HYDROGEN RECYCLING BY *RHODOPSEUDOMONAS CAPSULATA*

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1. Introduction

The photosynthetic bacterium, *Rhodopseudomonas capsulata* evolves large quantities of molecular H_2 via an energy-dependent nitrogenase activity when grown photoheterotrophically and supplied with certain amino acids, e.g., glutamate, as nitrogen source [1,2]. In addition, these bacteria are capable of utilizing H_2 as a reductant for photoautotrophic growth. H_2 uptake is mediated by a classical hydrogenase activity [3,4]. Aspects governing the control of H_2 metabolism in this bacterium have been recently reported by Hillmer and Gest [4]. However, as yet it has not been established whether there is a direct link between H_2 evolution and utilization under physiological conditions.

The coupling between nitrogenase and hydrogenase, as proposed by Dixon [5] in 1972, has since been demonstrated in a variety of N_2-fixing organisms including root nodule associations [6], *Azotobacter chroococcum* and *Klebsiella pneumoniae* [7], *Anabaena cylindrica* [8]; it has been briefly reviewed by Dixon [9]. Furthermore, a recent report [10] has shown that recycling of H_2 by hydrogenase enhances nitrogenase activity in cyanobacteria.

In the present communication, we have investigated H_2 production by *Rhodopseudomonas capsulata* under physiological conditions, using gas chromatographic and continuous amperometric techniques. We show that H_2 produced by nitrogenase is directly recycled by hydrogenase under conditions of low substrate concentration.

2. Materials and methods

2.1. *Cells and cultures*

Rhodopseudomonas capsulata strain B10 and W15 were generous gifts from the Photosynthetic Bacteria Group, Department of Microbiology, Indiana University, Bloomington, IN, 47401, USA. W15 is a nif$^-$ mutant derived from the wild type strain B10, and is incapable of light-dependent production of H_2 [3].

Cultures were grown in an RCV medium [2,11] supplemented with DL-lactate (30 mM) and L-glutamate (7 mM) or ammonium sulphate (7 mM) as carbon and nitrogen sources, respectively. Cultures were grown photosynthetically under anaerobic conditions at 32°C in modified 60 ml glass syringes [2] illuminated laterally with two, 100 W incandescent lamps (ca. 11 000 lux).

2.2. *Preparation of resting cell suspensions*

Unless otherwise stated, the following procedures were carried out under argon. Cultures were harvested at late exponential phase and centrifuged (25 000 × *g*, 30 min). The pellets were washed once with 10 mM K phosphate buffer (pH 6.8) and recentrifuged as above before finally resuspending in either a mineral base solution [2] or in the phosphate buffer. All solutions were pre-gassed (10 min) with argon before use. The resting cell suspension was stored under argon at 0°C until required.

2.3. *Measurement of H_2*

H_2 in the aqueous phase was measured continuously

using an amperometric technique as described by Wang et al. [12]. A Clark-type oxygen electrode (Hansatech Ltd, King's Lynn, Norfolk, England) was used. The cuvette (1 ml) was maintained at 27°C by circulated water and was stirred continuously. A potential of 0.6 V was continuously maintained across the electrode [6,12]. The apparatus was housed in a light-proof box and the cuvette illuminated with a slide projector from one side (ca. 1500 lux).

H_2 in the gas phase was monitored by gas chromatography. Reaction vessels were shaken at 30°C in a Warburg bath (B. Braun, Melsungen AG, FRG) uniformly illuminated by fourteen, 40 W incandescent lamps from below (ca. 5000 lux). Aliquots (50 μl) were withdrawn from the gas phase of the vials and injected into an Intersmat IGC 120 gas chromatograph (Intersmat Instruments, 93320 Pavillons-sous-Bois, France) having a thermal conductivity detector and provided with a Porapak Q column (80–100 mesh, 2 m × 3.2 mm). The results were quantitated by relating the peak heights to a calibration curve. Boiled bacterial cell controls, supplied with H_2, were included to correct for possible H_2 leakage.

2.4. *Other determinations*

Bacterial concentrations, expressed as dry weight of centrifuged cells, were determined according to Hillmer and Gest [2].

Lactate concentrations were determined enzymatically using lactate dehydrogenase [13].

3. Results

3.1. *H_2 production by growing cells*

Growing cultures of *Rhodopseudomonas capsulata* actively evolved H_2 during photosynthetic growth when supplemented with lactate (30 mM) and glutamate (7 mM) as carbon and nitrogen sources, respectively. There was no H_2 production in cultures growing with ammonium salts (7 mM) as nitrogen source. Similarly, a nif$^-$ mutant strain of *Rhodopseudomonas capsulata* (strain W15) did not produce H_2 when grown under optimal conditions for H_2 evolution. These results are in agreement with recent observations [2–4] that hydrogen evolution is mediated by nitrogenase in this bacterium.

3.2. *H_2 production and uptake by resting cells*

Light-dependent H_2 evolution was observed in washed resting cell suspensions of *Rhodopseudomonas capsulata* in the absence of added substrate, e.g., lactate (fig.1). This is presumably due to the presence of small amounts of endogenous substrate. Continuous amperometric monitoring revealed that the cessation of H_2 production (presumably due to substrate exhaustion) was followed by an immediate and rapid uptake of H_2 (fig.1). H_2 was produced and reutilized on subsequent additions of small amounts of substrate to the same sample, as shown in fig.1. The addition of ammonium salts (1 mM) resulted in a rapid cessation of the H_2 evolving activity with a corresponding uptake of H_2. There was no H_2 production after subsequent additions of substrate to the cuvette (fig.1).

A stoichiometry between lactate added and H_2 evolved was not always observed. Although the bacteria are grown photosynthetically, they always retain a small but significant respiratory activity as already reported by Lampe and Drews [14]. Thus, H_2 produced by nitrogenase could support respiration if trace amounts of oxygen were present in the cuvette. Prolonged incubation of the same sample (fig.1) would soon exhaust the O_2 present in the cuvette thus reducing H_2 uptake and permitting greater H_2 production by similar additions of lactate.

To verify this apparent H_2 recycling process, H_2

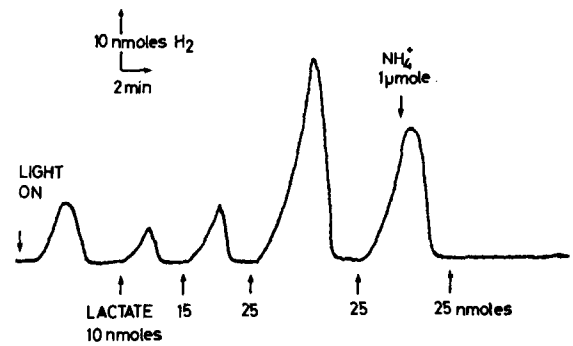

Fig.1. Continuous amperometric measurement of H_2 recycling by washed, resting, cell suspensions of *Rhodopseudomonas capsulata*. The trace is read from left to right. H_2 recycling due to endogenous substrate is shown at the left hand side of the trace. The reaction cuvette (1 ml) contained 3.1 mg dry wt bacterial suspension pre-gassed with argon. Additions to the cuvette were made via syringe in as small a volume as possible. Further details are described in Materials and methods.

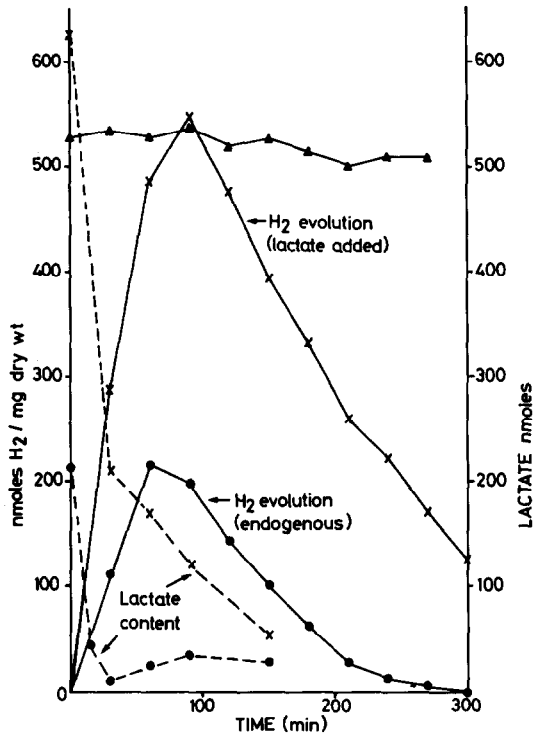

Fig.2. H_2 recycling by washed resting cell suspensions as monitored by gas chromatography. Reactions were carried out under argon 11 ml flasks fitted with gas-tight rubber septa (see Materials and methods). Reaction mixtures (2 ml) contained 9.8 mg dry wt bacteria plus lactate (200 μM) as indicated. Additional flasks were incubated under identical conditions and the reaction stopped and lactate concentration determined [13] at the indicated times: (●———●) H_2 production by cells without lactate (endogenous rate) and (● - - - - ●) corresponding lactate determination; (X———X) H_2 production by cells supplied with lactate (200 μM) and (X- - - - X) corresponding lactate determination. (▲———▲) represents a boiled bacterial cell control supplied with H_2.

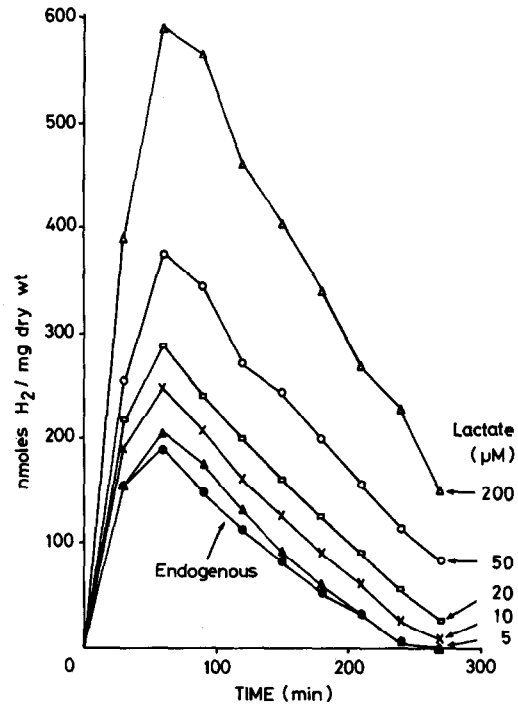

Fig.3. Effect of increasing concentrations of lactate on H_2 recycling by washed, resting, cell suspensions. Reaction mixtures (2 ml) contained 8.6 mg dry wt bacteria together with lactate at the indicated concentrations. Experimental conditions are as described for fig.2. (●———●) No added lactate (endogenous); (▲———▲) lactate (5 μM); (X———X) lactate (10 μM); (□———□) lactate (20 μM); (○———○) lactate (50 μM); (△———△) lactate (200 μM).

evolution by resting cells was monitored independently by gas chromatography. Figure 2 shows the H_2 output by bacteria with or without supplied lactate (200 μM). Residual lactate concentrations were determined under identical conditions of incubation (fig.2). H_2 recycling was again observed although over a longer time period in comparison to that measured by the amperometric technique. This may be a reflection of the lower availability to the cells of H_2 in the gaseous phase, as measured by gas chromatography, when compared to dissolved H_2 measured by the H_2-electrode (fig.1).

H_2 production by bacteria with or without added lactate (200 μM) was mirrored by a decrease in lactate concentration although small quantities of lactate are still present when photoproduction of H_2 has ceased (fig.2).

The effect of increasing concentrations of lactate on H_2 production and utilization is shown in fig.3. Increasing concentrations of lactate up to 200 μM resulted in higher rates of H_2 production but did not extend the time of H_2 evolution. At all lactate concentrations tested, H_2 utilization commenced after similar periods of H_2 evolution.

3.3. *Effect of CO*

In order to study the effect of CO on H_2 uptake, nitrogenase mediated H_2 production was blocked by

Table 1
Effect of CO on H_2 uptake by washed, resting, cell suspension of *Rhodopseudomonas capsulata*

%Co in gas phase	%Inhibition of H_2 uptake
0	0
5	7.5
10	9.7
15	16.1
20	19.9
25	22.6

Reaction mixtures (2 ml) contained 10.2 mg dry wt bacteria, lactate (200 μM) and ammonium sulphate (1 mM) to block nitrogenase activity [4]. The gas phase was argon together with CO at the indicated concentrations. The reaction was started by adding 1.1% H_2 to the gas phase. Experimental conditions are as described in fig.2.

including ammonium sulphate (1 mM) in reaction mixtures [4]. Increasing concentrations of CO resulted in increasing inhibition of H_2 utilization (table 1), which indicates that H_2 uptake is catalysed by a hydrogenase activity [15].

4. Discussion

Dixon [5] proposed that the efficiency of nitrogenase might be enhanced by a hydrogenase recycling electrons from substrate, lost through the H_2 evolving function of nitrogenase. There have since been several reports that there is a link between hydrogenase and nitrogenase in various N_2-fixing organisms [6–10]. The present study demonstrates that in the photosynthetic bacterium, *Rhodopseudomonas capsulata*, H_2 lost by nitrogenase can be reutilized via a hydrogenase activity. It has yet to be established whether this process may enhance nitrogenase activity in this bacterium. The recent reports of Schubert and Evans [6] and Tel-Or et al. [10] provide direct evidence that an uptake hydrogenase may support the overall nitrogenase activity of the organism. The large hydrogen output by *Rhodopseudomonas capsulata* during photoheterotrophic growth might suggest that if H_2 recycling does occur throughout the growth period, it represents a small or extremely inefficient process. However, under in vivo conditions, the nitrogenase would largely function to fix atmospheric N_2, thus greatly decreasing the H_2 output.

At present, it is not known whether hydrogen reutilization can occur throughout growth when excess substrate is present. Hillmer and Gest [4] have demonstrated that the utilization of H_2 for the photoreduction process in this bacterium is inhibited by substrate e.g., lactate. We have found that H_2 uptake is only observed at low substrate concentrations. This could be of physiological importance to the bacteria during growth in natural habitats. Rapid depletion of substrate could allow the expression of an autotrophic mode of growth through reutilization of the H_2 produced by nitrogenase. Thus, the interplay between nitrogenase and hydrogenase could also be of importance for cell maintenance under conditions of substrate limitation and in the transition between photoheterotrophic and photoautotrophic growth.

Acknowledgements

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