Changes of C-Phycocyanin in *Synechococcus* 6301 in Relation to Growth on various Sulfur Compounds

Ahlert Schmidt, Ingrid Erdle, and Hans-Peter Köst

Botanisches Institut, Universität München, Menziger Str. 67, D-8000 München 19, Bundesrepublik Deutschland

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The cyanobacterium *Synechococcus* 6301 is able to use a limited number of sulfur compounds as the only source of sulfur supply such as sulfate, thiosulfate, thioacetic acid, mercaptoacetic acid, thioacetamide, L-cysteine and glutathione. Compounds containg thioether linkages such as methionin or S-methylcysteine and all compounds investigated so far containing sulfonic acid structures do not support growth. Growth inhibiton was observed by addition of aminomethanesulfonic acid or cysteamine.

When non-growth sustaining sulfur compounds are added as sulfur source, the C-phycocyanin content of the Synechococcus cultures decreased drastically, causing a shift in color from blue-green to yellow-green. An analysis reveals the degradation of C-phycocyanin whereas chlorophyll formation still proceeds to a certain degree in growing sulfur-starved cells. Supplementation of a suitable sulfur source induces a period of intense and preferential C-phycocyanin synthesis prior to resumption of normal growth.

Materials and Methods

1) Organism

Synechococcus 6301 was obtained from the collection of algal cultures from Prof. Dr. R. Stanier (Pasteur Institute, Paris, France). Cells were grown in the BG-11 medium [1] as axenic culture as described earlier [2] at 37 °C and 4000 lux.

2) Modification of the original medium for growth on different sulfur sources

Magnesium sulfate of the original medium was replaced by magnesium chloride; the sulfur compound to be analyzed was added to a final concentration of 3×10^{-4} M/1. Prior to the actual addition, all sulfur compounds were checked for the presence of free sulfate using barium chloride. If sulfate was present it was removed by precipitation as $BaSO_4$ and excess barium was removed by binding to Dowex-50. All amino acid dervatives tested were free of inorganic sulfate.

3) Growth measurements

Growth of *Synechococcus* 6301 was followed as increase in optical density (OD_{680}) at 680 nm. Measurements were carried out using samples of cells in media with the respective sulfur compound and blanks containing no sulfur source.

4) Absorption spectra

Spectra from media containing whole cells were recorded with a Perkin-Elmer spectrophotometer with the cuvette positioned close to the photomultiplier to avoid light scattering. The spectra obtained are specific for the spectrophotometer used (different distances from the photomultiplier tube result in different recordings).

5) Sulfur sources

The sulfur compounds tested were purchased from the following companies: B = Boehringer, Mannheim; E = EGA-Chemie, Steinheim; F = Fluka AG, Buchs, Switzerland; M = Merck, Darmstadt; P = Pierce, Eurochemie, Rotterdam, Netherlands; H = Riedel de Haen, Seelze-Hannover; S = Sigma, München; V = Serva, Heidelberg; U = Unfriedchemie, München.

The compounds listed as K were synthesized in our labs according to established procedures. Contaminating sulfate was removed as described above. Details of the synthesis will be published elsewhere; structures were verified by NMR and related methods.

Reprint requests to Prof. Dr. A. Schmidt and Priv. Doz. Dr. H.-P. Köst.

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Results

a) Sulfate requirement of Synechococcus 6301

Growth of *Synechococcus* 6301 is dependent on the sulfate concentration in the medium (Fig. 1). Data were obtained by transferring aliquots of cells to sulfur-free medium and monitoring the growth as OD 680 until the stationary phase was reached. Different amounts of sulfate were added and the OD 680 was plotted against the sulfate concentration. The dotted line represents the amount of sulfate carried over with the inoculum; thus the difference between the dotted line and the solid line corresponds to the "sulfur reserve" within the cells. From these data it was calculated that 8 µmol of sulfate per liter will allow *Synechococcus* growth corresponding to an increase of $OD_{680} = 1$. However, *Synechococcus* material thus obtained is in a sulfur-deficient state and contains only minute amounts of phycocyanin (see b).

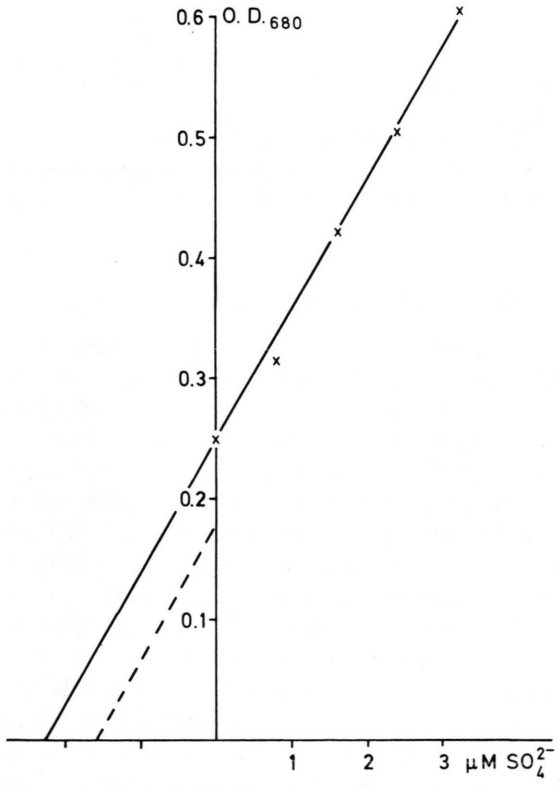

Fig. 1. Growth of *Synechococcus* 6301 as function of the sulfate concentration. The dotted line indicates the sulfate content of the inoculum.

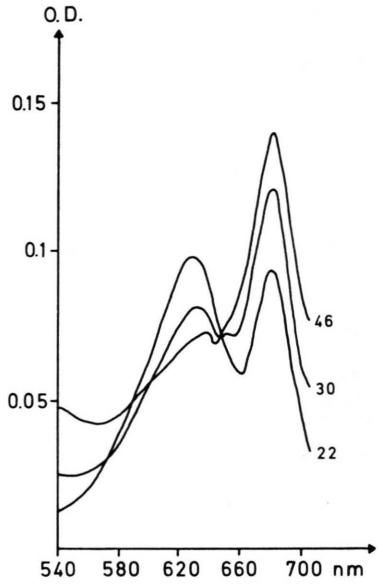

Fig. 2. C-Phycocyanin degradation during growth under sulfur starvation. For details see text.

b) Degradation of C-phycocyanin under sulfur starvation

Synechococcus 6301 growing in medium with limited sulfur content as first exhibit normal growth characteristics. After about 22 h the sulfate carried over from the inoculum is used up. During this time the chlorophyll and C-phycocyanin content remains normal (Fig. 2). After this time a decrease in C-phycocyanin content is observed, while the amount of chlorophyll a is still increasing. This demonstrates a C-phycocyanin degradation paralleled by a chlorophyll a formation; a situation uniquely suited for studies of C-phycocyanin and/or chlorophyll biosynthesis and degradation. After 46 h of growth under sulfur-limited conditions *Synechococcus* cultures contain only minute amounts of C-phycocyanin but still exhibit a little peak at 650 nm, the area of allophycocyanin absorption.

c) Regeneration of C-phycocyanin

Upon addition of a sulfur source allowing good growth (sulfate, thiosulfate, L-cysteine) C-phycocyanin is rapidly regenerated whereas the speed of chlorophyll a biosynthesis remains basically unchanged (Fig. 3). After about 20 h normal growth is resumed.

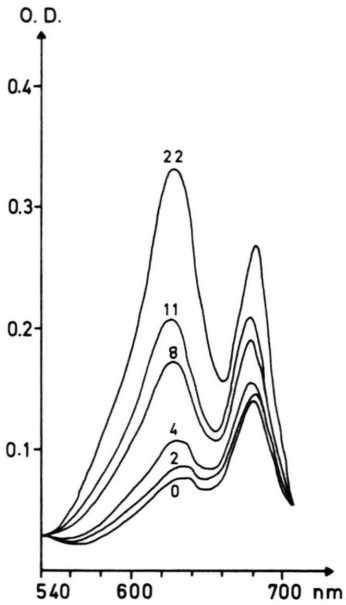

Fig. 3. Regeneration from sulfur starvation after addition of sulfate. Recordings were taken after hours indicated. For further details see text.

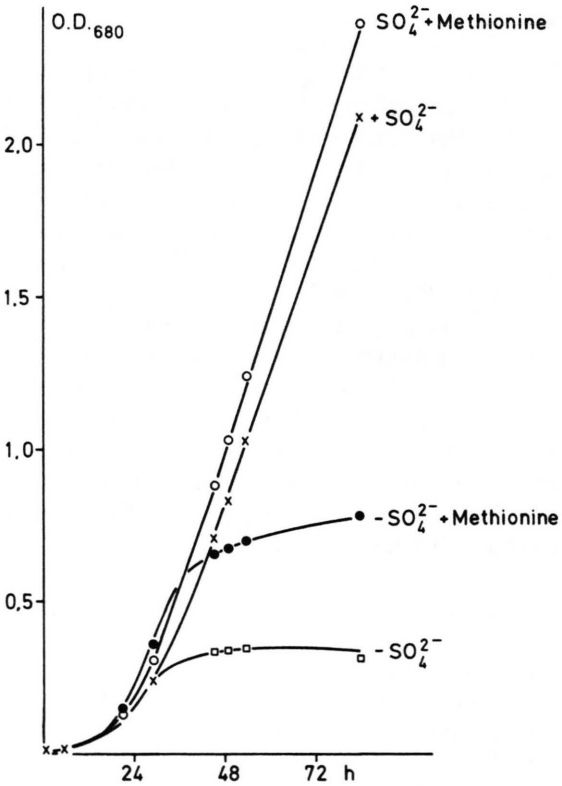

Fig. 4. Growth of *Synechococcus* 6301 in sulfur-deficient media after addition of sulfate, methionine, or both.

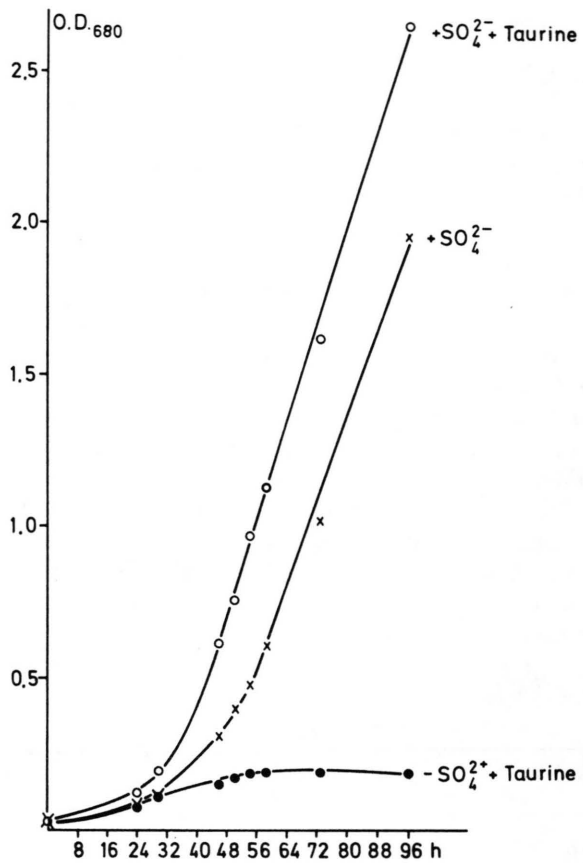

Fig. 5. Growth of *Synechococcus* 6301 in sulfur-deficient media after addition of taurine, sulfate, or both.

d) Sulfur sources sustaining growth

The observation of C-phycocyanin degradation under conditions of sulfur starvation and the recovery in biliprotein content after addition of either sulfate, thiosulfate, or L-cysteine showed that the C-phycocyanin content of cells and sulfur sources allowing good growth are related to each other. About 80 sulfur compounds were analyzed for their ability to support growth of *Synechococcus* 6301. A typical example is given in Figs. 4, 5, and Table I.

Only a limited number of sulfur compounds allowed good and rapid growth with doubling times between 4 and 6 h. These sulfur compounds are sulfate, thiosulfate, L-cysteine, glutathione, thioacetamide, mercaptoacetic acid and thioacetic acid. Methionine will not support growth when used as the only sulfur source. It does, however, enhance the "sulfur efficiancy" allowing additional growth rep-

Table I. Sulfur sources tested for growth of Synechococcus 6301

1) *Compounds allowing good growth*

Sodium sulfate (M) $Na_2SO_4 \times 10\ H_2O$
Sodium thiosulfate (M) $Na_2S_2O_3$
Thioacetic acid (F) C_2H_4OS
Mercaptoacetic acid (F) $C_2H_4O_2S$
Thioacetamide (E) C_2H_5NS
L-Cysteine (M) $C_3H_7NO_2S$
L-Cystine (S) $C_6H_{12}N_2O_4S_2$
Glutathione$_{red}$ (M) $C_{10}H_{17}N_3O_6S$

2) *Compounds allowing limited growth with reduced C-phycocyanin content*

Elemental sulfur (U) S_8
Sodium thiocyanate (M) NaSCN
Sodium tetrathionate (M) $Na_2S_4O_6 \times 2\ H_2O$
D-Cysteine (M) $C_3H_7NO_2S$
2,3-Dimercaptopropanol-1 (M) $C_3H_8OS_2$
2,2-Thiodiacetic acid sulfoxide (K) $C_4H_6O_5S$
2,2-Thiodiacetic acid (E) $C_4H_6O_4S$
2,2-Thioacetic acid sulfone (K) $C_4H_6O_6S$
4-Carboxyl-L-thiazolidine (E) C_4H_7NOS
L-Homocysteine (S) $C_4H_9NO_2S$
N-Acetyl-L-Cysteine (V) $C_5H_8NO_3S$
Benzenesulfinic acid (F) $C_6H_6O_2S$
Phenole-4-sulfonic acid (F) $C_6H_6O_4S$
D-Cystine (S) $C_6H_{12}N_2O_4S_2$
p-Toluenesulfinic acid (F) $C_7H_8O_2S$
Djencolic acid (S) $C_7H_{14}N_2O_4S_2$
Lipoic acid amide (V) $C_8H_{15}NOS_2$

3) *Sulfur compounds not sustaining growth*

a) elevated background

Thiourea (S) CH_4N_2S
Mercaptoethanol (M) C_2H_6OS
Ethanesulfonic acid (F) $C_2H_6O_3S$
1-Thioglycerol (F) $C_3H_8O_2S$
3-Amino-1-propanesulfonic acid (S) $C_3H_9NO_3S$
S-Methyl-L-cysteine (V) $C_4H_9NO_2S$
N-Acetyl-L-cysteine (V) $C_5H_9NO_3S$
L-Methionine (M) $C_5H_{11}NO_2S$
S-Ethylcysteine (S) $C_5H_{11}NO_2S$
L-Methionineamide (S) $C_5H_{12}N_2OS$
Sulfosalicilic acid (M) $C_7H_6O_6S$
D-Biotine (S) $C_{10}H_{16}N_2O_3S$

b) normal background

Iron sulfide (M) FeS
Sulfamic acid (V) H_3NO_3S
Sulfoacetic acid (K) $C_2H_4O_5S$
Coenzyme M (P) $C_2H_6O_3S_2$
2-Hydroxyethane sulfonic acid (M) $C_2H_6O_4S$
Dimethylsulfide (M) C_2H_6S
Dimethylsulfoxide (M) C_2H_6OS
Dimethylsulfone (M) $C_2H_6O_2S$
Taurine (S) $C_2H_7NO_3S$
Cysteic acid (S) $C_3H_7NO_5S$
3-Amino-1-propane sulfonic acid (S) $C_3H_9NO_3S$
S-Methyl-L-cysteine (V) $C_4H_9NO_2S$
DL-Homocysteic acid (S) $C_4H_9NO_5S$
2-Ethylmercaptoethanol (E) $C_4H_{10}OS$
2-Ethylmercaptoethanolsulfoxide (K) $C_4H_{10}O_2S$
2-Ethylmercaptoethanolsulfone (K) $C_4H_{10}O_3S$
O-Ethylcysteine (S) $C_5H_{11}NO_2S$
L-Methionine sulfoxide (S) $C_5H_{11}NO_3S$
L-Methionine sulfone (S) $C_5H_{11}NO_4S$
Sulfanilic acid (F) $C_6H_7NO_3S$
Orthanilic acid (F) $C_6H_7NO_3S$
Benzenesulfonic acid (F) $C_6H_6O_3S$
Lanthionine (S) $C_6H_{12}N_2O_4S$
DL-Ethionine (S) $C_6H_{13}NO_2S$
TES (S) $C_6H_{15}NO_6S$ (23)
Thiosalicilic acid (V) $C_7H_6O_2S$
p-Toluenesulfonic acid (M) $C_7H_8O_3S$
TAPS (S) $C_7H_{17}NO_8S$ (23)
Glutathione sulfonic acid (K) $C_{10}H_{17}N_3O_9S$
Glutathione$_{ox}$ (B) $C_{20}H_{32}N_6O_{12}S_2$

4) *Growth inhibition*

Aminoiminomethanesulfinic acid (S) $CH_4N_2O_2S$
Aminomethanesulfonic acid (F) CH_5NO_3S
Cysteamine (S) C_2H_7NS
Methional (S) C_4H_9NOS
O-Methylcysteine (S) $C_4H_9NO_2S$
DTE (B) $C_4H_{10}O_2S_2$
O-Ethylcysteine (S) $C_5H_{11}NO_2S$
Sulfanilamide (V) $C_6H_8N_2O_2S$
S-Methyl-L-methionine (S) $C_6H_{14}NO_2S^+$

resented by a twofold chlorophyll content in the stationary phase compared to controlls without addition of a sulfur source (Fig. 4). Addition of methionine to cultures growing in sulfate containing media enhances growth.

A different pattern can be found for sulfur compounds which can not be used as a sulfur source for growth such as taurine and other sulfonic acid containing compounds. If such compounds contain additionally nitrogen to the sulfonic acid group a growth increase is observed, suggesting that the nitrogen can be used for growth.

The compounds tested are listed in Table I under four different categories:

1) Compound allowing good growth: Best growth was achieved with reduced glutathione.

2) Compounds allowing limited growth with reduced C-phycocyanin content: Good sulfur sources of this category are L-homocysteine and thiazolidine-4-carbonic acid.

3) Sulfur compounds not sustaining growth: Some of these compounds as methionine or mercaptoethanol will cause an increased chlorophyll content in *Synechococcus* cultures compared to cultures containing no sulfur compound, however these compounds did not support growth when added as only sulfur source.

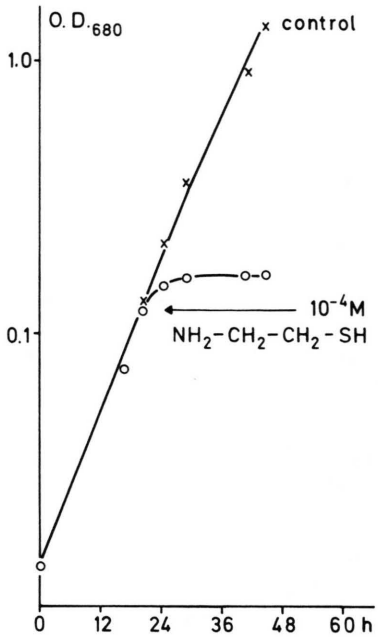

Fig. 6. Growth inhibition of *Synechococcus* 6301 by cysteamine. Cysteamine was added as indicated (10^{-4} M) with a second culture running as control.

4) Growth inhibiton: Some sulfur compounds listed in Table I inhibited growth when added to cultures in the absence or presence of sulfate during inoculation; these compounds did not allow any growth if cultures were started with the normal inoculum. The inhibition caused by cysteamine was analyzed in more detail. Cysteamine inhibited growth of *Synechococcus* 6301 completely at 10^{-4} M/l when added during log phase growth within 4 h. This effect of cysteamine seems to be related to the structure, since neither taurine, mercaptoethanol, nor coenzyme M inhibited growth at the same concentration.

Cysteamine	$HS-CH_2-CH_2-NH_2$
Taurine	$HO_3S-CH_2-CH_2-NH_2$
Mercaptoethanol	$HS-CH_2-CH_2-OH$
Coenzyme M	$HS-CH_2-CH_2-SO_3H$

A SH-group is essential for the inhibitory activity; oxidation to the sulfonic group abolishes the inhibition. The amino group is also essential for this inhibition, since replacement by either a sulfonic group as in coenzyme M or by a hydroxy group as in mercaptoethanol leads to compounds not inhibiting growth of *Synechococcus* 6301. The target point for this inhibiton is not known.

Discussion

Cyanobacteria (blue green algae) are procaryotic organisms with photosystems I and II [3–5]. Photosystem II is associated with characteristic light-harvesting pigments, the phycocyanins [4, 6]. These C-phycocyanins are bile pigment complexes covalently linked to the apoprotein moiety by a thioether linkage [7–10]. In order to fulfill their light-harvesting function, the biliproteins of *Synechococcus* 6301 are aggregated to complex antenna, the so-called phycobilisomes [6, 11–13], containing C-phycocyanin (λ_{max} at 630 nm); allophycocyanin (λ_{max} at 650 nm); and a number of colorless proteins [6, 14].

It is known for about 10 years that C-phycocyanin formation is dependent on the availability of reduced nitrogen [15–18]. Under nitrogen limitation C-phycocyanin is degraded [15, 18, 19]. In nitrate starved *Anacystis nidulans* (*Synechococcus*) cells, *de novo* C-phycocyanin synthesis was observed upon addition of nitrate [15, 17, 18]. These observations led to the postulate of a nitrogen storing function of C-phycocyanin besides its function as a light harvesting pigment [15].

C-phycocyanin formation and degradation in *Synechococcus* 6301 (*Anacystis nidulans*) is governed also by the availability of sulfur compounds which can be used for growth. This was surprising considering the amino acid composition of *Synechococcus* 6301 C-phycocyanin. The α-subunit contains 3 cysteine residues, two of these are linked to phycocyanobiline chromophores at positions 81 and 152 [20, 21]. The β-subunit contains cysteine at position 84 linked to phycocyanobilin as the only sulfur containing amino acid. Thus about 47 nitrogen atoms per sulfur atom are present in C-phycocyanin of *Synechococcus* 6301 which is a low ratio compared to normal proteins with about 15 to 20 nitrogen atoms per sulfur atom. This finding seems to exclude the possibility that C-phycocyanin should function as a storage protein for reduced sulfur.

Chlorophyll *a* formation still proceeds under C-phycocyanin degrading conditions. If C-phycocyanin degradation induced by nitrogen starvation is identical to C-phycocyanin degradation induced by sulfur starvation is not known. Chlorophyll, however, is not degraded under conditions of sulfur starvation, whereas the chlorophyll pool is small under nitrogen limitation (data not shown). When a sulfur starved culture is supplied with a sulfur

source allowing good growth such as sulfate, thiosulfate, cysteine or thioacetamide, an initial preferential synthesis of C-phycocyanin is observed before cells start to resume normal growth. A careful analysis of regeneration data obtained from different cultures using different sulfur sources has shown that cells preferentially synthesize C-phycocyanin compared to synthesis of chlorophyll *a*; after about 16 to 20 h normal growth occurs.

The usefulness of this system of *Synechococcus* 6301 for the elucidation of C-phycocyanin biosynthesis regulation depends on the question whether sulfur-starved cyanobacteria do in fact regenerate C-phycocyanin upon addition of sulfate prior to growth. Cell doubling times in regenerating algae are longer than those of normally growing cells (22 h *versus* about 6 h). This could be due to the presence of large amounts of unfunctional cells causing virtually longer doubling times. Thus we can assume the following situation:

$$OD_{total} = OD_{unfunctional\ cells} + OD_{living\ cells} \times e^{kt}$$

with OD = optical density at 680 nm or 630 nm; doubling time = Ln $2/k$.

For different assumed optical densities of unfunctional cells a curve fitting was conducted. The mean deviation of OD for living cells and k was smallest if the OD for unfunctionally cells was assumed to be zero. Therefore only a small negligible fraction of dead cells should be present. Unfortunately plating efficiency is low for *Synechococcus*, so that no direct viability counts can be made [22].

The increase in cell number runs in parallel with the chlorophyll *a* content per volume of the medium; the chlorophyll content of a single cell therefore can be regarded as constant; the C-phycocyanin content however, may vary widely. For analysis the C-phycocyanin content therefore has been calculated on the basis of constant chlorophyll concentrations. From C-phycocyanin concentrations thus calculated synthesis rates of C-phycocyanin can be obtained. The data of Fig. 3 demonstrate that the maximum C-phycocyanin synthesis was found after a regeneration time of 6 to eight hours. This however means that *Synechococcus* regenerates the C-phycocyanin first, thereafter the algae start growth. This suggests that the highest activities of C-phycocyanin- (and probably prophyrin-) synthesizing enzymes should be observed after a regeneration time of 6 to 8 h compared to "normally" grown or bleached cells.

The different sulfur compounds have been tested of their ability to support growth of *Synechococcus* 6301 with a twofold goal:

a) to elucidate which sulfur sources allow good growth,

b) to screen for sulfur compounds allowing limited growth.

Furthermore, we wanted to investigate how these compounds affect the biosynthesis of C-phycocyanin. From the data summarized in Table I some generalisations can be drawn. Only some few compounds will allow good growth. Surprisingly methionine (and other thioethers such as S-methylcysteine) can not be used for growth in this organism. One exception is a thioether having two C-2 carboxylic groups (thiodiacetic acid). Surprisingly mercaptoethanol is not used for growth, although mercaptoacetic acid is a very good sulfur source for growth; this suggests that either the uptake of mercaptoethanol or the oxidation of the alcohol to the acid is the rate limiting step.

Synechococcus will not grow on sulfonic acids so far analyzed such as taurine, cysteic acid or buffer substances with sulfonic acids (Good-buffers, [23]) although a growth stimulation was sometimes evident with some sulfonic acids, particularly with sulfoacetic acid. Thus effects other than that of nitrogen sources can be observed with some sulfonic acids.

Some sulfur compounds listed in group d inhibited growth of *Synechococcus* 6301. This was analyzed in more detail using cysteamine. This inhibition with cysteamine is dependent on the reduced nitrogen group and the thiol group of this compound, since replacement of the nitrogen group by a sulfonic group (coenzym M) did not inhibit growth, whereas the cell will grow on mercaptoacetic acid. This inhibitory effect is also completely abolished by oxidation of the thiol group to the corresponding sulfonic acid (taurine) proving that reduced nitrogen and reduced sulfur are needed for this inhibition. Cystamine, the oxidized disulfide of cysteamine, has the same inhibitory effect. The exact nature of this cysteamine inhibition is still unknown.

Acknowledgement

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