Relation between pH, Hydrogenase and Nitrogenase Activity, NH_4^+ Concentration and Hydrogen Production in Cultures of *Rhodobacter sulfidophilus*

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Rhodobacter sulfidophilus, a sulphide-tolerant, salt-tolerant member of the *Rhodospirillaceae*, has been shown to produce molecular hydrogen on a mineral medium with lactate as electron donor and glutamate as nitrogen source. A maximum production of about 1.1 mmol H_2 (mmol added lactate)⁻¹ was found at pH 6.75. The *in vivo* activity of hydrogenase and nitrogenase showed that hydrogenase activity (and thus the H_2 recycling system) was greatest at pH 6.5 and 6.75 and thus, at least partially, was responsible for the lack of H_2 production below 6.75, but that nitrogenase activity was greatest at between pH 6.5 and 7.0, and decreased to zero at pH 8.0, resulting in reduced H_2 production at pH 7.5 and none at pH 8.0. When sufficient NH_4^+ had accumulated in the culture, nitrogenase activity remained below the maximum value and no H_2 production occurred.

INTRODUCTION

The photosynthetic bacterium *Rhodospirillum rubrum* was first shown to produce molecular hydrogen under photosynthetic conditions by Gest & Kamen (1949). Since then a lot of research has been undertaken concerning hydrogen metabolism and in particular the enzymes involved, hydrogenase and nitrogenase. The results of these studies have been reviewed several times as cited by Vignais *et al.* (1985). Most of the research has been on *Rhodobacter capsulatus*, a rather vigorous hydrogen producer.

However, *Rhodobacter sulfidophilus* is also of interest, largely because of its growth capability with sulphides as electron donor and its high tolerance towards sulphides often present in industrial waste waters (Hansen & Veldkamp, 1973). Although they share common morphological and nutritional characteristics, the above-mentioned physiological differences between these *Rhodobacter* species are supported by other characters. DNA:rRNA hybridization data have shown that the two organisms are located at opposite borders of the same branch of rRNA superfamily IV (Gillis *et al.*, 1982), and their hydrogen metabolism differs with regard to H_2 production (Kelley *et al.*, 1979). Indeed, no net molecular hydrogen production had been observed with *Rh. sulfidophilus*; an active H_2 recycling system has been suggested to account for this (Kelley *et al.*, 1979). An H_2 recycling system, together with a high oxygen sensitivity of its nitrogenase, implied that *Rh. sulfidophilus* was not the cause of sunlight-dependent H_2 production from sulphide-rich waste waters (Kelley *et al.*, 1979). However, we (Stevens *et al.*, 1986) have shown that *Rh. sulfidophilus* is indeed able to produce H_2 , although to a lesser extent than *Rh. capsulatus*.

The role of hydrogenase and nitrogenase – the two key enzymes in H_2 metabolism – is not yet

Abbreviation: MB, methylene blue.

clear because the data available (Kelley *et al.*, 1979; Madigan *et al.*, 1984) refer to activities of the two enzymes in conditions that inhibit H_2 -evolution.

The present paper deals with the *in vivo* activity of the two enzymes at different pH values and at different stages of growth. The correlation between these activities and the presence of NH_4^+ in the culture was also studied.

METHODS

Bacterial strain and culture media. *Rhodobacter sulfidophilus* LMG 5202 (= W4) was used throughout. It was grown anaerobically at 30 °C in 1 l of a mineral salts medium (Stevens *et al.*, 1986) with 10 mM-potassium phosphate buffer, supplemented with 30 mM-sodium lactate and 7 mM-sodium glutamate. The fermenter was illuminated by six 60 W tubular tungsten lamps (Stevens *et al.*, 1986). During inoculation the culture was flushed continuously with helium; afterwards it was flushed for a further 30 min. The pH was kept constant at the desired value by a pH controller (LH Engineering). Samples were taken periodically; an equal amount of fresh, sterile medium containing 10 mM-sodium lactate was added at the same time.

Hydrogen detection. The gas produced was captured under a graduated cylinder inverted in water, and was analysed by gas chromatography (De Vos *et al.*, 1983).

Bacterial growth and cell mass determination. Bacterial growth was followed by measuring the optical density at 660 nm of the cell suspension (Beckman spectrophotometer G-2400). Dry weight determination was done according to the method described by Stevens *et al.* (1986).

Nitrogenase assay. This was done as follows. A culture sample (0.5 ml) was injected, under continuous argon sparging, into a 9 ml vial containing 0.5 ml 40 mM-sodium lactate, preflushed with argon. The vials were then fitted with rubber septa, thoroughly flushed with argon, vented to atmospheric pressure and incubated in a water bath at 30 °C, illuminated from below by seven 40 W incandescent lamps (about 6000 lx). After 10 min preincubation, 0.9 ml acetylene was injected into the gas phase. The vials were shaken periodically for a few minutes. At intervals, 25 µl gas samples were analysed by a gas chromatograph fitted with a flame ionization detector (Intersmat 120 M) [3.0 m stainless steel column, packed with Porapak R (80–100 mesh); carrier gas nitrogen (30 ml min⁻¹) at 50 °C; injector and detector temperature 120 °C]. Pure acetylene and ethylene were used as calibration standards.

Hydrogenase assay. H_2 -uptake hydrogenase activity was assayed by following the reduction of methylene blue (MB) in a spectrophotometer (Turner Model 350). A culture sample (1 ml), sparged continuously with argon, was diluted five to ten times with 10 mM-potassium phosphate buffer (pH 7.0); 0.5 ml of the diluted sample was injected into 9 ml optically homogeneous glass vials fitted with rubber septa and flushed with H_2 for about 10 min. Then 4 ml of a H_2 -saturated dye solution (0.2 mM-MB in 20 mM-Tris/HCl buffer (pH 8.0), or in 10 mM-potassium phosphate buffer (pH 6.5, 6.75, 7.0, 7.5 or 7.75) was added to the vial, which was then vented to atmospheric pressure. The reduction of MB was followed at 570 nm. Blanks flushed with argon instead of H_2 were included to evaluate non-specific reduction of MB.

Lactate determination. This was done according to the method described by Stevens *et al.* (1983).

NH_4^+ determination. After centrifugation of the culture samples at 12000 r.p.m. (Eppendorf 3200 microcentrifuge) for 2 min, the supernatants were stored at -20 °C. The NH_4^+ concentration was determined according to the phenol-hypochlorite method (Chaykin, 1969).

RESULTS AND DISCUSSION

All experiments were done at least twice. The results were reproducible. The results shown in Fig. 1(a–f) represent individual experiments, not means.

Growth

On the whole the shape of the growth curve was very similar at all pH values tested. The optical density always reached a maximum value of up to 2.5; the lag phase was less than 50 h; visually growth was greatest at pH 8.0. In all experiments the exponential phase was about 40 h. The mean value of the final dry weight of culture in each experiment was 4.5 mg ml⁻¹, except at pH 7.75 and 8.0 where dry weights of 5.3 and 5.7 respectively were measured.

Lactate consumption

Gas production started at the end of the exponential phase (Fig. 1) when only a reduced amount of the lactate added was available as electron donor; e.g. at pH 6.75 (Fig. 1) about half

Fig. 1. *Rh. sulfidophilus* growth (●), hydrogenase (▲) and nitrogenase (△) activity, and lactate consumption (○). Arrows indicate the start of H₂ production at different pH values (6.5–8.0, a–f) in a mineral salts medium containing 7 mM-glutamate and 30 mM-lactate.

the lactate was present in the culture when H₂ production started. This may contribute to the inability of *Rh. sulfidophilus* to produce as much H₂ as *Rh. capsulatus*, because with the latter H₂ production starts immediately at the beginning of the exponential phase (not shown) when all the electron donor is available.

H₂ production

We have already reported on net H₂ production by *Rh. sulfidophilus* (Stevens *et al.*, 1986). We have now confirmed and extended our previous conclusions on the pH dependence of this H₂ production, but with an improved experimental design. External conditions (e.g. fermenter design and thus culture mixing) seemed to affect optimum H₂ production. In our experiments H₂ production was limited to a very narrow pH range – 6.75 to 7.75 (Fig. 2). It was at a maximum [about 1.1 mmol H₂ (mmol added lactate)⁻¹] at pH 6.75, and decreased towards pH 7.75. Gas production occurred neither at pH 6.5 (even for incubation periods of 1 week) nor at pH 8.0.

The two most important enzymes for hydrogen metabolism in the photosynthetic bacteria are a hydrogenase responsible for hydrogen uptake and a nitrogenase responsible for production of molecular hydrogen. If both systems are expressed, the balance between the activities of the enzymes will determine the total amount of H₂ produced and its production rate. We followed therefore hydrogenase and nitrogenase activities during growth at different pH values.

Fig. 2. H₂ production as a function of pH during growth of *Rh. sulfidophilus*.

Hydrogenase and nitrogenase activity

We determined hydrogenase activity (by MB reduction) at the same pH as the fermentation experiment, and at pH 8.0, which is near the *in vitro* optimum for the H₂-uptake hydrogenase activity of *Rh. capsulatus* (Colbeau *et al.*, 1978). The activities were always the same at the two pH values and only the former determinations are shown (Fig. 1). The hydrogenase activity [given as nmol MB reduced min⁻¹ (mg dry wt)⁻¹] correlated with the pH of the fermentation. At the lower end of the pH range tested (pH 6.5 and 6.75) the hydrogenase had a maximum activity of 220; it decreased to about 70 at pH 7.0 or 7.5 and to about 30 at pH 7.75 or 8.0. When a hydrogenase activity of 220 (pH 6.5 and 6.75) was observed, maximum H₂ recycling could be expected. In both experiments this would result in lower H₂ production if the hydrogenase and nitrogenase activities and the lactate consumption all changed similarly as a function of time. This was true only for lactate consumption (Fig. 1*a, b*) and not for hydrogenase and nitrogenase activities. Indeed, at pH 6.5 hydrogenase activity reached a maximum at the end of the exponential phase; in fact the activity curve was completely parallel with the growth curve. At pH 6.75 hydrogenase activity remained low during the whole fermentation and the (high) maximum value was reached when lactate starvation had occurred in the late exponential phase. The nitrogenase activity was also quite different in the two experiments. It was greater at pH 6.75 than at pH 6.5 (Fig. 1*a, b*) during the whole fermentation. As a result, the H₂ recycling system was more active than the H₂ producing system at pH 6.5 and the opposite was true at pH 6.75; therefore net H₂ production occurred at pH 6.75 and not at pH 6.5.

At the other pH values, the maximum hydrogenase activities were lower: three times lower at pH 7.0–7.5 and seven times lower at pH 7.75–8.0 (Fig. 1*c–f*). H₂ recycling was thus less important under these conditions. However, the maximum nitrogenase activity was rather high only at pH 7.0 [3.4 nmol ethylene min⁻¹ (mg dry wt)⁻¹]. This resulted in significant H₂ production (17.3 mmol) at the latter pH. In spite of low nitrogenase activity at pH 7.5 [1.74 nmol ethylene min⁻¹ (mg dry wt)⁻¹] a considerable amount (22.4 mmol) of H₂ was produced. The reason for this may be a slower breakdown of lactate. Indeed, when gas production started, the lactate concentration was 16 mM at pH 7.5 but only 2 mM at pH 7.75. This might explain the low H₂ production at pH 7.75. At pH 8.0 no nitrogenase activity was observed, and therefore no net H₂ production occurred.

Effect of NH₄⁺

Low NH₄⁺ concentrations (30 μM) inhibit nitrogenase activity in *Rh. capsulatus* (Meyer *et al.*, 1978). During growth of *Rh. sulfidophilus* on lactate and glutamate substantial amounts of NH₄⁺ were found during the exponential phase at all pH values tested. From pH 6.5–7.75 NH₄⁺ concentrations varied from 17–71 μM at the beginning of the exponential phase. The NH₄⁺

concentration decreased gradually to zero; then H₂ production started except at pH 6.5 where no net H₂ production could be detected for the reasons stated above. At pH 8.0, the NH₄⁺ concentration increased from 52 µM (beginning of the exponential phase) to 214 µM (end of the experiment). Therefore the nitrogenase was totally inhibited. As *Rh. sulfidophilus* can grow on glutamate as sole nitrogen and carbon source (data not shown), NH₄⁺ probably originates from the deamination of glutamate, thus resulting in lower H₂ production by inhibition of the nitrogenase activity.

The overall regulation of H₂ production in *Rh. sulfidophilus* is therefore similar to that in *Rh. capsulatus*. Net production of molecular hydrogen will occur if (1) the balance of the activities of the two enzymes involved – hydrogenase and nitrogenase – is in favour of nitrogenase, (2) the NH₄⁺ concentration is low enough to allow nitrogenase synthesis and substantial activity, and (3) an electron and proton donor is still available for H₂ production. These conditions are only realized for *Rh. sulfidophilus* in an appropriate medium with lactate as electron and proton donor in a narrow pH range between 6.75 and 7.75; it is therefore not surprising that previous research overlooked the expression of this character in this photosynthetic bacterium.

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