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Regulation of Sulfate Assimilation by Light and *O*-Acetyl-L-Serine in *Lemna minor* L.[1]

Urs Neuenschwander, Marianne Suter, and Christian Brunold*

Pflanzenphysiologisches Institut der Universität Bern, Altenbergrain 21, Bern, Switzerland

ABSTRACT

The effect of 0.5 millimolar *O*-acetyl-L-serine added to the nutrient solution on sulfate assimilation of *Lemna minor* L., cultivated in the light or in the dark, or transferred from light to the dark, was examined. During 24 hours after transfer from light to the dark the extractable activity of adenosine 5′-phosphosulfate sulfotransferase, a key enzyme of sulfate assimilation, decreased to 10% of the light control. Nitrate reductase (EC 1.7.7.1.) activity, measured for comparison, decreased to 40%. Adenosine 5′-triphosphate (ATP) sulfurylase (EC 2.7.7.4.) and *O*-acetyl-L-serine sulfhydrylase (EC 4.2.99.8.) activities were not affected by the transfer. When *O*-acetyl-L-serine was added to the nutrient solution at the time of transfer to the dark, adenosine 5′-phosphosulfate sulfotransferase activity was still at 50% of the light control after 24 hours, ATP sulfurylase and *O*-acetyl-L-serine sulfhydrylase activity were again not affected, and nitrate reductase activity decreased as before. Addition of *O*-acetyl-L-serine at the time of the transfer caused a 100% increase in acid-soluble SH compounds after 24 hours in the dark. In continuous light the corresponding increase was 200%. During 24 hours after transfer to the dark the assimilation of $^{35}SO_4^{2-}$ into organic compounds decreased by 80% without *O*-acetyl-L-serine but was comparable to light controls in its presence. The addition of *O*-acetyl-L-serine to *Lemna minor* precultivated in the dark for 24 hours induced an increase in adenosine 5′-phosphosulfate sulfotransferase activity so that a constant level of 50% of the light control was reached after an additional 9 hours. Cycloheximide as well as 6-methylpurine inhibited this effect. In the same type of experiment *O*-acetyl-L-serine induced a 100-fold increase in the incorporation of label from $^{35}SO_4^{2-}$ into cysteine after additional 24 hours in the dark. Taken together, these results show that exogenous *O*-acetyl-L-serine has a regulatory effect on assimilatory sulfate reduction of *L. minor* in light and darkness. They are in agreement with the idea that this compound is a limiting factor for sulfate assimilation and seem to be in contrast to the proposed strict light control of sulfate assimilation.

Light (3, 21, 26) and the rate of nitrate assimilation or the availability of amino acids (4, 16, 17, 25) have been described as regulatory factors of assimilatory sulfate reduction. The light dependence of assimilatory sulfate reduction has been demonstrated by the finding that spinach chloroplasts form [^{35}S]cysteine from $^{35}SO_4^{2-}$ in the light (26). In agreement with this, extracts from spinach chloroplasts synthesize [^{35}S]cysteine from [^{35}S]APS[2] in a light and ferredoxin-dependent reaction sequence (21), and in spinach, APSSTase, a key enzyme of the pathway of sulfate assimilation, is predominantly or even exclusively localized in chloroplasts (7). Several studies have shown that this enzyme activity is very susceptible to various regulatory signals (3). Because of the convergence of nitrate and sulfate assimilation in the synthesis of proteins (17), regulatory effects from nitrogen metabolism on APSSTase activity could be anticipated. Indeed in *Lemna minor* the lack of a nitrogen source induces a rapid loss of APSSTase activity (4), whereas ammonium (4) and asparagine, glutamine, or arginine (25) cause a parallel increase of *in vivo* sulfate assimilation and in the extractable activity of APSSTase.

A regulatory effect on sulfate assimilation and APSSTase activity can also be expected from OAS, which has been shown to be the substrate for cysteine synthesis in bacteria (14). OAS has been identified as a limiting factor for cysteine formation in leaf discs from pumpkin kept in the light (16), suggesting that plants use the same substrate for cysteine formation as bacteria. OAS is one product of assimilatory nitrate reduction. This process has also been shown to be light dependent (10, 12). In the present paper, the effect of OAS on assimilatory sulfate reduction is examined during a light-dark transition and after preincubation in the dark for 24 h. *L. minor* was chosen as plant material because it can be cultivated under sterile conditions and takes up amino acids added to the nutrient solution directly into its green fronds.

MATERIAL AND METHODS

Plant Material

Lemna minor L. was cultivated as previously described (4). Before the experiments the plants were precultivated under controlled conditions of constant light (light intensity 100 μE m^{-2} s^{-1}, Philips TL 40 W/33), 25°C, 80% RH, and 340 ppm CO_2. Aeration was at 40 L h^{-1}. Dark treatments were performed by wrapping the culture vessels with aluminium foil. Amino acids and $^{35}SO_4^{2-}$ were dissolved in nutrient solution E-NO_3^- (4), the pH was adjusted to 6, and aliquots were added to the medium in a sterile fashion.

Extraction of the Plant Material for Enzyme Measurement

Plants were washed for 1 min with H_2O at 4°C, and extracts were prepared by grinding in a glass homogenizer cooled with

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[2] Abbreviations: APS, adenosine 5′-phosphosulfate; APSSTase, adenosine 5′-phosphosulfate sulfotransferase; OAS, *O*-acetyl-L-serine.

ice water in 0.1 M Tris-HCl (pH 8.0), containing 100 mM KCl, 20 mM $MgCl_2$, and 10 mM DTE. Routinely, 200 mg plant material was homogenized in 2 mL buffer solution. The homogenate was centrifuged for 10 min at 10,000*g*, and the supernatant was immediately used for the enzyme assay. APSSTase was measured as the production of [^{35}S]sulfite, assayed as acid-volatile radioactivity from [^{35}S]APS in the presence of DTE (5). Nitrate reductase activity (EC 1.6.6.1.) was determined by measuring the NO_2^- formed from NO_3^- (13). ATP sulfurylase (EC 2.7.7.4.) was estimated in the back reaction by measuring the ATP formed using an ATP meter (20). OAS sulfhydrylase activity (EC 4.2.99.8.) was determined by measuring the cysteine formed from sulfide and OAS (15).

Determination of the *in Vivo* Assimilation of $^{35}SO_4^{2-}$

About 100 fronds of *L. minor* L. were cultivated on 30 mL NO_3-medium with or without OAS in the presence of carrier-free $^{35}SO_4^{2-}$ for 24 h under sterile conditions. The plants were rinsed twice for 15 min with ice water and routinely homogenized in the buffer used for separation by HPLC described below using a glass homogenizer cooled with ice; 1 mL of buffer was used per 100 mg of plant material. The resulting extract was centrifuged for 20 min at 30,000*g* and 4°C.

The supernatant was degassed with argon, and 100 μL were used for the separation of thiols by isocratic reversed phase HPLC with a C_{18} 4.6 × 250 mm column, particle size 5 μm

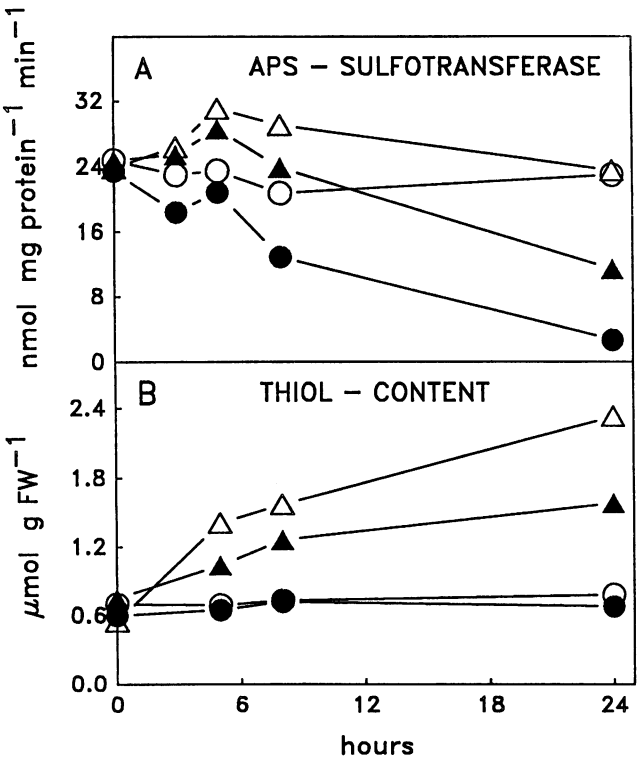

Figure 1. Extractable activity of APSSTase (A) and acid-soluble thiols (B, thiol content) of *L. minor* in continuous light (○), in continuous light with 0.5 mM OAS (△), and after transfer to the dark with (▲) and without (●) 0.5 mM OAS.

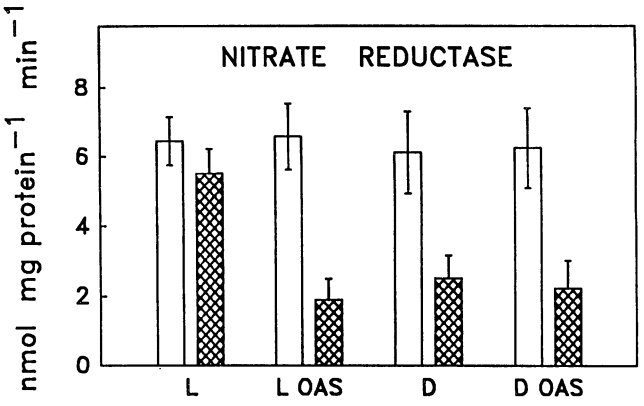

Figure 2. Extractable activity of nitrate reductase of *L. minor* in continuous light with (LOAS) and without (L) 0.5 mM OAS, and after the transfer into the dark with (DOAS) and without (D) 0.5 mM OAS. The open and the hatched columns represent the enzyme activity at the beginning and 24 h after the transfer to the dark or/and the addition of OAS, respectively. Mean values and SD from four to six experiments are presented.

(Stagroma, Wallisellen, Switzerland). The running buffer consisted of 96% 0.1 M monochloracetic acid (pH adjusted to 3.0 using NaOH) containing 2 mM sodium octyl sulfate and 4% methanol (v/v) (18). Separation and detection took place in a system made oxygen-free by flushing with helium. The flow rate was 1.0 ml min^{-1}. Reduced glutathione and cysteine were identified using an Au-Hg electrode and an amperometric detector (LC-4B, BAS West Lafayette, IN) and radioactivity in the eluent was measured using a radio-detector (LB 504 Berthold, Wildbach, Germany).

For quantification of the labeled protein fraction, 100 mg of plant material were homogenized in 1 mL of EDTA (0.1 mM):TCA (10%, w/v) = 10:2 (v/v) using a glass homogenizer cooled with ice water. The resulting extract was centrifuged for 20 min at 30,000*g* and 4°C and the pellet was resuspended in 2 mL Tris-HCl (pH 9.0). After 1 h the suspension was centrifuged at 20,000*g* and 4°C. The radioactivity in the supernatant was designated as ^{35}S in the protein fraction.

Measurement of Total Acid-Soluble Thiols

Plant material was homogenized as described for the enzyme assays but EDTA (0.1 mM):TCA (10%, w/v) = 10:2 (v/v) was used as the extraction medium. The measurement of thiols was according to Grill and Esterbauer (11).

Protein Determination

The protein content was measured according to Bradford (2) with BSA as a standard.

Chemicals

APS, OAS, *N*-acetyl-L-serine, and acetyl-CoA were from Sigma. All other chemicals were from Fluka, Buchs (Switzerland).

RESULTS

In a first set of experiments the effect of 0.5 mM OAS on assimilatory sulfate reduction in *L. minor* transferred from

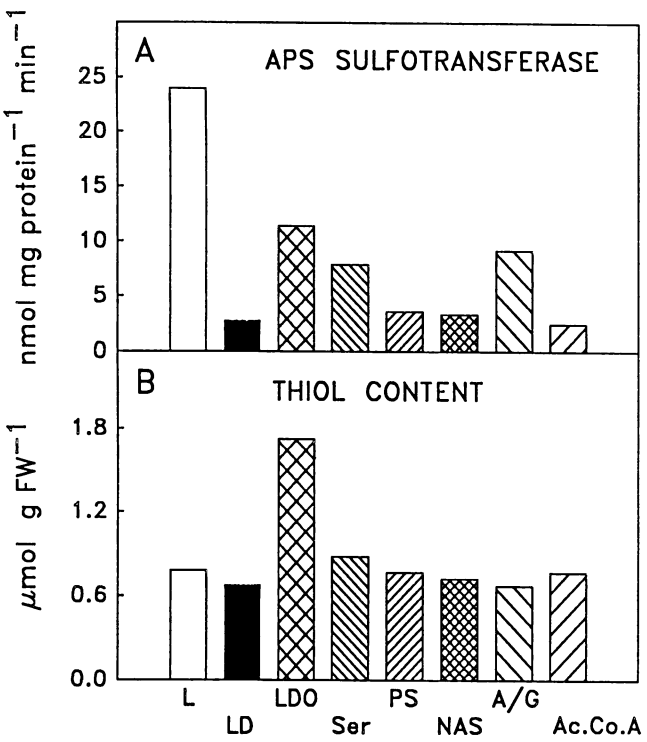

Figure 3. Extractable activity of APSSTase (A) and acid-soluble SH content (B, thiol content) of *L. minor* in continuous light (L) and after 24 h in the dark without addition (LD) or with addition of 0.5 mM OAS (LDO), 0.5 mM L-serine (Ser), 0.5 mM DL-phosphoserine (PS), 0.5 mM *N*-acetyl-L-serine (NAS), 2 mM each of L-asparagine and L-glutamine (A/G), and 0.5 mM acetyl-CoA (Ac.Co.A).

light to darkness was examined. Growth measurements showed that in the light OAS increased the doubling time of the *Lemna* plants from 34 ± 6 to 44 ± 6 h but had no effect on the dry weight per plant. Plants transferred to the dark still produced tiny new fronds, but after 24 h in darkness their total dry weight was estimated to be approximately 20% lower than at the time of the transfer. In Figure 1 the changes in APSSTase activity and in the thiol content are presented during 24 h after this transfer. Extracts from plants cultivated in the light without OAS contained a constant activity of approximately 24 nmol mg protein^{-1} min^{-1}. In the presence of OAS the value in the light increased initially, but then decreased to the normal light level. In extracts from *L. minor* transferred to the dark APSSTase activity decreased and was at 10% of controls in the light after 24 h. The presence of OAS during the dark period slowed down this decrease in activity so that after 24 h the value was still 50% of that measured in the light. When extracts from plants cultivated under the various conditions were combined, the activities of APSSTase were additive for all combinations indicating that no activating or inactivating substance was involved.

Under the various conditions the fresh weight of roots was about 25% of fresh weight of intact *Lemna* plants. This could mean that the APSSTase activity detected in extracts from whole plants kept in darkness for 24 h was exclusively localized in the roots and therefore not susceptible to light-dark regulation. APSSTase and OAS sulfhydrylase activity was therefore measured in intact plants and in plants with excised roots 24 h after the various treatments. These measurements gave no significant difference calculated on a fresh weight basis, indicating that in our system both enzyme activities were regulated in a very similar way in leaves and roots. Without OAS a constant level of the acid soluble thiols was maintained in the dark as well as in the light. Addition of OAS induced a 100 and 200% increase in the dark and in the light, respectively.

In constant light the concentration of OAS in the nutrient solution decreased from 0.5 mM at the beginning of the experiment to 0.45 mM after 24 h. It can be assumed, therefore, that changes in the concentration of OAS in the nutrient solution during the experiment were negligible.

Transfer of *L. minor* to the dark also induced a decrease in nitrate reductase activity, which was less pronounced after 24 h, however, than that of APSSTase activity (Fig. 2). Addition of OAS to plants cultivated in the light caused a similar decrease in this enzyme activity but did not decrease it further in plants transferred to darkness. The activity of OAS sulfhydrylase was neither affected by the transfer to the dark nor by the presence of OAS after 24 h (data not shown). Transfer to the dark also did not affect the activity of ATP sulfurylase, the first enzyme of assimilatory sulfate reduction during the first 24 h in darkness (data not shown). The decrease in APSSTase activity of *L. minor* transferred to the dark was also slowed down during the first 24 h by serine and a combination of L-asparagine and L-glutamine, whereas phosphoserine, *N*-acetyl-L-serine and acetyl-CoA had no effect (Fig. 3). A significant increase in the thiol content, however, was only detected in the plants treated with OAS.

Table I shows the effect of the transfer to the dark and the simultaneous addition of OAS on the uptake and incorporation of $^{35}SO_4^{2-}$ into *L. minor*. Compared to the control kept

Table I. *^{35}S Content in Sulfate, Cysteine, Glutathione, the Protein Fraction and Nonidentified Thiol Compounds of L. minor after 24 h with $^{35}SO_4^{2-}$ in Continuous Light, in the Dark, and in the Dark with 0.5 mM OAS*

Treatment	^{35}S Content[a]				
	Sulfate	Cysteine	Glutathione	Protein	Nonidentified
	nmol (g fresh wt)$^{-1}$				
Light	2,328	52	320	1,298	360
Dark	2,058	52	125	221	63
Dark + OAS	3,523	867	577	317	326

[a] Before the addition of $^{35}SO_4^{2-}$ the plants were cultivated in continuous light.

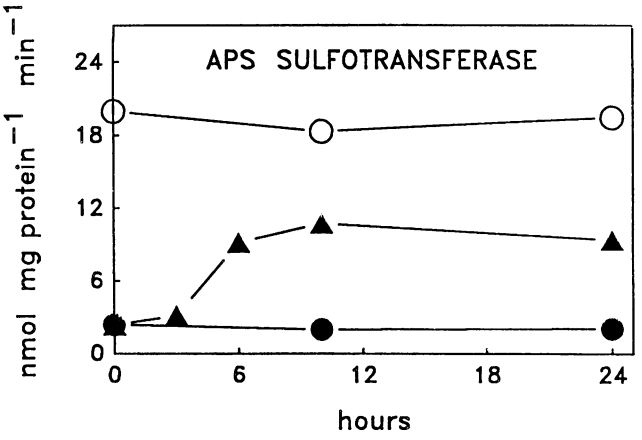

Figure 4. Extractable activity of APSSTase of *L. minor* cultivated in continuous light (○) or in the dark with (▲) or without (●) addition of 0.5 mM OAS. The cultures in the dark were precultivated in darkness for 24 h.

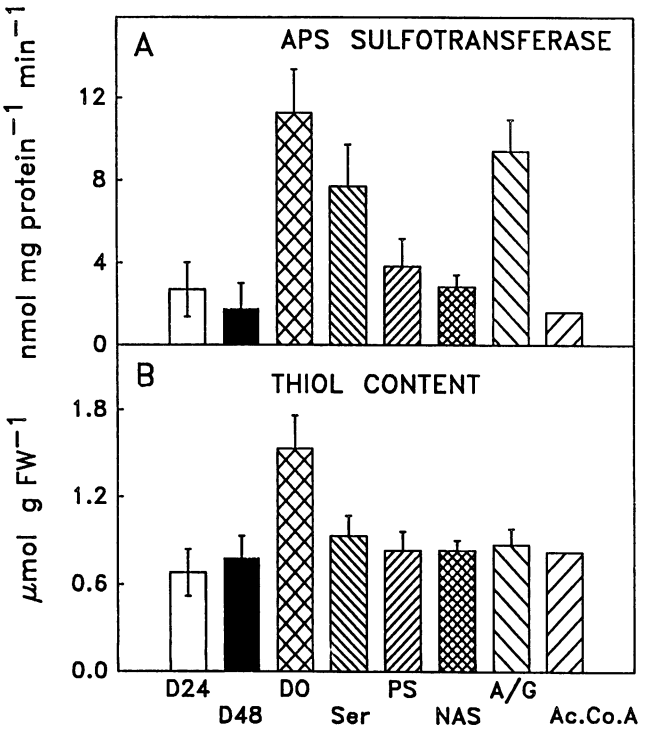

Figure 5. Extractable activity of APSSTase (A) and acid-soluble SH contents (B, thiol content) of *L. minor* after 24 h preincubation in the dark (D24) and after further 24 h in the dark without addition (D48) or with 0.5 mM OAS (DO), 0.5 mM L-serine (Ser), 0.5 mM DL-phosphoserine (PS), 0.5 mM *N*-acetyl-L-serine (NAS), 2 mM each of L-asparagine and L-glutamine (A/G), and 0.5 mM acetyl-CoA (Ac.Co.A). Mean values and SD of three to six measurements are presented except for acetyl-CoA, where the mean of two measurements is presented.

in the light the transfer to the dark induced a 40% decrease in total ^{35}S uptake, whereas addition of OAS parallel to the transfer caused an increase of 30%. After all three treatments, most of the radioactivity was detected in the sulfate fraction. In plants transferred to the dark, OAS caused a massive increase of the radioactivity in cysteine and glutathione. This indicates that the increase in the thiol content presented in Figures 1 and 3 was at least partly due to *de novo* synthesis of cysteine and glutathione from SO_4^{2-}. The incorporation of radioactivity into the protein fraction was greatly reduced in plants cultivated in the dark, indicating that protein synthesis was inhibited.

In a second set of experiments, the effect of the addition of OAS to the nutrient solution on sulfate assimilation was studied with *L. minor* that had been precultivated in the dark for 24 h. Under these conditions, an increase of the extractable activity of APSSTase occurred within 9 h so that a level was reached and maintained up to 24 h that was 50% of the controls kept in continuous light (Fig. 4). A higher level of APSSTase activity was also induced by serine or a combination of L-asparagine and L-glutamine, but no appreciable effect was detected with phosphoserine, *N*-acetyl-L-serine or acetyl-CoA (Fig. 5A). The content of acid-soluble SH compounds in the plants was doubled 24 h after addition of OAS (Fig. 5B), whereas the other compounds had no effect.

The increase in the extractable activity of APSSTase in the dark induced by OAS was inhibited by the simultaneous

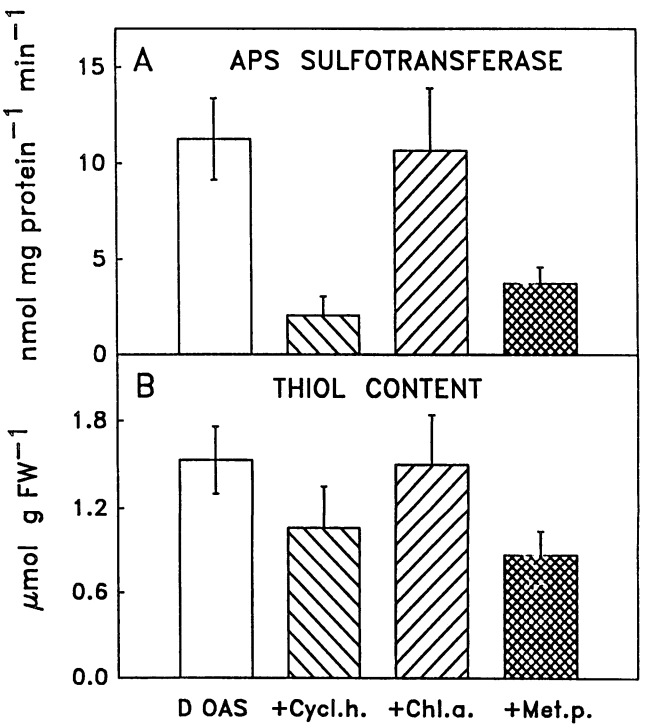

Figure 6. Extractable activity of APSSTase (A) and acid-soluble SH contents (B, thiol content) of *L. minor* after 24 h preincubation in the dark and further 24 h in the dark after addition of 0.5 mM OAS (DO), together with 4 µg ml^{-1} cycloheximide (Cycl.h.), 50 µg ml^{-1} chloramphenicol (Chl.a.), or 150 µg ml^{-1} 6-methylpurine (Met.p.). Mean values and SD of three to six measurements are presented.

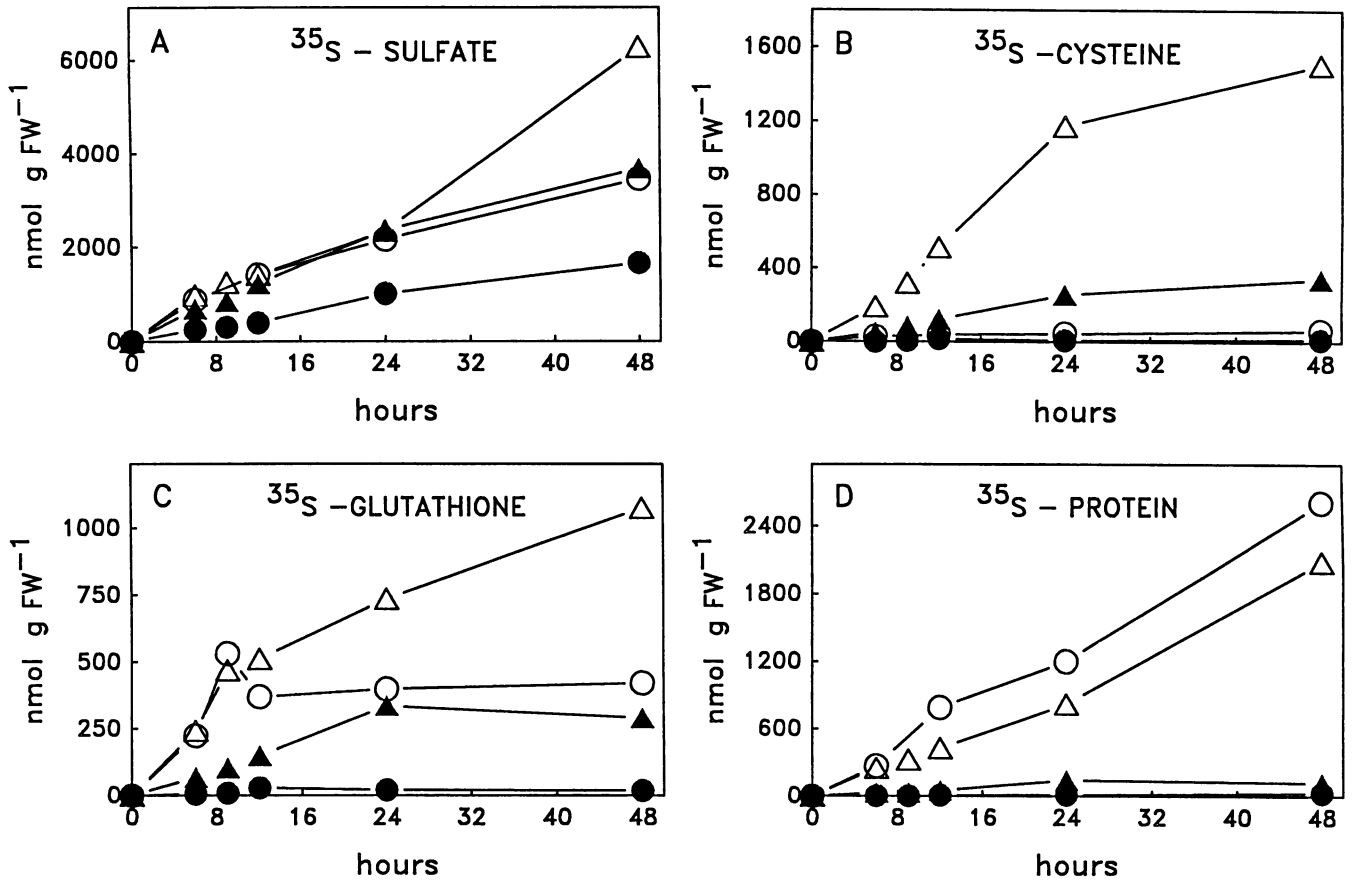

Figure 7. ^{35}S content in sulfate (A), cysteine (B), glutathione (C), and the protein fraction (D) of *L. minor* cultivated in continuous light in the presence of $^{35}SO_4^{2-}$ and with (△) or without (○) 0.5 mM OAS or after 24 h preincubation in the dark, subsequent addition of $^{35}SO_4^{2-}$ together (▲) or without (●) 0.5 mM OAS and further cultivation in darkness for 24 h.

addition of 4 μg ml^{-1} cycloheximide (an inhibitor of translation on 80S cytoplasmic ribosomes) or 150 μg ml^{-1} 6-methylpurine (an inhibitor of transcription) (Fig. 6A). The activity of the enzyme measured in the presence of these inhibitors corresponds to that of the dark control after 48 h (Fig. 4). Chloramphenicol at 50 μg ml^{-1}, an inhibitor of chloroplast protein synthesis, did not affect the increase of APSSTase activity induced by OAS.

In line with the results presented in Figure 6A, OAS together with cycloheximide and 6-methylpurine caused an increase in the thiol content that was smaller, however, than the increase with OAS alone or in combination with chloramphenicol (Fig. 6B). The incorporation of label from $^{35}SO_4^{2-}$ into sulfate (Fig. 7A), cysteine (Fig. 7B), glutathione (Fig. 7C) and the protein fraction (Fig. 7D) of *L. minor* is presented from 24 to 72 h in the dark or in continuous light. In plants kept in continuous light the addition of OAS to the nutrient solution caused an increase in total assimilation of ^{35}S even though the protein fraction contained about 30% less label than the controls. This lower labeling of the protein fraction is consistent with the increase in doubling time in the presence of OAS. In the dark cultures, OAS addition caused an increase in the label in all fractions as compared to the appropriate controls. After 24 h a 100-fold increase of the label in the cysteine fraction was detected, whereas the content of [^{35}S] glutathione increased 10-fold and was found to be stable at a value comparable to the light control. These results show that after a precultivation of 24 h in the dark $^{35}SO_4^{2-}$ was reduced in the dark and incorporated into amino acids. OAS greatly enhanced this sulfate assimilation in the dark.

DISCUSSION

When cucurbit cells are fed OAS in presence of 25 mM sulfate, the rate of hydrogen sulfide emission in response to sulfate declines and the incorporation of [^{35}S]sulfate into cysteine increases (16). Inhibition of the synthesis of the OAS precursor acetyl-CoA by 3'fluoropyruvate enhances hydrogen sulfide emission but inhibits cysteine synthesis. These observations indicate that the availability of OAS is the rate-limiting factor for cysteine synthesis in the light. From the results presented here, it can be concluded that OAS limits assimilatory sulfate reduction in *L. minor* cultivated on 0.88 mM sulfate both in the dark and in the light.

The dependence of sulfate assimilation on light is well established (3, 21, 26). With cucumber leaf discs, however, it has been shown that under dark conditions in the presence of 25 mM sulfate ^{35}S is incorporated into organic compounds at 60% of the rate in the light (24). When cultivated on 50 mM sulfate, leaf discs of spinach produce glutathione in the dark

at a rate which is 59% of that in the light (6). Our own results demonstrate a *de novo* synthesis of cysteine and glutathione from $^{35}SO_4^{2-}$ in the dark under physiologically relevant sulfate concentrations. These findings taken together seem in contrast to the idea of a strict control of sulfate assimilation by the light reaction of photosynthesis (23), and show that sulfate assimilation in green tissues can also proceed in the dark, albeit at a reduced rate.

From the finding that in *L. minor* Chl and ribulosebisphosphate carboxylase activity were stable up to 8 d in darkness, Ferreira and Davies (8) concluded that these plants do not appear to be programmed to senesce, or at least that darkness does not induce senescence in these plants. If this view is adopted for the discussion of the results presented here, the decrease in APSSTase of *L. minor* after transfer to the dark is not based on senescence. The relatively slow decrease suggests that regulation by the thioredoxin system of the chloroplasts (22) is not involved. This substantiates previous findings that showed that APSSTase activity is not dependent on thioredoxin (21). The results from inhibitor experiments with *L. minor* precultivated for 24 h in the dark show that the OAS induced increase in APSSTase activity is prevented either by the simultaneous addition of cycloheximide or 6-methylpurine. These findings suggest an *in vivo* light-dark regulation of APSSTase activity by protein turnover.

The parallel decrease in APSSTase and nitrate reductase activities after transfer of *L. minor* to the dark may be taken as an indication of the regulatory interaction between assimilatory sulfate and nitrate reduction. A light dependence of nitrate reductase activity in plants has been shown in different systems (10, 12). Several studies have established regulatory interactions between the pathway of assimilatory sulfate and nitrate reduction (1, 3, 9, 17, 25). Our results indicate that in *Lemna* OAS could be a positive signal from the pathway of nitrate assimilation to that of sulfate assimilation, thus establishing a coordination of both pathways. A corresponding function of OAS has been proposed previously for bacteria (14).

Taken together the results presented here support the idea previously put forward (3, 4, 24, 25) that APSSTase is a key control enzyme of sulfate assimilation and that OAS is a limiting substrate of this pathway. In combination with previously published findings (3, 19), a scheme of light-dark regulation of sulfate assimilation in chloroplasts emerges. After transfer to the dark, this pathway is rapidly downregulated because the decreased pH of the stroma and the increased level of 5′-AMP affect APSSTase activity negatively; because this enzyme has a pH optimum around 9 and is competitively inhibited by 5′-AMP (19). A further and slower decrease in the flux through the pathway is induced by the loss of APSSTase activity and the restricted availability of OAS. Further experiments are necessary to examine if the *in vivo* concentration of OAS and changes thereof are compatible with this scheme.

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