Effect of pH on Glycolate and Ammonia Excretion in L-MSO Treated Chlorella Cells

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The effect of pH changes on the excretion of ammonia and glycolate from algal cells into the medium was investigated in L-MSO (final concentration, 0.5 mM) – treated High- and Low CO₂-cells of Chlorella vulgaris 211-11h. The excretion was analyzed in the condition in which the cells were continuously gassed with air at 25 °C. At the values tested, generally more ammonia was excreted in L-MSO-treated Low CO₂-cells than in L-MSO treated High CO₂-cells. In both kinds of algal cells more ammonia was excreted at low pH-values and absolutely no ammonia was excreted at pH 8. In the dark, no or only slight ammonia excretion was observed in both L-MSO-treated High and Low CO₂-cells. Under all these conditions no or only very low glycolate excretion was observed in both L-MSO treated High and Low CO₂-cells.

In High CO₂-cells rates of photosynthesis were high at pH 6 and lower at higher pH values. On the other hand Low CO₂-cells showed practically little dependence of photosynthetic rates on the pH. This result might indicate that the major part of the ammonia excretion observed, was not due to the inhibition of photosynthesis at acid pH values. It is known that ammonia excretion in L-MSO treated algal cells is due to the inhibition of the fixation of ammonia which originates from the glycolate pathway. Our results demonstrate that glycolate production and glycolate metabolism are more intense at low pH values when compared to high pH values. This is valid for both High and Low CO₂-cells. Low CO₂-cells in Chlorella vulgaris 211-11h exhibit a more active glycolate metabolism than High CO₂-cells.

Introduction

It is well known that unicellular algae synthesize during photosynthesis glycolate via the glycolate pathway and may excrete an appreciable amount of glycolate into the culture medium [1-4]. It is generally thought that the conditions which favor the production and excretion of glycolate are high O₂ partial pressure, low CO₂ concentration and high light intensity. Moreover, some investigators have reported that ¹⁴C-incorporation into glycolate during short-term photosynthetic ¹⁴CO₂ fixation was much higher at high pH values than at low ones. This was valid if the concentration of total inorganic carbon (NaHCO₃) was kept constant at all tested pH-values.

Abbreviations: L-MSO, L-methionine-DL-sulfoximine; Low CO₂-cells, algal cells grown in ordinary air, i.e. with 330 ppm CO₂; High CO₂-cells, algal cells grown in air supplemented with 3% CO₂; a-HPMS, a-hydroxy-2-pyridyl methanesulfonate; INH, isonicotinyl hydrazide; GS-GOGAT, glutamine synthetase – glutamine – 2-oxo glutarate-aminotransferase.

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This was investigated with cells of Chlamydomonas and Scenedesmus grown under high CO₂ concentrations (High CO₂-cells) [5, 6]. However, it has been reported later that the High CO₂-cells of these algae utilized, as the substrate for photosynthesis, only free CO₂ dissolved in the solution and not the bicarbonate ion [7-9]. Therefore, equal concentrations of free CO₂, rather than of bicarbonate should be maintained at the pH values to be tested since the ratio of CO₂ to bicarbonate ions in any solution is strongly dependent on the pH of the solution.

The experiments described in the present paper are made with algal cells that were kept at different pH-values under the same CO₂ tension by bubbling the suspension with ordinary air containing approx. 0.03% CO₂. These experiments showed that in contrast to the general opinion appreciably more glycolate and ammonia is formed at low pH values than at higher ones.

Materials and Methods

Chlorella vulgaris 211-11h was obtained from Prof. W. Kowallik (University of Bielefeld, W. Germany but originates from the algal collection of the Department of Agricultural Biology, University of Göttingen, Germany).
University of Göttingen, W. Germany). The algal cells were axenically grown at 25 °C in an inorganic medium according to Hogetsu and Miyachi [10]. The medium did not contain ammonia and was constantly bubbled with ordinary air enriched with 3% CO₂. After several days, the algal suspension was divided into two portions. One was kept at the same conditions (High CO₂-cells), whereas the other portion was bubbled with ordinary air containing ~ 0.03% CO₂ (Low CO₂-cells). During growth the algae were illuminated by a bank of fluorescent lamps. The cells were harvested and the High and Low CO₂-cells were centrifuged at 3000 rpm for 5 min and the pellets resuspended in 100 ml of 20 mM MES (at pH 6.0) or HEPES (at pH 7.0 and 8.0) buffer containing 5% of the culture medium and L-MSO to give a final concentration of 0.5 mM. These suspensions were first kept in the dark for 3 h with constantly gassing the cells with ordinary air containing ~ 0.03% CO₂. The dark period was supposed to permit incorporation of L-MSO into the cells and to produce full inhibition of glutamine synthetase (GS) [11]. After 3 h dark the cells were illuminated by fluorescent lamps (approx. 10 klux). At chosen intervals, portions of the two types of algal suspensions were harvested and immediately transferred for the measurement of photosynthetic O₂ evolution to the reaction vessel of a Clark type O₂ electrode (Rank Brothers, Co. Ltd., London). A another portion of the harvested suspension was centrifuged at 3000 rpm for 5 min at 4 °C in order to obtain the supernatant of the suspension which was used for ammonia and glycolate determination. Concentrations of glycolate and ammonia were determined colorimetrically using the methods of Calkins and Weatherburn [12, 13], respectively.

Results

Fig. 1 shows the effect of three different pH values on the time course of ammonia excretion in the presence of a final concentration of 0.5 mM L-MSO in High and Low CO₂-cells of Chlorella vulgaris 211-11 h. The experiment has been carried under a condition in which the cells were constantly gassed with ordinary air containing ~ 0.03% CO₂. In Low CO₂-cells, the rate of ammonia excretion was approx. 40% lower at pH 7.0 than at pH 6.0. It is seen that ammonia is excreted without a lag at these pH values. In High CO₂-cells the rate of ammonia excretion was approx. 25% lower at pH 7.0 than at pH 6.0. The figure clearly shows that ammonia was excreted at pH 6.0 and a lag of approx. 2.5 h at pH 7.0. No ammonia excretion was observed at pH 8.0 neither in High nor in Low CO₂-cells. The calculated rates of ammonia excretion at pH 6.0 were 24 and 6 μmol·ml⁻¹·pcv⁻¹·h⁻¹ for Low and High CO₂-cells, respectively. During these experiments, no or only a very low amount of glycolate was detected in the supernatant of both High and Low CO₂-cells. Only in L-MSO treated Low CO₂-cells some ammonia was slowly excreted into the medium during the dark period.

Fig. 2 shows the photosynthetic O₂ evolution pattern in cells which were harvested from the same algal suspension as that used in Fig. 1. The algal suspension was quickly transferred to the O₂ electrode vessel which was quickly closed in order to maintain the CO₂ concentration in the suspension at the same level as in the culture medium before the measurement of photosynthetic O₂ evolution. After the measurement of the rate of photosynthetic O₂ evolution, without addition of any external inorganic carbon,
NaHCO$_3$ was injected with a microsyringe in order to measure the maximal rate of photosynthesis. In L-MSO treated Low CO$_2$-cells the rates of photosynthetic O$_2$ evolution with or without the addition of external NaHCO$_3$ were almost the same at the different values tested and did practically not change in the course of the experiment. On the other hand, actual rates of photosynthesis in the absence of external NaHCO$_3$ were greatly increased in L-MSO treated High CO$_2$-cells at all pH values by the transfer of the algae to the bubbling conditions in which ordinary air, containing ~0.03% CO$_2$, was used whereas maximal photosynthetic rates increased only slightly. The data shows that due to the transfer to the low CO$_2$ concentration the affinity characteristics for inorganic carbon were gradually changed to those of Low CO$_2$-cells. The observed rates in the absence of external NaHCO$_3$ were higher at every pH value in L-MSO treated Low CO$_2$-cells (at the time 5.5 h) than those in L-MSO treated High CO$_2$-cells (at 5 h) with the maximal rates of photosynthesis in the presence of external NaHCO$_3$ being almost the same at every pH value in both types of cells.

**Discussion**

Fig. 1a and b show the time course of ammonia excretion into the medium at the pH values 6, 7 and 8 measured under ordinary air (low CO$_2$ conditions) in L-MSO treated High and Low CO$_2$-cells of Chlorella vulgaris 211-11h. L-MSO is a well-known inhibitor
Table I. Effect of pH on changes in the rate of photosynthesis and dark respiration after incubating L-MSO treated High (H) and Low (L) CO₂-cells of *Chlorella vulgaris* 211-11h in ordinary air (0.03% CO₂).

<table>
<thead>
<tr>
<th>Incubation time [h]</th>
<th>pH</th>
<th>Rate of dark respiration [µmol·ml⁻¹pcv·h⁻¹]</th>
<th>Rate of photosynthetic O₂ evolution [µmol·ml⁻¹pcv·h⁻¹] without addition of external NaHCO₃ (A)</th>
<th>with 10 mM NaHCO₃ (B)</th>
<th>A/B × 100</th>
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<td>L</td>
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<td>L</td>
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<td>858</td>
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</table>

Algal cells harvested at intervals were immediately transferred into the Clark type O₂ electrode vessel for measuring dark respiration and photosynthetic O₂ evolution.

* Algal cells were first kept in the dark during 3 h.

*Low CO₂-cells* (L) were harvested after 5.5 h.

of glutamine synthetase (GS) [14] and inhibits the assimilation of ammonia via the GS-GOGAT pathway [15]. According to the photorespiratory nitrogen cycle described by Keys et al. [16] in photorespiration of higher plants the rate of ammonia release seems to correspond to that of CO₂ release with the ammonia being reassimilated in the reactions of the GS-GOGAT pathway. Ogren showed in his review that ammonia as well as CO₂, is produced by the glycine-serine aminotransferase reaction in the glycolate pathway in mitochondria [17]. From there ammonia is transported into the chloroplast and reassimilated by the GS-GOGAT reactions. This explains why in higher plants ammonia is excreted from the cells when the GS-GOGAT pathway is inhibited by the addition of L-MSO under conditions which are favorable to photorespiration [18]. The same is true for *Chlamydomonas* [11]. Due to these observations the activity of serine formation in the glycolate pathway can be estimated by measuring the rate of ammonia excretion in the presence of L-MSO. This seems to be valid in the light in the presence of L-MSO for *Chlorella vulgaris* 211-11h. The rate of ammonia excretion was the same as that of glycolate excretion in the presence of INH or α-HPMS at pH 6.0 [25 °C] under ≈ 0.03% CO₂ in air (data not shown). It seems as if the rate of ammonia excretion together with that of glycolate excretion observed in the presence of L-MSO was a measure of glycolate synthesis in the cells.

According to Miyachi and Shiraiwa *Chlorella vulgaris* 211-11h cells utilize only free CO₂ as a substrate for photosynthesis [19]. In order to maintain the concentration of free CO₂ constant at the pH-values measured, *High* and *Low* CO₂-cells of *Chlorella vulgaris* 211-11h, suspended in the respective buffers at pH 6 to 8, were constantly gassed with air containing ≈ 0.03% CO₂ at 25 °C during the experiments. Under these conditions, the concentration of free CO₂ is kept at approx. 10.2 µM at the respective pH. As shown in Fig. 1 under these conditions, ammonia formation was higher at lower pH values than at higher pH values. This was valid for both *High* and *Low* CO₂-cells. If one correlates in the present paper ammonia excretion with glycolate formation, the results reported here seem to be in contrast with the known effect of pH on glycolate formation. For *Chlamydomonas* grown under 0.2–0.5% CO₂ Orth et al. [5] has reported that the ¹⁴C-incorporation into glycolate during photosynthetic ¹³CO₂ evolution was higher at high pH values than at low pH values. The measurements of Orth et al. [5] have been carried out
at the same concentration of NaH\textsubscript{14}CO\textsubscript{3} at the different pH-values. Under these conditions the concentration of bicarbonate was 1.25 times higher at pH 8.8 than at pH 7, whereas the concentration of free CO\textsubscript{2} was 50-fold higher at pH 7 when compared to pH 8.8. On the other hand, it has recently been reported that high CO\textsubscript{2}-grown \textit{Chlamydomonas} cells utilize as a substrate for photosynthesis only free CO\textsubscript{2}, dissolved in the solution but not the bicarbonate ion. In contrast to this \textit{Low CO\textsubscript{2}-cells} can apparently utilize bicarbonate ions [8, 9] although later it was also reported that \textit{Low CO\textsubscript{2}-cells} absorb only free CO\textsubscript{2} by the action of carbonic anhydrase located on the cell surface [20]. From these observations it can be concluded that the data reported by Orth et al. [5] are at least partially due to a decrease of the concentration of free CO\textsubscript{2}. Therefore, attention should be given to the type of active species of inorganic carbon in photosynthesis and its concentration in the solution when pH and temperature effects are measured.

At every pH measured it appeared that the rate of glycolate formation was higher in \textit{Low CO\textsubscript{2}-cells} than that in \textit{High CO\textsubscript{2}-cells}. On the other hand, the rate of photosynthetic O\textsubscript{2} evolution without addition of external NaHCO\textsubscript{3} is higher in \textit{Low CO\textsubscript{2}-cells} than in \textit{High CO\textsubscript{2}-cells} at any time (Table I, 0.3 and 5 h) whereas the maximal rate of photosynthesis measured in the presence of external NaHCO\textsubscript{3} was almost identical in \textit{High} and \textit{Low CO\textsubscript{2}-cells} at all pH values tested. When \textit{High CO\textsubscript{2}-cells} are transferred to low CO\textsubscript{2} conditions (0.03\% CO\textsubscript{2} in air) the characteristics of \textit{High CO\textsubscript{2}-cells} are gradually changed to those of \textit{Low CO\textsubscript{2}-cells}. Thus, at pH 8.0 carbonic anhydrase activity and the affinity of algal cells for CO\textsubscript{2} in photosynthesis nearly reaches the level of \textit{Low CO\textsubscript{2}-cells} around 3 h after the transfer to the low CO\textsubscript{2} conditions [21]. The ratio of the rate of photosynthetic O\textsubscript{2} evolution under CO\textsubscript{2} limiting and CO\textsubscript{2} saturating conditions which permits to estimate the affinity for CO\textsubscript{2} in \textit{High CO\textsubscript{2}-cells}, increases gradually to the values of \textit{Low CO\textsubscript{2}-cells} in the course of 9 h, i.e. 6 h after the onset of the illumination (Table I). The increase of this ratio, which describes the changes of photosynthetic characteristics of \textit{High CO\textsubscript{2}-cells} to those of \textit{Low CO\textsubscript{2}-cells} was faster at pH 6 than at pH 7 and 8 (Table I). This is thought to be due to the longer induction time of ammonia excretion in L-MSO treated \textit{High CO\textsubscript{2}-cells} at pH 7 when compared to the induction time at pH 6. This data also shows that the low activity of the glycolate pathway in \textit{High CO\textsubscript{2}-cells} is not due to an inhibition or low activity of photosynthesis and that the induction of carbonic anhydrase is essential for an active glycolate production and metabolism.

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