Hydrogen metabolism of mutant forms of *Anabaena variabilis* in continuous cultures and under nutritional stress

D.A. Sveshnikov a, N.V. Sveshnikova b, K.K. Rao a, D.O. Hall a, *

* Division of Life Sciences, King’s College London, Campden Hill Road, London W8 7AH, UK
b Department of Plant Physiology, Biological Faculty, M.V. Lomonosov Moscow State University, Vorobyevy Gory, 119899 Moscow, Russian Federation

Received 20 September 1996; revised 10 December 1996; accepted 20 December 1996

Abstract

Nitrogenase activity and H₂ evolution were studied in two mutants of the cyanobacterium *Anabaena variabilis* ATCC 29413 which are impaired in molecular H₂-related metabolism. Evidence was obtained that mutants deficient in uptake and reversible hydrogenases were suitable for biotechnological research on H₂ production. H₂ production by the mutant PK84 in continuous cultures was 4.3 times higher compared to the wild-type. Enhancement in H₂ evolution by all the cultures under N₂ (1.8–1.9 times) and CO₂ starvation (1.4–1.5 times) was observed.

**Keywords:** Hydrogen metabolism; *Anabaena variabilis*; Mutant form

1. Introduction

H₂ production using biological materials is one of the goals of renewable energy technology. H₂ evolution is a conservative process inherent in most N₂-fixing microorganisms and involves the two enzymes – nitrogenase and reversible hydrogenase [1]. Cyanobacteria are suited for biotechnological H₂ production as they are the only photoautotrophic N₂-fixers capable of producing molecular H₂ with H₂O as the electron source and they are stable towards various stress factors [2–7].

Several species of cyanobacteria are presently being used in H₂ photoproduction research and development, *Anabaena variabilis* among them [8]. In addition to simple selection of the strains, genetic methods are being applied to create mutants with altered nitrogenase or hydrogenase systems for enhanced H₂ production capacity. Furthermore, physiological manipulation can be used for optimising H₂ production [9].

Nitrogenase activity and H₂ production in three strains of *A. variabilis* (the wild-type and two mutant forms) are presented here using continuous cultures during the exponential growth phase in a steady-state mode. This phase is characterised by the highest activities of the enzymes involved in H₂ metabolism and is of interest for the practical cultivation of various cyanobacterial species [5,6,8,10,11]. In the present research CO₂ or N₂ starvation was used to establish the possibility of regulation of the H₂ metabolism of *A. variabilis* both for basic knowledge and as a background for applied biotechnology.
2. Materials and methods

2.1. Bacteria used

Three strains of the cyanobacterium *A. variabilis* ATCC 29413 were used: the wild-type (the initial form) and two new chemically generated mutant forms, PK84 and PK17R, provided by Professor S.V. Shestakov and Dr. L.A. Mikheeva of Moscow State University, Department of Genetics. The mutations affected regulation of the enzymes of H₂ metabolism, thereby enhancing yields of molecular H₂ evolution. Reversible hydrogenase activity was impaired in the mutant PK84, and both forms were deficient in uptake hydrogenase [12]. However, physiological parameters such as nitrogenase activity, growth rates, heterocyst frequency, etc. were similar in all three forms [12].

2.2. Growth conditions

Continuous growth of the cyanobacteria was carried out in thermostated 350 ml glass bioreactors equipped with a pH-control system and permanently illuminated with daylight fluorescent lamps at a limiting light intensity of 90 μE m⁻² s⁻¹ [13]. The dilution rate was 0.03 h⁻¹. The gas mixture contained 25% N₂, 2% CO₂, and 73% Ar. Total gas flow was 250 ml min⁻¹. Metabolic stress conditions were achieved by lowering the N₂ content to 5% or switching off the CO₂ supply. Modified nitrogen-free Allen and Arnon medium [14] was used for N₂-fixing growth. A pH of 7.5 was maintained automatically by addition of NaOH.

2.3. Heterocyst frequency, assays of chlorophyll, protein and enzyme activities

Heterocysts were counted visually under a microscope taking not less than 500 cells at a time. The frequency in Tables 1–3 is given in percent related to the total number of cells counted.

Chlorophyll content was determined in methanol extracts prepared by incubation of cells in 90% methanol for 3 min at 75°C [15]. An optical density (OD₆₆₅) of 1 corresponded to 13.4 μg Chl a ml⁻¹ of the culture.

Protein content was determined by the method of Bradford [16]. One unit of optical density (OD₅₉₅) corresponded to 1.3 μg of protein ml⁻¹. All the reagents were the highest grades commercially available.

Enzyme activities in whole cells were determined by assaying H₂ photoproduction and C₂H₂ reduction. The gases were monitored by gas chromatography (Hewlett Packard 5890) after incubation of the samples in glass vials at 30°C for 30 min under daylight lamp fluorescent illumination (140 μE s⁻¹ m⁻²). The gas phase for H₂ evolution assay contained only Ar, whereas C₂H₂ was added at 10% (v/v) for the C₂H₂ reduction assay and the C₂H₄ produced by nitrogenase was detected.

3. Results and discussion

Continuous cultivation is often used in biotechnology for maintaining cultures in the exponential phase of growth, obtaining higher yields of biomass and higher enzyme activity. We have chosen this method since the maximal activity of nitrogenase occurs during the exponential growth phase [8], and it is known that hydrogenase activities in the mutants increased

<table>
<thead>
<tr>
<th>Strain</th>
<th>Heterocysts (%)</th>
<th>Chl (μg ml⁻¹)</th>
<th>Protein (μg ml⁻¹)</th>
<th>Dry weight (μg ml⁻¹)</th>
<th>H₂ production (nmol μg prot⁻¹ h⁻¹)</th>
<th>C₂H₂ production (nmol μg prot⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W.T.</td>
<td>6.4</td>
<td>2.75</td>
<td>34.4</td>
<td>151</td>
<td>1.62</td>
<td>2.84</td>
</tr>
<tr>
<td>PK84</td>
<td>6.8</td>
<td>3.07</td>
<td>33.1</td>
<td>145</td>
<td>6.91</td>
<td>3.66</td>
</tr>
<tr>
<td>PK17R</td>
<td>6.6</td>
<td>2.93</td>
<td>33.5</td>
<td>148</td>
<td>2.24</td>
<td>3.25</td>
</tr>
</tbody>
</table>
Table 2
Maximal effect of nitrogen starvation (5% N₂) upon growth parameters and enzymatic activity of the wild-type (W.T.) and two mutants (PK84 and PK17R) of *Anabaena variabilis* measured at 48 h after starting the treatment (standard deviation did not exceed 10% of the values)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Heterocysts (%)</th>
<th>Chl (µg ml⁻¹)</th>
<th>Protein (µg ml⁻¹)</th>
<th>Dry weight (µg ml⁻¹)</th>
<th>H₂ production (nmol µg prot⁻¹ h⁻¹)</th>
<th>C₂H₂ production (nmol µg prot⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W.T.</td>
<td>10.2</td>
<td>1.93</td>
<td>28.4</td>
<td>121</td>
<td>3.07</td>
<td>6.32</td>
</tr>
<tr>
<td>PK84</td>
<td>10.0</td>
<td>2.12</td>
<td>28.2</td>
<td>116</td>
<td>12.60</td>
<td>6.44</td>
</tr>
<tr>
<td>PK17R</td>
<td>10.0</td>
<td>2.03</td>
<td>29.3</td>
<td>118</td>
<td>4.10</td>
<td>6.32</td>
</tr>
</tbody>
</table>

at later stages of growth [12]. The wild-type and two mutants were maintained for not less than three doubling times in chemostats as light-limited continuous cultures in order to establish equivalent conditions for the cultures before the treatment with low levels of N₂ and CO₂.

3.1. Growth parameters in continuous cultures

The data on biomass accumulation (dry weight), protein content, chlorophyll concentration and heterocyst frequency showed that both mutants did not exhibit any noticeable changes in their physiological properties compared to the wild-type while growing as continuous cultures under the same conditions (Table 1). The results were in agreement with the preliminary data obtained for the strains in batch cultures [12] and confirmed the viability of the mutants.

3.2. Hydrogen metabolism

3.2.1. Nitrogenase activity of the cells during continuous cultivation

Two aspects of the reducing function of nitrogenase were studied – the reduction of C₂H₂ (as a substrate equivalent to molecular N₂) to C₂H₄ and molecular H₂ evolution.

The data showed clear differences between the three strains of *A. variabilis* in the quantitative yield of the gases produced by nitrogenase in continuous cultures (Table 1). Both mutant forms showed higher rates of H₂ evolution than the wild-type. The most pronounced differences were obtained between the mutant PK84 and the wild-type: this mutant evolved 4.3 times more H₂ than the wild-type (Table 1), whereas the amounts of H₂ evolved by the PK17R mutant strain differed from the wild-type by 1.4 times (Table 1).

In the case of C₂H₂ reduction (Table 1) the differences between the enzyme activities in different strains during continuous cultivation were less pronounced, although the nitrogenase activity in the mutants was still slightly higher than in the wild-type (about 1.2 times). The impaired H₂ uptake activity of hydrogenases [12] is thus the probable cause of the higher H₂ evolution rates observed in the mutants.

3.2.2. The influence of nitrogen and carbon deficiency

Stress factors (including starvation by the main nutrients – carbon and nitrogen) can lead to a temporary increase of the activities of enzymes involved in the main metabolic pathways. This hypothesis was tested by placing the cyanobacteria under nutritional stress of N₂ and CO₂ starvation.

Table 3
Maximal effect of carbon starvation (0% CO₂) upon growth parameters and enzymatic activity of continuous cultures of wild-type (W.T.) and two mutants (PK84 and PK17R) of *Anabaena variabilis* measured at 24 h after starting the treatment (standard deviation did not exceed 10% of the values)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Heterocysts (%)</th>
<th>Chl (µg ml⁻¹)</th>
<th>Protein (µg ml⁻¹)</th>
<th>Dry weight (µg ml⁻¹)</th>
<th>H₂ production (nmol µg prot⁻¹ h⁻¹)</th>
<th>C₂H₂ production (nmol µg prot⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W.T.</td>
<td>7.2</td>
<td>1.76</td>
<td>30.9</td>
<td>136</td>
<td>2.37</td>
<td>4.77</td>
</tr>
<tr>
<td>PK84</td>
<td>7.4</td>
<td>1.98</td>
<td>29.5</td>
<td>131</td>
<td>10.85</td>
<td>5.30</td>
</tr>
<tr>
<td>PK17R</td>
<td>7.0</td>
<td>1.89</td>
<td>29.9</td>
<td>133</td>
<td>3.39</td>
<td>5.52</td>
</tr>
</tbody>
</table>
3.2.2.1. Nitrogen deficiency. After stable growth in continuous cultures had reached the N₂ content of the gas phase was decreased from 25% to 5%. This decrease in N₂ supply produced its effect after 24 h with a maximal response after 48 h (Table 2). The nitrogenase activity increased rapidly with the patterns similar for each strain. H₂ production was 1.9 times higher in the wild-type and about 1.8 times higher in PK84 and in PK17R. The patterns of C₂H₂ reduction were also quite similar: 2.2 times increase in the wild-type, and about 1.8 times increase in both mutants (Table 2 versus Table 1).

All the other features – dry weight, protein content, chlorophyll concentration – decreased considerably 48 h after the beginning of the N₂ starvation treatment (Table 2 versus Table 1). All these changes were reversible upon the addition of N₂ to the gas phase and the cultures could restore all the measured parameters to the levels shown in Table 1.

These results show the possibility of the regulation of H₂ metabolism in cyanobacteria by N₂ supply in order to improve the efficiency of light energy conversion for H₂ production.

3.2.2.2. Carbon deficiency. The procedure for changing the growth conditions in order to study carbon starvation was similar to that described for N₂. The carbon stress effect reached its maximum within 24 h after stopping the CO₂ supply, but the rate of H₂ evolution and C₂H₂ reduction (Table 3) was somewhat lower than that achieved after 48 h of N₂ deficiency (Table 2). With decreased CO₂ the H₂ evolution activity increased about 1.5 times in both the wild-type and the mutants. The C₂H₂ reduction activity of the nitrogenase increased also, but to a lesser extent than that observed in the absence of N₂ (a 1.5–1.7 times increase in the wild-type and in the mutants). CO₂ depletion effects after 24 h resulted in an irreversible decrease in the other measured physiological parameters (Table 3 versus Table 1).

3.2.3. Conclusion

A comparison of the physiological effects of carbon and nitrogen stress on H₂ metabolism in three strains of Anabaena variabilis indicated that the PK84 mutant is the most appropriate form for use in photobioreactors designed for H₂ production. Deficiencies in N₂ and CO₂ resulted in substantial increases in H₂ evolution activity. Continuous cultures with optimal nitrogenase activity can be established for long-term H₂ production.

Acknowledgments

The project was supported by RITE (Japan), INTAS (Brussels) and the Royal Society (UK).

References

