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Bradhyrhizobium japonicum hydrogen-ubiquinone oxidoreductase activity: quinone specificity, inhibition by quinone analogs, and evidence for separate sites of electron acceptor reactivity

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Abstract

The purified H_2-uptake hydrogenase of *Bradyrhizobium japonicum*, containing no cytochrome *b*, catalyzed efficient H_2-ubiquinone oxidoreductase activity. Hydrogen-oxidizing membranes also catalyzed H_2-ubiquinone oxidoreductase activity, and the site of ubiquinone reduction was localized to the H_2-quinone oxidoreductase complex based on comparative antimycin A and HQNO titrations of both H_2-ubiquinone-1 oxidoreductase and ubiquinol-1 oxidase activities. A variety of quinones could function as electron acceptors of both pure or membrane-bound hydrogenase, including ubiquinone-0 (Q_0), ubiquinone-1 (Q_1), duroquinone and menadione, indicating relatively loose substrate specificity with regard to the quinone head group. Both the redox potential and the quinone structure determined the efficiency of hydrogenase turnover. Among short-chain ubiquinones, the isoprenoid chain length had a profound affect on K_m, with each additional isoprenoid unit resulting in the K_m of the membrane-bound enzyme to decrease more than an order of magnitude. For pure enzyme, the K_m values for Q_0, Q_1 and Q_2 were 1.97 mM, 68.8 μM and 3.1 μM, respectively. V_{max} was also influenced by the substrate isoprenoid chain length for the pure enzyme. The inhibition patterns of H_2-dependent Q_1 versus MB reduction by the quinone analogs (2-n-heptyl-4-hydroxyquinoline *N*-oxide and Antimycin A) were significantly different, and clear differences in pH optima for the two activities were observed. In addition, the two hydrogen-dependent electron acceptor activities (Q_1 and MB) exhibited different time-dependent inactivation patterns by the chemical modification reagent diazobenzene sulfonate. Ubiquinone and MB therefore react by different mechanisms (perhaps at different sites) within the hydrogenase complex in situ. The inhibition pattern of hydrogen-ubiquinone oxidoreductase activity by antimycin A was clearly different than antimycin A inhibition of ubiquinol oxidation at the bc_1 complex. This is, to our knowledge, the first report of antimycin A inhibition of a hydrogenase complex, and also of a quinone reducing site of a primary dehydrogenase. When pure hydrogenase is assayed in the absence of dithionite, a delay (lag phase) is observed prior to attainment of full activity. The length of this lag period (in minutes) was inversely dependent on ubiquinone concentration, and was greatly reduced (but not eliminated) at saturating ubiquinone levels. These effects were obtained with both Q_1 and MB as electron acceptor, and the lag phases with Q_1 were significantly longer than with MB. Electron acceptor binding to hydrogenase is thus required for reductive activation of hydrogenase during turnover.

Keywords: Hydrogenase; Quinone; Methylene blue; Electron transport; (*B. japonicum*)

Abbreviations: BSA, bovine serum albumin; DABS, diazobenzene sulfonate; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMN, 2,3-dimethyl-1,4-naphthoquinone; Duroquinone, 2,3,5,6-tetramethyl-1,4-benzoquinone; EMS, ethylmethane sulfonate; EPR, electron paramagnetic resonance spectroscopy; HQNO, 2-n-heptyl-4-hydroxyquinoline *N*-oxide; Menadione, 2-methyl-1,4-naphthoquinone (menaquinone-0); MK, menaquinone; MB, Methylene blue; PMSF, phenylmethylsulfonyl fluoride; Q_1, ubiquinone-1; Q, ubiquinone; For Q and MK, the number of prenyl units in the side chain is indicated by the subscript following the abbreviation.

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1. Introduction

Hydrogenases are a diverse group of H_2-activating enzymes which occur in a variety of prokaryotic organisms [1–3]. A large subset of these enzymes, the NiFe-hydrogenases, contain two subunits, one each of approx. 65 and 33 kDa, and Ni and two or three Fe-S clusters as prosthetic groups. The NiFe-hydrogenases from diverse species of aerobic N_2-fixing bacteria, aerobic H_2-oxidizing bacteria, purple non-sulfur bacteria, green sulfur bacteria, methylotrophs, enteric bacteria, and sulfate-reducing bacteria have been shown to be immunologically related [4–7]. The structural genes encoding the membrane-bound hydrogenase from many of these bacteria have been sequenced and show a high degree of homology (see [8] and [9] for reviews). Membrane-bound NiFe-hydrogenases generally couple H_2-oxidation to an energy-generating, membrane-bound electron transport chain. A lipophilic quinone functions in electron transport between hydrogenase and the terminal reductase in these electron transport chains; the specific quinones used (menaquinone, ubiquinone, or plastoquinone) varies with the particular system [10–13].

The identity of the specific redox components functioning between H_2 and quinone in these organisms remains controversial. In the aerobic, N_2-fixing bacterium *Bradyrhizobium japonicum* we have recently demonstrated efficient H_2-ubiquinone oxidoreductase activity by purified heterodimeric hydrogenase, containing no detectable cytochromes. The enzyme catalyzed H_2-Q_1^1 oxidoreductase activity at rates up to 47% of H_2-linked MB reducing activity [14] and was the first direct demonstration of H_2-ubiquinone oxidoreductase activity by a purified hydrogenase. However, H_2-linked quinone reduction was demonstrated in a trimeric hydrogenase complex from the anaerobic rumen bacterium *Wolinella succinogenes*. In addition to a 60 kDa large subunit and a 30 kDa small subunit, this complex contained a *b*-type cytochrome. The cytochrome *b* of the complex was proposed to be the direct electron donor to menaquinone, since H_2-dependent reduction of the cytochrome *b* and the menaquinone analog DMN was observed [15]. Previous proposals for the possible direct electron acceptor of membrane-bound hydrogenases include ubiquinone [11,13], cytochromes [16–18] and an unidentified electron carrier [19]. The proposed involvement of all of these carriers were based on experiments with membrane vesicles containing many redox-active components, rather than with purified or reconstituted systems, so the direct electron acceptor from hydrogenase has not been rigorously identified.

Many membrane-bound heterodimeric NiFe hydrogenases, including the *B. japonicum* enzyme, have been purified and extensively studied using artificial electron acceptors such as MB [1–3,20–22], the most commonly used electron acceptor for hydrogenase assays. Because of the lack of available information on the H_2-quinone oxidoreductase reaction and the likelihood that it represents a more 'physiological' reaction as compared with the H_2-MB oxidoreductase reaction, it is important to characterize and compare the two reactions by both membranes and purified hydrogenase. Here we present data which indicate that both the redox potential and structure of quinones play a role in determining the substrate specificity of different quinones with hydrogenase. In addition, H_2-dependent reduction of ubiquinone and MB behave differently with respect to pH optima and inhibition by quinone analogs, and the two activities were differentially inactivated by chemical modification with DABS. These results indicate that MB and ubiquinone react by different mechanisms with the hydrogenase complex in situ. Finally, experiments on H_2-dependent activation of purified hydrogenase during turnover demonstrated that the electron acceptor concentration dictates the delay period (lag phase) required to achieve maximal rates of both (H_2-dependent) ubiquinone and MB reduction. The length of the lag phase is significantly decreased at saturating levels of electron acceptor, demonstrating that acceptor binding is a necessary step in hydrogenase activation during turnover.

2. Materials and methods

2.1. Bacterial strains and culture conditions

B. japonicum strain SR473, an EMS-induced regulatory mutant which expresses hydrogenase constitutively [23], was grown aerobically and heterotrophically at 29° C. Cells were grown in 15-liter batches to a density of $(6–8) \cdot 10^8$ cells/ml in modified Bergerson's medium [24] supplemented with 5 μM $NiCl_2$ [25], and washed in 50 mM potassium phosphate, 2.5 mM $MgCl_2$ (pH 7.0). Cells were used immediately for membrane preparations and stored at −80° C for hydrogenase preparations. The hydrogenase in these cells is indistinguishable from the enzyme in derepressed wild-type cells [25].

2.2. Preparation of membrane vesicles

Membrane vesicles were prepared from 15 L batches of freshly harvested and washed cells as previously described [26]. Membranes were aliquotted and rapidly frozen, and stored in liquid N_2. Membranes stored in this way retained over 95% of their H_2 uptake activity to O_2 over a period of months. Membranes were kept at 0° C under Ar during experiments.

2.3. Purification of hydrogenase

Hydrogenase was solubilized from extracts of 15–30 g of heterotrophically grown cells and purified aerobically as previously described [25]. All buffers contained 0.2–1.0 mM PMSF. The eluate of the Reactive Red 120-agarose column was concentrated by ultrafiltration using an Ami-

con concentrator with a YM-10 membrane, rapidly frozen and stored in liquid N_2. Hydrogenase stored this way was stable for 1 year. The apparent quality of the purified preparation affected the delay period (lag phase) required to reach full activity (Fig. 7); the lag phase for pure hydrogenase was somewhat variable from preparation to preparation, ranging from 5 to 12 min under identical assay conditions with 200 μM Q_1 as electron acceptor. It is noteworthy that preparations with higher specific activities showed short lag phases upon assay.

2.4. Measurement of H_2 and O_2 uptake

H_2 and O_2 were measured simultaneously amperometrically. A model YSI-5331 Clark-type O_2 electrode (Yellow Springs Instruments, Yellow Springs, OH) was used for amperometric O_2 measurement and modified for amperometric H_2 measurement [27,28]. The H_2 electrode preconditioning protocol and interfacing circuitry for both electrodes were modified from that previously devised for use with the YSI-4004 O_2 electrode [27], which was discontinued by the manufacturer. In order to precondition the probe for H_2 measurement, AgCl is deposited on the Ag electrode by immersing the tip in 0.1 M HCl and applying a voltage of +9 V from a battery for approx. 30 min. During this procedure, H_2 bubbles rapidly from the tip of the Pt electrode. Electrode lifetime is adversely affected by prolonged periods of preconditioning. The electrode is then assembled as per manufacturer's instructions and inserted into the experimental chamber; baseline equilibration occurs over approx. 1 h. Unlike the previously described protocols for H_2 measurement using the YSI-4004 electrode, no initial pretreatment with H_2 gas is necessary to increase electrode sensitivity or decrease response time; in fact, such treatment actually decreases the sensitivity of the electrode. 90% of full response is attained in 10 s; full response occurs over 60–90 s. The signal is quite stable except for slow leakage of H_2 from the chamber ($< 5\%$ over 10 min); this background rate was subtracted in order to normalize the H_2 consumption rates. The signal is linear with H_2 concentrations from 0.4 μM to 100 μM in solution.

H_2 uptake assays with quinones or MB as electron acceptors were conducted as previously described [14]. All activities were calculated from the linear phase on the H_2 uptake progress curve [14]. One unit represents 1 μmol/min/mg. Assays with O_2 as electron acceptor (H_2-O_2 activity or Q_1H_2-O_2 activity) were done under microaerobic conditions (15–30 μM O_2); maximal levels of H_2-O_2 activity were observed under these conditions (data not shown). Buffer was sparged with Ar at a constant rate during the course of an experiment, then transferred (without introducing air bubbles) to the chamber, which was immediately sealed. O_2 levels (monitored amperometrically) varied less than 5 μM during the course of an individual experiment. For Q_1H_2 oxidase assays 200 μM Q_1 was reduced by 2.0 mM dithioerythritol during the assay.

2.5. Inhibition by quinone analogs

Components were added to the assay buffer [14] composed of 50 mM potassium phosphate, 2.5 mM $MgCl_2$ (pH 6.2) in the following order: sample, glucose oxidase and catalase, glucose, H_2 (10.8 μM), NaCN (250 μM) and inhibitor. Reactions were initiated immediately by addition of electron acceptor. Inhibitors were added as ethanolic aliquots, such that the total concentration of ethanol was less than 1%. This concentration of ethanol did not effect H_2 uptake rates by membranes or pure hydrogenase. HQNO was soluble in the assay buffer at all concentrations used; antimycin A was partially insoluble above 150 μM. The antimycin A used in these experiments is a mixture of the A_1 and A_3 isomers.

2.6. H_2-ubiquinone kinetics

Various soluble and hydrophobic ubiquinone homologs were studied in steady-state kinetics experiments in order to determine their K_m and V_{max} values. A single preparation of enzyme or membranes was used for these experiments in order to avoid variation in specific activity among different preparations. All assays were done as previously described, using the glucose oxidase system to remove O_2 [14]. NaCN (250 μM) was present in all assay mixtures; and assay mixtures were identical for membranes and purified hydrogenase. NaCN inhibits $> 98\%$ of O_2-linked H_2 uptake in membranes (data not shown), thus all H_2 uptake measured was due to quinone reduction. H_2 uptake rates were calculated from the linear phase of H_2 uptake (following the non-linear lag period of reductive activation) and data were fit to the Michaelis-Menten equation

$$V = \frac{[Q]V_{max}}{(K_m + [Q])}$$

by non linear least-squares regression. Non-linear regression was done with the curve-fitting function of SigmaPlot (Jandel Scientific, Corte Madera, CA), which uses the Marquardt-Levenberg algorithm.

2.7. pH optima determination and chemical modification experiments

H_2-Q_1 or H_2-MB oxidoreductase activity was assayed in membranes or purified hydrogenase as described above (Section 2.4), except that the buffer was 25 mM Mes, 25 mM Hepes, 25 mM Tris and 25 mM glycine adjusted to the appropriate pH (4.5–9.0) with HCl or NaOH. All points represent averages of two or three determinations. The chemical modification experiments using DABS were performed exactly as described [29], except that samples

were incubated at 0° C for 10 min rather than 5 min prior to initiation of the modification reaction by the addition of DABS. DABS was synthesized as previously described [30] and stored in aliquots at −80° C. DABS was used at a final concentration of 1.0 mM, in 50 mM potassium phosphate (pH 8.0). Membranes were diluted at least 1:3 from their storage buffer (Buffer B in Ref. [14]) and activities (H_2-Q_1 or H_2-MB) were determined as described above at different time points.

2.8. Synthesis of ubiquinone-2

Ubiquinone-2 (Q2) was synthesized by a modification of a previously described method [31]. The modifications were as follows: [1] diethyl ether was treated with $FeCl_2$ to remove peroxides prior to use, [2] following reaction of Q_0H_2 with linalool, the reaction mixture was diluted in 150 ml diethyl ether and washed six times with 50 ml of 5% $NaHCO_3$. In addition, following the washing and drying steps, the product was purified by preparative chromatography over two successive Silica-gel 60 (Whatman) columns developed in hexanes/diethyl ether (5:1, v/v) and toluene/acetone (97:3, v/v), respectively. Column fractions were identified by analytical thin-layer chromatography on silica gel (Whatman K5) in an optimized solvent system of toluene/acetone (97:3, v/v), which cleanly separated Q_1, the product and Q_4 (data not shown). The product migrated with an intermediate R_F between Q_1 and Q_4: R_F values for Q_1, product and Q_4 were 0.38, 0.44 and 0.52, respectively. Ubiquinones were identified on the TLC plates by their yellow color, and purity of the product was confirmed by I_2 staining. The identity of the product as a ubiquinone was confirmed by UV/visible redox difference spectrophotometry ($NaBH_4$-reduced minus air-oxidized); the concentration of the product was calculated using $\Delta\epsilon_{red-ox,\ 278\ 300} = 10.6\ mM^{-1}\ cm^{-1}$ [32], assuming the Q_2 had a $\Delta\epsilon_{red-ox,\ 278\ 300}$ identical to that of Q_1. The product was further identified by fast-ion bombardment mass spectrometry (m/z of the product, 319; corresponding to $C_{19}H_{27}O_4$).

2.9. Protein determination

Total protein was measured with a modified Lowry assay [33], using BSA as a standard.

2.10. Chemicals and reagents

Q_1 and Q_4 were kind gifts of Hoffman-LaRoche, Inc., Nutley, NJ. Q_0, decyl-Q, Q_{10}, menadione, duroquinone, vitamin K_1, vitamin K_2, DBMIB, DCMU, HQNO, antimycin A (Catalog No. A-8674) and myxothiazol were obtained from Sigma; as were glucose oxidase (Type X-S), bovine liver catalase, DNase, PMSF, butylated hydroxytoluenes. Linalool was obtained from Aldrich. Argon (type HP), nitrogen (type HP) and hydrogen (type PP) were obtained from Linde Gases of Baltimore, Baltimore, MD. All other chemicals were of reagent grade and were obtained from Sigma or J.T. Baker Chemical Co., Phillipsburg, NJ.

3. Results

3.1. H_2-dependent ubiquinone reduction in membranes occurs at the hydrogenase complex

H_2-Q_1 oxidoreductase activity is catalyzed by both purified heterodimeric H_2-uptake hydrogenase (containing no cytochrome *b*) and membrane vesicles from *B. japonicum* [14]. In order to characterize and compare the H_2-quinone oxidoreductase activity of the in situ hydrogenase complex with that of purified hydrogenase, it was important to ascertain that exogenous Q_1 is actually reduced at the hydrogenase complex in situ. *B. japonicum* contains a cytochrome bc_1 complex which is expressed in both bacteroids and free-living cells, and mutants lacking this complex are deficient in H_2-O_2 respiration (17% of the wild-type level) [34]. Although this complex catalyzes the net oxidation of one quinol molecule, the currently accepted Q-cycle model for cytochrome bc_1 complex catalysis states that one molecule of quinone is reduced at center N of the complex for every two molecules of quinol oxidized at center P [35,36], thus it was possible that Q_1 reduction was occurring via center N of the cytochrome bc_1 complex. Q_1 reduction at center N could thus occur by two different mechanisms, which were tested in separate experiments. In order to test the possibility of center N-dependent Q_1 reduction, coupled to $Q_{10}H_2$ oxidation at center P, we tested for inhibition with myxothiazol, which specifically inhibits quinol oxidation at center P [35]. No inhibition of Q_1 reduction was found by 20 μM myxothiazol, despite the fact that the same concentrations of inhibitor dramatically inhibited Q_1H_2- and H_2-linked O_2 uptake (see Table 1). Since myxothiazol inhibits 76% of the H_2-O_2 oxidoreductase activity and 96% of the Q_1H_2-O_2

Table 1
Effect of myxothiazol on quinone reduction and quinol oxidation

Assay	Specific activity		% Inhibition
	control	myxothiazol [a]	
H_2-Q_1	556 ± 26 (3)	575 ± 11 (3)	−3.4
H_2-O_2	44 ± 6.5 (3)	10.3 ± 3.1 (4)	76.4
Q_1H_2-O_2	74 ± 6.7 (3)	3.2 ± 0.2 (3)	95.7

H_2 and O_2 uptake in membranes (86–172 μg protein) were assayed amperometrically as described in Materials and Methods. Specific activities (nmol/min/mg) are expressed as the mean ± S.E. (*n* determinations). For H_2-Q_1 activity H_2 uptake was assayed in the presence of 200 μM Q_1. For H_2-O_2 activity and ubiquinol (Q_1H_2) oxidation activity, oxygen uptake was monitored [14] microaerobically as described in Materials and Methods. Q_1H_2 (200 μM) was reduced with 2.0 mM dithioerythritol
[a] Myxothiazol was added to a concentration of 20 μM.

activity, most electron flow to O_2 probably proceeds through a myxothiazol-sensitive cytochrome bc_1 complex. Since myxothiazol does not inhibit H_2-linked Q_1 reduction, reduction of Q_1 at center N is not coupled to Q_{10} H_2 oxidation at center P.

The second possible mechanism of Q_1 reduction at the bc_1 complex involves a quinol-quinone transhydrogenation at center N via a ping-pong mechanism, as reported for the cytochrome bc_1 complexes from *Neurospora crassa*, bovine heart and potato [37–39]. This possibility was tested by performing separate titrations of H_2-dependent Q_1 reduction and Q_1H_2-O_2 oxidoreductase activities with inhibitors HQNO and antimycin A. These inhibitors effectively inhibited H_2-Q_1 oxidoreductase activity of *B. japonicum* membranes (see below and [14]), and inhibit center N but not center P of the bc_1 complex [40]. If H_2-linked Q_1 reduction occurred at center N, then inhibitor titration curves of H_2-Q_1 and Q_1H_2-O_2 activities should be very similar. The results indicated, however, that inhibitory concentrations of both antimycin and HQNO were significantly lower for Q_1H_2-O_2 oxidoreductase activity than for H_2-Q_1 activity (Fig. 1). For the inhibitor Antimycin A the I_{50} for Q_1H_2-O_2 activity was approximately three orders of magnitude less than that for H_2-Q_1 (about 10^{-9} vs. 10^{-6}, Fig. 1A) and one order of magnitude less with HQNO (about $5 \cdot 10^{-8}$ vs. $5 \cdot 10^{-7}$, Fig. 1B). All these results, taken together, indicate that H_2-linked Q_1 reduction occurs 'upstream' of the center N of the bc_1 complex; and the most likely site is an antimycin- and HQNO-sensitive site on the hydrogenase complex.

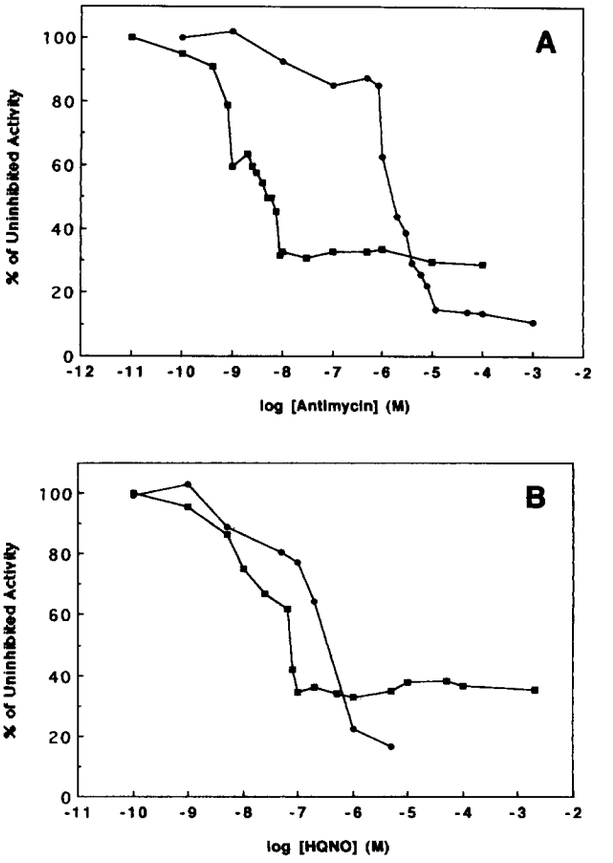

Fig. 1. Differential inhibitor titrations of H_2-Q_1 and Q_1H_2-O_2 oxidoreductase activities. H_2 uptake activity (■) was assayed amperometrically with Q_1 (200 μM) as electron acceptor, and O_2 uptake (●) was assayed amperometrically with Q_1H_2 as electron donor, as described in Experimental Procedures. H_2-Q_1 oxidoreductase assays were initiated by addition of Q_1. Q_1H_2-O_2 oxidoreductase assays were conducted under microaerobic conditions ($[O_2] = 15$–30 μM) and initiated by addition of dithioerythritol (2 mM). Inhibitors were added from stock solutions in ethanol immediately before assay. (A) Inhibition by antimycin A. Assay mixtures (H_2-Q_1) contained 210 μg membranes, and uninhibited specific activity (H_2-Q_1) was 0.95 μmol min^{-1} mg^{-1} (B) Inhibition by HQNO. Assay mixtures (Q_1H_2-O_2) contained 2.1 mg of membranes, and uninhibited specific activity (Q_1H_2-O_2) was 42.5 nmol min^{-1} mg^{-1}.

3.2. Specificity of H_2-quinone oxidoreductase activity

Various quinones were tested as electron acceptors with purified hydrogenase and with membranes. The activities with different quinones were compared to the rates with MB, which is the most effective artificial electron acceptor for both membranes and purified hydrogenase. The ubiquinones Q_0 and Q_1, menadione (MK_0) and the plastoquinone analog duroquinone all functioned effectively as electron acceptors with membranes (Table 2), although activity to duroquinone was approximately half the others at the two concentrations tested. Phylloquinone was essentially an ineffective substrate, and MK_4 functioned poorly as a substrate; these quinones had the lowest midpoint potentials (see Table 2). Q_4 functioned effectively as electron acceptor with membranes: its V_{max} of 0.12 μmol/min per mg (data not shown), was 39% that of the maximal rate for Q_1 reduction by membranes (Table 3). A similar pattern of substrate specificity was observed with purified hydrogenase. However, duroquinone and menadione were significantly more effective as electron acceptors with pure hydrogenase than with membranes, relative to Q_1. In addition, only low levels of activity were observed with pure hydrogenase using 400 μM Q_0 as electron acceptor, in contrast to the situation with membranes. This is undoubtedly due to the high K_m (2.0 mM) for Q_0 by pure enzyme (Table 3). No activity was observed with pure hydrogenase using Q_4 or Q_{10} as an electron acceptor (data not shown).

3.3. Kinetic characterization of activity with different quinone homologs

Initial investigations of H_2-quinone oxidoreductase activity by hydrogenase showed that the apparent K_m for Q_1 was much lower than that for Q_0. Due to this difference, and the observed quinone-dependent specific activity differences in H_2-linked quinone reduction (for both purified hydrogenase and membranes) (Table 2), we further exam-

Table 2
Effectiveness of different quinones as electron acceptors

Electron acceptor	E'_0	Concentration (μM)	Specific activity	
			membranes (nmol/min per mg)	pure enzyme (μmol/min per mg)
Q_1	+110	80	117 ± 4	1.2 ±0.9
		400	198 ± 5	5.1 ±0.2
Q_0	+162	80	111 ± 4	n.d.
		400	197 ± 7	0.13 ± 0.01
Duroquinone	+ 5	80	57 ± 3	1.5 ±0.1
		400	102 ± 7	3.7 ±0.1
Menadione	− 1	80	113 ± 10	7.6 ±0.2
		400	212 ± 26	10.6 ±1.0
Phylloquinone	− 50	400	1.6 ± 1.0	0.02 ± 0.01
Menaquinone-4	− 72	80	6.0 ± 5.1	0.01 ± 0.0
		400	12.2 ± 3.8	
MB	+ 11	180	1144 ± 88	9.8 ±0.7

Membranes (29.5 μg protein) or pure enzyme (4.5 μg protein) were assayed amperometrically at 25° C. Specific activity of membranes with 15–30 μM O_2 was 44.5 nmol min^{-1} mg^{-1}. The values are the means ± S.E. of four determinations.

ined the steady-state kinetic parameters of hydrogenase for a number of ubiquinones that varied in their isoprenoid chain length. K_m and V_{max} values for Q_0, Q_1 and Q_2 were determined for both the pure enzyme or membranes by steady-state kinetic experiments. Among the short-chain ubiquinones Q_0, Q_1 and Q_2, isoprenoid chain length was found to have a profound effect on the K_m (Table 3). With membranes, each additional isoprenoid unit caused the K_m to decrease approximately an order of magnitude. V_{max}, however, was largely independent of chain length, with the exception that Q_2 had an approx. 2-fold higher V_{max} than Q_1. Even more dramatic effects of isoprenoid chain length on K_m were observed with purified hydrogenase. The K_m for Q_1 was 22-fold higher than that for Q_2; and the K_m for Q_0 was 28-fold higher than that for Q_1 (Table 3). Unlike the case of membranes, however, the V_{max} with purified hydrogenase was greatest using Q_1 as the electron acceptor; it was 7-fold higher than the V_{max} for Q_0. The K_m for Q_4 with membranes was estimated at 0.063 μM, and no activity with Q_4 was observed with pure hydrogenase (data not shown).

Q_1 was used as electron acceptor for additional investigations of H_2-quinone oxidoreductase activity, because of its high solubility, low K_m and high V_{max}. The latter two properties suggest that it reacts as the physiological ubiquinone binding site. In order to relate H_2-ubiquinone oxidoreductase to the previous studies of hydrogenase which used other electron acceptors, both H_2-Q_1 and H_2-MB oxidoreductase activities were compared in several biochemical parameters.

3.4. pH optima for H_2 oxidation

Initially, the pH dependence of H_2-Q_1 and H_2-MB oxidoreductase were investigated. For membranes, H_2-MB activity was observed over a broad pH range from 4 to 9, with an optimum at pH 6.5, while H_2-Q_1 activity showed a somewhat narrower range of activity and an optimum pH of 7.0 (Fig. 2A). Oxygen-dependent H_2 oxidation activity in *B. japonicum* membranes has been previously shown to have an optimum pH of approx. 7.2 [41], similar to the value obtained for H_2-Q_1 activity. Like the membranes, purified hydrogenase demonstrated significantly different pH optima for H_2-Q_1 and H_2-MB activities. H_2-Q_1 and H_2-MB activities showed pH optima of 5.5 and 6.0, respectively. High levels of H_2-Q_1 activity were observed at pH 4.5–5.0, where virtually no H_2-MB activity was observed (Fig. 2B). The difference in pH optimum with Q_1

Table 3
Steady-state kinetics of H_2-ubiquinone oxidoreductase activity

Ubiquinone	Membranes		Pure hydrogenase	
	K_m (μM)	V_{max} (U/mg)	K_m (μM)	V_{max} (U/mg)
Q_0	30.5 ±5.2	0.30 ± 0.03	1974 ±31	0.71 ± 0.04
Q_1	2.9 ±0.3	0.31 ± 0.02	68.8 ± 13.9	5.2 ±0.5
Q_2	0.44 ± 0.08	0.64 ± 0.04	3.1 ± 1.2	2.1 ±0.4

Results were determined by non-linear least-squares regression to the Michaelis-Menten equation, as described in 'Materials and Methods,' and are expressed as mean ± S.D. Results are representative of at least two independent experiments.

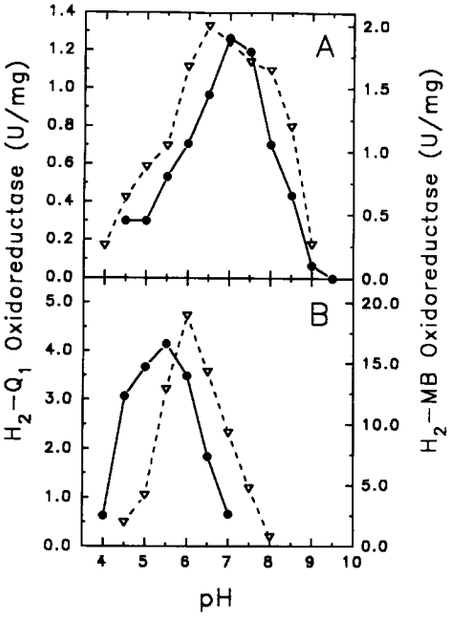

Fig. 2. pH optima for H_2 uptake activity with Q_1 and MB. Membranes (A, 52 μg) or purified hydrogenase (B, 1.6–5.5 μg) were assayed amperometrically in 25 mM Mes, 25 mM Hepes, 25 mM Tris, 25 mM glycine at the stated pH. H_2 uptake by membranes was assayed with Q_1 (100 μM, ●) or MB (180 μM, ▽). H_2 uptake by purified hydrogenase was assayed with Q_1 (250 μM, ●) or MB (180 μM, ▽). Points represent the average of two or three determinations.

or MB as electron acceptors may be due to different mechanisms of electron transfer to the two electron acceptors; this is most easily explained by reaction of the electron acceptors at separate binding sites. This possibility was further examined by the use of inhibition and chemical modification experiments.

3.5. Inhibition of H_2 uptake by quinone analogs

Since HQNO and antimycin significantly inhibited Q_1-dependent H_2 uptake, we tested the effects of other inhibitors which are known to inhibit at quinone binding sites. However, DBMIB, DCMU, and myxothiazol (all tested at 50 μM) did not inhibit H_2-Q_1 oxidoreductase activity. Inhibition by HQNO and antimycin were then characterized in more detail. Inhibition of H_2-Q_1 oxidoreductase activity by HQNO was observed at saturating (200 μM) and sub-saturating (10 μM) Q_1 concentrations (Fig. 3). Maximal inhibition was about 80%. A very similar inhibition profile was observed for H_2-O_2 activity (Fig. 3). In contrast, H_2 uptake to MB was not inhibited, consistent with previous observations with free-living cells [42] and root nodule bacteroids [43]. Indeed, in our experiments, HQNO was consistently found to enhance H_2-MB oxidoreductase activity in membranes by 30–40%; this enhancement was dependent on inhibitor concentration (Fig. 3). The concentration dependence of this activation

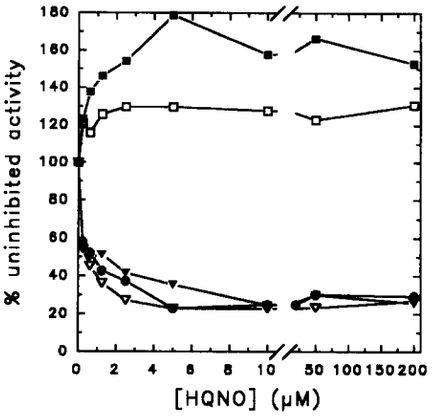

Fig. 3. HQNO inhibitor titration of H_2 uptake by membranes. H_2 uptake in membranes was assayed amperometrically with O_2 (15–30 μM, ●), Q_1 (10 μM, ▽, or 200 μM, ▼) or MB (12 μM, □, or 180 μM, ■) as electron acceptor. HQNO was added from stock solutions in ethanol immediately before assay. Reaction mixtures contained 28.4 μg membranes for assays with MB (180 μM), and 56.7 μg for other assays. Reactions were initiated by the addition of electron acceptor (Q_1 or MB) or H_2 (for H_2-O_2 activity assays). Uninhibited specific activity of membranes was 0.186 μmol min^{-1} mg^{-1} (H_2-O_2); 0.56 μmol min^{-1} mg^{-1} (H_2-10 μM Q_1); 3.38 μmol min^{-1} mg^{-1} (H_2-200 μM Q_1); 1.81 μmol min^{-1} mg^{-1} (H_2-12 μM MB); and 7.07 μmol min^{-1} mg^{-1} (H_2-180 μM MB).

effect and HQNO inhibition of H_2-O_2 and H_2-Q_1 activities were very similar (Fig. 3).

Inhibition experiments on H_2 uptake were also performed with Antimycin A (Fig. 4). Efficient inhibition of membrane-bound H_2-Q_1 and H_2-O_2 oxidoreductase activities were observed with this inhibitor with an I_{50} similar to that observed with HQNO (about 0.5 μM). The maximal inhibition observed was also approximately the same as

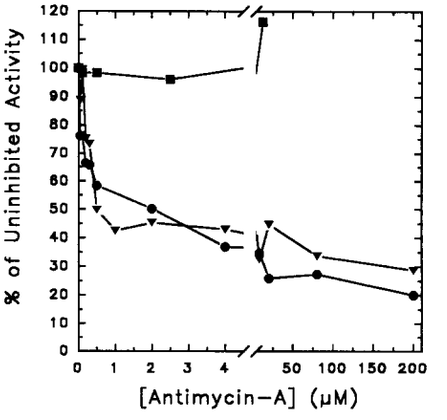

Fig. 4. Antimycin A inhibition of H_2-Q_1 oxidoreductase activity by membranes. Inhibitor was added to membranes (20 μg protein) immediately prior to the addition of H_2, and the reaction was initiated by the addition of electron acceptor (Q_1, O_2, or MB). Uninhibited H_2 uptake was 0.31 μmol min^{-1} mg^{-1} with O_2 (15–30 μM), 0.44 μmol min^{-1} mg^{-1} with Q_1 (10 μM), and 0.62 μmol min^{-1} mg^{-1} with MB (18 μM). (●), H_2-O_2 activity (▼), H_2-Q_1 activity; (■) H_2-MB activity.

with HQNO (70–80%) (Fig. 3), although Antimycin A is insoluble in this assay system at concentrations above 100 μM. Antimycin, like HQNO, clearly did not inhibit H_2-MB activity in membranes (Fig. 4), but no activation was observed.

When HQNO-dependent inhibition of H_2-Q_1 and H_2-MB activities by purified hydrogenase were examined by similar titration experiments, very different inhibition profiles were observed as compared with the results for membrane-associated activity. With subsaturating electron acceptor concentrations (40 μM Q_1 and 12 μM MB), both H_2-Q_1 and H_2-MB activities were significantly inhibited. However, much higher concentrations of HQNO (about 200 μM) were required for 50% inhibition of pure enzyme (Fig. 5) compared to the membrane-bound enzyme.

3.6. Differential effects of the membrane-impermeant reagent DABS on H_2 uptake by membranes

Although the results of the pH optima and inhibition experiments described above indicated that MB and Q_1 react at different sites of hydrogenase in situ, more direct evidence was obtained by use of the chemical modification reagent DABS. DABS has previously been shown to cause a redox-dependent differential inactivation of H_2-MB activity in *B. japonicum* membranes: activity is rapidly inactivated under a H_2-containing atmosphere but not under an O_2-containing atmosphere [29]. DABS-induced inactivation rates of the two acceptor (MB and Q_1) activities were compared. H_2-Q_1 activity exhibited redox-dependent differential inactivation similar to H_2-MB activity: rapid and complete DABS-dependent inactivation was only observed when the membranes were H_2-reduced in the absence of O_2 (data not shown). However, when the DABS

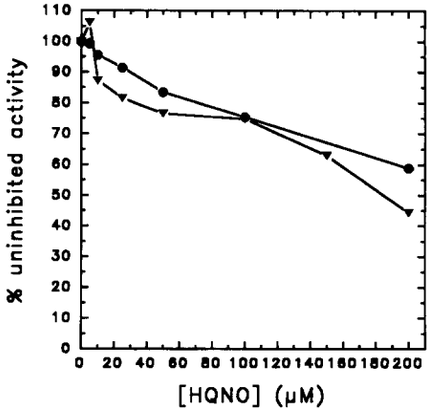

Fig. 5. HQNO inhibition of H_2 uptake by purified hydrogenase. H_2 uptake by purified hydrogenase (3.3 μg with MB, 8.2 μg with Q_1) was assayed amperometrically as described in Materials and Methods. HQNO was added immediately prior to assay and the reaction was initiated by the addition of electron acceptor. Uninhibited specific activity with Q_1 (40 μM, ●) and MB (12 μM, ▼) as electron acceptor was 0.38 μmol min^{-1} mg^{-1} and 2.33 μmol min^{-1} mg^{-1}, respectively.

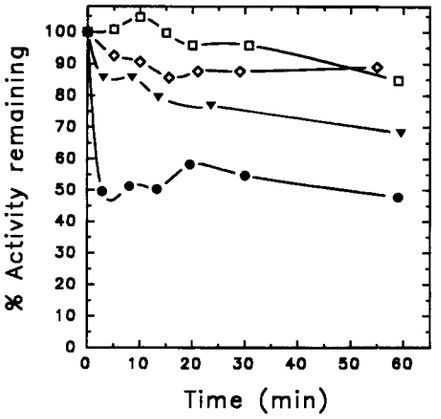

Fig. 6. Differential DABS-induced inactivation of H_2-Q_1 and H_2-MB oxidoreductase activities in membranes. Membranes were diluted in 50 mM potassium phosphate, 2.5 mM $MgCl_2$ (pH 8.0) as described in 'Materials and Methods'; equilibrated under a gas phase of 90% Ar/10% O_2; and treated with DABS (1.0 mM) under the same atmosphere. Aliquots (25 μl or 50 μl) were removed and assayed immediately at the indicated time points, as described in Experimental Procedures. The data are the pooled results of two experiments. Time course of inactivation of control membranes (4.64 mg/ml) assayed with 200 μM Q_1 (◇) or 180 μM MB (□) as electron acceptor. Specific activities were 0.124 μmol min^{-1} mg^{-1} to Q_1 and 0.184 μmol min^{-1} mg^{-1}, to MB. (●, ▼) Time course of inactivation of DABS-treated membranes (4.5 mg membrane protein per ml) assayed with 200 μM Q_1 (●) or 180 μM MB (▼) as electron acceptor. Specific activities were 0.13 μmol min^{-1} mg^{-1} to Q_1 and 0.20 μmol min^{-1} mg^{-1}, to MB.

reaction was conducted in an O_2-containing atmosphere, H_2-Q_1 activity showed a rapid phase of DABS-dependent inactivation ($t_{1/2}$ ~ 1 min), followed by a period of prolonged stability (Fig. 6). In contrast, H_2-MB activity was much more stable to DABS inactivation under these (oxygen-incubated) conditions, as was previously observed [29]. DABS inhibition therefore causes rapid alterations at one or more site(s) involved in H_2-ubiquinone-1 activity but not for H_2-MB activity.

3.7. Effects of electron acceptor on activation of hydrogenase

NiFe hydrogenases are generally isolated in an inactive but activatable form [44], and reductive activation of hydrogenase by H_2 or other reductants has been studied with several of these enzymes [22,45–47]. When aerobically purified *B. japonicum* hydrogenase activity is assayed in the absence of dithionite, a lag phase of several minutes is observed prior to achieving maximal activity [14]. This phenomenon was observed with Q_1 or MB as electron acceptor [14], and is further examined here. In these experiments, the lag phase is defined as the time prior to attainment of the maximal rates of H_2 uptake (Fig. 7A). At constant saturating concentrations of H_2 (10.7 μM), the lag time prior to reaching full activation was inversely dependent on Q_1 concentrations and was reduced but not

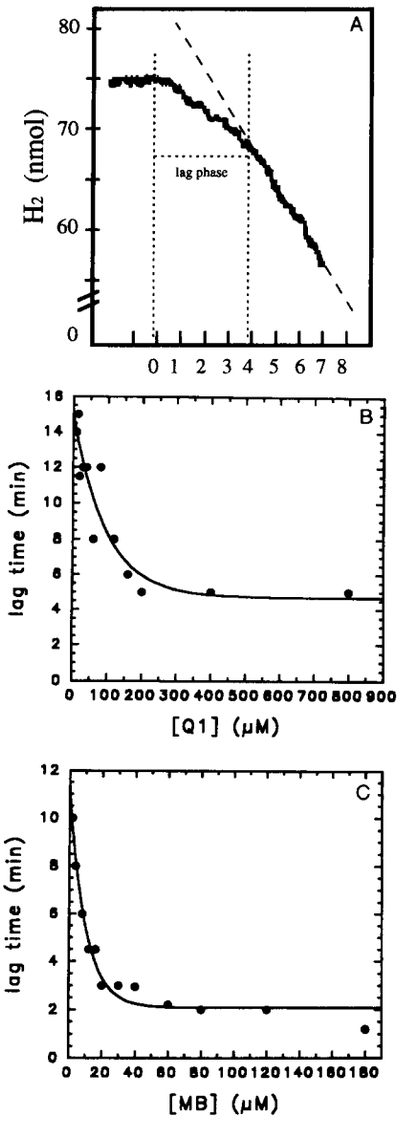

Fig. 7. Dependence of hydrogenase activation rate on electron acceptor concentration. Pure hydrogenase (with an activity of 6.1 μmol min mg^{-1} to 200 μM Q_1, and 11.2 μmol min mg^{-1} to 180 μM MB) was obtained following Reactive Red-120 affinity chromatography. O_2 was removed enzymatically and assays were performed as described in 'Materials and Methods,' except that reactions were initiated by addition of enzyme (2.2 μg). The lag time (see A) refers to the length (in min) of the non-linear phase in the H_2 uptake progress curve. (B) Lag time as a function of Q_1 as electron acceptor. Specific activities ranged from 1.02 μmol min mg^{-1} with 10 μM Q_1 to 6.07 μmol min mg^{-1} with 200 μM Q_1. (C) Lag time as a function of MB as electron acceptor. Specific activities ranged from 1.00 μmol min mg^{-1} with 2 μM MB, up to 11.2 μmol min mg^{-1} with 180 μM MB. All assays were performed at 25° C.

eliminated at saturating Q_1 concentration (Fig. 7B). A similar inverse concentration dependence was observed with MB as electron acceptor (Fig. 7C); however, the minimal lag period (at saturating electron acceptor levels) was significantly and reproducibly less than that with Q_1 as electron acceptor (2 min vs. 5 min in Figs. 7B and 7C, respectively).

4. Discussion

Although a good deal of data are available regarding the mechanism of interaction of NiFe hydrogenases with H_2, there is a relative paucity of data on the mechanisms of reaction of membrane-bound hydrogenases with electron acceptors (see [3] and [44] for reviews). This is especially true for quinones, the apparent physiological electron acceptors. Since ubiquinone functions as an electron acceptor of purified *B. japonicum* hydrogenase [14], and is known to function in electron transport during H_2 oxidation in situ [2], a systematic study of the H_2-Q oxidoreductase activity by both purified hydrogenase and H_2-oxidizing membrane particles was undertaken. These data provide new evidence which indicates that exogenous quinones and the artificial electron acceptor MB are reduced at separate sites on the hydrogenase complex in situ, and clarifies enzymological aspects of the quinone specificity of a bacterial NiFe hydrogenase. Such results can most likely be extrapolated to other hydrogen-uptake NiFe hydrogenases, due to their highly-conserved nature [8].

4.1. Specificity of interaction of hydrogenase with quinones

The reactivity of quinones with different head group structures and redox potentials was examined, and the kinetic parameters of ubiquinones with different length isoprenoid side chains were determined. Ubiquinones, the plastoquinone analog duroquinone, and the menaquinone menadione were all reduced by the enzyme, indicating a relatively loose specificity with regard to the structure of the quinone headgroup. However, the two quinones with the lowest redox potentials, phylloquinone and MK_4 functioned poorly as electron acceptors. Although the lack of reactivity of these quinones may have been due at least in part to their hydrophobicity and consequent insolubility in the assay buffer (each has a 20 carbon lipophilic side chain), it is more likely that they are unreactive due to unfavorable interaction with an electron donating redox center on the enzyme which is poised at a relatively high potential. Two pieces of data support this conclusion: [1] Q_4, which has an identical isoprenoid chain as MK_4 but a midpoint redox potential 186 mV more positive (+110 mV vs. −74 mV), functioned effectively as electron acceptor with membranes, and [2] it is well established that artificial electron acceptors with $E'_0 < 0$ function poorly with *B. japonicum* hydrogenase [2].

Low substrate specificity with regard to the quinone headgroup has also been observed in other quinone-reactive enzymes, but has not been previously observed for any hydrogenases. Bovine NADH-ubiquinone reductases reduce rhodoquinone and duroquinone [48,49], and both duro- and menaquinol are oxidized by the mitochondrial ubiquinol-cytochrome *c* oxidoreductase (Complex III) [50,51]. Benzoquinones, naphthoquinones and anthraquinones can also be incorporated into bacterial photo-

synthetic reaction center depleted of the 'Q_B' quinone, where they function to oxidize the 'special pair' bacteriochlorophylls [52]. The effect of substituents on the 1,4-benzoquinone ring on ubiquinone reduction and oxidation in the succinate-cytochrome *c* region has also been studied by the use of synthetic decyl-ubiquinone derivatives substituted in the 2, 3, and 5 positions of the ring [53]. Substrate specificity was relatively loose for quinol-cytochrome *c* reductase, but somewhat stricter for succinate-ubiquinone reductase. It is clear from previous reports in the literature that although specificity of quinone-reactive enzymes can be quite loose with regard to the head group, some degree of structural specificity occurs [49,51,53,54].

The membrane bound respiratory chain of *B. japonicum* is branched, terminating with at least two terminal cytochrome oxidases, cytochromes aa_3 and *o* [2]. Since *B. japonicum* and other aerobic bacteria contain ubiquinone but no menaquinone [55,56], there is no need for hydrogenases from these organisms to reduce a lower redox potential substrate. The situation is different, however, in bacteria which carry out anaerobic H_2-fumarate respiration, such as *W. succinogenes* [57] and *E. coli* [58]. Menaquinone, not ubiquinone, functions in H_2-linked electron transport in vivo in these organisms, and membrane-bound and purified hydrogenases from these organisms react effectively with low potential electron acceptors such as benzyl viologen, DMN and even the hydrophobic phylloquinone [15,57,59]. These differences among hydrogenases in electron acceptor specificity based on redox potential have not been sufficiently addressed in the literature. It is likely that homologous hydrogenases in different organisms which use different quinones have subtle yet important differences in their quinone-reactive sites and intramolecular electron transport mechanisms, despite their overall structural similarities. Subtle differences between homologous quinone-reactive enzymes have been observed among succinate-quinone reductases [60,61], fumarate reductases [61], and among bacterial photosynthetic reaction centers [62].

The role of the isoprenoid side chain of ubiquinone in the H_2-Q oxidoreductase reaction was investigated using ubiquinone homologs with different alkyl chains. All of the soluble ubiquinone homologs exhibited low K_m values (< 40 μM with membranes) indicating tight binding, and the K_m was dramatically decreased with increasing isoprenoid chain length. Similar effects were noted with purified heterodimeric hydrogenase, although the K_m values were significantly higher. Since Q_2 and Q_1 partition into hydrophobic phases to a much greater degree than Q_0 [63], this differential partitioning may play a role in the different apparent K_m's observed between Q_0 and the other ubiquinones with membranes (Table 3). Differential partition coefficients do not, however, explain the different K_m values between Q_1 and Q_2 with membranes, since both are expected to partition overwhelmingly into the lipid phase based on partition studies with model solvents [63]. Similarly, the partition coefficients do not explain the dramatically decreased K_m values for longer chain quinones observed with purified hydrogenase. Our data therefore indicate that the isoprenoid side chain plays a crucial role in binding of ubiquinone to hydrogenase, and suggest that structurally specific interactions of hydrogenase with the isoprenoid side chain are likely to occur. Similar correlations between isoprenoid chain length of soluble ubiquinones and a decrease in K_m (in the μM range) have been observed in the case of the respiratory D-glucose dehydrogenases of *Acinetobacter calcoaceticus* and *Gluconobacter suboxydans* [64,65]. The decreased V_{max} for pure hydrogenase observed in our experiments for Q_2 (relative to Q_1), as well as the lack of reactivity with Q_4, may indicate that the quinol off-rate is the rate-limiting step of the catalytic cycle with pure enzyme, at least in our assay system. The solubility of longer chain quinones in the phospholipid milieu probably facilitates turnover, as suggested by the increased relative electron acceptor activity of Q_2 and Q_4 with membrane bound enzyme. Although activity of membrane-bound hydrogenase to added Q_{10} (the physiological ubiquinone in *B. japonicum*) was observed [55], we were unable to obtain accurate kinetic parameters due to the insolubility of this ubiquinone in our assay system.

4.2. Ubiquinone reduction is the rate-limiting step in the H_2-O_2 electron transport chain and is inhibited by HQNO and antimycin A

Since Q_1H_2-O_2 oxidoreductase activity is inhibited by much lower concentrations of antimycin and HQNO then H_2-Q_1 oxidoreductase (Fig. 1), and since myxothiazol inhibits the former activity but not the latter (Table 1), it is clear that exogenous ubiquinone reduction occurs 'upstream' of the cytochrome bc_1 complex, presumably at the H_2-ubiquinone oxidoreductase complex. The very similar concentration dependence of HQNO-induced inhibition of H_2-Q_1 oxidoreductase activity and HQNO-induced activation of H_2-MB oxidoreductase activity (Fig. 3) also supports this conclusion, since MB is known to accept electrons directly from hydrogenase [1–3,14].

Our results suggest that H_2-linked ubiquinone reduction is the rate-limiting step in the H_2-O_2 electron transport chain, when H_2 and O_2 are present in excess. HQNO and antimycin titration curves of H_2-O_2 and H_2Q_1 activities were very similar, while Q_1H_2 activity was inhibited at significantly lower concentrations of both inhibitors (Fig. 1). Therefore, the partial inhibition (about 70%) of the bc_1 complex by the lower concentrations of these inhibitors does not cause inhibition of O_2-linked H_2 uptake (compare Figs. 1, 3 and 4), but inhibition of H_2-quinone oxidoreductase activity by higher levels of the inhibitors slows the rate of H_2-O_2 electron transfer (Figs. 3, 4). Both HQNO and antimycin A have been previously demonstrated to inhibit H_2-O_2 respiratory activity in membranes from *B.*

japonicum free-living cells [13] and bacteroids [43]. HQNO inhibition curves in both cases are monophasic; and similar K_i values of 7 μM [13] and 5.6 μM [43] have been determined for free-living cells and bacteroids, respectively. Based upon a more detailed analysis, a two-site model of HQNO inhibition of H_2 respiration can now be proposed which is consistent with the data presented here, the previous results on H_2-linked electron transport in *B. japonicum* [42,43,13], and with accepted models of cytochrome bc_1 catalysis. Since turnover at the Q-reducing site of a H_2-Q oxidoreductase complex is rate-limiting, HQNO inhibition at this site would account both for the observed monophasic inhibition curves, and inhibition of H_2-linked cytochrome reduction [13,42,43]. The second site of HQNO inhibition is at presumably center N of the cytochrome bc_1 complex. Since most of the electron flux to O_2 in free-living *B. japonicum* membranes (about 83%) goes through the cytochrome bc_1 complex [34], inhibition at this site in membranes would account for the previously observed H_2-linked accumulation of quinol in HQNO-inhibited *B. japonicum* membranes [13].

Although HQNO is known to inhibit both quinone reduction and quinol oxidation with various enzymes [40,66,67], antimycin A has been assumed to be a specific inhibitor of cytochrome bc_1 complexes. Our results demonstrate for the first time that antimycin A also inhibits H_2-linked ubiquinone reduction. To our knowledge, these data constitute the first report of antimycin inhibition of quinone reduction at a primary dehydrogenase. These results argue for caution in interpreting antimycin inhibition experiments with intact membrane particles: for example, antimycin cannot be assumed to bind only to the cytochrome bc_1 complex.

4.3. Evidence for separate sites of reactivity in situ for ubiquinone and methylene blue

Several pieces of evidence suggested that Q_1 and MB reacted at different sites in a H_2-ubiquinone oxidoreductase complex in situ. The pH profile for the two activities were markedly different, and both antimycin A and HQNO dramatically inhibited Q_1 reduction but did not inhibit MB reduction. In addition, HQNO-induced activation of H_2 uptake with MB as electron acceptor was observed in membranes; the concentration dependence was indistinguishable from that for the inhibition of H_2-Q_1 oxidoreductase (Fig. 3). Activation of hydrogenase by HQNO has not been previously reported, and suggests a working model in which HQNO binding at a quinone-reducing site in the enzyme complex induces a redox and/or conformational change which increases the electron transport to MB at a separate site. The chemical inactivation experiments with DABS provide further direct evidence for separate sites of MB and ubiquinone reactivity in situ. H_2-Q_1 oxidoreductase activity was significantly more sensitive than H_2-dependent MB reduction in an O_2-containing atmosphere (Fig. 6). This result indicates that at least one DABS-reactive residue exists which is necessary for electron transfer to ubiquinone and not to MB.

Since the kinetics data indicate that the heterodimeric enzyme clearly contains a high-affinity ubiquinone-reactive site, it is likely that this site has physiological significance. Nevertheless, significant differences in pH optima, inhibition patterns, and substrate specificity were observed between purified heterodimeric hydrogenase and membranes. The high affinity site for HQNO inhibition of Q_1 reduction is not present in the purified enzyme (compare Figs. 3 and 5), and the K_m and V_{max} with Q_0, Q_1, Q_2 differ significantly between membrane-bound and purified enzyme (Table 3). Differences in O_2 inhibition of activity and the reductive activation of hydrogenase were also observed between membranes and pure hydrogenase [14]. These differences indicate that the purified heterodimeric hydrogenase differs significantly from the in situ hydrogenase complex. Since a trimeric cytochrome-*b*-containing hydrogenase was recently purified from *Wolinella succinogenes*, and the putative cytochrome-*b*-encoding third ORF of hydrogenase operons is conserved in *B. japonicum* and other bacteria [15,68–74], it is likely that the heterodimeric hydrogenase is a portion of a trimeric or multimeric hydrogenase complex that functions in situ. The putative cytochrome *b* and/or other polypeptides may modulate reactivity with ubiquinone. In addition, the redox state and/or integrity of FeS clusters may differ between the purified heterodimeric and membrane-bound forms of the enzyme, resulting in altered inhibitor binding and altered reactivity with quinones.

4.4. Electron acceptor binding is necessary for optimal reductive activation

Reductive activation of purified NiFe-hydrogenases by H_2 has been studied in detail and is proposed to occur in two stages [44]. Following O_2 removal from the inactive oxidized enzyme, the enzyme is reduced (by H_2) with a concomitant slow conformational shift to its active form. Several of our observations are consistent with this general scheme of activation. Purified *B. japonicum* hydrogenase, like other hydrogenases, is active only in the absence of O_2 [14]. Even though the assays are performed anaerobically (Fig. 7), there is a lag phase prior to achieving maximal turnover, indicating that deoxygenation is not sufficient for activation. In addition to deoxygenation and reduction, however, the dependence of the activation rate on electron acceptor concentration indicates that the activation process involves binding of electron acceptor and possibly enzyme turnover. A similar observation has been made in studies of the periplasmic NiFe hydrogenase from *D. gigas*; the presence of the soluble electron acceptor cytochrome c_3 during H_2-induced activation, increased the rate of enzyme activation and also the appearance of the 'Ni-C' EPR signal that is correlated with the active state [47].

Dye-dependent redox cycling caused a greater activation of *A. eutrophus* heterodimeric hydrogenase than reduction alone, and could be correlated with altered EPR spectra [46]. Reductive activation has also been studied with *Azotobacter vinelandii* hydrogenase, where extended H_2 incubations resulted in increased activity and faster activation during assay [22]. Our observations indicate that activation is accelerated when either ubiquinone or artificial electron acceptor is bound, consistent with the artificial electron acceptor studies of *D. gigas* and *A. eutrophus* hydrogenase [45–47]. In addition to electron acceptor binding, it is likely that oxidation of the enzyme by electron acceptor is in fact necessary for the transition (during turnover) to the active form of the enzyme, as implied by the term 'turnover activation' [22]. If this were the case, then ubiquinone binding might cause specific changes in EPR spectroscopic properties of H_2 uptake NiFe hydrogenases.

Acknowledgements

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