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Localization of hydrogenase in *Thiocapsa roseopersicina* photosynthetic membrane

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The photosynthetic cell membrane is impermeable to the oxidized redox dyes Methyl Viologen and Benzyl Viologen, whereas the reduced forms easily penetrate into the cells. By exploiting this permeability difference, the orientation of the membrane-bound hydrogenase has been determined.

Hydrogenase enzymes (EC class 1.12) from different micro-organisms have been purified and extensively studied recently (Adams *et al.*, 1981), particularly since their role as components of potential biological and bio-analogue solar-energy-conversion systems has been recognized. The enzymes are usually associated with the plasma membrane.

Although the location of hydrogenase within the membrane and thus its relationship with the electron-transport chain is bioenergetically significant, very little progress has been made in elucidating this relationship. The enzyme has been identified as a transmembrane protein in both *Paracoccus denitrificans* (Sim & Vignais, 1978) and *Escherichia coli* (Jones, 1980), which accords with its assumed respiratory proton-translocating role. The active centre of the enzyme, however, is probably on the periplasmic side of *P. denitrificans* membrane, whereas it is on the cytoplasmic face in *E. coli*.

In the present paper a relatively simple and general method is described for the determination of the previously unknown location of the hydrogenase in the photosynthetic membrane.

Materials and methods

The phototrophic purple sulphur bacterium *Thiocapsa roseopersicina* strain BBS was generously provided by Professor E. N. Kondratieva (Department of Microbiology, Moscow State University, Moscow, U.S.S.R.). Cultures were grown anaerobically in a modified Pfennig's medium by the method of Gogotov *et al.* (1978). NH_4Cl was added as nitrogen source to repress nitrogenase synthesis (Kondratieva *et al.*, 1979). Light-intensity was 1500 lx.

Hydrogenase activity was determined by H_2-evolution or H_2-uptake measurements.

For H_2-evolution assay, 2 ml of hydrogenase-containing material (cells, spheroplasts or pure enzyme) and 1 ml of 2 mM-MV^{2+} were pipetted into a calibrated reaction vessel (about 23 ml) having a side arm where 10 mg of solid $Na_2S_2O_4$ was placed. After sealing of the vessel with a rubber stopper and replacing the atmosphere with argon, H_2 evolution was initiated by mixing the $Na_2S_2O_4$ in the side arm with the solution in the main chamber. H_2 production was measured with a gas chromatograph built in this laboratory (Bagyinka *et al.*, 1981).

H_2 uptake was measured in 15 ml screw-cap vials (no. 13043; Pierce, Rotterdam, The Netherlands). Cell or spheroplast suspension (5 ml) was mixed with 1 ml of 2 mM-MV^{2+} or -BV^{2+} solution. The appearance of reduced Viologens was recorded with respect to time at 600 nm on a Spectromom 203 spectrophotometer (MOM, Budapest, Hungary). Molar absorption coefficients were taken from Jones & Garland (1977).

For permeability measurements 4 ml portions of cell or spheroplast suspension ($A_{650} = 1.0$/cm, equivalent to approx. 10^7 cells/ml) were pipetted into 15 ml screw-cap glass vials (Pierce no. 13043), and 1 ml of 2 mM-MV^{2+} or -BV^{2+} was added. Samples were treated as shown in Fig. 1. The A_{600} of reduced Viologens was measured in an anaerobic cuvette (optical path length 1 cm) on a Spectromom 203 spectrophotometer after their reduction by $Na_2S_2O_4$.

Spheroplasts were prepared as follows. Cells were

Abbreviations used: BV^{2+}, oxidized Benzyl Viologen; MV^{2+}, oxidized Methyl Viologen; BV^{+}, reduced radical of Benzyl Viologen; MV^{+}, reduced radical of Methyl Viologen.

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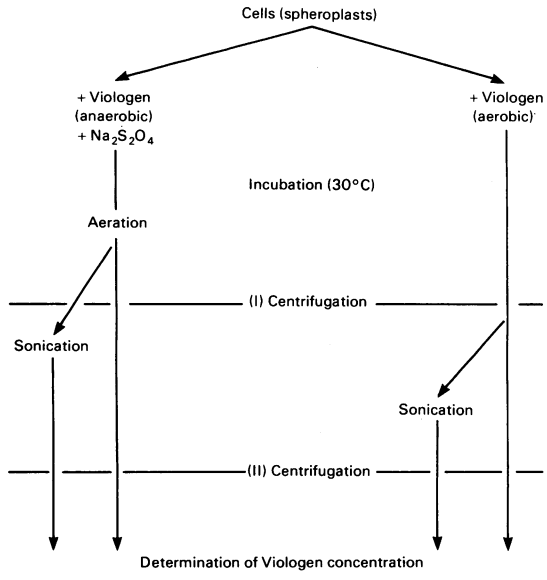

Fig. 1. *Scheme of Viologen permeability measurements* Anaerobic samples were reduced by 0.5 ml of 10 mM-$Na_2S_2O_4$. Sonication was with an MSE ultrasonicator (medium power; setting-2 amplitude for 4 x 2 min).

collected and resuspended in 0.5 M-sucrose and digested by lysozyme (0.5 mg of lysozyme/ml). After incubation at 30°C for 20 min the suspension was centrifuged (10000 *g* for 10 min) and resuspended in 20 mM-phosphate buffer containing 160 mM-NaCl (pH 7.3).

Cell number was estimated from light-scattering at 650 nm. A calibration curve of light-scattering versus cell number was made by parallel counting of the cells in a Coulter counter model ZB_I (Coulter Electronics, Harpenden, Herts., U.K.) and of colonies on anaerobically grown agar plates (BBL Gas Pak anaerobic systems; Becton Dickinson and Co., Cockeysville, MD, U.S.A.).

Protein was assayed by the method of Hartree (1972), with bovine serum albumin as standard.

Hydrogenase was purified by the method of Gogotov *et al.* (1978).

Methyl Viologen and Benzyl Viologen were purchased from Fluka (Buchs, Switzerland) and from BDH Chemicals (Poole, Dorset, U.K.) respectively. Bovine serum albumin (fraction V) was obtained from Sigma Chemical Co. (Kingston upon Thames, Surrey, U.K.). Lysozyme (EC 3.2.1.17), $Na_2S_2O_4$, sucrose and buffer materials (KH_2PO_4) were from Reanal Fine Chemicals (Budapest, Hungary).

Results and discussion

Cells as well as spheroplasts of *T. roseopersicina* show hydrogenase activity (Table 1). There is practically no activity loss during spheroplast preparation. Therefore the hydrogenase is located in or within the cell membrane. After osmotic shock at least 70% of the activity sediments in the pellet. The supernatant contains about 30% of the original activity, presumably because light membrane fragments do not sediment during the centrifugation (10000 *g* for 10 min). The results corroborate that hydrogenase in *T. roseopersicina* is predominantly membrane-bound (Gogotov *et al.*, 1978).

The activity measurements also imply that the active centre of the enzyme should be located on the periplasmic side of the cell membrane, unless both oxidized and reduced Viologens freely permeate the membrane. To resolve this ambiguity, the permeability properties of oxidized and reduced Viologens were further investigated.

The A_{600} of re-reduced supernatants of the

Table 1. *Hydrogen-evolution activity of T. roseopersicina preparations*

Sample	Hydrogenase activity (μmol of H_2/h per mg of protein)
Intact cell	3.0 ± 0.2
Spheroplast	3.1 ± 0.2
Spheroplast supernatant	0.2 ± 0.2
Broken-spheroplast pellet	2.2 ± 0.2
Broken-spheroplast supernatant	1.4 ± 0.2
Pure enzyme	51.3 ± 0.3

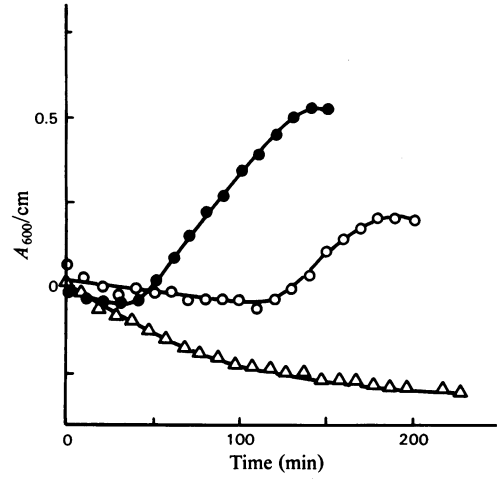

Fig. 2. *H_2-uptake activity of intact cells* (●, *BV^{2+} outside;* △, *BV^{2+} inside*) *and spheroplasts* (○, *BV^{2+} outside*) *of T. roseopersicina*
BV^{2+}-loaded cells were incubated in 1 M-BV^+, then oxidized by aeration and washed in phosphate buffer.

Table 2. *Methyl Viologen uptake by T. roseopersicina*
Each measurement was repeated three times. Sonicated samples give a measure of Viologen uptake, and the non-sonicated controls show the extent of back diffusion during aeration and second centrifugation.

	Incubation time (h)	MV^{2+}		MV^{+}	
		A_{600}/cm	MV^{2+} uptake (%)	A_{600}/cm	MV^{+} uptake (%)
Sonicated	0.33	0.03	0.30	0.15	1.80
	4	0.03	0.30	0.08	0.90
	24	0.03	0.30	0.05	0.60
Non-sonicated	0.33	0.03	0.30	0.05	0.60
	4	0.03	0.30	0.02	0.30
	24	0.01	0.15	0.01	0.15

Table 3. *Benzyl Viologen uptake by T. roseopersicina*
Experimental conditions were as described in the legend to Table 2.

	Incubation time (h)	BV^{2+}		BV^{+}	
		A_{600}/cm	BV^{2+} uptake (%)	A_{600}/cm	BV^{+} uptake (%)
Sonicated	0.33	0.03	0.60	0.23	4.65
	4	0.04	0.75	0.22	4.50
	24	0.07	1.35	0.21	4.20
Non-sonicated	0.33	0.04	0.75	0.20	4.05
	4	0.02	0.45	0.20	4.05
	24	0.03	0.60	0.20	4.05

sonicated samples (see Fig. 1) gives a measure of Viologen uptake by the cells. Data are collected in Tables 2 and 3 for MV^{2+} and MV^{+} and for BV^{2+} and BV^{+} respectively. The experimental error estimated from several repetitions is $\pm 0.01 A_{600}$/cm. The main source of error is the remaining extracellular Viologen solution in the pellet of the first centrifugation.

The data indicate that neither of the oxidized Viologen cations are able to cross the membrane (in agreement with the results of Jones *et al.*, 1976; Jones & Garland, 1977; Sim & Vignais, 1978). In addition, no adsorption effect was detectable for oxidized Viologens.

Both MV^{+} and BV^{+}, however, can easily penetrate through the cell membrane, conceivably owing to their increased hydrophobicity.

In addition to MV^{2+} and BV^{2+}, biological membranes are also impermeable to $Na_2S_2O_4$ (Keilin & Hartree, 1946; Kimmelberg & Lee, 1970; Jones & Garland, 1977). Consequently Viologens entrapped in non-sonicated cells cannot appear in the second supernatant, unless (*a*) the dye is only adsorbed on the outer surface of the membrane of (*b*) incomplete oxidation of reduced Viologens on aeration permits backward movement of the radicals during the second centrifugation.

The first possibility can be directly checked experimentally. Cells containing MV^{2+} (or BV^{2+}) should display H_2-uptake activity (see below) after the first centrifugation if a significant amount of dye has been adsorbed on their surface. No H_2 uptake was observed in such samples.

The exact orientation of hydrogenase within the cell membrane can be determined by exploiting the permeability difference of oxidized and reduced forms of Viologen dyes. One can prepare cells loaded with MV^{2+} (or BV^{2+}) and also cell suspensions where only the outer surface of the membrane has access to the dye. Typical H_2-uptake curves are presented in Fig. 2. The hydrogenase activities estimated from the slopes are 0.83 μmol of BV^{+}/h per mg of protein for intact cells and 0.76 μmol of BV^{+}/h per mg of protein for spheroplasts. These values agree well with the activities measured by the H_2-evolution assay, indicating that the hydrogenase is truly reversible.

BV^{2+}- or MV^{2+}-loaded cells failed to show H_2-uptake activity, although control experiments (H_2-evolution assay) gave positive evidence of an active hydrogenase. Conversely, when BV^{2+} or MV^{2+} was in the external medium, both H_2-uptake and H_2-evolution activity could be measured. This is clearly direct proof of the outer-surface location of the enzyme or at least of its active centre. The results also indicate that the 30% activity in the supernatant of osmotically shocked spheroplasts is not due to the existence of both soluble and membrane-bound hydrogenases (see Zorin & Gogotov, 1980).

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