Adenosine Triphosphate Sulfurylase and O-Acetylserin Sulfhydrylase in Photoheterotropically and Heterotrophically Cultured Tobacco Cells

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Photoheterotrophic and heterotrophic cell suspension cultures of *Nicotiana tabacum* have been examined for changes in specific activity of ATP-sulfurylase (EC 2.7.7.4) and OAS-sulfhydrylase (EC 4.2.99.8) during growth on different nitrogen sources. During exponential growth the specific activity of ATP-sulfurylase and OAS-sulfhydrylase remained constant and was on the same level in cells with high and with low rates of sulfate assimilation. The specific activity of both enzymes rapidly increased in green photoheterotrophic cells as well as in chloroplast-free heterotrophic cells after the sulfur from the medium had been used up. This increase was reversed when the cells were transfered back to a sulfate supplemented nutrient solution. The changes in enzyme activity due to sulfur depletion seem to indicate a regulatory mechanism for these enzymes.

Introduction

Reuveny and Filner [1] recently have shown that the biosynthesis of ATP-sulfurylase in heterotrophic tobacco cell suspension cultures is repressed* during growth on readily assimilated sulfur sources but becomes derepressed during growth on slowly assimilated sulfur sources or during sulfur starvation. They, therefore, suggested that the sulfate assimilation in higher plants can be controlled by the regulation of ATP-sulfurylase synthesis. The authors also reported that the nitrogen pathway exercises a positive control on the biosynthesis of ATP-sulfurylase allowing the absolute rates of sulfur assimilation to rise and to fall with the demand for net protein synthesis.

In green cells, however, the product of the reaction catalysed by ATP-sulfurylase, adenosine-5′-phosphosulfate, is needed not only as an intermediate in sulfur reduction but also for the synthesis of chloroplast sulfolipids. On this basis it is difficult to conceive a regulation of sulfur assimilation in chloroplast containing cells by a mechanism adjusting the rate of ATP-sulfurylase formation only to meet the needs of protein synthesis. As sulfate assimilation in photoautotrophic plants is confined mainly to the leaves [2, 3], it seems important to obtain data on the response of ATP-sulfurylase to changes in sulfur nutrition in green cells.

In this paper we present evidence which shows that ATP-sulfurylase and O-acetylserin sulfhydrylase in green, chloroplast containing cells are repressed during growth on sulfate and become derepressed during sulfur starvation as in heterotrophic, chloroplast-free cells. Our data also show that the activity of ATP-sulfurylase does not run parallel to the rate of sulfate assimilation in the cells which points to a different mechanism for the fine regulation of sulfate assimilation.

* The molecular bases of changes in the rates of development of total extractable ATP-sulfurylase activity are not known. Therefore, changes in the ATP-sulfurylase activity found in the tobacco cell system should be considered cases of apparent induction, repression or derepression.

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Materials and Methods

Growth of cell suspension cultures

Cell suspension cultures of *Nicotiana tabacum* var. Samsun have been cultured as described by Berg-

mann *et al.* [4] in a modified liquid M+S medium [5] and grown at 23 °C under continuous illumination (3000 lx) or in darkness on a rotary shaker (100 rpm). In suspensions grown with nitrate as sole nitrogen source NH_4NO_3 was replaced by equimolar amounts of $Ca(NO_3)_2$. Glutathione and sulfate in the media, cell protein and dry weight of the suspensions were measured as described before [6, 7].

Extraction of soluble protein and assay of the enzymes

Cells from 3 to 4 cultures were separated from the medium by filtration and subsequent washing with cold Tris-HCl buffer (pH 8.4) 50 mM, containing 2 mM $MgCl_2$ and 0.5 mM β-mercaptoethanol. An aliquot of the cell material was taken and first homogenized in a potter homogenizer (1 g fresh weight/2 ml Buffer). The resultant slurry was sonicated with a Brason sonifier for 4×15 s with intervals of 15 s. The temperature was kept below 4 °C during sonification and all further treatment. A clear crude protein extract was obtained after 15 min of centrifugation at 18000 × *g*. 5 ml of this extract were layered on a Biogel P-2 column. The column was eluated with Tris-HCl buffer (pH 8.4) 25 mM and 2 ml fractions were collected. Enzyme activity was normally confined to fraction 5–8 and these fractions were used to assay ATP-sulfurylase and OAS-sulfhydrylase activity. The protein content was measured by following basically the method of Lowry *et al.* [8].

The enzymatic activity of ATP-sulfurylase was determined spectrophotometrically as the APS-dependent generation of ATP in the presence of inorganic pyrophosphate according to Schwenn *et al.* [9]. OAS-sulfhydrylase activity was assayed by measuring the formation of cysteine in the presence of O-acetylserine and Na_2S according to Becker *et al.* [10].

Results

The tobacco cell cultures used in the experiments grow in darkness as chloroplast-free heterotrophic cell suspensions and in light develop photosynthetically active chloroplasts [11]. If cultured heterotrophically with ammonium and nitrate as nitrogen sources the cells release large amounts of glutathione into the medium whereas only small amounts of glutathione are found in the medium of cells cultivated either photoheterotrophically with nitrate alone or heterotrophically with ammonium plus nitrate [7]. Since nitrate grown cells exhibit a lower rate of protein synthesis than cells supplied with ammonium plus nitrate [4] enzyme activities of cells with different rates of sulfur and nitrogen assimilation can be compared in this cultivated tobacco cell system.

Under the conditions used the photoheterotrophic and heterotrophic cultures grew on ammonium plus nitrate with nearly the same vigor doubling their dry matter every two days (Table I). Starting with 30 mg DW/100 ml and 40 micromoles sulfate the cultures reached a dry weight of 250 to 300 mg before the

Table I. Dependence of growth rates, glutathione production and enzymatic activities of photoheterotrophic and heterotrophic cell suspension cultures of *N. tabacum* on nitrogen sources.

	Light $NH_4^+ + NO_3^-$	Dark $NH_4^+ + NO_3^-$	Light NO_3^-
Growth Rate [a]	40.3%	40%	33.5%
Increase in Protein mg Protein, g DW^{-1}, 24 h^{-1}	88.05 ± 3.2	88.62 ± 8.58	33.45 ± 3.66
GSH-Production [b] µmol GSH, g DW^{-1}	130.6 ± 6.8	4.1 ± 1.0	10.85 ± 2.9
Sulfate Uptake nmol SO_4^{2-}, g DW^{-1}, 24 h^{-1}	128.8 ± 11.6	47.9 ± 5	–
ATP-Sulfurylase nmol min^{-1} mg $Prot^{-1}$ µmol min^{-1}, g DW^{-1}	90.24 ± 13.76 7.64 ± 0.36	92.25 ± 11.9 7.42 ± 0.67	94.9 ± 10.78 4.50 ± 0.65
OAS-Sulfhydrylase µmol min^{-1} mg $Prot^{-1}$	0.86 ± 0.08	1.12	1.70 ± 0.08

[a] % increase in dry weight per day = (antilog ¼ log (g d. w. day 7/g d. w. day 3) − 1) × 100.
[b] GSH – in the medium after 10 days of culture.

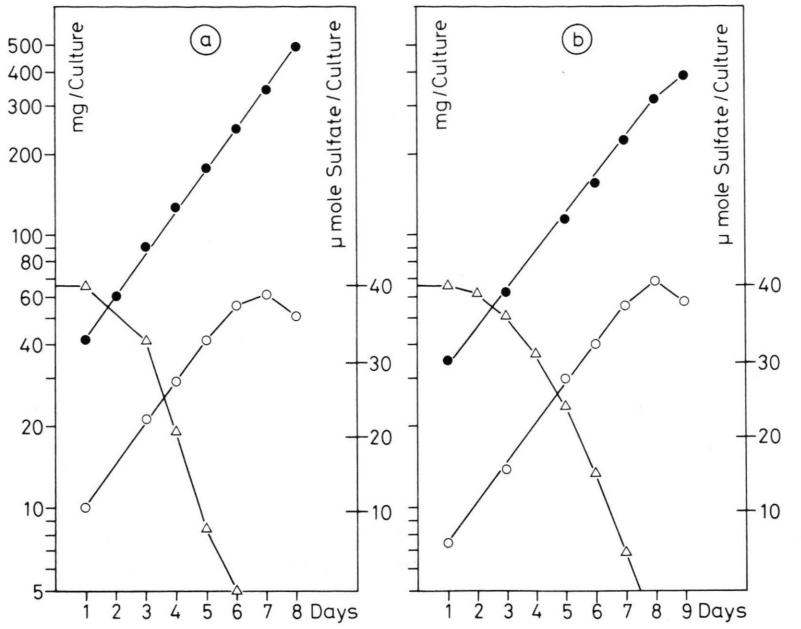

Fig. 1. Changes in dry weight (●), protein (○) and in the sulfate levels (▲) in the medium during the growth cycle of photoheterotrophically (a) and heterotrophically (b) cultured cell suspensions of *N. tabacum* grown on M + S medium with ammonium nitrate.

sulfate in the medium became limiting (Fig. 1). During exponentional growth the cells of both cultures exhibit protein contents between 23 and 25% of the dry weight and produced 88.0 and 88.6 mg protein per g DW and day. After depletion of the sulfur in the medium the protein content of the cells dropped below 10% of the dry matter. The protein increase of the cultures came to a stop usually after 7 days. Thereafter a decline of the content of protein was observed which may indicate a damage to the cells caused by the increasing sulfur starvation.

Cells cultivated on nitrate as sole nitrogen source exhibit a lower growth rate than the controls on ammonium plus nitrate and showed a lower protein content. During exponentional growth the cells doubled their dry matter every 58 h and produced 32.8 mg protein per g DW and day, which is approximately a third of the net-protein synthesis of cells grown on ammonium plus nitrate. After exhaustion of the sulfur supply in the medium protein synthesis decreased and the protein increase of the cultures came to a standstill (Fig. 3). In contrast to cultures grown on ammonium plus nitrate the protein level of the nitrate-cultures did not decline during sulfur starvation but kept constant for four or more days after the onset of starvation.

Under various culture conditions the tobacco cells release quite different amounts of glutathione into the medium (Table I). In photoheterotrophic cells grown an ammonium plus nitrate 130 µmol GSH per g D.W. are found after 10 days of culture. Heterotrophic cells grown in the dark produced only 4.1 µmol GSH per g D.W. during the same period, and in photoheterotrophic cultures grown with nitrate as sole nitrogen source 10.8 µmol GSH per g D.W. were released into the media. Since heterotrophically and photoheterotrophically grown cells produce the same amount of protein during exponentional growth the rates of sulfur assimilation in the photoheterotrophic cells is nearly two times higher than in heterotrophic cells. This difference between the rates of sulfate assimilation is reflected in the rates of sulfate uptake which were 128 µmol and 48 µmol per g D.W. and day, respectively.

During exponentional growth on sulfate containing media the level of ATP-sulfurylase and OAS-sulfhydrylase activity in the tobacco cells remained constant and at a low level (Fig. 2). Despite the pronounced differences in the rates of sulfur assimilation between cells cultivated photoheterotrophically with ammonium plus nitrate and with nitrate, or grown photoheterotrophically and heterotrophically on ammonium nitrate, no differences were observed in the specific activities of ATP-sulfurylase (Table I). Also the amount of enzyme activity per g dry weight was the same in cells grown either photohetero-

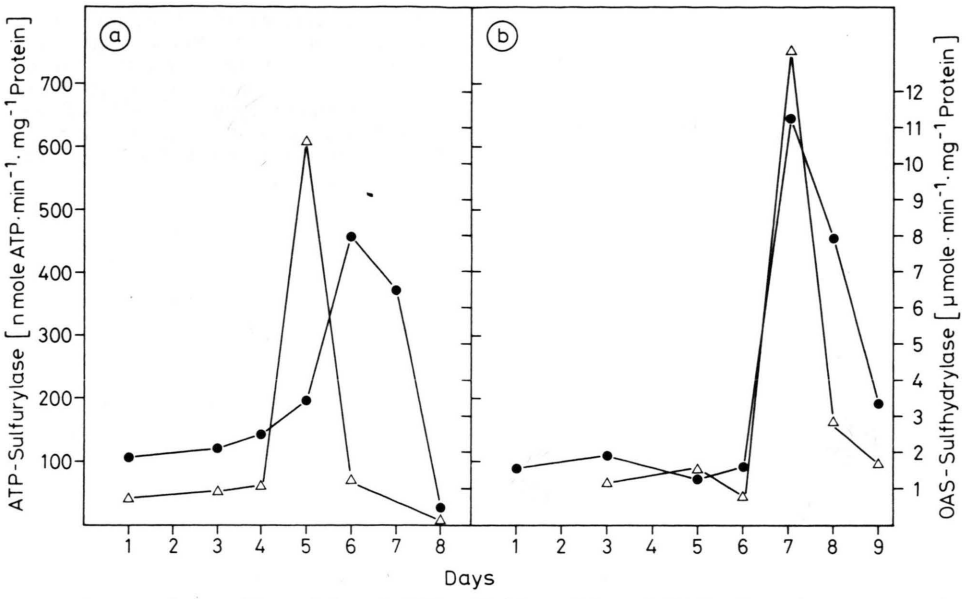

Fig. 2. Changes in specific activity of ATP-sulfurylase (●) and OAS-sulfhydrylase (△) during the growth cycle of cultured cell suspensions of *N. tabacum* grown photoheterotrophically (a) and heterotrophically (b) on M + S medium with ammonium-nitrate. (For growth data *cf.* Fig. 1.)

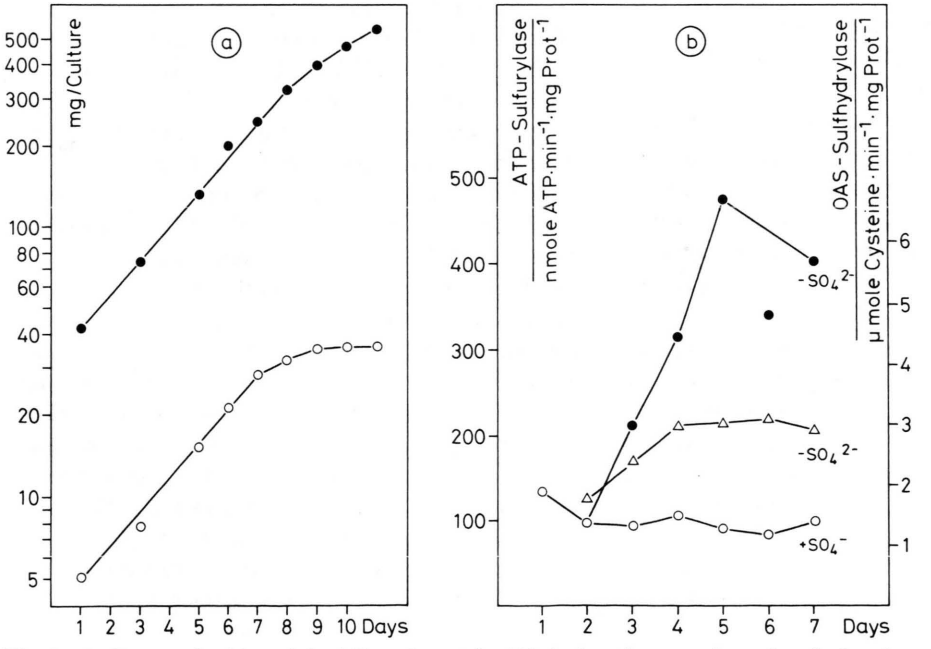

Fig. 3. a) Changes in dry weight (●) and protein (○) during the growth cycle of photoheterotrophically cultured cell suspensions of *N. tabacum* grown on M + S medium with NO_3^- and 0.4 mM sulfate. b) Effect of sulfate starvation on ATP-sulfurylase activity (●) and OAS-sulfhydrylase activity (△) in photoheterotrophically cultured cells suspensions of *N. tabacum* grown on sulfate-free M + S medium with nitrate.

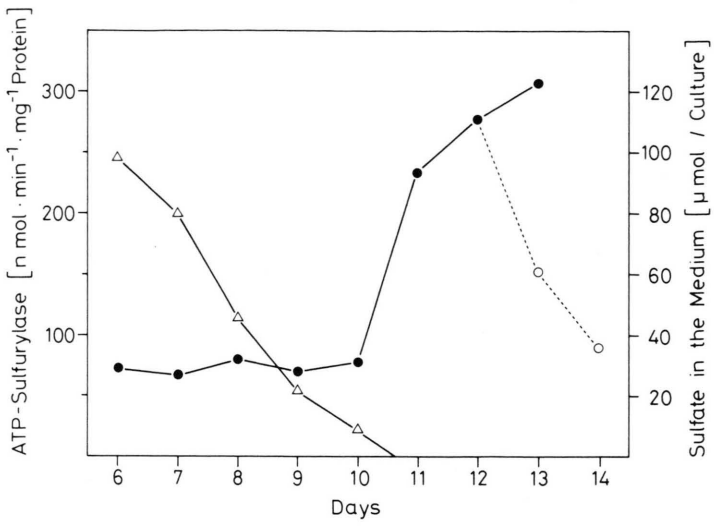

Fig. 4. Development of ATP-sulfurylase activity in photoheterotrophic cell suspension cultures of *N. tabacum* grown with limiting amounts of sulfate on M + S medium. ●—● ATP-sulfurylase activity; ○—○ ATP-sulfurylase activity after transfer of cultures to fresh sulfate-containing media; △—△ sulfate in the medium.

trophically or heterotrophically on ammonium plus nitrate, whereas the enzyme activity per dry weight in nitrate grown cells was nearly half of those, which is in accordance with the lower protein content of the nitrate cells.

Determinations of OAS-sulfhydrylase activities gave similar results; the data also revealed no correlation between the rate of sulfur assimilation and the enzyme activity (Table I).

After depletion of the sulfate supply in the medium the specific activities of ATP-sulfurylase and OAS-sulfhydrylase began to rise and increased up to 6–10 fold above the initial level (Fig. 2), which indicates that both enzymes in the green and non-green tobacco cells are subjected to a regulatory mechanism.

The activity of ATP-sulfurylase and OAS-sulfhydrylase decayed in cells cultivated on ammonium 24–48 h after this increase which may probably be due to ammonium becoming toxic to the cells during sulfur starvation. The enzyme activities in the nitrate grown cells remained at the increased level for a longer period and declined more slowly when the sulfur starvation become severe (Fig. 3). Transfered back to fresh, sulfate containing media the starved cells resumed growth and protein synthesis again during which the activity of ATP-sulfurylase and OAS-sulfhydrylase declined as described by Reuveny and Filner for their system (Fig. 4).

Discussion

The effect of sulfur deficiency on the activities of ATP-sulfurylase and OAS-sulfhydrylase in the chloroplast-containing and chloroplast-free tobacco cells clearly indicates that both enzymes are repressed during growth on sulfate and became derepressed by sulfate starvation. The changes observed in the activities of ATP-sulfurylase were somewhat smaller but in the same order than those reported by Reuveny and Filner [1] and by Schwenn *et al.* [9] for the Catharanthus cell system. The changes in the level of OAS-sulfhydrylase on the other side were much more pronounced than the differences found by Smith [12] in the roots of sulfur-deficient beans, which may be due to the fact that in cell suspension cultures the symptoms of sulfur deficiency are more regular inducible than in whole plants. The increase in the enzyme activities of ATP-sulfurylase and OAS-sulfhydrylase in our tobacco cells most likely depends on de novo enzyme synthesis as indicated by the simoultaneous decrease of the specific activity of 5-oxo-prolinase in the enzyme extracts (Polle and Rennenberg, pers. communication).

Despite the proved ability of the tobacco cells to adapt their level of ATP-sulfurylase to the sulfur supply, no differences were found in the enzyme activity between cells with high and low rates of sulfur assimilation. Obviously the enzyme activities in the repressed state were sufficient to support

assimilation of quite different amounts of sulfate. The failure to demonstrate a correlation between the enzyme activities and the sulfate assimilation indicates that the rate of sulfate assimilation in the tobacco cells can not be controlled only by changes in the level of ATP-sulfurylase in the cells but must be regulated by other systems.

In view of the fact that chloroplasts contain the enzymes necessary to reduce sulfate to cysteine it is noteworthy that the specific activities of the enzymes tested in the extracts from chloroplast-containing and chloroplast-free cells were of the same magnitude. This would only be understandable if the plastids in the dark-grown cells also contain the enzymatic machinery for sulfate assimilation.

Acknowledgement

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