Photoproduction of Hydrogen by Purple Bacteria: A Critical Evaluation of the Rate Limiting Enzymatic Steps

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The enzymatic mechanisms and energetics of nitrogenase-catalyzed photoproduction of hydrogen from organic C-compounds by purple bacteria are discussed in respect to the question of which of the following three enzymes or enzyme systems are rate limiting for H_2-production: (a) the nitrogenase-complex; (b) the enzymes and electron transport proteins involved in hydrogen transfer from the organic substrate(s) to nitrogenase; and (c) the system of photosynthetic ATP-regeneration. Calculations of maximum *in vivo* rates of photosynthetic ATP-regeneration (q_{ATP}-values derived from growth rates), and of ATP-consumption by nitrogenase-catalyzed H_2-production, and comparison of these rates with the specific activities of the enzymes of hydrogen or electron transfer from the C-substrate to the nitrogenase-complex, make it very likely that, in *Rhodospirillum rubrum,* nitrogenase-catalyzed H_2-formation is limited by the availability of ATP and, possibly, of reducing power. In two other nonsulfur purple bacterial species (*Rhodobacter capsulatus* and *Rhodobacter sphaeroides*), H_2-photoproduction is probably not energy-limited.

Introduction

The biocatalyst responsible for photosynthetic H_2-liberation from organic C-compounds in purple bacteria is the N_2-fixing nitrogenase complex [1, 2]. This enzyme system is composed of two functionally and structurally different components (dinitrogenase reductase and N_2-reductase) and catalyzes the following reaction:

$$8\,H^+ + 8\,e^- + N_2 + 16\,ATP \longrightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,P_i.$$

As shown in the equation, nitrogenase-activity consumes a large amount of ATP which, under light-anaerobic conditions, is produced by the cellular photosynthetic apparatus. The reducing equivalents required for N_2-reduction are derived from the organic substrate(s) and are transported to the nitrogenase system by the catalytic action of special dehydrogenases and electron transport carriers. As a by-product of N_2-reduction, about a quarter of the reducing power is liberated as gaseous hydrogen. In the absence of N_2, all reducing equivalents transported to the nitrogenase complex are liberated as H_2.

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Metabolic antagonist of the nitrogenase system is the H_2-uptake hydrogenase (Hup), a membrane-bound Ni-dependent enzyme in all purple bacterial species investigated so far [3–7]. In the presence of cell-internal electron sinks (CO_2, a.o.), the Hup hydrogenase is able to "recycle" a considerable fraction of the hydrogen evolved by the nitrogenase system [8, 9].

The two purple bacteria *R. rubrum* and *Rb. capsulatus* were shown to contain two different N_2-fixing enzymes: the classical molybdenum-dependent (Nif) and a heterometal-free alternative system (Anf) [10, 11]. In *Rb. capsulatus,* more than 30 different genes were found to be involved in the biosynthesis of the Nif complex [12]. The *nif* genes are regulated by ammonium, oxygen, and, in the case of the alternative nitrogenase system, also by molybdenum. The initiation of *nif* expression seems to be activated by a specific regulatory protein (NifA), the biosynthesis of which is in turn controlled by a specific gene system repressed by ammonium [13].

Rate Limiting Steps in Photosynthetic H_2-Evolution

At nearly saturating illumination with tungsten-lamps (light-intensity of 10 klux, corresponding to about 400 W/m^2), wild type strains of purple bacteria catalyze H_2-photoproduction with rates of

30–130 ml H_2/h × g biomass and H_2-yields of 50–70% in relation to the H-content of the organic C-compound used as electron donor [14–16].

From a biotechnical point of view, it would be of great interest to achieve an increase of rate and yield of H_2-photoproduction by optimizing the culture conditions and/or the genetic constitution of appropriate bacterial strains. While elimination of Hup hydrogenase, either by mutation of *hup* genes or by growing the cells in the presence of 0.5 mM EDTA, increases the yield of H_2-photoproduction by *Rb. capsulatus* and *R. rubrum* [17, 18], mutations which lead to the inactivation of the poly-β-hydroxybutyric acid synthesizing enzyme system do not have much effect on H_2-photoproduction by *R. rubrum* and *Rb. sphaeroides* [19]. Similarly, the capacity of cells to synthesize polyglucosides has no great inhibitory influence on rate and yield of photosynthetic H_2-liberation [20].

Under conditions of missing Hup activity (either by cultivation of cells in the presence of EDTA or by using *hup*$^-$ strains), the rate of H_2-photoproduction is governed by the activity of one or more of the following enzyme systems: (i) the nitrogenase complex; (ii) the electron transport-chain to nitrogenase; and (iii) the photosynthetic ADP-phosphorylation system. The presence of an active nitrogenase system (either Nif or Anf) is absolutely necessary for H_2-photoproduction. Wild type species without nitrogenase (for example, *Rhodocyclus purpureus* [21]) as well as nitrogenase-negative mutants [2, 16] are unable to photoproduce hydrogen. In addition, wild type strains of nitrogenase-positive species have been isolated which express nitrogenase at a very low level (for example, some *Rb. capsulatus*-strains [22]). It is reasonable to assume that, in these strains, the rate of H_2-photoproduction is limited by the restricted nitrogenase activity. However, in organisms with a normally high nitrogenase-activity, the *in vivo*-rates of electron transfer to nitrogenase and of photosynthetic ATP-regeneration may be rate-limiting for H_2-production.

The cellular electron donors to the nitrogenase complex (N_2ase) must be compounds with redox potentials of about −400 mV (ferredoxins or flavodoxins). Presently, most researchers agree that a special ferredoxin (Fd) is the reductant of the nitrogenase complex [23, 24]. Since the light-reaction of purple bacteria seems to be incapable of directly reducing a stable compound with a potential more negative than −200 mV [25], the electron flow from the growth substrate or from a compound of intermediary metabolism to ferredoxin (E_o of −420 mV) is, very likely, energy-dependent. The following sequence of dehydrogenases and/or electron carriers may be postulated:

Substrate → NADH →
NADH-Fd-oxidoreductase → Fd →
N_2ase complex.

Some of the dehydrogenases at the "substrate-site" have been studied with respect to structure, function and genetic mechanisms. As examples, the enzymes required for lactate metabolism by *R. rubrum* shall be discussed. *R. rubrum* contains two membrane-bound lactate dehydrogenases (one specific for D-lactate, the other for L-lactate) which are independent of NAD(P) and are present in cell extracts at moderately high activities (0.02 and 0.035 μmol/min × mg protein, respectively, at 30 °C) [26]. A specific activity of about 0.03 μmol/min × mg protein would be sufficient to ensure carbon assimilation from lactate by a *R. rubrum* population growing at maximal rate (0.15 h^{-1}). The breakdown of the C_3-skeleton to CO_2 proceeds *via* operation of pyruvate dehydrogenase and the anaerobic tricarboxylic acid cycle (TCC). The pyruvate dehydrogenase complex of *R. rubrum* is associated with the intracytoplasmatic membrane fraction (specific *in vitro* activity of about 0.05–0.10 μmol/min × mg protein at 30 °C) and resembles the enzyme complex from other bacteria in respect to its catalytic and regulatory properties as well as its molecular architecture [27, 28]. Other TCC enzymes analyzed in *R. rubrum* (citrate synthase, succinate dehydrogenase, malate dehydrogenase [29–31]) are present at specific activities (about 0.20, 0.15 and 1.20 μmol/min × mg protein) which are not rate limiting for growth and H_2-production at maximum velocities (see Table I). Final proof of the existence of a NADH-Fd-oxidoreductase in purple bacteria is still lacking. Although a novel FAD-protein which effectively catalyzes reduction of methyl viologen by NADH was purified from *R. rubrum* [32], the possible connection of that enzyme with one or both of the two cytoplasmatic ferredoxins of that organism [33] remains obscure. Furthermore, the specific *in vitro* activity of the FAD-protein (about

0.03 μmol/min × mg protein) would be too low to ensure *in vivo* rates of nitrogenase-catalyzed acetylene reduction of about 0.08 μmol/min × mg biomass (see Table I).

Calculation of Cellular Rates of ATP-Regeneration (q_{ATP}) and Comparison of q_{ATP}-Values with Experimentally Measured Rates of H_2-Formation

It can be anticipated that, when nitrogenase activity and electron flow to nitrogenase are not limiting, H_2-photoproduction is controlled by the rate of photosynthetic ATP-regeneration. In order to make an estimation of cellular rates of photosynthetic ATP-regeneration in purple bacteria, the *in vitro*-rates measured with fragments (= "chromatophores") of the intracytoplasmatic membranes are not very useful because of the uncertainty concerning the extent of irreversible inactivation of the phosphorylation system occurring during cell rupture.

Cellular rates of ATP-regeneration (q_{ATP}) can be calculated from the growth rates (μ) of the bacterial cultures by using an appropriate conversion factor. For two purple bacteria (*Rb. capsulatus* and *R. rubrum*), the relationships between light intensity and rates of growth and of H_2-photoproduction are known [14, 15, 34, 35]. By plotting rate against light intensity, one obtains hyperbolic curves (Fig. 1) from which the maximum rates can be extrapolated by using double-reciprocal plots.

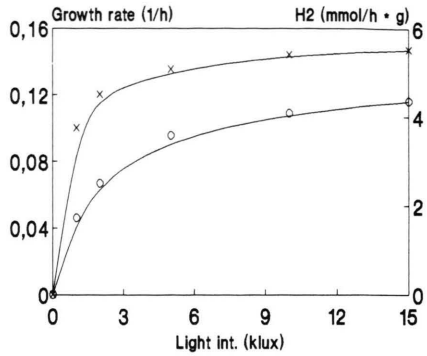

Fig. 1. Growth rate (μ) and rate of H_2-photoproduction by *Rhodospirillum rubrum* S 1 measured at 30 °C with various light intensities (klux) provided by tungsten lamps. Data refer to own measurements [34] and experiments of other authors [15, 35]. Upper curve (×): growth rate (h^{-1}); lower curve (○): H_2-photoproduction (mmol H_2/h × g biomass) measured with resting cells harvested from cultures grown at the same light intensity as used for the H_2-production experiment. Reproducibility of data is within a 10 to 15% range.

Table I. Maximum growth rates, rates of H_2-photoproduction and nitrogenase (N_2ase) activities of non-sulfur purple bacteria.

Species	Maximum growth rate[a] [h^{-1}]	Maximum rate of H_2-photoproduction[b] [mmol/h × g biomass]	N_2ase activity[c]	Reference(s)
R. rubrum S 1	0.15 ± 0.02	4.9 ± 0.6	4.7 ± 0.36	[15, 34, 35]
Rb. capsulatus Z 1	0.38 ± 0.05	6.0 ± 0.7	–	[14]
Rb. capsulatus J 2 (deriv. of B 10)	0.32 ± 0.04	3.6 ± 0.4	5.1 ± 0.38	[14]
Rb. sphaeroides 2.4.1	0.18 ± 0.02	1.9 ± 0.2	2.2 ± 0.11	[21, 43]

[a] Maximum growth rates (μ_{max}) were calculated from experimental data showing the relation between growth rate and light intensity (compare Fig. 1) during photosynthetic growth (*R. rubrum, Rb. capsulatus*) or taken directly as the value measured at a light intensity of 10 klux (*Rb. sphaeroides*). The organisms were grown at 30 °C (*R. rubrum, Rb. sphaeroides*) or 34 °C (*Rb. capsulatus*) in defined media with 30–50 mM lactate as C-source and 7–10 mM glutamate as N-source.

[b] Maximum rates of H_2-photoproduction (q_{H_2}) from lactate were calculated from experimental data showing the relation between rate of H_2-photoproduction and light intensity at a given temperature (*R. rubrum, Rb. capsulatus*) or taken directly as the value measured at a light intensity of 10 klux (*Rb. sphaeroides*). H_2-photoproduction was measured with resting cells at the temperatures used for cellular growth.

[c] N_2ase activities of whole cells were determined by measuring the capacity of resting cells to reduce acetylene to ethylene with lactate as electron donor (30 °C; light intensity: 10 klux, tungsten lamps).

As shown in Fig. 1, the growth rate μ and the rate of H_2-photoproduction (q_{H_2}) of *R. rubrum* S 1 approach their maximum values at illumination with tungsten lamps at intensities of greater than 10 klux (corresponding to about 400 W/m²). The maximum rates of photosynthetic growth (0.15–0.38 h^{-1}) of 3 different species of nonsulfur purple bacteria *(R. rubrum, Rb. capsulatus, Rb. sphaeroides)* are compared with the maximum rates of H_2-photoproduction and cellular nitrogenase activities (Table I). Rates of H_2-photoproduction vary from about 2 to 6 mmol H_2/h × g biomass and correlate quite well with the nitrogenase-activities (2.2–5.1 mmol ethylene/h × g biomass) measured at near-saturating light intensity (10 klux) with resting cells.

According to Pirt [36] the relationship between the energy requirement R and the growth rate μ of a microbial population can be described by the following equation:

$$R = R_{min} + m_e/\mu.$$

The energy requirement per gram of biomass synthesized consists of the energy fraction required for the formation of new cell material (R_{min}) and the fraction required for maintenance (m_e/μ). The maintenance coefficient (m_e) is a constant specific for a given organism growing under given conditions. By using the derivative formula

$$q_{ATP} = \mu \times 1/Y(ATP)_{max} + m_e$$

(where q_{ATP} is the product of R and μ, and $Y(ATP)_{max}$ is the reciprocal of R_{min}) one can calculate the cellular rate of ATP-production (q_{ATP}) for any growth rate, provided the numerical values of m_e and $Y(ATP)_{max}$ are known. For heterotrophic bacteria growing in lactate-minimal medium, $Y(ATP)_{max}$ was calculated to be 13.4 g biomass/mol ATP [37]. For phototrophic bacteria, the latter value has been corrected to be 8 g biomass/mol ATP [38]. As a fairly good approximation for m_e, a value of 4 mmol/h × g biomass will be used [38].

As shown in Table II, the q_{ATP}-values vary from 23 ± 3 to 52 ± 6 mmol ATP/h × g biomass. Using the specific bacteriochlorophyll (BChl) content of the organism (μg BChl per mg biomass) under the given culture conditions, one can convert the q_{ATP}-values to the more familiar rates of photosynthetic phosphorylation in terms of μmol ATP/h × mg BChl. For *R. rubrum* S 1 with a specific BChl-content of 2 μg/mg biomass at light-saturation [34], phosphorylation rates of about 10×10^3 μmol ATP/h × mg BChl are obtained. Note that maximal *in vitro*-rates of cyclic photophosphorylation with chromatophore preparations from *R. rubrum* and *Rb. capsulatus* are only about 800 or 450 μmol ATP/h × mg BChl, respectively [39, 40].

Table II. Maximum cellular rates of photosynthetic ATP-production (q_{ATP}), calculated from the μ_{max}-values, and minimum energy requirement for maximum rates of H_2-photoproduction from lactate in non-sulfur purple bacteria. q_{ATP}-Values are calculated from the growth rates (μ) given in Table I by using the formula: $q_{ATP} = \mu \times 1/Y(ATP)_{max} + m_e$. (For values of constants $Y(ATP)_{max}$ and m_e, see last chapter.)

Species	q_{ATP}	Energy requirement for maximum rate of H_2-photoproduction [mmol ATP/h × g biomass]
R. rubrum S 1	23 ± 3	19.6 ± 2.2
Rb. capsulatus Z 1	52 ± 6	24.0 ± 2.9
Rb. capsulatus J 2 (B 10)	46 ± 5	14.4 ± 1.7
Rb. sphaeroides 2.4.1	28 ± 3	7.6 ± 0.9

To see if rates of cellular ATP-regeneration could be limiting for H_2-photoproduction at light saturation, one should remember that at least 4 mol ATP is required per mol of H_2 liberated by the nitrogenase complex [41]. Thus, the minimum energy requirement for H_2-photoproduction can be calculated by multiplying the q_{H_2}-values of Table I by a factor of 4. Inspection of Table II shows a clear difference between *R. rubrum* S 1 and the *Rhodobacter* strains. In *R. rubrum* S 1, the maximum q_{ATP} (23 ± 3 mmol/h × g biomass) is of the same order of magnitude as the energy requirement of H_2-production (minimum of 19.6 ± 2.2 mmol ATP/min × g biomass). However, in the three *Rhodobacter* strains, the q_{ATP}-values are significantly higher (factors of 2 to 3) than the maximum energy requirements of H_2-photoproduction. This may be interpreted to mean that, in *R. rubrum* S 1, H_2-photoproduction at light-saturating conditions is probably limited by the rate of cellular ATP-regeneration. Thus, by using this organism, there would be no great chance to increase the H_2-output by genetic or physiological improvements of nitrogenase expression. However, in organisms of the second group with much higher maximum *in vivo* rates of cellular ATP-regeneration, an

increase of the nitrogenase activity, possibly by enhancing *nif* (*anf*) expression and by mutational inactivation of the systems responsible for repression of nitrogenase genes, and/or an optimization of the energy and electron flow to nitrogenase, would probably improve the capacity for photosynthetic H_2-production.

The calculations show that, in purple bacteria with high cellular levels of nitrogenase (like *R. rubrum*), the rate of H_2-photoproduction depends primarily on the rate of cellular ATP-regeneration. Biotechnical efforts to increase the efficiency of bacterial H_2-photoproduction must therefore not only focus on a genetic engineering of the nitrogenase system but must also take into account the optimization of the energy and electron flux to the nitrogenase system. By carefully optimizing the genetic constitution of appropriate bacterial strains and by controlling the cultural and biophysical conditions it may be possible to increase the maximum productivity rates of purple bacterial H_2-bioreactors from, at present, 2–4 [42] to about 8–12 l $H_2/h \times m^2$ at subsaturating light intensity.

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