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HupUV Proteins of *Rhodobacter capsulatus* Can Bind H_2: Evidence from the H-D Exchange Reaction

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The H-D exchange reaction has been measured with the D_2-H_2O system, for *Rhodobacter capsulatus* JP91, which lacks the *hupSL*-encoded hydrogenase, and *R. capsulatus* BSE16, which lacks the HupUV proteins. The *hupUV* gene products, expressed from plasmid pAC206, are shown to catalyze an H-D exchange reaction distinguishable from the H-D exchange due to the membrane-bound, *hupSL*-encoded hydrogenase. In the presence of O_2, the uptake hydrogenase of BSE16 cells catalyzed a rapid uptake and oxidation of H_2, D_2, and HD present in the system, and its activity (H-D exchange, H_2 evolution in presence of reduced methyl viologen [MV^+]) depended on the external pH, while the H-D exchange due to HupUV remained insensitive to external pH and O_2. These data suggest that the HupSL dimer is periplasmically oriented, while the HupUV proteins are in the cytoplasmic compartment.

The photosynthetic bacterium *Rhodobacter capsulatus* contains a membrane-bound hydrogenase, encoded by the *hupSL* genes, which is part of the respiratory chain (2, 16), functions physiologically in H_2 uptake (18), and has Ni at its active site (5). The *R. capsulatus* enzyme is dimeric and belongs to the class of [NiFe]hydrogenases (20). The *hupTUV* operon (8, 9) is part of the cluster of *hup* and *hyp* genes necessary for the synthesis of hydrogenase (4). The *hupUV* genes are expressed from the *hupT* promoter and are involved, with *hupT*, in negative control of hydrogenase synthesis (9). The *hupT* gene product belongs to the superfamily of protein histidine kinases, which act as sensors in bacterial two-component regulatory systems. The product of *hupU* has 20% similarity to the small subunit (HupS), and that of *hupV* has 29% similarity to the large subunit (HupL) of *R. capsulatus* hydrogenase. HupU lacks the signal peptide of HupS, and HupV lacks the C-terminal sequence of HupL, which is cleaved during hydrogenase processing, so the HupUV dimer is expected to be a soluble cytoplasmic protein. The *hupU* and *hupV* gene products also share a significant degree of identity with the cytoplasmic [NiFeSe]hydrogenase of *Desulfovibrio baculatus* (now termed *Desulfomicrobium baculatus*) (15). The HupU protein, rich in cysteine, contains all of the Cys residues involved in the binding of the three Fe/S clusters in the small subunit of *Desulfovibrio gigas* hydrogenase (19). The HupV protein contains at its N-terminal and C-terminal ends (9) the Cys arrangements found at the same locations in the large subunit of [NiFe]hydrogenases and shown by X-ray diffraction to bind Ni (19). Therefore, the HupUV dimer might be expected to have hydrogenase activity or at least bind H_2. Hydrogenase activity can be measured by the proton-deuterium (H-D) exchange reaction. In the D_2-H_2O system, molecular D_2 is split at the active site of the enzyme and is replaced by HD and H_2 by exchange with protons of the solvent, H_2O. This method was used for the first time by Yagi et al. (21) to determine the intrinsic activity of the purified membrane-bound hydrogenase from *Desulfovibrio vulgaris*. The H-D exchange activity of the HupUV proteins was tested in vivo. It is shown here that the H-D exchange due to HupUV is distinguishable from the H-D exchange resulting from HupSL activity; in particular, it is insensitive to the external pH.

The *R. capsulatus* strains used were the wild-type strain, B10 (14); the *hupSL* mutant, JP91 (3); and the Hup(UV)$^-$ mutant, BSE16, which exhibits hydrogenase activity (9). Plasmid pAC206, which carries the *hupTUV* operon, can complement in *trans* Hup(UV)$^-$ mutants (9); it was introduced into JP91 by triparental mating (7). All of the strains were grown anaerobically in the light with 30 mM DL-malate as a C source and 7 mM ammonium sulfate as an N source (MN medium) (6) to prevent the synthesis of nitrogenase, the activity of which would otherwise interfere with the H-D exchange measurements, since nitrogenase can reduce protons and catalyze the formation of H_2 (12). The H-D exchange reaction, measured at 30°C, was monitored continuously in the aqueous phase of cell suspensions by a mass spectrometric method (12, 22). Cells grown overnight in MN medium were collected by centrifugation, resuspended in 1/10 volume of water, and stored under H_2. The reaction vessel was filled with 1.7 ml of the cell suspension (optical density at 660 nm of 10) and then sparged with Ar and then with D_2 until saturation. Changes in gas concentration, D_2 (mass, 4 Da), HD (mass, 3 Da), H_2 (mass, 2 Da), and, when used, O_2 (mass, 32 Da) were automatically scanned by a peak jumping system and continuously recorded in a PC computer, immediately after the vessel was closed. The rates of D_2 consumption and of H_2 and HD production were corrected for simultaneous consumption by the mass spectrometer (first-order kinetics) with the velocity constants $k_{D2} = 0.0990 \cdot \text{min}^{-1}$ and $k_{H2} = 0.1086 \cdot \text{min}^{-1}$ determined for D_2 and H_2 consumption, respectively, in the absence of cells. The buffers (50 mM)

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TABLE 1. Rate of the H-D exchange reaction in *R. capsulatus* cells at three pH values[a]

Strain	D_2 consumption (μmol/min) at pH			H_2 formation (μmol/min) at pH		
	5	7	9	5	7	9
B10	0.37	0.23	0.19	0.20	0.12	0.09
BSE16	0.56	0.31	0.17	0.30	0.13	0.08
JP91	0.12	0.12	0.13	0.07	0.05	0.07
JP91(pAC206)	0.47	0.49	0.47	0.16	0.17	0.15

[a] D_2 consumption and H_2 formation result from the H-D exchange reaction. The values are initial rates corrected for gas consumption by the mass spectrometer.

used were phosphate-HCl at pH 5 and 7 and Tris-HCl at pH 9. O_2 was generated by additions of 3% H_2O_2 (5 μl).

The observed rates of HD and H_2 formation with JP91(pAC206) cells were similar at the three external pH values of 5, 7, and 9 (Table 1 and Fig. 1A and B). Since in the H-D exchange reaction, D_2 gives rise to HD plus H_2, the rate of exchange can be determined from the rate of D_2 uptake. In JP91 cells harboring plasmid pAC206, this D_2 uptake rate was three- to fourfold higher than that of JP91 cells, in agreement with the fact that the latter contain a single chromosomal copy of the *hupUV* genes, while in JP91(pAC206) there are four to five additional copies brought by the plasmid. The insensitivity of the reaction to external pH indicates that the HupUV proteins are located in the cytoplasmic compartment. In one experiment, Triton X-100 was added to equilibrate the pH between the cytoplasmic and the external compartments, and the H-D exchange was remeasured at pH 5. The rates of HD and H_2 formation and of D_2 uptake were found to be slightly lower than those before Triton X-100 addition, an indication that Triton might affect the stability of the putative HupUV dimer besides permeabilizing the membrane. The rate of H-D exchange in B10 and BSE16 was highest at acid pH (Table 1).

The patterns of H_2 and HD formation in BSE16 are different from those obtained with cells containing the HupUV proteins (Fig. 1). In the H-D exchange reaction catalyzed by the *hupSL*-encoded hydrogenase of B10 (not shown) and BSE16 (Fig. 1C and D), H_2 was formed at a rate higher than HD. An H_2/HD ratio higher than 1.0, already reported when chromatophores from *R. capsulatus* were used (12), has also been observed with the cytoplasmic hydrogenase from *D. baculatus* (10). It has been speculated (10, 17) that an H_2/HD ratio greater than 1 is a signature of the [NiFeSe]hydrogenase, since H_2/HD ratios lower than 1 were obtained with [NiFe]hydrogenases from *Desulfovibrio* species. In the [NiFeSe]hydrogenase of *D. baculatus*, one of the sulfur ligands to nickel is replaced by the selenium atom of a selenocysteine (11), and this selenocysteine has been suggested to be the real intermediate involved in the exchange of H atoms from hydrogen gas with the protons of the solvent (1). However, the [NiFe]hydrogenase from *R. capsulatus* is shown here to catalyze an exchange which leads to a H_2/HD ratio greater than 1, although the nucleotide sequence of the gene encoding the large subunit (HupL), which provides the ligands to Ni, does not indicate that a Cys has been replaced by a SeCys (13). The rate of H_2 formation is linked to the rate of exchange with the solvent, as illustrated by the strong isotope effect observed for the *R. capsulatus* hydrogenase in the H_2-D_2O system, in which the rate of HD formation became higher than that of D_2 (12). It is not known yet whether it is the structure of the active site and of a putative proton channel or the nature of the amino acids

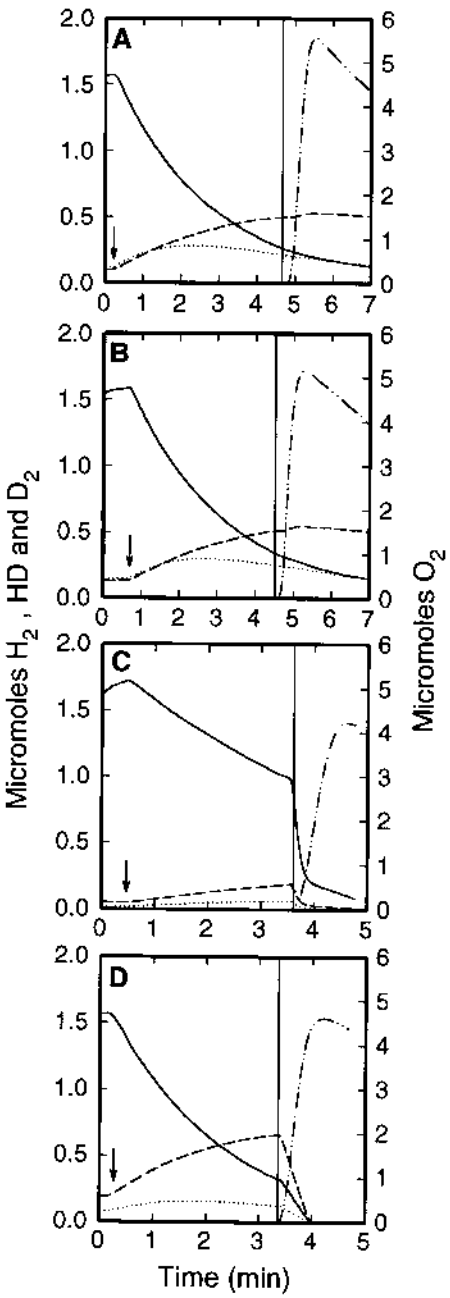

FIG. 1. H_2 and HD production in exchange with D_2 uptake catalyzed by whole cells of JP91(pAC206) at pH 9 (A) and 5 (B) and of BSE16 at pH 9 (C) and 5 (D). At the time indicated by arrows, the vessel was closed, and the concentrations of H_2 (– – –), HD (. . .), and D2 (–) were recorded. The vertical line indicates the time of H_2O_2 addition; the increase in O_2 concentration (– · · –) was monitored at mass 32 Da. The figure shows the real concentrations of the hydrogen species and of O_2 present in the vessel.

liganding the metal atoms (Ni and Fe) of the active site which is responsible for that isotope effect. Unexpectedly, it was the *hupSL*-encoded hydrogenase of *R. capsulatus* which exhibited H-D exchange kinetics of the type produced by the cytoplasmic hydrogenase from *D. baculatus* (10) and not the HupUV proteins, which share significant similarity with the *D. baculatus* enzyme. The H-D exchange catalyzed by HupUV was rather of the type exhibited by the periplasmic hydrogenases from *D.*

gigas (10) or from *Thiocapsa roseopersicina* (22). The fact that the H-D exchange due to HupSL activity was sensitive to the external pH (it increased from pH 9 to pH 5) (Table 1) suggests that the *hupSL*-encoded hydrogenase is oriented towards the periplasm of *R. capsulatus* cells.

In presence of oxygen or an appropriate electron acceptor, the membrane-bound hydrogenase, encoded by *hupSL*, can quickly oxidize the three species of hydrogen present in the system. Upon O_2 addition, H_2 and HD, which had accumulated by exchange of D_2 with protons, were quickly absorbed (oxidized) and D_2 uptake, due also to D_2 oxidation, was considerably enhanced (Fig. 1C and D). D_2 oxidation by cells from BSE16 (Fig. 1) and from B10 (not shown) already rapid at pH 5, was 4.1 and 3.3 times as fast, respectively, at pH 9. Under the same conditions, the H-D exchange catalyzed by the HupUV proteins remained unchanged in the presence of O_2 (Fig. 1C and D). The H-D exchange reactions catalyzed by the HupSL proteins, on the one hand, and by the HupUV proteins, on the other hand, could therefore be distinguished from the difference in response to O_2.

The absence of H_2 and HD uptake after O_2 addition to JP91 cells confirms that the strain lacks the membrane-bound uptake hydrogenase. The rapid O_2 consumption by JP91(pAC206) cells (Fig. 1A and B) indicates that O_2 is used for the oxidation of endogenous substrates (other than hydrogen). Although the data of Fig. 1 suggest that the H-D exchange catalyzed by the HupUV proteins is insensitive to O_2, it is very probable that because of the very rapid oxygen consumption by the dense cell suspensions, the internal compartment has remained anaerobic during the first minutes of the test.

In conclusion, by the use of strain JP91 devoid of the *hupSL*-encoded hydrogenase and of plasmid pAC206 from which the *hupUV* genes are expressed, it has been possible to demonstrate that the HupUV proteins catalyze in vivo an H-D exchange reaction. Thus, the HupUV proteins can detect and bind hydrogen gas, a prerequisite for HupUV to function as an H_2 sensor. The H-D exchange reaction catalyzed by the HupUV proteins is distinguishable from the exchange catalyzed by HupSL in strains B10 and BSE16: (i) the former is insensitive to changes in the external pH, while the H-D exchange linked to HupSL activity was found to be higher at pH 5 than at pH 9, and (ii) the former did not respond to oxygen, while in the presence of O_2 the membrane-bound hydrogenase (*hupSL* encoded) catalyzed a very rapid hydrogen uptake and oxidation. Finally, the hydrogenase activity of HupSL, which responds to the external pH, suggests that the *R. capsulatus* membrane-bound hydrogenase is oriented towards the periplasmic space.

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