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Regulation by Amino Acids of Photorespiratory Ammonia and Glycolate Release from *Ankistrodesmus* in the Presence of Methionine Sulfoximine[1]

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ABSTRACT

Methionine sulfoximine induced release of ammonia from illuminated cells of *Ankistrodesmus braunii* (Naegeli) Brunnth, in normal air, but less in air enriched to 3% CO_2. In normal air, methionine sulfoximine also induced glycolate release. Addition of either glutamate, glycine, or serine suppressed glycolate release, whereas glutamate and glycine at the same time stimulated ammonia release. The results indicate that inhibition of glutamine synthetase and thereby inhibition of photorespiratory nitrogen cycling restricts the sink capacity for glycolate in the photorespiratory carbon cycle. An external supply of glutamate, glycine, or serine seems to stimulate glyoxylate transamination and thus partly restores the sink capacity. Calculations of total glycolate formation rates in air from glycolate and ammonia release rates in the presence of methionine sulfoximine and glutamate revealed values of approximately 20 micromoles glycolate per milligram chlorophyll per hour on the average. Similar calculations led to an estimated rate of photorespiratory ammonia release in air, in the absence of methionine sulfoximine, of about 10 micromoles per milligram chlorophyll per hour on the average, a value comparable to the primary nitrogen assimilation rate of 8 micromoles per milligram chlorophyll per hour.

During photosynthetic CO_2 fixation in air, in most algae and higher plants a substantial portion of the carbon passing through the reductive pentose phosphate cycle enters the photorespiratory pathway via phosphoglycolate and glycolate (18–20). Glycolate is then converted to glyoxylate, glycine, and serine. The formation of 1 mol serine from 2 mol glycine in the reactions of glycine decarboxylase-serine hydroxymethyl transferase is accompanied by the liberation of 1 mol each of CO_2 and NH_3, a process located in the mitochondria (1, 7, 10). As shown by Keys *et al.* (8), the liberated NH_3 is reassimilated to glutamate via the GS[3]/GOGAT pathway. Much of the evidence for the involvement of GS in the reassimilation of NH_3 has been provided by experiments with MSO, an irreversible inhibitor of GS (17). Inhibition or lack of active GS/GOGAT induce accumulation of NH_3 in photorespiring tissue to toxic levels (5, 13, 16) and cause liberation of $^{15}NH_3$ from ^{15}N-labeled glycine fed previously to the tissue (8). In intact algae, MSO induces NH_3 release to the medium (3). In all of these cases, glutamate dehydrogenase does not play a significant role in reassimilation of NH_3.

If the photorespiratory pathway operates also under conditions of inhibited NH_3 primary assimilation and recycling, the loss of NH_3 from the cycle must be completely accounted for by endogenous N sources. Indications for a coupling between photorespiration and protein catabolism were obtained in experiments with *Chlamydomonas* (3). In *Chlamydomonas*, the rates of NH_3 release suggested that photorespiratory N cycling attains maximally 40% of that of primary NH_3 assimilation (3), in contrast to higher plants, where photorespiratory N cycling seems to exceed primary assimilation by an order of magnitude (8).

It will be shown in this paper that treatment of *Ankistrodesmus* with MSO not only induces NH_3 release, but also stimulates the release of glycolate. The rates of NH_3 and glycolate release can be considerably influenced by external application of amino acids that serve as suppliers of amino groups or are as such involved in the photorespiratory pathway. This interaction indicates a close coupling between the photorespiratory C and N cycles and suggests that improved estimates of photorespiratory N cycling and even of glycolate turnover can be made by measuring both NH_3 and glycolate release in the presence of MSO.

MATERIALS AND METHODS

The unicellular green alga *Ankistrodesmus braunii* (Naegeli) Brunnth, (*Monoraphidium braunii* Legnerová), strain 202-7d, was obtained from the Pringsheim Algae Collection, University of Göttingen. The cells were grown synchronously at 2.5% CO_2 according to Eisele and Ullrich (4). For experiments, the algae were collected by filtration 2 h after the beginning of the light period, washed, and finally resuspended in N-free nutrient solution (pH 6.0) to a density of approximately 50 μg Chl ml^{-1}. The Chl content was determined according to Senger (14). The experiments were performed in 30 ml temperature-controlled (30°C) cuvettes flushed with normal air or air enriched to 3% CO_2. For the experiments with varied MSO concentration, wide centrifuge tubes (25 ml), containing 1 ml algal suspension each and placed in a photo-Warburg apparatus were used. The irradiance during the experiments was approximately 400 w m^{-2} (white light). For determinations of NH_3 and glycolate in the medium, 0.5- to 1-ml samples were removed from the experimental vessels and rapidly centrifuged at 0°C. The content of NH_3 in the supernatant was then measured colorimetrically according to Solórzano (15). The absorbance of the indophenol formed was measured at 644 nm. Glycolate was determined according to Calkins (2). After centrifugation of the samples, 0.2 ml supernatant was mixed with 0.05 ml 10 N H_2SO_4. An aliquot of 2.5 ml freshly prepared reagent

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[3] Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; MSO, methionine sulfoximine.

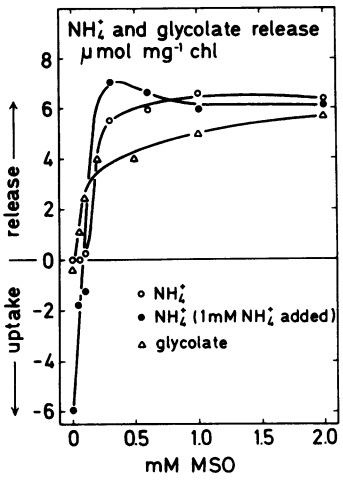

FIG. 1. NH_4^+ exhange and glycolate release in air as a function of MSO concentration. The algae were preincubated for 30 min with 1 mM MSO ± 1 mM NH_4^+. The data were calculated as the difference in NH_4^+ and glycolate concentration of the medium between 30 and 90 min of incubation. The values for glycolate represent mean values of data obtained in the absence and presence of NH_4^+. Combination of representative experiments.

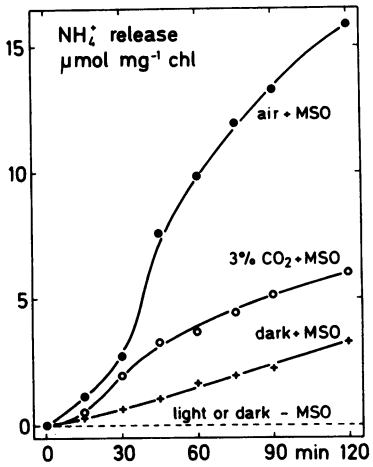

FIG. 2. NH_4^+ release as affected by MSO, light and CO_2 concentration. MSO was added at zero time to a final concentration of 1 mM. Means of four experiments.

(obtained by dissolving 10 mg 2,7-dihydroxynaphthalene in 100 ml concentrated H_2SO_4) was then added, and the mixture was subsequently heated to 90° for 3 h. After cooling to room temperature, the absorbance was measured at 546 nm. Glutamate, glycine, and serine did not interfere with this assay, but slightly elevated the background in the NH_3 determinations (as did also MSO); however, with the NH_3 concentrations obtained, these interferences were negligable.

Due to daily variations in the rates of NH_3 and glycolate release, usually results from single representative experiments performed with one batch of algae are presented. In those cases (Figs. 3–7), three to five independent experiments have been performed.

RESULTS

MSO was used as a tool to reveal photorespiratory N (and C) cycling by preventing the reassimilation of NH_3, produced in the mitochondrial serine hydroxymethyltransferase reaction. In *Ankistrodesmus* in the light in air, net NH_4^+ assimilation was completely inhibited by MSO at concentrations between 0.1 and 0.2 mM. At higher MSO concentrations, the cells released NH_3 at

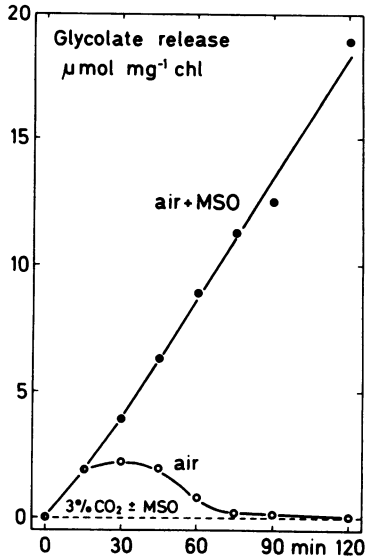

FIG. 3. Glycolate release in the light as affected by MSO (1 mM) and CO_2 concentration. MSO was added at zero time. Representative experiment.

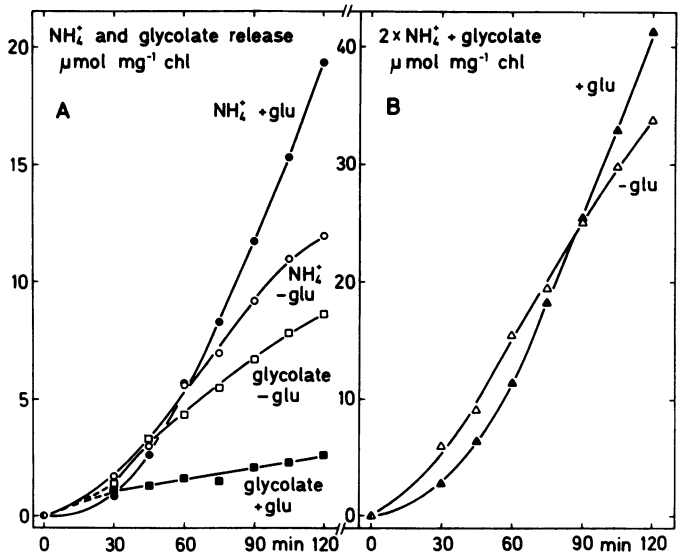

FIG. 4. A, Effects of 1 mM glutamate on MSO (1 mM) -induced NH_4^+ and glycolate release. MSO was added at zero time. Representative experiment. B, Recalculated values from A, showing NH_4^+ and glycolate release plotted as (2 × NH_4^+ + glycolate).

quantities comparable to those taken up in the absence of inhibitor (Fig. 1). Simultaneously, MSO induced a similar quantity of glycolate release (Fig. 1). Since the sensitivity of the cells to lower MSO concentrations varied, 1 mM of the inhibitor was used as the standard concentration for all further experiments.

Coupling of a process to photorespiration can be demonstrated by its light dependence and by its response to CO_2 or O_2. In Figure 2, the dependence of NH_3 release on illumination and CO_2 concentration is presented. Whereas in the absence of MSO no NH_3 was released in light or dark, either at low or at high CO_2 concentration, the NH_3 release in the dark with MSO showed a rate of 1.5 to 2 µmol mg^{-1} Chl h^{-1}. In the light at 3% CO_2, the rate was higher initially, but then after 40 min decreased more or less to the dark rate. In the light with air (0.03% CO_2), however, the release rate was severalfold higher, but the time course was still sigmoidal. The values for Figure 1 were calculated from the differences after 30 and 90 min incubation and thus represent the

time interval of the highest rates.

The corresponding dependence of glycolate release on MSO, light, and on the CO_2 concentration is shown in Figure 3. No glycolate was excreted at 3% CO_2 irrespective of whether MSO was present or not. In air (0.03% CO_2) in the absence of MSO, glycolate release was transient and followed by a complete reassimilation. Under the same conditions, however, MSO induced

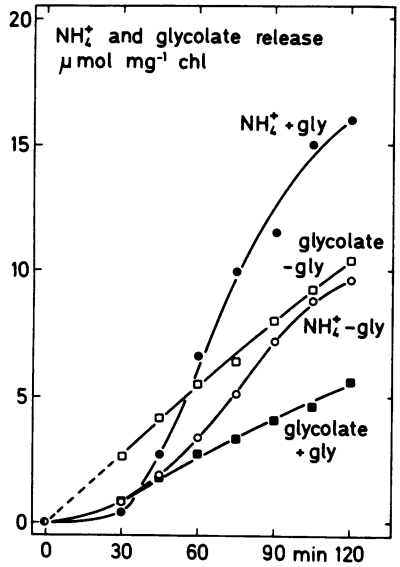

FIG. 5. Effects of 1 mM glycine on MSO (1 mM) -induced NH_4^+ and glycolate release. Details as in legend to Figure 4A.

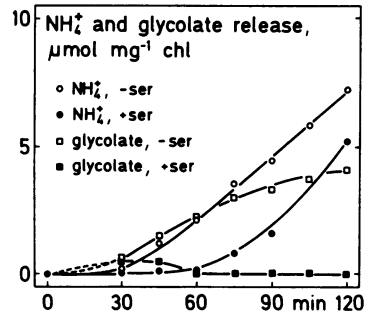

FIG. 6. Effects of 1 mM serine on MSO (1 mM) -induced NH_4^+ and glycolate release. Details as in legend to Figure 4A.

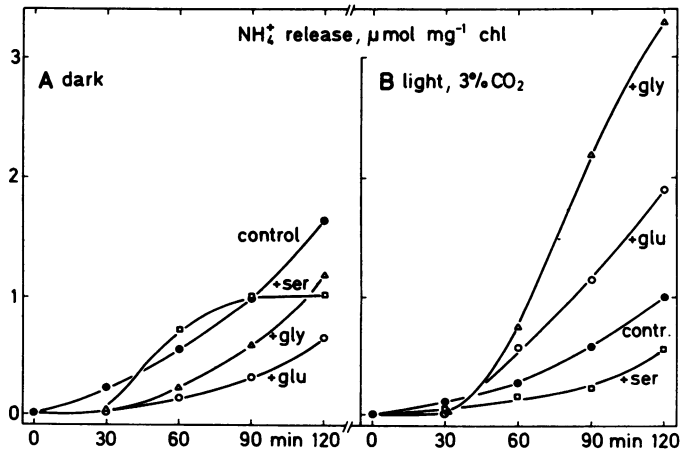

FIG. 7. Effects of glutamate, glycine, and serine on MSO (1 mM) -induced NH_4^+ release in the dark (A) and in the light at 3% CO_2 (B). MSO and amino acids were added at zero time. Representative experiment.

rather constant rates of glycolate release for the whole experimental period of 2 h. The rates of NH_3 and glycolate release were not pH dependent between pH 5 and 8, except that the initial lag phase in NH_3 release was more pronounced at higher pH (data not shown).

Glutamate is known as substrate of glutamate:glyoxylate aminotransferase, an essential enzyme of the photorespiratory nitrogen cycle (9, 18, 19). Addition of 1 mM glutamate to *Ankistrodesmus* cells in the light in air and in the presence of MSO considerably changed the efflux pattern: glycolate release was reduced to about one-third, whereas NH_3 release was increased by a factor of 1.5. After an initial lag, the rates were constant, in contrast to those in the absence of glutamate (Fig. 4A). According to the reactions involved in the photorespiratory pathway, the rate of total glycolate formation should equal the sum of glycolate released plus the double rate of NH_3 release, if there are no leaks in the cycle (*cf.* "discussion"). The data of Figure 4A were recalculated in this way (2 × NH_3 released + glycolate released) which is shown in Figure 4B. The recalculated values yielded plots that were initially parallel after which the rate declined in the absence of glutamate, whereas it remained constant in the presence of glutamate.

Glycine is not only a supplier of amino groups, but also acts as an intermediate of the N cycle located directly at the site of NH_3 liberation. Similarly to glutamate, it induced a reduction in the rate of glycolate release and, as expected, it stimulated the release of NH_3 (Fig. 5). The rates were less constant than with glutamate, and the stimulation of NH_3 release was not stoichiometric with the reduction in glycolate release (calculations not shown).

An even more pronounced response was observed upon addition of serine. In the presence of MSO, serine almost completely abolished glycolate release (Fig. 6), or limited its release time to the first 30 min, after which it was reconsumed. NH_3 release attained similar or somewhat higher rates in the presence of serine than in its absence, but only after a lag of about 60 min (Fig. 6).

As shown in Figure 7A, glutamate, glycine, and serine did not much affect the dark rate of NH_3 release in the presence of MSO. However, in the light and under 3% CO_2, both glutamate and glycine stimulated NH_3 release in the presence of MSO (Fig. 7B), although the rates were low compared to the rates obtained in air.

DISCUSSION

The results presented in this paper can be interpreted with the action of MSO on refixation of NH_3 produced in protein catabolism and in the photorespiratory N cycle. Although there is no unequivocal evidence, it can be assumed that the basic rate of NH_3 liberation in protein turnover is reflected by the NH_3 release in the dark and in the presence of MSO (Fig. 2). The rate is approximately the same during illumination at 3% CO_2, but increases drastically when the cells are illuminated in air (0.03% CO_2). This effect can be attributed to degradation of recirculating amino acids or to increased protein turnover coupled to photorespiration as suggested from experiments with *Chlamydomonas* (3). Protein turnover will supply amino groups for transamination of glyoxylate to glycine, and these amino groups are subsequently liberated as NH_3 when glycine is converted to serine. In *Ankistrodesmus*, apparently, increased protein turnover cannot completely sustain the demand for amino groups for recycling of all glycolate formed, and glycolate is thus released from the cells (Figs. 1 and 3). This implies a fundamental importance of GS for operation of not only the photorespiratory N cycle as shown previously (8, 21) but also the photorespiratory C cycle.

Compensation for the loss of GS activity by exogenous glutamate, the product of the GS/GOGAT reactions, relieves the inhibitory effect of MSO on glycolate recycling, as shown by the reduced rates of glycolate release (Fig. 4). The role of glutamate in this context is most likely to serve as a substrate for the

glutamate:glyoxylate transaminase reaction, thus increasing the capacity for internal recycling of glycolate. A stoichiometric relation of 2:1 between glycolate consumed and NH_3 released is expected since only one glycine molecule out of two is disintegrated in the hydroxymethyl transferase reaction (19). Hence, the total glycolate formation can be calculated according to the equation:

$$\text{glycolate formed} = \text{glycolate released} + 2 \times NH_3 \text{ released}$$

When this equation was applied to the results of Figure 4A, a discrepancy between the control without glutamate and the +glutamate curve was obtained, although the lines appeared initially parallel (Fig. 4B). The incapability of the algae to supply amino groups rapidly enough in the absence of an external source are regarded as the cause for the progressive decline in NH_3 release which is prevented in the presence of glutamate. In some experiments, a 1:1 ratio between glycolate consumption and NH_3 release yielded better parallelism (data not shown). This could mean that the stoichiometric significance of NH_3 release is overestimated in the above equation. However, a direct comparison of the rates in the presence and absence of glutamate may be ambiguous for metabolic reasons. Glutamate may stimulate the conversion of glycine to serine and thus improve the sink effect in the photorespiratory cycle and stimulate even glycolate formation. On the other hand, glutamate may stimulate deamination processes occurring in the light but not tightly coupled to photorespiration as indicated in Figure 7B. Also, leakage in the photorespiratory cycle as decarboxylation of glyoxylate (6, 11) would affect stoichiometric relations, but in the reverse sense by leaving less glyoxylate for glycine formation and NH_3 release. It could be of importance here only if substantially influenced by glutamate. The data do not confirm observations by Oliver and Zelitch (12) in which glutamate at higher concentrations caused a decrease in the total glycolate turnover.

The effect of external glycine resembles that of glutamate, but glycine is not only a source for amino groups but also a direct member of both the photorespiratory N and C cycles. A stimulation of NH_3 release by the immediate amino group donor must be expected. Reduced glycolate release at the same time indicates an increased flow of endogenous glyoxylate towards the glycine-serine conversion, *i.e.*, an increased rate of serine formation, the formed serine serving as the substrate for transamination of glyoxylate. Calculations of $2 \times NH_3$ + glycolate resulted in much higher values in the presence of glycine than in the control experiments (results not shown), probably due to conversion of excess glycine to serine. Accordingly, glycine drastically increased NH_3 release also at 3% CO_2 (Fig. 7B).

The explanation for the effect of glycine is further supported by the experiments with serine. Addition of serine completely suppresses glycolate release suggesting a considerably enlarged sink capacity of the photorespiratory C cycle. Probably, serine is used as a substrate for transamination of glyoxylate. The longer lag phase in NH_3 release in the presence of serine is not completely understood as yet, but may be related with interference of serine with the uptake of MSO. This may explain that, during the 1st h of incubation with serine and MSO, the release pattern closely resembles that without MSO, showing some initial release of glycolate followed by complete reassimilation, and no NH_3 release (*cf.* Figs. 2, 3, and 6).

In conclusion, the results show a close coupling between the photorespiratory N and C cycles. In the presence of MSO but in the absence of external amino group donors, the flow of carbon from glyoxylate to glycine and serine seems to function as the bottle neck. Calculations of ($2 \times NH_3$ + glycolate) release in the presence of glutamate yield a value of 23 ± 6 ($n = 4$) μmol mg^{-1} Chl h^{-1}. After compensation for NH_3 release that is not tightly coupled to photorespiration (Figs. 2 and 7), the value for glycolate formation in *Ankistrodesmus* in air would be in the order of 20 μmol mg^{-1} Chl h^{-1}. The corresponding rate of internal NH_3 liberation would then be approximately 10 μmol mg^{-1} Chl h^{-1}, which is comparable to the rate of primary N assimilation of 8 ± 2 μmol mg^{-1} Chl h^{-1} ($n = 5$) under the same conditions, but in the absence of MSO.

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