Regulation and Partly Purification of the ATP-Sulfurylase from the Cyanobacterium *Synechococcus* 6301

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ATP-sulfurylase from the cyanobacterium *Synechococcus* 6301 was regulated *in vivo* during growth in batch culture. The activity was highest at the third day after inoculation, declining afterwards to a level found in resting cells. During growth with air supplemented with 2% CO_2 this activity increased 3-fold compared to controls grown with normal air as CO_2 source. Addition of either nitrite or urea enhanced ATP-sulfurylase activity about 2-fold, whereas cysteine and especially methionine decreased ATP-sulfurylase activity to 5% of controls without treatment.

The ATP-sulfurylase was purified by conventional techniques using DEAE-cellulose chromatography and further separation on blue sepharose achieving a 250-fold increase in the specific activity. An apparent K_M of 5 μM for APS and of 40 μM for pyrophosphate was determined with the purified enzyme fraction.

Introduction

Sulfate, the most oxidized sulfur source, is adenylated before it is reduced and eventually incorporated into cell constituents. The first step of sulfate assimilation is the activation catalyzed by the ATP-sulfurylase (ATP: sulfate adenylyltransferase; EC 2.7.7.4). This enzyme is present in virtually all types of organisms [1–3] including cyanobacteria: *Anabaena cylindrica* [4, 5], *Spirulina platensis* [6], and *Synechococcus* 6301 [7–9]. Highly purified enzyme was obtained from yeast [10, 11], rat liver [12, 13], *Penicillium chrysogenum* [14–16], *Euglena* [17] and higher plants [18, 19]. ATP-sulfurylase has been partly purified (10-fold) from *Anabaena cylindrica* and characterized [4, 5]. However, no attempt has been made, as yet, to completely purify and characterize this enzyme in cyanobacteria. Being the first step of sulfate metabolism, this enzyme is expected to be under thight regulation. For example, net sulfate entry, net ATP generation and relative levels of reduced sulfur products could operate and regulate the enzymatic reaction. However, no information on the regulatory properties of the ATP-sulfurylase in cyanobacteria is available so far.

Materials and Methods

Organism and growth conditions

Synechococcus 6301 axenic cultures (obtained from the algal collection Pasteur Institut, Paris) were grown in BG-11 medium [20] with nitrate ($NaNO_3$ 10 mM) and sulfate ($MgSO_4$ 0.3 mM) as normal constituents cultured in 750 ml Pirson flasks at 27 °C and 14,000 lux light intensity under constant aeration. Unless otherwise mentioned, 2% CO_2 was sparged with air from 2nd day following inoculation.

Sulfur starvation experiments were done in a medium containing 0.3 mM $MgCl_2$ instead of $MgSO_4$. All additions were made aseptically following filter sterilization (10 μm pore size, Sartorius) of the stock solution.

Extraction of the enzyme

Unless otherwise mentioned 5 day grown cultures were harvested by centrifugation (10,000 rpm for 15 min at 4 °C in a Sorvall centrifuge), washed and resuspended in 20 mM Tris-HCl buffer pH 8.0 containing 0.01 M $MgCl_2$ (breaking buffer) in a ratio of 1:5 (w:v). The cell suspension was passed twice through a french press cell (15,000 PSI) to

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break the cells. The supernatant resulting from 12,000 rpm centrifugation was used for the assays.

Enzyme assay

The ATP-sulfurylase was determined by the backward reaction forming ATP from APS and pyrophosphate using the firefly method [21, 22].

The reaction mixture for ATP-sulfurylase contained in 0.2 ml: Adenosine-5′-phosphosulfate (APS) 2 nmol; pyrophosphate (Na-salt) 160 nmol; 50 µl luciferin-luciferase reagent and an appropriate amount of the extract. Pyrophosphate was added 10 sec after stabilization of the reaction mixture to start the assay. The difference of ATP produced between 60 sec and 120 sec at 30 °C was considered for the activity determination. The specific activity of the ATP-sulfurylase is defined as 1 µmol ATP produced min^{-1} mg^{-1} protein.

Analytical processes

ATP was estimated by the luciferin-luciferase reagent (Boehringer, Mannheim). Protein measurements were done with the Biorad reagent (Biorad, München) using bovine serum albumin as a standard.

Purification steps of the ATP-sulfurylase

Step 1: Crude extract

Frozen cells (50 g) were suspended in 250 ml of 100 mM Tris-HCl buffer pH 8.0 containing 0.01 M $MgCl_2$. The cell-free extract was made as described above.

Step 2: Ammonium sulfate precipitation

The proteins from the crude extract fractionated between 35% and 50% ammonium sulfate were resuspended and subsequently dialyzed against Tris-HCl buffer pH 9.0 (15 mM).

Step 3: DEAE-cellulose chromatography

The dialyzed fraction was applied to a DEAE-cellulose column (4 × 30 cm) equilibrated with 0.05 M Tris-free base. The column was washed with 100 ml of this buffer and the enzyme was eluted with a linear NaCl gradient (0–2 M) in Tris-free base. The active fractions were collected, pooled and concentrated by ultrafiltration.

Step 4: Blue sepharose affinity column chromatography

The enzyme was further purified by a second chromatography on blue sepharose (Amicon) column (0.5 × 20 cm) equilibrated previously with 0.05 M Tris-free base. The column was washed with the equilibrium buffer, and the enzyme was eluted with a linear gradient of NaCl (0.2 M in Tris-free base). Active fractions were combined and concentrated as above.

Step 5: PAGE electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemli on a linear gradient (8 to 18%) containing 0.1% SDS.

Chemicals

The chemicals used were purchased as described earlier [23].

Results

Effects of growth conditions and CO_2 on ATP-sulfurylase activity

Light-grown aerated cultures were sparged with/without CO_2 (2% addition) on the 2nd day following inoculation and ATP-sulfurylase activity was monitored in a kinetic experiment. The enzyme activity appeared after 24 h of growth and attained its peak on the third day. Addition of CO_2 on the 2nd day enhanced the enzyme level on the following day approximately 3-fold compared to normal air grown cultures. Subsequently the enzyme activities of both, CO_2-enhanced and normal air-gased cultures decreased (Fig. 1).

Light and dark regulation of the enzyme

Light-grown cultures were inoculated and incubated under dark conditions. In another experiment cultures were kept for 12 h in darkness and then illuminated. Dark-incubated cultures had approximately 53% of the enzyme activity of the light control. Upon illumination the ATP-sulfurylase activity increased after a lag period of 2 h to approximately to 85% of light-grown cultures. On the other hand 30% inactivation was observed after a shift to darkness. Therefore a weak light regulation of the enzyme activity is implied (Fig. 2).

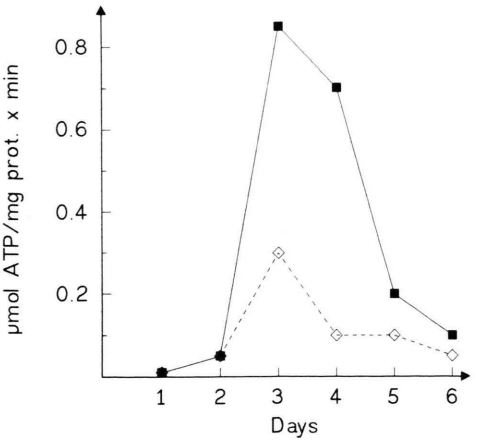

Fig. 1. Development of the specific activity of the ATP-sulfurylase during growth of *Synechococcus* 6301 in batch culture gassed with normal air or supplemented with additional 2% CO_2. Aliquots were taken as indicated and the specific activity was determined as described in Materials and Methods. ◇ = growth with air; ■ = growth on air supplemented with 2% CO_2.

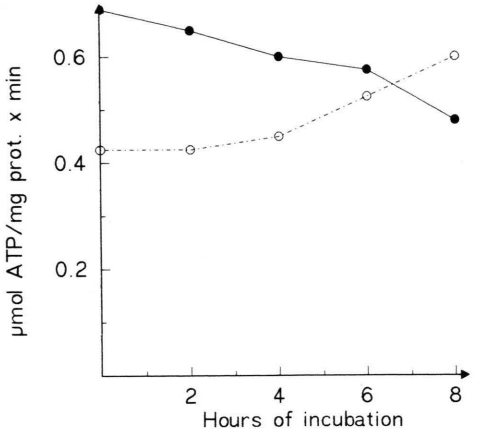

Fig. 2. Development of the ATP-sulfurylase activity after a light/dark or a dark/light transition. Cultures were grown for 3 days with CO_2 and one culture was kept dark for 10 h before illumination. Samples were taken in the intervals indicated and the specific activity of the ATP-sulfurylase was determined. ● = change light/dark; ○ = change dark/light.

Effect of sulfur starvation

Quitting the sulfur source in sulfate-grown cultures resulted in an initial minor increase of ATP-sulfurylase activity after 4 h. There was, however, a sharp decline in this enzymatic activity after prolonged sulfate starvation for 24 h (Fig. 3).

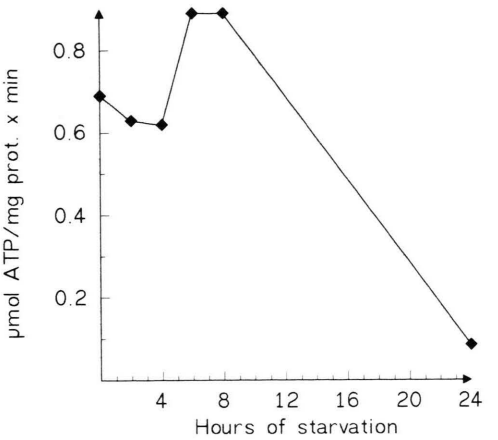

Fig. 3. Development of the ATP-sulfurylase activity after sulfur starvation. A culture grown for 3 days with normal sulfate nutrition was harvested, washed with sulfur-free medium and than resuspended in sulfur-free medium for further growth under sulfur starvation conditions. Aliquots were taken at the time intervals indicated.

Influence of sulfur amino acids on the ATP-sulfurylase activity

A variety of sulfur amino acids were analyzed for a possible effect on the ATP-sulfurylase of *Synechococcus* 6301. Cultures grown with normal sulfate for 84 h were supplemented with the sulfur amino acids listed in Table I. The ATP-sulfurylase was determined 5 h afterwards; the data of these experiments are summarized in Table I.

Cysteine reduced the ATP-sulfurylase to about 18% when added at 0.1 mM to a growing culture. Cysteine derivatives such as S-carboxymethyl-L-cysteine, L-cysteine ethyl ester, taurine, β-mercaptopyruvate, thiazolinin-4-carboxylic acid, cystathionine, N-acetyl-L-cysteine, L-cysteic acid and lanthionine caused some inhibition on the ATP-sulfurylase activity. Strong inactivation was observed with the addition of cysteamine, which caused growth inhibition in this cyanobacterium [24].

Methionine – either alone or in combination with lysine and or threonine – reduced this enzyme activity to about 5%. Homocysteine inhibited this enzyme activity to 60%, thus being less efficient compared to L-methionine. Reduced activities were also observed when lysine and threonine or methionine and isoleucine were added. Growth

Table I. Influence of sulfur and nitrogen compounds on the activity of the ATP-sulfurylase of *Synechococcus* 6301. The compounds stated above were added to *Synechococcus* cultures growing with sulfate (0.3 mM) at a final concentration of 0.1 mM. The activity of the ATP-sulfurylase was measured 5 h later.

Compound used	Activity of the ATP-sulfurylase [μM ATP × mg^{-1} protein × min^{-1}]	% of control
Control sulfate grown	0.89	100
Ammonium chloride	0.66	74
Urea	1.61	181
Sodium nitrite	1.60	180
L-Cysteine	0.16	18
L-Cysteine ethyl ester	0.67	75
L-Cysteine methyl ester	0.30	34
N-Acetyl-L-cysteine	0.36	40
S-Carboxymethyl-L-cysteine	0.75	84
Thiazolidine-4-carboxylic acid	0.55	62
β-Mercaptopyruvate	0.55	62
Cysteic acid	0.17	19
Taurine	0.63	71
Cysteamine*	0.05	6
L-Cystathionine	0.43	48
DL-Lanthionine	0.10	11
L-Homocysteine	0.30	34
L-Methionine	0.05	6
L-Methionine + L-lysine	0.07	8
L-Methionine + L-threonine	0.42	47
L-Methionine + L-isoleucine	0.21	24
L-Methionine + L-lysine + L-threonine	0.01	1
L-Lysine + L-threonine	0.31	35
Reduced glutathione	0.71	80
Oxidized glutathione	0.44	49

* Causes growth inhibition [24].

of *Synechococcus* was not restricted by addition of methionine [24]. Cysteamine and methionine did not affect the ATP-sulfurylase assay in the cell-free system.

Addition of glutathione (oxidized or reduced) to sulfate-grown cultures decreased the activity to 53% and 82% respectively (Table I).

In a kinetic experiment the effects of methionine and cysteine on the ATP-sulfurylase were tested on sulfate-grown cultures (Fig. 4). After addition of methionine, the enzyme activity was sharply reduced to nearly zero level after about 2 h and remained constantly low thereafter. Addition of L-cysteine initially lowered the activity to about 50%, which subsequently increased to 125% of the zero time value. After 3 h of incubation there was a sharp decline in the ATP-sulfurylase activity comparable to the methionine-incubated cultures. Neither cysteine- nor methionine-inhibited growth of *Synechococcus* 6301 in the concentration range used.

Effect of nitrogen sources

Addition of $NaNO_2$ and urea to cultures with normal nitrate and sulfate nutrition enhanced the ATP-sulfurylase activity to 180% of the control. In contrast, addition of NH_4Cl reduced this activity to about 74% of the nitrate-grown cultures (Table I).

Purification of ATP-sulfurylase

ATP-sulfurylase could be purified by ammonium sulfate fractionation followed by DEAE-cellulose and blue sepharose chromatography (Table II). A 4- to 5-fold purification was achieved when freshly prepared crude extract (12,000 × *g* supernatant) was fractionated between 35% to 50% of solid ammonium sulfate. This preparation was further purified during subsequent chromatography on DEAE-cellulose with an increasing NaCl gradient. Almost 225-fold purification was obtained after blue sepharose step in the fractions eluted with 0.5 M NaCl.

Table II. Purification profile of the ATP-sulfurylase.

Step	Total activity	Total protein [mg]	Specific activity [units/mg]	Purification factor	Recovery [%]
Crude extract	2899	785	3.7	1	100
Ammoniumsulfate cut	3482	219	15.9	4.3	120
DEAE-cellulose	3874	43	90	24.4	133
Blue sepharose	1875	2.25	833	226	65

SDS gel electrophoresis of the purified preparation exhibited two bands with a molecular weight of 41,000 and 44,000 daltons and the molecular weight by gel chromatography was estimated to about 85,000 daltons suggesting that this enzyme is active as a dimer.

This purified enzyme preparation was used to determine its basic properties. The enzyme could be stabilized in crude extracts by either low pH values around a pH of 5 or under alkaline conditions in Tris buffers above a pH of 9, suggesting a degradation of the ATP-sulfurylase (in crude extracts) by proteases, which are removed on DEAE-cellulose chromatography. Optimal pH for the ATP-sulfurylase in the fire-fly assay was around 9.0. The substrate saturation constants for APS and pyrophosphate in the reverse direction have been determined to an apparent K_M of 40 μM for pyrophosphate and an apparent K_M of 5 μM for APS. The Lineweaver-Burk plots for the two substrates are given in Fig. 5 and 6.

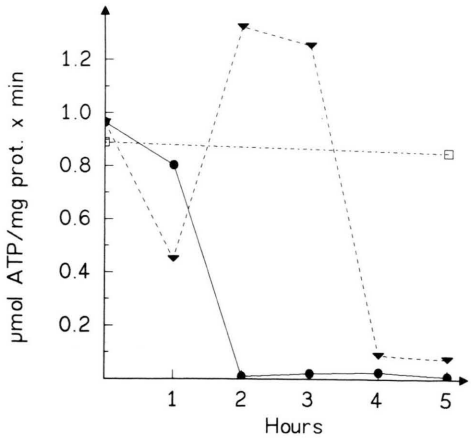

Fig. 4. Decrease of ATP-sulfurylase activity in *Synechococcus* 6301 after addition of L-cysteine or L-methionine. These compounds were added aseptically to a final concentration of 0.1 mM and the ATP-sulfurylase activity was determined at the time intervals indicated. ● = addition of 0.1 mM L-methionine; ▼ = addition of 0.1 mM L-cysteine; □ = control.

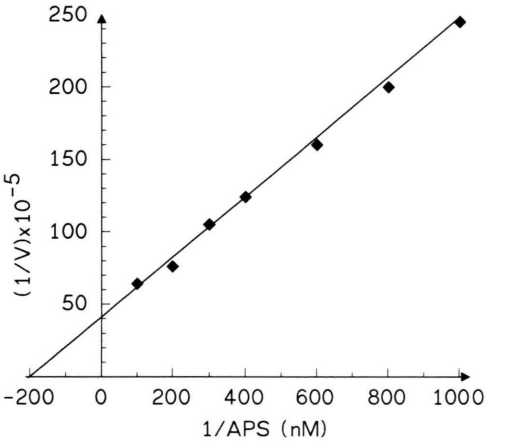

Fig. 5. K_M determination for APS using the purified enzyme fraction after the blue sepharose column (step 4).

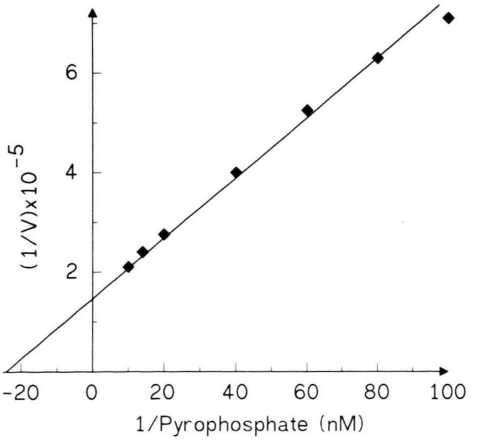

Fig. 6. K_M determination for pyrophosphate using the purified enzyme fraction after the blue sepharose column (step 4).

Discussion

Regulation of sulfate uptake and control of its further reduction is necessary for growth in all assimilatory sulfate reducers. Sulfate uptake and its regulation have been analyzed in some detail and described in recent reviews [2, 3, 25–28].

The ATP-sulfurylase was analyzed in this study to understand possible regulatory signals for sulfate transformation to sulfolipids and protein biosynthesis in cyanobacteria. *Synechococcus* 6301 was chosen, because it is an unicellular, fast growing strain, which has been used by us for several studies related to sulfur metabolism in our laboratory [3, 8, 9, 24, 29–33].

Our results demonstrate, that the ATP-sulfurylase can be regulated by signal(s) monitoring the metabolic activity.

a) During fast growth the ATP-sulfurylase is derepressed and it is repressed it during slow growth or resting cultures.

b) Enhancing the CO_2 concentration increased growth of *Synechococcus* 6301 and the specific activity of the ATP-sulfurylase.

c) The ATP-sulfurylase activity was influenced to some extent by light or darkness; however, the observed changes are only moderate, suggesting a control by the growth status of the cell, excluding a direct thioredoxin-activated step at this site. This was proven later with the purified enzyme, which could not be activated by any thiol- and or thioredoxin combination from *Synechococcus, Escherichia coli, Spinacia oleracea* or *Chlorella vulgaris* [8, 9, 32 34].

d) Addition of nitrite or urea to growing cultures of *Synechococcus* increased the ATP-sulfurylase level. This effect could be due to a higher demand of reduced sulfur compounds to balance the ratio of reduced nitrogen to reduced sulfur [35, 36]. However, ammonia did not show such a dramatic effect, possibly due to its uncoupling effect on photophosphorylation.

Regulation of the ATP-sulfurylase activity by growth conditions and sulfur availability was observed in tobacco tissue cultures, where this enzyme responded to sulfur and nitrogen treatments [35–37] and similar observations were made in the higher plant *Ipomea* sp. [38]. Thus, the increase of the ATP-sulfurylase activity after nitrite or urea addition fits well into regulatory aspects known from higher plants.

The data compiled in Table I demonstrate, that the ATP-sulfurylase of *Synechococcus* 6301 responds to the addition of externally supplied sulfur sources. Especially methionine decreased the ATP-sulfurylase level to about 5% of the sulfate-grown control.

Cysteamine had been shown previously to stop growth of *Synechococcus* within 15 min [24], thus we can not deduce from the cysteamine effect, if this is due to a specific control of the ATP-sulfurylase or if this represents a normal consequence of growth inhibition.

Kinetic analysis of the decrease of the ATP-sulfurylase activity caused by cysteine or methionine at a final concentration of 0.1 mM showed a fast correspondence to methionine decreasing this activity *in vivo* within 2 h (Fig. 4), whereas addition of methionine to the purified ATP-sulfurylase *in vitro* did not change its activity (data not shown). Addition of L-cysteine also decreased ATP-sulfurylase activity. However, an inactivation comparable to that of methionine could be measured only after 4 h, whereas the inactivation caused by methionine was complete within 2 h (Fig. 4). The early 1-h drop of the ATP-sulfurylase after addition of L-cysteine was measured repeatedly and it is not an "accident" of this specific experiment. Thus methionine or a derivative of methionine seems to be necessary for a repression of the ATP-sulfurylase in *Synechococcus*. This is in contrast to other regulatory control systems of assimilatory sulfate reduction in *Escherichia coli* [39] and *Salmonella typhimurium* [40], where cysteine seems to be the signal for this regulation.

The ATP-sulfurylase of *Synechococcus* 6301 can be stabilized and further purified. Stabilization could be achieved by keeping the enzyme at a high pH above 9 during storage and purification. Obviously proteases are inactive at this high pH leaving the ATP-sulfurylase active. Addition of glycerol (20% w:v) allowed storage at −20 °C for a period of 20 days without loss of activity.

Separation on DEAE-cellulose and gel chromatography columns could be performed without any difficulty. An effective purification step could be achieved using dye ligand chromatography on blue sepharose (Cibacron blue), which allowed a specific and rapid purification with a high yield and an over 200-fold increase in the specific activity. The purified enzyme can be separated on native

gels with a yield of about 30%. Only two bands were detected in this enzyme preparation after the blue sepharose treatment; thus methods are available now for the purification of this enzyme from a cyanobacterium.

The ATP-sulfurylase from *Synechococcus* seems to be a "normal" enzyme compared to data available from ATP-sulfurylases from bacteria and higher plants. An apparent K_M of 5 μM for APS and of 40 μM for pyrophosphate is in the range measured for other ATP-sulfurylases. This activity was not effected by thiols or thioredoxins. Furthermore the high activity under alkaline storage conditions suggests, that thiols should not be needed for activation/inactivation, which is in agreement with our observation that this enzyme is not affected dramatically by light changes *in vivo*.

The data presented here indicate that the ATP-sulfurylase of *Synechococcus* 6301 is controlled by a repression/derepression mechanism with evidence for a methionine-related regulatory signal.

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