PHOSPHORYLATION COUPLED TO H_2 OXIDATION BY CHROMATOPHORES FROM *RHODOPSEUDOMONAS CAPSULATA*

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1. Introduction

The phototrophic bacterium, *Rhodopseudomonas capsulata* can use H_2 as electron donor for either photoautotrophic or chemoautotrophic growth [1,2]. When grown in the presence of H_2, cells contain a relatively high hydrogenase activity of the H_2-uptake type [3]. This hydrogenase is located in the membrane [3], which also contains the redox components of the respiratory chain. Cells of *Rps. capsulata* grown anaerobically in the light have been shown to have respiratory activities [4] similar to those found in aerobic cells [5]. The purpose of the experiments reported here was to show that hydrogenase formed in the membranes of photosynthetically grown cells can feed electrons to the respiratory chain and that H_2 oxidation is linked to ATP synthesis.

2. Materials and methods

2.1. *Bacterial strain and culture*

Rps. capsulata, strain B10, was a gift from the Photosynthetic Bacteria Group, Department of Microbiology, Indiana University, Bloomington, IN 47401, and was cultured photosynthetically at 30°C for 16 h in a mineral salts medium [6,7] supplemented with 30 mM malate and 7 mM glutamate as carbon and nitrogen sources, respectively. Cultures were illuminated by 100 W incandescent lamps (~10 000 lux). The same medium was used for chemoheterotrophic growth but cells were cultured in the dark in a flask plugged with cotton wool and shaken continuously.

Abbreviation: FCCP, carbonylcyanide, *p*-fluoromethoxyphenylhydrazone

2.2. *Preparation of membranes*

Cells were harvested at late logarithmic phase, washed once with 100 mM Tricine–NaOH buffer (pH 7.5), 8 mM $MgCl_2$ and broken by a French press at 20 000 lb. in^{-2} at 5°C. Unbroken cells were eliminated by centrifugation at 20 000 × *g* for 15 min before sedimenting the membranes in a Spinco centrifuge (140 000 × *g* for 30 min). Membranes (~15 mg protein/ml) resuspended in the above-mentioned buffer and kept in ice were used within a few hours of preparation. All operations were performed at 2–5°C. In some experiments, cells were broken by sonication in a Branson sonifier (80 W, 1 min, twice).

2.3. *O_2 consumption*

O_2 consumption was measured amperometrically at 25°C in the dark with a Clark-type oxygen electrode polarised at −0.8 V. The chamber was filled with 1.6 ml 2.7 mM $MgCl_2$, 33 mM Tricine–NaOH buffer (pH 7.5). For NADH- or succinate-oxidase assays, chromatophores (1–1.5 mg protein corresponding to 50–75 μg bacteriochlorophyll) were introduced through the rubber stopper closing the chamber and the reaction was started by injection of the substrate. For hydrogen-oxidase assays, the chamber was filled with a mixture of air-saturated and H_2-saturated buffer (0.4 ml and 1.2 ml, respectively) after calibration with air-saturated buffer and the reaction initiated by chromatophores. Potassium cyanide solutions were freshly prepared in Tricine–$MgCl_2$ buffer and the pH adjusted to 7.5.

2.4. *Oxidative phosphorylation*

To measure the phosphorylation coupled to respiration, the medium used in oxygraphic experiments was supplemented with 30 mM glucose and 1.6 units hexokinase/ml. Incubation was carried out in the oxygraphic chamber. Chromatophores and substrates were added first as described for O_2 consumption, followed by 30 μl of a mixture of 0.2 M ADP/0.4 M $P_i/^{32}P_i$ (1:1:1, by vol.) (final spec. act. 100–150 cpm/nmol P_i). All measurements were made for periods of time during which both the O_2 uptake and ATP synthesis were linear. The incubation was ended by adding 0.1 ml 2.5 N perchloric acid and the vessel immediately placed in crushed ice. After centrifugation of the membranes, unreacted $^{32}P_i$ was extracted from the supernatant as phosphomolybdate complex by isobutanol benzene according to [8]. Glucose-6-[^{32}P]phosphate remaining in the aqueous phase was then counted in a scintillation counter. Control assays without substrate or with argon instead of H_2 were run in parallel to estimate ^{32}P-incorporation into ATP due to exchange reactions or substrate-level phosphorylation.

2.5. *Photophosphorylation*

Chromatophores (~1 mg protein) in the same buffer as for oxidative phosphorylation were placed in 10 ml flasks covered with aluminium foil, closed with rubber septa and flushed with argon for 20 min; the ADP–$^{32}P_i$ mixture described above (30 μl) was then added. Flushing with argon was carried on for an additional 5 min. The final volume was 1.7 ml. The flasks maintained in the dark were placed in the waterbath of a photosynthetic Warburg apparatus (B. Braun AG, Melsungen) uniformly illuminated by 40 W incandescent lamps from below (~5000 lux). Photophosphorylation was initiated by light. After 10 min incubation at 30°C, the reaction was stopped by 0.1 ml 2.5 N perchloric acid and glucose-6-[^{32}P]-phosphate formed was determined as already described.

2.6. *Hydrogenase activity*

Hydrogenase activity (H_2-uptake) was estimated from the reduction of benzylviologen followed in a Zeiss PMQII spectrophotometer. The membrane suspension (0.2 ml containing 50 μg protein) supplemented with 0.5% Triton X-100 was placed in 2 ml glass tubes fitted with rubber septa and gassed with H_2. The H_2-saturated dye solution (1.5 ml 5 mM benzylviologen in 20 mM Tris–HCl (pH 8) equilibrated at 30°C was added. Control tubes gassed with N_2 were included to evaluate non-specific reduction of the dyes. The reduction of benzylviologen was read at 555 nm (ϵ = 7.55 $mM^{-1}.cm^{-1}$) [9].

2.7. *Other methods*

Proteins were assayed by the method in [10] or [11] and the bacteriochlorophyll content of chromatophores by the method in [12] using ϵ = 75 mM^{-1} at 775 nm.

2.8. *Materials*

NADH, rotenone and hexokinase (14 units/mg) were purchased from Sigma Chemical Co, St Louis, MO, carbonylcyanide, *p*-fluoromethoxyphenylhydrazone (FCCP) from Pierce, Rockford, IL. Other reagents were of the highest purity commercially available.

3. Results and discussion

3.1. *Hydrogenase-linked respiration on H_2*

Chromatophores were prepared from cells of *Rps. capsulata* grown anaerobically in the light. When incubated in the dark in an air-saturated buffer, these chromatophores showed very small rates of O_2 consumption (fig.1 (a) and (d) before addition of NADH). This indicates that the chromatophore preparation did not contain large amounts of endogenous substrates. The rate of respiration increased markedly when a pulse of H_2-saturated buffer was introduced into the incubation medium (fig.1(b)). Quantitative measurements of H_2 and O_2 uptake allowed one to evaluate that 2 mol H_2/mol O_2 were consumed (H_2O_2 ratio 2.05, fig.1(b)).

Figure 1 shows that respiration on H_2 is linked to the presence of hydrogenase. Indeed membranes from aerobically grown cells which have a very low hydrogenase activity [3], were unable to use H_2 as respiratory substrate (fig.1(c)). The rate of respiration on H_2 was comparable to that on NADH (fig.1(d)) (note the different scales used for (b) and (c)). However, while the NADH-oxidase remained fairly similar from one experiment to another

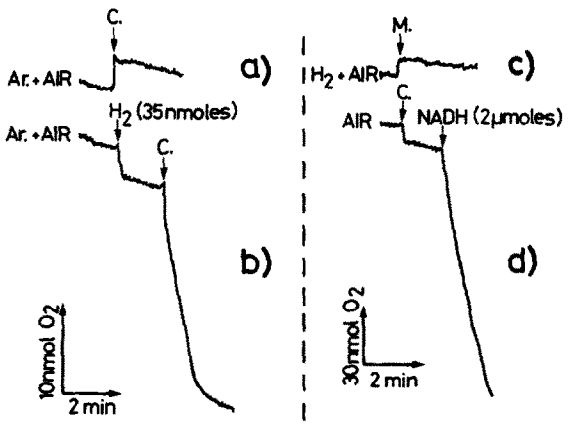

Fig.1. O_2-uptake by membranes from *Rps. capsulata*. Chromatophores (C) from photosynthetically grown cells or membranes (M) from chemoheterotrophic cells were used. (a) Chromatophores (~1 mg protein) were added to 1.6 ml of a mixture of argon- and air-saturated buffer (2:1, v/v). (b) To 1.6 ml of a mixture of argon- and air-saturated buffer (2:1, v/v) were added, as indicated, 50 μl H_2-saturated buffer followed by 20 μl of the chromatophore preparation (~0.3 mg protein). (c) Membranes from chemoheterotrophic cells (~1 mg protein) were injected into 1.6 ml of a mixture of H_2- and air-saturated buffer (2:1, v/v) (final H_2 0.5 mM). (d) Chromatophores (1 mg protein) were added to 1.6 ml air-saturated buffer and respiration initiated by addition of 2 μmol NADH. Other conditions as in section 2. Note the difference in scale used for the 2 sets of experiments.

(~4 μatoms O_2 $.h^{-1}$ .mg $protein^{-1}$), the activity of H_2-oxidase varied quite markedly with the different membrane preparations (from 1.5–5 μatoms O_2 $.h^{-1}$ .mg $protein^{-1}$); the higher the hydrogenase activity, the faster the hydrogen-oxidase activity. It is to note that the rate of electron transfer from H_2 to O_2 was much lower than that from H_2 to benzylviologen (table 1). This would indicate that activation of H_2 by hydrogenase is not likely the rate limiting step in H_2 oxidation. Nevertheless hydrogenase is a labile enzyme, sensitive to air and protected by H_2 [3]. While hydrogenase activity was measured under H_2 atmosphere, H_2 oxidation was measured in the presence of air. The real hydrogenase activity during incubation with air is not known. More experimental data are therefore needed to elucidate the location of the rate-limiting step in H_2 oxidase.

3.2. *Titration of H_2 oxidase activity by KCN – effect of rotenone*

The biphasic nature of the inhibition of NADH oxidation by KCN has already been demonstrated [5] with membrane fragments from aerobically grown cells of *Rps. capsulata* (strain St Louis). These authors concluded that alternative terminal oxidases with different sensitivities to cyanide existed [5]. The occurrence in *Rps. capsulata* of a branched respiratory chain with two terminal oxidases was confirmed [4] using membrane fragments from photosynthetically grown cells of strain Z-1 and mutants of Z-1 lacking either one of the two terminal oxidases.

Membrane fragments from photosynthetically grown cells of strain B10 showed a biphasic inhibition by KCN of the respiration on H_2 as well as on NADH or on succinate (fig.2). The concentrations of KCN producing 50% inhibition of the O_2 consump-

Table 1
Effect of the mode of cell breakage on the hydrogenase and hydrogen-oxidase activities and on ATP synthesis in chromatophores from *Rps. capsulata*

Cell breakage	Hydrogenase[a] activity	Hydrogen-oxidase[b] activity	Phosphorylation[c] activity
French press	59 ± 11	3.4 ± 1.4	1.5 ± 0.5
Sonication	49 ± 8	1.9 ± 0.4	0.4 ± 0.1

[a] μmol benzylviologen reduced $.h^{-1}$ .mg $protein^{-1}$
[b] μatoms O_2 consumed $.h^{-1}$ .mg $protein^{-1}$
[c] μmol ATP formed $.h^{-1}$ .mg $protein^{-1}$
Values are mean values from 3–7 exp. ± SEM

Photoheterotrophically grown cells were disrupted by passage through a French pressure cell or by sonication. Hydrogenase, hydrogen-oxidase and phosphorylation activities were measured as in section 2

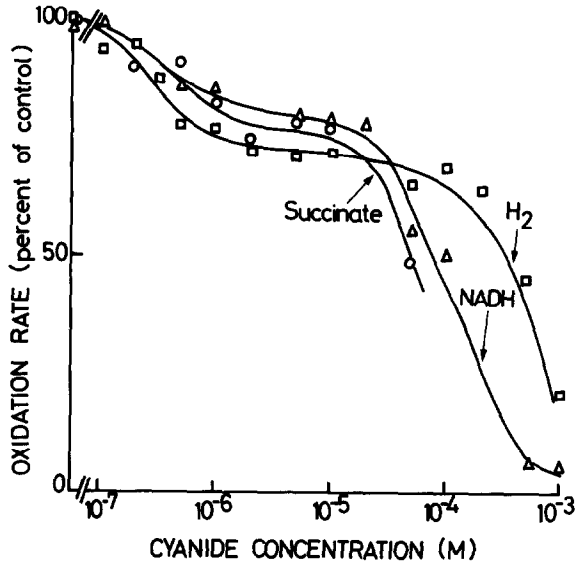

Fig.2. Oxidase activities of chromatophores as a function of cyanide concentration. (△) NADH oxidase, (○) succinate-oxidase, (□) hydrogen-oxidase. The control values, without inhibitor, were 3.85, 2.2 and 2.5 μatoms O_2 consumed $.h^{-1}$ .mg $protein^{-1}$ for respiration on 1.4 mM NADH, 14 mM succinate and 0.5 mM H_2, respectively.

tion were 4×10^{-7} M for the more sensitive and 10^{-4} M for the chain less sensitive to KCN. These inhibitory concentrations of KCN are the same for H_2, NADH or succinate oxidation; they are ~10-fold lower than those obtained with other strains of *Rps. capsulata* [4,5]. The fact that the same KCN concentrations produced the same degree of inhibition of the respiration on the 3 substrates indicates that electrons from H_2 follow the same respiratory pathways as those already demonstrated for NADH and succinate oxidation [4,5] and suggest that electrons coming from either of the 3 substrates, H_2, NADH or succinate, are entering the electron-transport chain before the branching point from which the two terminal oxidases diverge.

Rotenone at a concentration which completely abolished NADH oxidation (10^{-5} M) had no effect on the oxidation of H_2 (not shown). This lack of inhibition by rotenone of H_2 oxidation has already been observed with *Paracoccus denitrificans* [9]. It indicates that electron paths from H_2 and NADH do not share the same first electron carrier. It fits with the observation that the hydrogenase from *Rps. capsulata*, as other membrane-bound hydrogenases found in aerobic bacteria such as *P. denitrificans* [9,13] or *Alcaligenes eutrophus* [13], do not catalyse the direct reduction of NAD from H_2 [2].

3.3. *Phosphorylation coupled to H_2 oxidation*

The phosphorylating capacity of the chromatophores depends strongly on the mode of their preparation. Table 1 shows that membrane fractions having roughly the same hydrogenase activity could exhibit a 3-fold difference in their phosphorylating capacity depending on the way they were prepared. It is obvious that sonication was more deleterious for the phosphorylating capacity of chromatophores than treatment by the French press.

The *P/O* values obtained in this study for the oxidation of NADH and succinate by chromatophores of *Rps. capsulata*, strain B10, are similar to those obtained [5] with membranes prepared from aerobic cells of *Rps. capsulata*, strain St Louis. Values of *P/O* obtained for H_2 oxidation varied from 0.3–0.5 depending on the membrane preparation. A typical experiment is reported in table 2 where the same preparation of chromatophores was used to measure the rate of ATP synthesis in the dark with H_2 compared to NADH or succinate as electron donors. With all 3 substrates phosphorylation was practically completely abolished in presence of 2.5 μM FCCP indicating that ATP synthesis resulted from oxidative phosphorylation. Table 2 shows also that the rate of photophosphorylation (anaerobic) was 5–20-times higher than the rate of oxidative phosphorylation. The *P/O* ratio for H_2 oxidation (0.42) is roughly similar to that for NADH and twice as high as for succinate oxidation. As for other bacterial systems, these *P/O* values are low and do not permit a precise evaluation of the number of energy conserving sites. However our results suggest that electron paths from H_2 and NADH may involve one coupling site in excess of the number of sites for succinate oxidation.

The fact that H_2 oxidation is linked to ATP synthesis is not an unexpected result. It has indeed been shown that *Rps. capsulata* can grow in darkness in autotrophic conditions [1,2]. In aerobic autotrophic conditions, H_2 is the source of energy and reducing power for CO_2 reduction, while O_2 allows energy transduction at the level of the respiratory chain.

In conclusion, it is shown here that chromato-

Table 2
Phosphorylation coupled to respiration in chromatophores from *Rps. capsulata*

Substrate	FCCP (2.5 μM)	Oxidation rate (*O*) μatoms O_2 .h^{-1} .mg protein^{-1}	Phosphorylation rate (*P*) μmol ATP .h^{-1} .mg protein^{-1}	*P/O*
Aerobiosis in the dark				
H_2	–	2.5	1.02	0.42
(0.5 mM)	+	2.5	0.06	0.02
NADH	–	4.3	2.25	0.52
(1.4 mM)	+	5.0	0.06	0.01
Succinate	–	2.1	0.41	0.20
(14 mM)	+	2.4	0	0
Anaerobiosis				
Light	–	–	11.4	–
(~5000 lux)	+	–	1.1	–

A single preparation of freshly isolated chromatophores obtained after disruption of photosynthetically grown cells, by passage through a French press, was used for this experiment. Incubation of chromatophores with the phosphorylating medium was carried out in the respiration chamber as in section 2. The amount of ATP formed in absence of added substrate (0.11 μmol ATP .h^{-1} .mg protein^{-1}) has been subtracted from the values reported above. 1 mg protein/assay was used

phores from *Rps. capsulata* can carry out oxidative phosphorylation using H_2 as electron and energy source. Oxidation of H_2 is linked to the activity of hydrogenase; thus only membranes with high hydrogenase activity can actively use H_2 as a respiratory substrate. Similarly to the hydrogenase of *P. denitrificans* [9] the hydrogenase of *Rps. capsulata* can feed electrons to the respiratory chain, in the dark, by a rotenone-insensitive pathway. *Rps. capsulata* has a branched respiratory chain. We demonstrated here that electrons from H_2 can use both terminal oxidases. This is in agreement with the fact that mutants from *Rps. capsulata* lacking either one of the terminal oxidases can grow in chemoautotrophic conditions [1].

Work in progress aims at defining the site of interaction between the hydrogenase and the redox components of the electron transport chain.

Acknowledgements

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