JOURNAL OF BACTERIOLOGY, Nov. 2000, p. 5997–6004 Vol. 182, No. 21
0021-9193/00/$04.00+0

Characterization of the Hydrogen-Deuterium Exchange Activities of the Energy-Transducing HupSL Hydrogenase and H_2-Signaling HupUV Hydrogenase in *Rhodobacter capsulatus*

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Received 26 May 2000/Accepted 16 August 2000

Rhodobacter capsulatus **synthesizes two homologous protein complexes capable of activating molecular H_2, a membrane-bound [NiFe] hydrogenase (HupSL) linked to the respiratory chain, and an H_2 sensor encoded by the *hupUV* genes. The activities of hydrogen-deuterium (H-D) exchange catalyzed by the *hupSL*-encoded and the *hupUV*-encoded enzymes in the presence of D_2 and H_2O were studied comparatively. Whereas HupSL is in the membranes, HupUV activity was localized in the soluble cytoplasmic fraction. Since the hydrogenase gene cluster of *R. capsulatus* contains a gene homologous to *hoxH*, which encodes the large subunit of NAD-linked tetrameric soluble hydrogenases, the chromosomal *hoxH* gene was inactivated and *hoxH* mutants were used to demonstrate the H-D exchange activity of the cytoplasmic HupUV protein complex. The H-D exchange reaction catalyzed by HupSL hydrogenase was maximal at pH 4.5 and inhibited by acetylene and oxygen, whereas the H-D exchange catalyzed by the HupUV protein complex was insensitive to acetylene and oxygen and did not vary significantly between pH 4 and pH 11. Based on these properties, the product of the accessory *hypD* gene was shown to be necessary for the synthesis of active HupUV enzyme. The kinetics of HD and H_2 formed in exchange with D_2 by HupUV point to a restricted access of protons and gasses to the active site. Measurement of concentration changes in D_2, HD, and H_2 by mass spectrometry showed that, besides the H-D exchange reaction, HupUV oxidized H_2 with benzyl viologen, produced H_2 with reduced methyl viologen, and demonstrated true hydrogenase activity. Therefore, not only with respect to its H_2 signaling function in the cell, but also to its catalytic properties, the HupUV enzyme represents a distinct class of hydrogenases.**

In the photosynthetic bacterium *Rhodobacter capsulatus*, the ability to use H_2 as an electron donor is conferred by an H_2-uptake hydrogenase, a membrane-bound [NiFe] hydrogenase linked to the respiratory chain (31) and encoded by the *hupSL* genes (21).

The *hupSL* genes are part of a cluster of *hup* (for hydrogen uptake) and *hyp* (for hydrogenase pleiotropic) genes necessary for the biosynthesis of the *hupSL*-encoded hydrogenase (7). The *hup* and *hyp* gene products bear significant structural identity to hydrogenase gene products from *Escherichia coli*, *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*), *Rhizobium leguminosarum*, *Bradyrhizobium japonicum*, and *Azotobacter vinelandii*. Some of these products are necessary for maturation of the enzyme, some for Ni insertion at the active site, and some for regulation of *hupSL* gene expression (reviewed in references 17 and 42).

The *hup-hyp* cluster comprises the *hupTUV* operon, the products of which exert a negative control on *hupSL* gene expression. The *hupU* gene product shares 20% amino acid sequence similarity with the small subunit (HupS) of the *hupSL*-encoded hydrogenase, and that of *hupV* shares 29% similarity with the large subunit (HupL) (12, 15). It is thought that HupU and HupV proteins function as a complex, since mutants with inactivated *hupU* or *hupV* or deleted *hupUV* genes have the same phenotype (12). The HupUV protein complex can catalyze the hydrogen-deuterium (H-D) exchange reaction in the presence of D_2 gas and was suggested to function as a cellular H_2 sensor (40). The *hupT* gene product is a protein histidine kinase (13, 15). With the response regulator HupR, it forms the two-component HupT-HupR system, which regulates the synthesis of HupSL hydrogenase in *R. capsulatus* (16). In the absence of H_2, HupT represses the transcription of hydrogenase (*hupSL*) genes by phosphorylating HupR (16).

We demonstrate in this study that the H-D exchange reaction catalyzed by the HupUV protein complex can be differentiated from that of the HupSL hydrogenase by different relative rates of H_2 and HD formation in exchange with D_2, a different sensitivity to acetylene, and a different in situ response to oxygen. Thus, this report defines specific features of a new type of hydrogenase, the H_2-signaling HupUV hydrogenase.

MATERIALS AND METHODS

Bacterial strains and cultures. The strains and plasmids used in this work are listed in Table 1. *R. capsulatus* strains were grown heterotrophically either anaerobically in the light or aerobically in darkness at 30°C as described previously (7) in minimal salts RCV medium (43) supplemented with 30 mM Na DL-malate as a carbon source and either 7 mM ammonium sulfate (MN medium) or 7 mM glutamate (MG medium) as a nitrogen source. The concentrations of the antibiotics used were as follows: kanamycin, 10 μg ml^{-1}, tetracycline, 1 μg ml^{-1}; and streptomycin, 25 μg ml^{-1}.

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TABLE 1. Bacterial strains and plasmids used in this study

R. capsulatus strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
B10	Wild type, Hup^+ Nif^+	26
BSE16	Δ*hupUV*	12
JP91	*hupSL*	6
RCC12	*hypD*	7
RCC44	Δ(*hoxH*, ORF2 *hupTUV hypF*)	This work
JBC12	Sm^r *hoxH*	This work
JBC13	Sm^r *hoxH hupSL*	This work
Plasmids		
pAC142	Tc^r; pPHU234 with 730-bp *Hin*dIII-*Xho*II insert containing a *phupS*::*lacZ* fusion	8
pAC206	Tc^r; pPHU231 with 4.8-kb *Sal*I-*Hin*dIII insert containing the *hupTUV* operon	12
pAC63	Tc^r; pRK292 with 9.6-kb *Hin*dIII-*Hin*dIII insert containing the *hypAB hupR hypCDE* genes	7
pAC171	Ap^r; pUC18 with a 2.2-kb *Hin*dIII-*Hin*dIII insert containing the *hoxH* gene	This work
pAC229	Tc^r, pPHU281 with a 6.0-kb *Bam*HI-*Pst*I deletion replaced by an omega cassette	This work

DNA manipulations and bacterial mating. DNA preparation and cloning were carried out according to reference 34. Restriction endonucleases and DNA-modifying enzymes were used by following the instructions of the manufacturers. Plasmids were introduced in *R. capsulatus* by the helper plasmid pRK2013 (10), as described earlier (5). The *hoxH* gene carried on plasmid pAC171 was inactivated by insertion of an omega cassette at the *Hin*dII site, and the mutated gene was exchanged with wild-type *hoxH* in the chromosome by double recombination. Two mutant strains having the chromosomal *hoxH* gene inactivated were isolated. The first, termed JBC12, was obtained from the wild-type strain B10, and the second, termed JBC13, was obtained from the *hupSL* mutant JP91. Strain JBC13 is therefore an $Hup(SL)^-$ $HoxH^-$ double mutant. The RCC44 mutant was obtained from B10 by exchange with the insert of plasmid pAC229, which has the 6.0-kb *Bam*HI-*Pst*I fragment carrying the *hoxH*, open reading frame 2 (ORF2), *hupTUV*, and *hypF* genes replaced by an omega cassette.

Enzyme assays and protein determination. Hydrogenase activity was assayed by the rate of H_2 (or D_2) uptake, H_2 production, or H_2 and HD formed in exchange with D_2 (H-D exchange). Hydrogen uptake was determined spectrophotometrically by using methylene blue (MB) (0.15 mM) (9) or by mass spectrometry with oxidized benzyl viologen (BV^{2+}) or MB (4 mM) as an electron acceptor. One unit of hydrogenase activity is 1 nmol of H_2 (D_2) consumed (produced)/min/mg of protein. The rates of H_2 uptake with BV^{2+}, of H_2 production by reduction of protons in the presence of Zn-reduced methyl viologen (MV^+), and of H_2 and HD formed in exchange with D_2, measured at 30°C, were monitored continuously in the aqueous phase of cell suspensions (either whole cells, membranes, or soluble cytoplasmic fraction) by the mass spectrometric method described in detail previously (19, 41).

β-Galactosidase activity was assessed from the rate of *o*-nitrophenol (ONP) released from *o*-nitrophenyl-β-D-galactopyranoside (ONPG) at 30°C, according to Miller (29) as described previously (8). One unit of β-galactosidase activity is 1 μmol of ONP formed min/mg of protein.

The protein concentration of whole cells was estimated by the empirical relationship optical density at 660 nm (OD_{660})/5 = mg of protein ml^{-1} (27), and that of membranes and cell free extracts (obtained by cell breakage in a French pressure cell followed by two successive centrifugations at 20,000 × *g* for 30 min and then 100,000 × *g* for 70 min) was estimated by the bicinchoninic acid protein assay (Bio-Rad Laboratories, Hercules, Calif.) with bovine serum albumin as a standard.

RESULTS

The hydrogenase *hup-hyp* gene cluster of *R. capsulatus* can encode more than one hydrogenase. The gene organization at the *hup* locus of the chromosome of *R. capsulatus* (strain B10) is shown in Fig. 1. The *hup-hyp* gene cluster comprises the structural hydrogenase genes for H_2 uptake (*hup*) and accessory genes for the synthesis of active hydrogenase(s) (*hup* and *hyp* genes). The *hupSLC* operon encodes the membrane-bound H_2-uptake [NiFe] hydrogenase (HupSL) (21) and HupC, a cytochrome *b*, which links HupSL to the respiratory chain (3). It is expressed from the *hupS* promoter (*phupS*) (8), which depends on σ^{70} factor (16). The *hupTUV* operon encodes proteins that negatively control *hupSL* gene expression (12). The *hupU* gene product, homologous to the small hydrogenase subunit HupS, lacks the long twin-Arg signal peptide present at the N terminus of HupS. This type of signal peptide has been

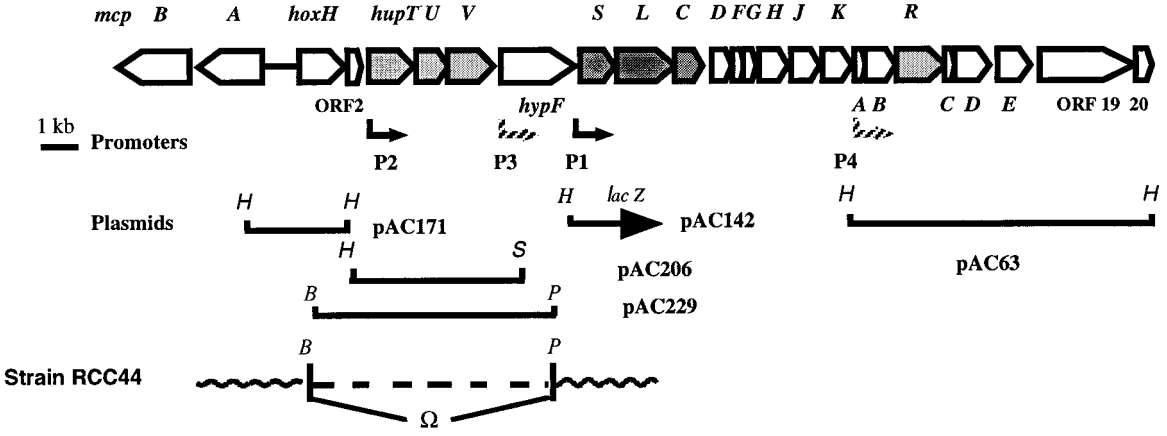

FIG. 1. Gene organization at the *hup* locus of the chromosome of *R. capsulatus*. The coding region at the *hup* locus of *R. capsulatus* chromosome comprises 21 ORFs, all contiguous and transcribed from the same strand. At the 5′ end, it is separated by around 500 nt from the *mcpA* and *mcpB* genes transcribed in the opposite direction (28). The positions of known promoters and plasmid inserts are shown. B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I.

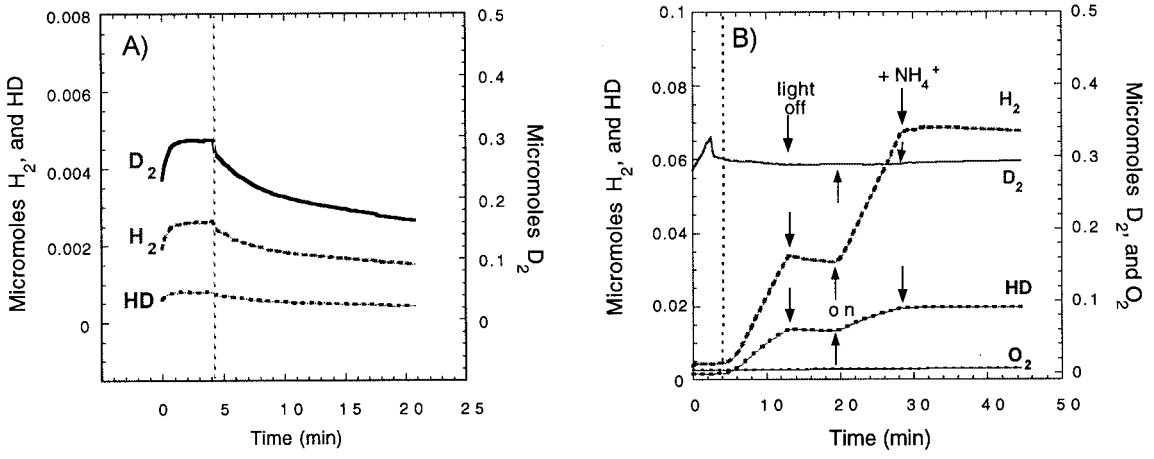

FIG. 2. Nitrogenase-mediated H_2 and HD production, in the presence of D_2 and H_2O, by the RCC44 mutant. The RCC44 mutant (lacking the *hoxH*, *hupTUV*, and the *hypF* genes) was grown photoheterotrophically in either MN medium (A) or MG medium (B). (A) The MN culture (1.5 ml, 0.7 mg of protein) was sparged with D_2, and the reaction vessel was closed at the time indicated by the vertical dotted line. The curves (not corrected for the consumption of gasses of the apparatus) exhibit the real changes with time in D_2 (——), H_2 (– – –), and HD (• • •) concentrations in the reaction chamber. (B) The MG culture (1.5 ml, 0.6 mg of protein) in the reaction chamber was sparged with D_2. At the time indicated by the vertical dotted line, the cell was closed and the H-D exchange reaction in whole cells was measured under light (light on) or in darkness (light off) and after addition of 10 mM ammonium sulfate, as indicated by arrows. The curves have been corrected for gas consumption by the mass spectrometer.

shown in *E. coli* to lead to the export of dimeric hydrogenase to the periplasm by the Tat system (33, 35). Indeed, evidence is given below that, in contrast to the membrane-bound, periplasmically oriented HupSL hydrogenase, the HupUV protein complex is localized in the cytoplasm. Upstream from the *hupTUV* operon lies an ORF, termed *hoxH*, whose predicted product shares significant similarity with the large subunit of [NiFe] hydrogenases, in particular with HoxH, the β-subunit of the tetrameric soluble NAD-linked hydrogenase (39). The genes encoding the other three subunits of the tetrameric NAD-linked hydrogenases were not found in the hydrogenase gene cluster. Downstream from *hoxH*, ORF2 can encode a protein of 181 amino acids, which shares no significant similarity with known proteins. Upstream from *hoxH*, separated by approximately 500 nucleotides (nt) and transcribed in the opposite direction, are the *mcpA* and *mcpB* genes capable of encoding methyl-accepting-type chemoreceptors (28).

It is not known whether *hoxH* is expressed and, if so, under what conditions. The genes encoding a tetrameric, reversible, NAD-linked hydrogenase have not yet been identified in the (nearly completely sequenced) genome of *R. capsulatus* (B. Billoud, A. Colbeau, and P. M. Vignais, unpublished data). However, it is possible that the product of *hoxH* can function with some subunits of complex I, as suggested by Appel and Schulz (1). To eliminate any interference with the putative HoxH protein, the *hoxH* gene was inactivated in the chromosome of *R. capsulatus*, and the mutants were used to study HupUV activity in the cell.

The H-D exchange activity catalyzed by the HupUV protein complex is not sensitive to oxygen. After the discovery of the presence of *hoxH* in the hydrogenase gene cluster, and because HupUV has a low level of activity (40), special care was taken to assess the cellular H-D exchange activity. A mutant strain, RCC44, with the DNA encompassing *hoxH*, *orf2*, *hupTUV*, and *hypF* deleted (Fig. 1), was constructed and used in control experiments. In the absence of the *hypF* gene product (4), there was no active membrane-bound (HupSL) hydrogenase. When grown under nitrogenase-repressing conditions (in MN medium), there was no H-D exchange reaction at all and no formation of HD and H_2 in exchange of D_2, and the curves displayed in Fig. 2A show only the consumption of D_2, H_2, and HD by the mass spectrometer (in this experiment, H_2 and HD were brought as contaminants of D_2—hence the difference in scales). On the other hand, RCC44 cells grown photoheterotrophically in MG medium (nitrogenase-inducing conditions) catalyzed an H-D exchange reaction due to nitrogenase activity (19). Figure 2B shows the pattern of H_2 and HD formation in exchange with D_2, and also some proton reduction, catalyzed by the nitrogenase complex. The typical features of the nitrogenase-catalyzed H-D exchange are that it requires light (for the regeneration by photophosphorylation of the ATP needed for nitrogenase activity) and it is completely inhibited by ammonia. These two simple tests (insensitivity to darkness and to ammonia) were used to check that the H-D exchange activities measured in our experiments represented hydrogenase and not nitrogenase activity. Moreover, for in vivo experiments, the cells were systematically grown in MN medium, although the synthesis of the HupSL hydrogenase is strongly repressed under such conditions.

The H-D exchange activity of HupUV was also determined in strain JP91 (*hupSL* mutant) and in its derivative, in which the *hoxH* gene has been inactivated, JBC13 (*hupSL hoxH* double mutant). Figure 3 shows that, although feeble, the rates of HD and H_2 formed in the course of the H-D exchange reaction catalyzed by HupUV in JBC13 cells could be determined. The initial rates of H_2 and HD formation were determined for three time intervals following gassing by D_2 and then closing of the reaction chamber: between 5 and 6 min (interval 1), between 22 and 23 min (interval 2), and between 35.3 and 36.5 min (interval 3). Light was off for intervals 2 and 3, and O_2 was present in interval 3 when the rates of HD and H_2 formation were determined. The initial rates were 0.8, 0.9, and 0.8 nmol of H_2 formed/min and 1.4, 1.5, and 1.4 nmol of HD formed/min for intervals 1, 2, and 3, respectively. This experiment shows that (i) the H-D exchange proceeded in the absence of light; (ii) the initial rates were reproducible and unchanged in the presence of O_2; and (iii) the H-D exchange reaction was due to HupUV, since JBC13 cells have no *hoxH* or *hupSL* genes. There were no significant differences between the cellular activities of JBC13 (*hoxH hupSL* mutant) and JP91 (*hupSL* mutant). Thus, either *hoxH* was not expressed in cells grown

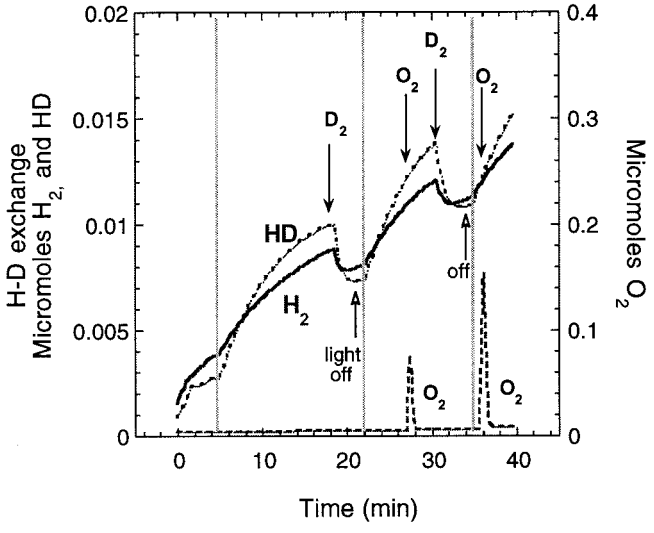

FIG. 3. Time course of H_2 and HD production in the D_2-H_2O system by the HupUV protein complex in JBC13 cells. Cells were grown anaerobically in the light in MN medium. The H-D exchange reaction in whole cells of JBC13 (*hupSL hoxH* double mutant) (3.7 mg of protein) was measured in 1.5 ml of 50 mM Tris-HCl buffer (pH 8.0). After gassing the cell suspension with D_2, the reaction chamber was closed (vertical grey lines), and the H-D exchange reaction was allowed to proceed. At 18 min, the reaction chamber was regassed with D_2; at 22 min, the light was turned off and the vessel was closed; at 27 min, H_2O_2 (5 μl, 0.3%) was added and O_2 was liberated by decomposition (the lower trace shows O_2 concentrations measured at a mass of 32 Da); at 30 min, the chamber was regassed with D_2; at 35 min, the light was turned off, the vessel was closed, and there was new addition of H_2O_2 (10 μl, 0.3%). The figure shows the real gas concentrations of H_2 (——), HD (• • • •), and O_2 (- - -) in the reaction chamber.

anaerobically in the light in MN medium or, if it was expressed, the level of expression was even lower than that of the *hupUV* genes and was not detectable in our tests.

Unlike the membrane-bound HupSL hydrogenase, the HupUV H_2 sensor is a soluble cytoplasmic enzyme. The lack of a signal peptide at the N terminus of HupU predicted that the *hupUV* gene products are cytoplasmically located. The experiments shown in Fig. 4 demonstrate that the HupUV protein complex in cells of the *hupSL* mutant JP91 cannot transfer H_2 electrons to O_2 or to the impermeant electron acceptor ferricyanide, in contrast to HupSL in the *hupUV* mutant, BSE16. Figure 4A shows that the initial rate of H_2 formation (14.6 nmol/min/mg of protein) was higher than that of HD (7.5 nmol/min/mg of protein) in the HupSL-catalyzed H-D exchange reaction (in BSE16 cells). On the other hand, for the HupUV-catalyzed H-D exchange reaction [in JP91(pAC206) cells], the reverse was observed (initial HD production rate, 5.3 nmol/min/mg of protein > initial H_2 rate, 3.7 nmol/min/mg of protein) (Fig. 4B). Upon addition of a small amount of O_2, there was immediate and rapid uptake by HupSL of the three isotopic forms of hydrogen gas, D_2, HD, and H_2. The formation of H_2 and HD resumed when all O_2 had been consumed, and a further addition of ferricyanide immediately reoxidized the three hydrogen species (Fig. 4A). Neither the addition of O_2 nor that of ferricyanide affected significantly the H-D exchange reaction catalyzed by the HupUV protein complex (Fig. 4B). (On the figure, the decrease in HD concentration is due to the further exchange of HD with protons and not to an inhibition by O_2 or ferricyanide.) The same types of results were observed with JP91 cells without pAC206 (not shown) and with JBC13 (*hupSL hoxH* double mutant) (Fig. 3). This is further evidence that HupSL transmits H_2 electrons to O_2 through the respiratory chain and that HupUV is not directly connected to the cytoplasmic membrane.

The use of detergents brought another piece of evidence of the cytoplasmic localization of HupUV. Whereas treatment of B10 or BSE16 cells by sodium dodecyl sulfate plus chloroform (the same treatment used for β-galactosidase assays) abolished the H-D exchange activity of HupSL hydrogenase, the activity of the HupUV protein complex only dropped from 3.9 to 3.3 nmol of HD produced/min/mg of protein after that treatment. Finally, it is demonstrated below that the HupUV H-D exchange activity was found in the soluble cytoplasmic fraction obtained after breakage of the cells and removal of the membranes by centrifugation.

The H-D exchange reaction catalyzed by HupUV is distinguishable from that of HupSL by its insensitivity to acetylene. Typically, as shown in Fig. 3 and 4, in the H-D exchange catalyzed by HupUV, the initial rate of HD formation was higher than that of H_2. Then, as the D_2 concentration decreased, HD further exchanged with protons and H_2 became predominant. We show now (Fig. 5) that acetylene inhibited the H-D exchange reaction catalyzed by HupSL, but not that catalyzed by HupUV. The *hupSL*-encoded hydrogenase of BSE16 (Δ*hupUV*) cells was 95% inhibited after a 1-h 40-min

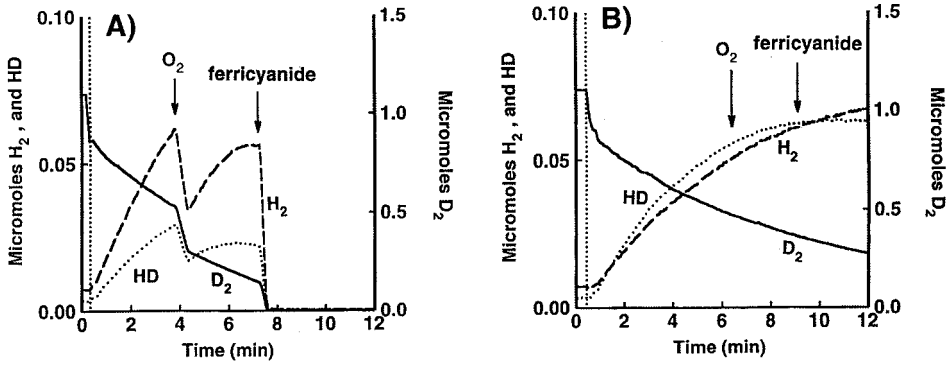

FIG. 4. Time course of H_2 and HD production and D_2 consumption in the D_2-H_2O system by the HupSL (A) and HupUV (B) protein complexes. Cells were grown anaerobically in the light in MN medium. The H-D exchange reaction in whole cells of BSE16, a Δ*hupUV* mutant (2.5 mg of protein) (A), and in whole cells of JP91(pAC206), a *hupSL* mutant, with the *hupTUV* operon-containing plasmid pAC206 (5.2 mg of protein) (B), was measured in 50 mM citrate-phosphate buffer (pH 7.0). At the time indicated by the vertical dotted line, the reaction vessel was closed, and the concentrations of D_2 (——), H_2 (- - - -), and HD (• • • •) were recorded. The arrows indicate the time of O_2 appearance in the medium after H_2O_2 addition (2 μl of 0.3% H_2O_2) and the time of ferricyanide addition (10 mM). The changes in O_2 concentration were monitored at a mass of 32 Da (data not shown). The figure shows the real concentrations of the hydrogen species present in the vessel.

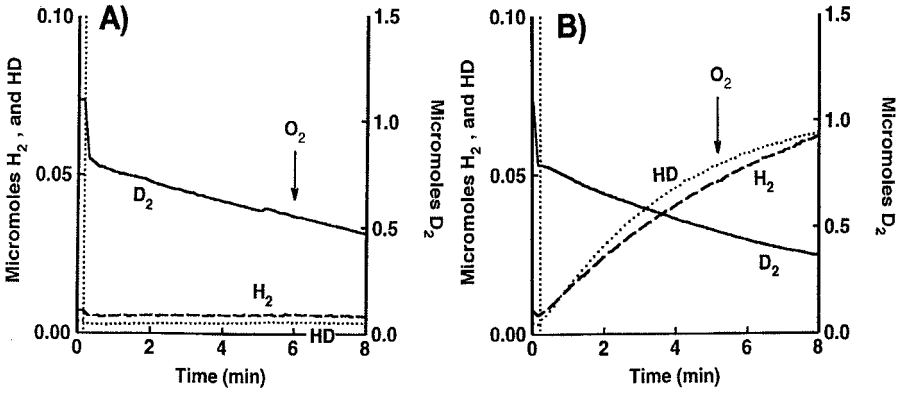

FIG. 5. Effect of acetylene on the H-D exchange reaction catalyzed by the *hupSL*-encoded hydrogenase (A) and by the *hupUV*-encoded hydrogenase (B). The conditions were the same as those in Fig. 4, with cells grown overnight anaerobically in the light in MN medium. H_2 (– – –) and HD (· · · ·) production in exchange with D_2 (– – – –) uptake catalyzed by whole cells of BSE16 (2.5 mg of protein) (A) and JP91(pAC206) (5.2 mg of protein) (B) was measured at pH 7 after the cells had been incubated for 1 h at room temperature under a gas phase of C_2H_2-Ar (1:1). The figure shows the real concentrations of the hydrogen species in the vessel.

incubation under a gas phase containing a 1:1 mixture of acetylene and argon (Fig. 5A), while under the same conditions, the H-D exchange activity of the HupUV protein was practically not inhibited by acetylene (Fig. 5B). The lack of an acetylene effect on HupUV was not due to a lack of acetylene penetration in the cytoplasmic compartment, since acetylene is the substrate commonly used to measure the activity of nitrogenase, which is also cytoplasmically located (19). Acetylene, which inhibits the H-D exchange activity of *Thiocapsa roseopersicina* hydrogenase, had been shown earlier to interact with the Ni atom of the hydrogenase active site (abolishing the electron paramagnetic resonance signal due to Ni-C and stabilizing it in an electron paramagnetic resonance-silent state) (44). Thus, apparently acetylene cannot reach the active site of HupUV as it can do for *T. roseopersicina* hydrogenase.

This ability of acetylene to specifically inhibit the *hupSL*-encoded hydrogenase was then used to demonstrate that the synthesis of the *hupUV*-encoded enzyme necessitates the product of the accessory *hypD* gene (24). The RCC12 mutant contains an in-frame deletion of 54 bp within the *hypD* gene. It is complemented by plasmid pAC63, which provides in *trans* the wild-type form of HypD. In the mutant, the *hupSL* genes are transcribed, but the hydrogenase is not processed and thus not active (7). It is shown here that the *hypD* RCC12 mutant also lacks the active HupUV protein complex and that complementation of the mutant restored HupUV activity. Table 2 provides the rates of H_2 and HD formation in cells of the wild-type strain B10, the *hupSL* mutant JP91, and the *hypD* mutant RCC12. The RCC12 cells have no H-D exchange activity; the experimental values given in Table 2 (experiment 4) indicate the sensitivity of the measurements. The presence of plasmid pAC63 in the *hypD* mutant restored H-D exchange activity to a level even higher than that in wild-type B10 cells, for the plasmid also provided additional copies of the transcriptional activator HupR (Fig. 1 and Table 2) (16, 38). The residual activity measured in RCC12(pAC63) cells incubated with C_2H_2-Ar (1:1), which was not significantly diminished by addition of O_2 (not shown), was attributed to HupUV (insensitive to acetylene; Fig. 5). The data demonstrate that the *hypD* gene product was required for the synthesis of mature and active HupSL and HupUV enzymes.

The catalytic properties of the HupUV protein complex are those of hydrogenase enzymes. Not only can HupUV activate the H_2 molecule, as indicated by the H-D exchange reaction, but it can also catalyze the other hydrogenase partial reactions, namely H_2 production and H_2 oxidation. Cell extracts were used to overcome the permeability barrier to electron acceptors or donors and to measure HupUV activity as a function of pH. To ascertain whether the activity was due to HupUV, it was systematically checked whether the presence of plasmid pAC206 containing the *hupTUV* operon that produces active

TABLE 2. Restoration of HupSL and HupUV activities in the complemented *hypD* mutant RCC12 as seen by the H_2 and HD production in exchange with D_2 uptake and the effect of acetylene[a]

Expt	Strain	Conditions	Formation (nmol · min^{-1} · mg of $protein^{-1}$) of:	
			H_2	HD
pH 4				
1	B10 (wild type)	Zero time	20.3	11.3
	B10	2 h under C_2H_2-Ar	0.9	1.7
2	JP91 (*hupSL*)		0.8	1.7
3	JP91(pAC206)	Zero time	2.5	5.6
	JP91(pAC206)	2 h under C_2H_2-Ar	2.6	5.7
4	RCC12 (*hypD*)		0.03	0.03
5	RCC12(pAC63)	Zero time	65.5	30.5
	RCC12(pAC63)	2 h under C_2H_2-Ar	4.9	4.2
pH 7				
6	B10 (wild type)	Zero time	3.1	2.8
	B10	1 h 30 min under C_2H_2-Ar	1.5	2.0
7	JP91(pAC206)	Zero time	3.7	5.2
	JP91(pAC206)	1 h 30 min under C_2H_2-Ar	3.9	5.1
8	RCC12(pAC63)	Zero time	11.1	6.4
	RCC12(pAC63)	1 h 30 min under C_2H_2-Ar	1.6	2.1
9	BSE16 (Δ*hupUV*)	Zero time	13.9	6.0
	BSE16	1 h 30 min under C_2H_2-Ar	0.3	0.2

[a] Cells were grown photoheterotrophically in MN medium to an OD_{660} of ca. 2.4 and collected by centrifugation. The initial rates of H_2 or HD formation were determined in whole cells, in 50 mM citrate-phosphate buffer, at time zero, and after incubation under C_2H_2-Ar (1:1) as indicated.

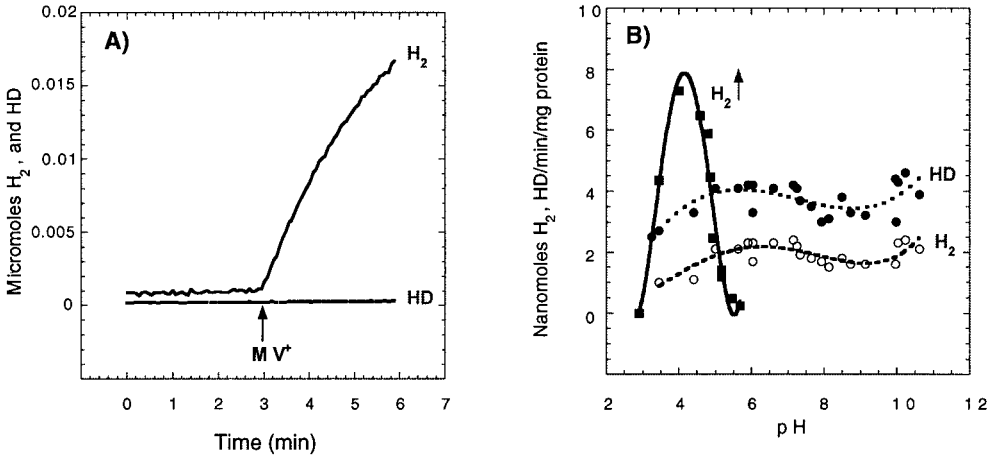

FIG. 6. HupUV hydrogenase activities in the soluble cytosolic fraction of JP91(pAC206) cells as a function of pH. JP91(pAC206) cells grown photoheterotrophically in MN medium were broken by passage through a French pressure cell. The soluble cytoplasmic fraction obtained by centrifugation at 100,000 × *g* for 70 min was used to determine HupUV hydrogenase activities. (A) H_2 production linked to MV oxidation at pH 4.0. The phosphate-citrate buffer (1.25 ml, final concentration of 100 mM) in the reaction chamber was first sparged with argon to remove O_2, and then the reaction vessel was closed, and 0.25 ml of soluble cytosolic fraction (0.9 mg of protein) was added. Two minutes later, MV^+ (50 μl, final concentration of 120 mM) was injected into the reaction vessel. (B) pH dependence of MV^+-mediated H_2 production and H_2 and HD formation in exchange with D_2. Initial rates determined for the first minute of H_2 (○) and HD (●) production (in 1.5 ml, 0.8 mg of protein) are plotted versus pH. To measure the H-D exchange, the reaction vessel was sparged first with D_2. H_2 (■) was formed by proton reduction with MV^+. The buffers used (final concentration of 100 mM) were phosphate-citrate (pH 2.9 to 7.0), phosphate-Tris (pH 6.6 to 8.5), phosphate-glycine-NaOH (pH 7.5 to 10), and glycine-NaOH (pH 9.0 to 12.8).

HupUV protein complex (12) resulted in an increase (three- to fourfold) in hydrogenase activity of JP91(pAC206) cells compared to JP91 cells. H_2 production by HupUV upon addition of MV^+ (Fig. 6A) was observed at acidic pH, even at pH 4, where proteins precipitated. It occurred within a narrow range of acidic pH, in contrast to the H-D exchange activity, which did not vary significantly between pH 5 and 11 (Fig. 6B). The lack of pH dependence of the H-D exchange reaction catalyzed by HupUV is in contrast with the sharp pH dependence of the H-D exchange reaction catalyzed by the HupSL hydrogenase (Fig. 7) and other membrane-bound hydrogenases, e.g., in *T. roseopersicina* (44) and in *Paracoccus denitrificans* (41). The rates of HupSL-catalyzed HD and H_2 formation peaked at around pH 4.5 and were close to zero at pH 9 with BSE16 membranes. The apparent maximal rate of H_2 production by HupSL was also around pH 4 to 5, in agreement with what was observed for other [NiFe] hydrogenases, such as the hydrogenase 1 of *T. roseopersicina* (44), but was sevenfold lower than the H-D exchange reaction (Fig. 7).

The H_2-uptake activities of HupSL and HupUV, as a function of pH, were determined by using BV^{2+} or MB as an electron acceptor. Maximal uptake activity of HupSL was in the range of pH 8.5 to 9.0, while the uptake activity of HupUV paralleled that of the HupUV H-D exchange activity (not shown). At pH 8.7, the initial rate of hydrogen uptake with MB as an electron acceptor was 260 nmol of D_2/min/mg of protein for HupSL in BSE16 (Δ*hupUV*) cells and 21 nanomoles of D_2/min/mg of protein for HupUV in the soluble fraction of JP91(pAC206) cells with BV^{2+} as an electron acceptor. If we take into account an estimated 3-fold increase in HupUV protein complex brought by plasmid pAC206, there would be a 30-fold difference in activity between HupSL and HupUV.

In short, the HupUV protein complex exhibited all of the typical hydrogenase reactions, and thus HupU and HupV form an active hydrogenase. However, the measured H_2 uptake and H_2-activating activities are very low, near the limit of detection, and more than 10 times lower than those measured for HupSL. Differences in the expression levels of the *hupTUV* operon and the *hupSLC* operon, monitored by the *lacZ* reporter gene, have also been observed. The ratio of *phupT*::*lacZ* to *phupS*::*lacZ* expression was between 1/50 and 1/100 (8, 12). Therefore the low HupUV activity found in situ may be due to a low level of protein, although it is also quite possible that HupUV has a low specific activity. The main feature of HupUV hydrogenase is the pH insensitivity of the H-D exchange reaction, while the H-D exchange catalyzed by HupSL showed a strong pH dependence with a sharp peak at around pH 4.5.

DISCUSSION

This work deals with the H-D exchange properties of the energy-transducing hydrogenase, HupSL, and the H_2 sensor, HupUV. Since a gene homologous to *hoxH* of *R. eutropha* (39) was found in the hydrogenase gene cluster of *R. capsulatus*, new mutants with an inactivated *hoxH* gene were constructed

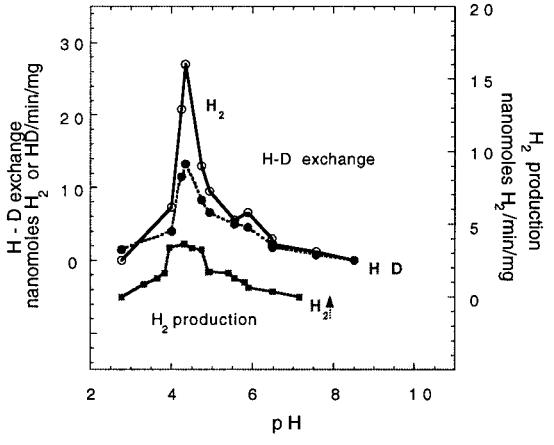

FIG. 7. pH dependence of the H-D exchange reaction and H_2 production catalyzed by the *hupSL*-encoded hydrogenase in BSE16 membranes. The experimental conditions were the same as those in Fig. 6. H_2 (○) and HD (●) were produced by the H-D exchange reaction and 0.4 mg of protein. Production of H_2 (■) was measured with MV^+ and 0.8 mg of protein.

and studied comparatively with the *hupUV* and *hupSL* mutants. No detectable change in H-D exchange was found in the *hoxH* mutants. Thus, either the *hoxH* gene was not expressed under the growth conditions used (repressing conditions for HupSL synthesis), or the activity of the *hoxH* gene product was too low to be detected.

HupUV has a low hydrogenase activity, but its activity is detectable with a mass spectrometer by the measurement of changes in the concentrations of D_2, H_2, and HD in cell suspensions. Production of H_2 by extracts of cells lacking HupSL [JP91, JP91(pAC206), and JBC13] could be observed at acidic pH values after addition of MV^+. The rate of H_2 production by HupUV at pH 4 was twice as high as the rate of H-D exchange (Fig. 6), unlike with HupSL, whose specific activity for H_2 production was sevenfold lower than the H-D exchange reaction at pH 4 (Fig. 7). The insensitivity of HupUV to acetylene and oxygen is probably due to limited gas access to the HupUV active site (30). The lack of pH effect on the H-D exchange reaction may also reflect the poor physical connection of the active site to the surface of the protein or a narrow putative proton channel. Thus, besides its specific cellular function in signal transduction, the HupUV hydrogenase displays catalytic (and probably structural) features specific to this new class of hydrogenases.

The product of the accessory *hypD* gene is necessary for the synthesis of active HupSL and HupUV hydrogenases (Table 2). This implies that HupUV requires the same posttranslation activation as other [NiFe] hydrogenases (24, 25) and thus has a Ni atom at its active site. Indeed, the presence of Ni and of the ligands to the Fe atom of the active site, CN and CO (18), has been demonstrated in the homologous HoxBC protein of *R. eutropha* (32). In a very recent report, Kleihues et al. (20) have proposed that these H_2 sensors form a new subclass of so-called "regulatory hydrogenases."

The demonstrated hydrogenase activity of HupUV is a first step for understanding how the HupUV H_2 sensor and the protein histidine kinase HupT communicate and interact to control the transcriptional induction of hydrogenase (*hupSL*) genes in response to H_2 (12, 15). H_2 sensing systems homologous to HupUV have been found in bacteria other than *R. capsulatus*. They include HupUV in *B. japonicum* (2), HoxBC in *Alcaligenes hydrogenophilus* (23), and HoxBC in *R. eutropha* (22), shown to be necessary, as in *R. capsulatus*, for hydrogenase gene expression in response to H_2.

In cells lacking *hupT*, maximal *hupSL* derepression was observed in the presence of O_2. A region of the *phupS* regulatory region similar or very close to the binding site of HupR appears to be involved in O_2 derepression of *hupSL* gene expression (38). Either the transcriptional factor RegA, which responds to redox and binds to the same *phupS* region (14), HupR, or both control *hupSL* expression in response to oxygen. It is not known whether the HupUV protein complex could also be involved. The sensor kinase HupT has been shown recently to belong to the PAS domain-containing superfamily of proteins (37). PAS domains are signaling modules that detect changes in light, redox potential, oxygen, small ligands, and overall energy level of a cell. According to Taylor and Zhulin (37), most PAS domains in prokaryotes are in histidine kinase sensor proteins, and their primary role is sensing oxygen, redox potential, and light. The sensing function of PAS proteins is commonly associated with the binding of specific cofactors. The flavoprotein NifL of *A. vinelandii*, a PAS protein, is a redox sensor which regulates nitrogen fixation by modulating the activity of the transcriptional factor NifA in response to oxygen and to fixed nitrogen (11). It is believed that the oxidation state of the prosthetic group flavin adenine dinucleotide (FAD) acts as a switch to control transcriptional activation by NifA. FAD binds to the N-terminal region of NifL in the PAS core region (36), alignable to the PAS core of HupT (37). Does HupT contain a redox-sensitive chromophore reducible by the HupUV hydrogenase, such as FAD or NAD, or is the PAS core of HupT a domain involved in protein-protein interaction between the histidine kinase HupT and the sensory HupUV protein complex?

The three-dimensional structure of the H_2-signaling HupUV hydrogenase should reveal the features able to account for the inaccessibility of acetylene to the active site and for the restricted proton access reflected by the lack of pH sensitivity of HupUV activity; hopefully, this structure will also provide molecular basis for the specific function of HupUV hydrogenase in signal transduction.

ACKNOWLEDGMENTS

This work was supported by the Commissariat à l'Energie Atomique and the Centre National de la Recherche Scientifique (CEA/CNRS/UJF UMR 5092). N. Zorin received financial support from the International Association for the Promotion of Cooperation with Scientists from the Independent States of the Former Soviet Union (INTAS) (grants 94-882 and 98-862) and partial support by a travel grant through the COST Action 8.18. M. Tomiyama (Tsukuba, Japan) was supported by the Special Coordination Funds for Promoting Science and Technology based on Japan-French Bilateral International Joint Research Programme.

We gratefully acknowledge Jacqueline Chabert for participating in the construction and verification by sequencing of the JBC12 and JBC13 mutants, Patrick Carrier for the mass spectrometric measurements, and J. C. Willison (UMR 5092) for critical reading of the manuscript.

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