_2 uptake activity (with O_2 as the electron acceptor) was recovered in the membrane fraction (data not shown). This is a substantially greater recovery of activity in membranes than was possible for similar fractionations from *Rhizobium japonicum* (17, 18). The preparation of membranes under anaerobic conditions and in the presence of antioxidants (17) did not improve the recovery of H_2 oxidation activity.

Spectrophotometric analysis. To identify cytochromes of the H_2 oxidation system, we performed absorption difference spectra of membranes with H_2 as the reductant. The H_2-reduced minus O_2-oxidized absorption difference spectrum is shown in Fig. 1A. H_2 reduced cytochrome *d* (peak at

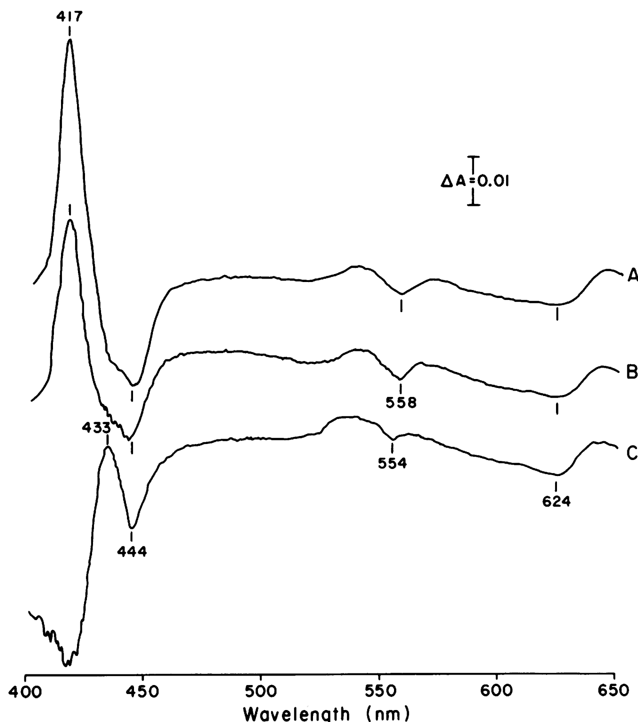

FIG. 2. Carbon monoxide difference spectra. (A) (Dithionite + CO) minus dithionite. Sample in the stoppered cuvette containing 1 mM dithionite was recorded and memorized as described in the text. The sample was then flushed with CO for 2 min. The absorption spectrum of dithionite-reduced sample was subtracted from the (CO + dithionite) minus reduced membrane spectrum. (B) (Malate + CO) minus malate. The procedure was the same as described in (A) except that 1 mM malate was used as the reductant. (C) (H_2 + CO) minus H_2. The H_2-reduced sample was recorded as described in the text, and then the stoppered cuvette was flushed with CO and H_2 simultaneously for 2 min. The sample was then equilibrated for 5 min in the dark before the (H_2 + CO) minus H_2 spectrum was recorded.

627 nm), cytochrome *b* (shoulder at 559 nm), and cytochrome *c* (peak at 550 nm). Reduced minus oxidized difference spectra with malate or dithionite as reductants are also shown in Fig. 1. Malate and dithionite reduced significantly more *b*-type cytochrome than did H_2 as seen by the increased absorption at 559 nm in Fig. 1B and C. H_2 was also unable to reduce cytochrome *a* (broad peak at 595 nm) or flavoprotein (trough at 455 nm); however, these features were observed in the malate- and dithionite-reduced spectra.

Figure 2 shows (CO + reduced) minus reduced difference spectra, using H_2, malate or dithionite as the reductant. Malate and dithionite (Fig. 2A and B) each reduced both cytochromes *o* and *d* as seen by the troughs at 558 and 624 nm. However, H_2 reduced cytochrome *d* only (Fig. 2C); it did not reduce any CO-reactive, *b*-type cytochrome. These data are consistent with the observation that malate and dithionite reduced quantitatively more *b*-type cytochrome than did H_2 (Fig. 1). Furthermore, the CO spectra in the presence of malate or dithionite showed a peak in the Soret region at 417 nm that was absent in the H_2-reduced spectrum. The 417-nm peak is indicative of cytochrome *o* (23); the absence of this feature in the (H_2 + CO) minus H_2 spectrum supports our observation that H_2 oxidation does not involve cytochrome *o*.

We did, however, observe a trough at 554 nm in the (H_2 + CO) minus H_2 spectrum (Fig. 2C). The presence of this trough, distinct from the 558-nm trough of cytochrome *b*, was consistently observed in over 20 independent CO spectra. This feature (554-nm trough) has not previously been observed in *A. vinelandii*. The spectrophotometric data pertaining to cytochrome *d* are limited to the α region of the spectrum, since this component has yet to be purified. Therefore, it is possible that the trough at 554 nm is due to the β peak of cytochrome *d*. The 554-nm trough which is seen in our studies may be detectable, since there is no interference from cytochrome *o*.

There were no features of our CO spectra that were clearly indicative of a cytochrome a_1-CO complex despite the observed resolution of cytochrome a_1 by malate and dithionite (Fig. 1). However, we cannot rule out the possibility

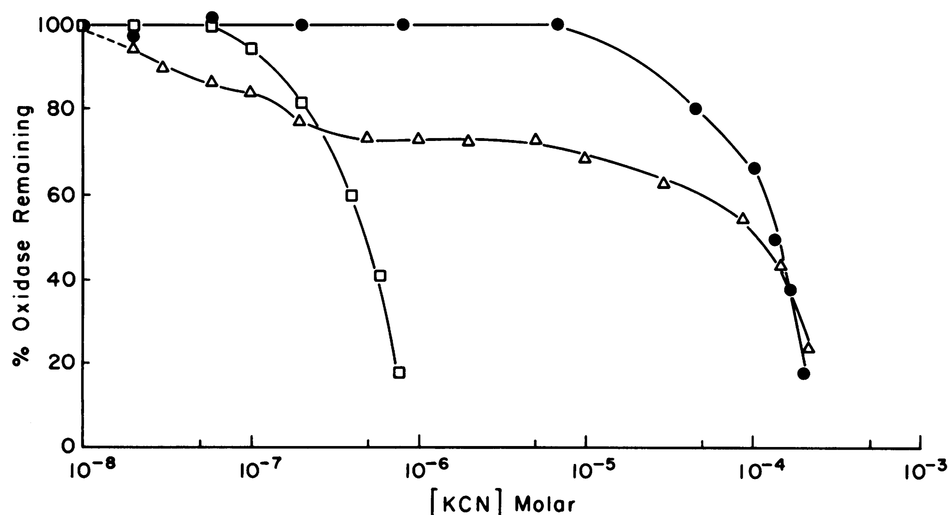

FIG. 3. Effect of CN on the oxidation of malate (△), TMPD-ascorbate (□), and H_2 (●). Assay procedure is described in the text. 100% activities were (in micromoles of O_2 consumed per min per milligram of protein) as follows: malate oxidation, 0.6; TMPD-ascorbate oxidation, 1.4; and H_2 oxidation, 0.14.

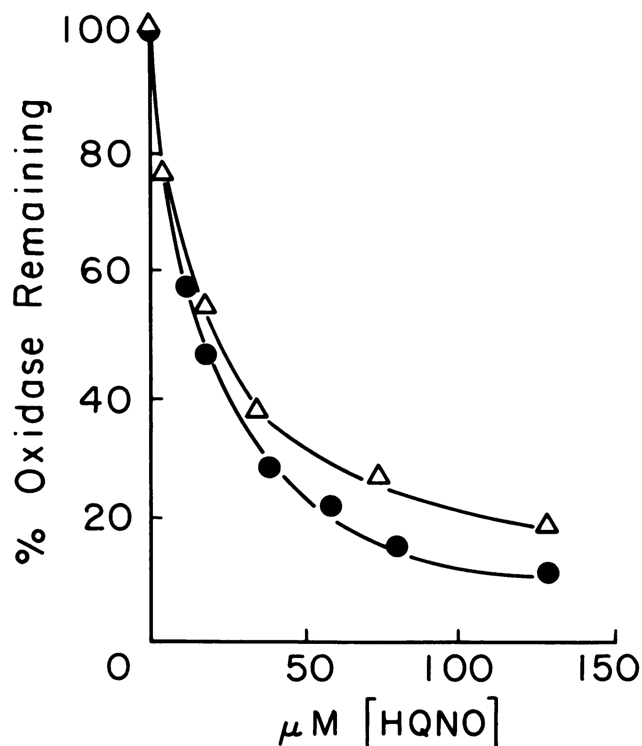

FIG. 4. Effect of HQNO on the oxidation of malate (△) and H_2 (●) in membranes. The initial specific activities for malate and H_2 oxidation were 0.36 and 0.17 μmoles of O_2 oxidized per min per mg of protein, respectively.

that the trough at 444 nm and the peak at 433 nm in the CO spectra (Fig. 2) are due to cytochrome a_1.

Cyanide inhibition studies. Jones and Redfearn concluded that cytochrome *d* is relatively insensitive to cyanide in *A. vinelandii* (11), whereas cytochrome *o* has a high binding affinity for cyanide (8). We therefore monitored the rate of H_2 uptake by membranes as a function of cyanide concentration to determine the CN-reactive oxidases involved in H_2 oxidation (Fig. 3). We observed that H_2 uptake was not sensitive to low concentrations of cyanide; this is consistent with our previous conclusion that H_2 oxidation uses only cytochrome *d* as the terminal oxidase. The K_i value of cyanide for H_2 uptake was 135 μM.

Conversely, ascorbate-TMPD oxidation (Fig. 3) was very sensitive to cyanide inhibition, with a K_i value of ca. 0.1 μM. It has been previously observed that ascorbate-TMPD oxidation involves cytochrome *o* (8, 14) and has a low K_i value (of 0.46 μM) for cyanide (8). Our results therefore are in good agreement with the previous studies. The results provide further support for the observation that H_2 oxidation does not involve cytochrome *o*, since H_2 uptake was not sensitive to low concentrations of cyanide.

Cytochromes *d*, a_1, and *o* have been reported as the terminal oxidases for malate oxidation in *A. vinelandii* (6, 8). Cyanide inhibition of malate oxidation revealed a curve with at least two inhibition phases (Fig. 3). One of the phases seems to correspond to the ascorbate-TMPD inhibition curve, with a K_i of 0.37 μM (probably due to cytochrome *o*). The other phase occurs at a high CN concentration, with a K_i of 133 μM. The latter K_i value corresponds very well with that found for H_2 oxidation and suggests that malate and H_2 oxidation share a common cyanide-insensitive oxidase. This component is probably cytochrome *d*.

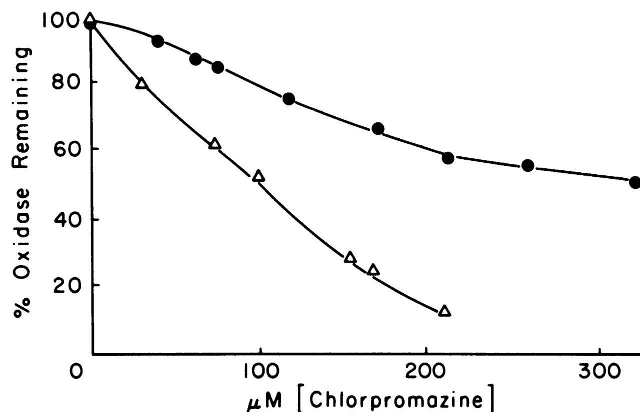

FIG. 5. Effect of chlorpromazine on the oxidation of malate (△) and H_2 (●) in membranes. The initial specific activity for malate and H_2 oxidation was 0.35 and 0.12 μmoles of O_2 oxidized per min per mg of protein, respectively.

HQNO inhibition. HQNO has been shown to be a potent inhibitor of *A. vinelandii* respiration (11, 13). It is believed to inhibit the reduction of cytochrome *b*. Our results (Fig. 4) showed that the H_2 oxidation activity in membranes was inhibited almost 90% by HQNO at 80 μM. HQNO also inhibited malate oxidation to approximately the same extent as H_2 oxidation.

Studies with chlorpromazine. The inhibitor chlorpromazine has been shown to be a potent inhibitor of D(−)-lactate oxidation in *A. vinelandii* (12). The specific site of inhibition is not known. Figure 5 shows the effect of chlorpromazine on H_2 and malate oxidation by membranes. Chlorpromazine inhibited malate oxidation ca. 80% at a concentration of 160 μM. However, H_2 oxidation was much less sensitive to this level of chlorpromazine; it was inhibited ca. 30%.

UV light treatment. Quinones are damaged by irradiation with UV light (4). We tested for the involvement of quinones in H_2 oxidation by irradiating membranes with UV light (Table 1). Of the H_2 oxidation activity, 65% was eliminated by UV irradiation. The activity was partially restored by the addition of ubiquinone extracted from *A. vinelandii* whole cells. UV-treated ubiquinone, however, could not effectively restore H_2 oxidation activity. The results suggest that the inhibition of H_2 uptake in membranes upon UV irradiation was due to damage of quinone.

DISCUSSION

We have found direct evidence for the involvement of cytochromes *b*, *c*, and *d* in H_2 oxidation in *A. vinelandii* by

TABLE 1. Effect of added ubiquinone on H_2 oxidation activity in UV-treated and untreated membranes

Addition	H_2 uptake activity[a]	
	Untreated membranes	UV-irradiated membranes
None	152	53
Q_8	162	108
UV-treated Q_8	158	78

[a] Nanomoles of H_2 consumed per minute per milligram of protein.

the use of H_2-reduced minus O_2-oxidized absorption difference spectra. The CO spectra with H_2 as reductant provided additional evidence for the involvement of cytochrome *d*. The CO spectra with other reductants (malate and dithionite) showed the presence of cytochrome *o* in the membrane; however, cytochrome *o* was not reduced by H_2.

Inhibitor studies also provided evidence for the involvement of cytochrome *d*, but not *o*, as an oxidase for H_2 oxidation. The cyanide inhibition curve of H_2 oxidation was monophasic with a high K_i. Since cytochrome *d* is known to be the most CN-resistant oxidase in *A. vinelandii*, we attributed the high K_i to cytochrome *d*. A low K_i component in the membranes was attributed to cytochrome *o*, and H_2 oxidation was not sensitive to low levels of cyanide. Chlorpromazine markedly inhibited malate oxidation but inhibited H_2 oxidation to a much lesser extent.

Although H_2 oxidation electron transport chains in other bacteria have been described previously (3), the selective reduction of a terminal oxidase by H_2, under fully reduced conditions, has not been reported. However, studies on *Paracoccus denitrificans* suggested differences in the involvement of cytochrome *o* compared with cytochromes aa_3 in H_2 oxidation (20). Also, *Arthrobacter* strain 11/x contains an additional oxidase, cytochrome *d*, when cells are cultured lithotropically with H_2 (1, 3).

Ultraviolet irradiation inhibited H_2 oxidation. Adding *A. vinelandii* ubiquinone Q8 to the UV-damaged membranes partially restored H_2 oxidation activity, whereas addition of UV-treated, isolated Q8 did not increase activity. These results suggest the involvement of ubiquinone in H_2 oxidation. Suggested H_2 oxidation electron transport pathways in other bacteria include quinone as an electron carrier (3, 18). As in some other H_2-oxidizing bacteria (3), no flavoprotein was reduced by H_2 in *A. vinelandii* membranes.

Currently, efforts are being made to study details of H_2 oxidation electron transport in *A. vinelandii*. The specific sequences of reduction will be examined more closely with stopped-flow spectrophotometry.

ACKNOWLEDGMENT

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