Anaerobic fermentation in *Cyanidium caldarium*

M.A. Lafraye and A. Betz

Botanisches Institut der Universität, Kirschallee 1, D-5300 Bonn 1, Federal Republic of Germany

Abstract. *Cyanidium caldarium* cells kept anaerobically in the dark have no detectable gas exchange and form exclusively D-(−)-lactate at the expense of their starch content. The addition of acetate enhances both starch breakdown and lactate accumulation by a factor of two. During prolonged anaerobiosis *Cyanidium* is able to keep its energy charge at a low, but fairly constant level. The adenylate-kinase equilibrium, however, undergoes considerable changes, indicative of a regulatory mechanism which maintains a high energy charge particularly by accumulating AMP instead of ADP.

Key words: Adenylate kinase (equilibrium in anaerobiosis) - *Cyanidium* - Energy charge - Fermentation (anaerobic) - D-(−)-Lactate formation.

Introduction

*Cyanidium caldarium*, a blue-green-coloured eucaryotic alga, lives in extremely warm and acidic water and can grow at high cell densities (Allen 1959; Fukuda 1981). As a photosynthetic organism, it does not suffer from lack of oxygen as long as the light intensity is high enough to compensate for respiration. In the dark, however, oxygen may become limiting. In an organism adapted to extreme conditions, control mechanisms for energy metabolism can be expected to be highly efficient. It seemed worthwhile, therefore, to follow carbohydrate breakdown and product accumulation in *Cyanidium* cells under anaerobic conditions in the dark. To gain insight into control mechanisms, endogenous fermentation was stimulated by feeding experiments and the adenylates were monitored during anaerobiosis.

Material and methods

*Cyanidium caldarium* was obtained from the Algensammlung Göttingen, FRG (108.79, No. C-14.1.2). The algae was kept on agar slants as described by Allen (1959). The medium recommended by Rigano et al. (1976) with (NH₄)₂SO₄ (50 mM) was used for mass culture. It contained 0.3 g KH₂PO₄, 0.3 g MgSO₄·7H₂O, 0.02 g CaCl₂, 6.6 g (NH₄)₂SO₄, 1.8 g H₂SO₄ in 1 l H₂O. From a stock solution of trace elements containing 11.93 g FeCl₃·6H₂O, 1.8 g MnCl₂·4H₂O, 2.9 g H₂BO₃, 0.05 g ZnCl₂, 0.08 g CuSO₄·5H₂O, 0.13 g (NH₄)₆Mo₇O₂₄·4H₂O, 1 ml was added to 1 l nutrient medium. The pH was adjusted to 3.5. Fermenter cultures were kept at 40°C, illuminated with fluorescence lamps (3000 lx at the surface), stirred continuously and aerated vigorously with CO₂ (3-5%) in air.

Cells from the stationary phase were collected by centrifugation, washed twice with 50 mM K-phosphate buffer (pH 4.6), and resuspended in the same buffer so that 1 ml contained 3-6·10⁸ cells. Fermentation was followed either using the Warburg technique in a N₂ atmosphere or in a temperature-controlled glass vessel flushed with oxygen-free nitrogen. Samples were taken from Warburg flasks after HC₁O₃ had been added from the side arm. Larger samples from fermentation flasks were quickly centrifuged under refrigeration, washed once with ice-cold buffer and resuspended in 7% HC₁O₃.

In the neutralized supernatant, the following metabolites were assayed enzymatically essentially as described in the cited papers: glucose with hexokinase and glucose-6-phosphate dehydrogenase: Bergmeyer et al. (1974); D-(−)-lactate: Gawehn and Bergmeyer (1974); L-(+)-lactate and L-(-)-malate: Gutmann and Wahlefeld (1974); pyruvate and phosphoenolpyruvate: Czok and Lamprecht (1974); formate: Höpner and Knappe (1974); ethanol: Bernt and Gutmann (1974); glycerol: Wieland (1974); oxalacetate: Wahlefeld (1974); acetaldehyde: Bernt and Bergmeyer (1974); acetate: Holz and Bergmeyer (1974); ATP: Lamprecht and Trautschold (1974); ADP and AMP: Jaworek et al. (1974). In the pellet, starch was estimated as glucose after digestion with amylase-α,1,4-α,1,6-glucosidase. *Cyanidium* cells are not freely accessible to digestion (Enami and Fukuda 1977) and it was necessary, therefore, to incubate them first with trypsin (1 mg/10 mg cells, wet weight) in 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8) for 6 h and subsequently, after the enzyme had been inactivated by boiling, with lipase (1 mg/10 mg pellet) at pH 7 for another 6 h. After denaturation of the enzyme in boiling water, starch was pelleted, resuspended in 4 ml 30 mM acetate buffer (pH 4.6) and incubated with amylase-α,1,4-α,1,6-glucosidase (10 µg/4 ml) for an-
Results and discussion

When *Cyanidium* cells were kept in a nitrogen atmosphere in the dark, no gas exchange was observable under any conditions. The only detectable fermentation product was D-(-)-lactate which was identified and estimated with D-(-)-lactate dehydrogenase (E.C. 1.1.1.28) and was unaffected by L-(+)-lactate dehydrogenase (E.C. 1.1.1.27). There was no detectable L-(+)-lactate, ethanol, formate, glycerol, fumarate, oxalacetate, acetate or acetaldehyde.

The amount of D-(-)-Lactate produced in a given time interval always covered the concomitant loss in glucosyl units from starch. Usually, the balance was somewhat higher than 100% (105-110%). This result is best explained by the assumption that *Cyanidium* contains, besides starch, some soluble carbohydrate which escaped as a result of our procedure for starch determination. This assumption is supported by the results of Nagashima and Fukuda (1981).

The anaerobic fermentation of *Cyanidium* is obviously a homofermentative process. The time course of fermentation is shown in Fig. 1. The rates of starch breakdown and D-(-)-lactate production were both highest immediately after exposure to N₂ and declined considerably after 3 h. This was not due to carbohydrate exhaustion, since the cells still contained more than 80% of their initial starch after 5 h.

In the presence of acetate (20-50 mM), fermentation was enhanced by a factor of two. As shown in Table 1, starch breakdown and D-(-)-lactate production were scarcely dependent on the pH. But at any pH, glycolysis was equally stimulated by acetate. A similar stimulation of respiration in *Cyanidium* by acetate has been explained by the assumption that an energy-dependent proton pump is activated by undissociated acetic acid permeating into the cells (personal communication by Westphal, Botanisches Institut der Universität Bonn, FRG). For aerobic respiration, this assumption is supported by its pH dependence. In our experiments with anaerobic cells, this was also the case as long as pH 6.5 and pH 4.6 were compared. There was no further stimulation, however, at pH 2.5. Perhaps acetate inflow is so high at this pH that inhibition becomes effective.

Acetate was taken up into the cells, but does not seem to have been metabolised. There were losses of 10-20% of the acetate from the medium; half of this loss could be found after extraction of the cells. An appreciable amount of acetate was driven out during incubation in the nitrogen stream and could be recovered in flasks containing KOH or Ba(OH)₂. Up to 5% could not be recovered, but this may reflect the limitations in accuracy and precision of the test methods.

In contrast to acetate, glucose did not stimulate fermentation, even in cells grown on glucose in the dark. The sugar was recovered almost completely after an incubation of 5-6 h, most of it in the supernatant and a smaller part in the HClO₄ extract. Neither glucose nor acetate altered the type of fermentation: starch was consumed exclusively — with the possible exception of a small quantity of soluble carbohydrate which was not assayed — and D-(-)-lactate was the only product of fermentation.

The unique position of D-lactate as the fermentation product in *Cyanidium* was established by enzymatic analysis as mentioned above, as well as with thin-layer chromatography. The following substances have been checked using the latter method: acetate, D-(−)- and L-(+)-lactate, malate, D-glucosamine, D-galactosamine, D-mannosamine, N-acetyl-D-glucosamine, D-glucuronic acid and the amino acids glycine, leucine, valine, all except D-(−)-lactate — with negative results.

The stimulation of glycolysis by the addition of acetate called for a detailed study of adenine nucleotides. The results of a series of experiments at pH 2.5, 4.6 and 6.5 are summarized in Table 2. For experiments at pH 4.6, the time course of production of adenine nucleotides during anaerobiosis is shown in Fig. 2. During glycolysis, ATP increased, especially in the first hour, whereas