A PAPS-Dependent Sulfotransferase in *Cyanophora paradoxa* Inhibited by 5′-AMP, 5′-ADP, and APS

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Z. Naturforsch. **34 c**, 222–228 (1979); received January 17, 1979

Cyanophora paradoxa, Sulfate Reduction, PAPS-Sulfotransferase, 3′-Phosphoadenosine-5′-phosphosulfate

A 3′-phosphoadenosine-5′-phosphosulfate (PAPS) dependent sulfotransferase was partly purified from *Cyanophora paradoxa*. This enzyme has a pH-optimum of 8.5 and was found to be specific for PAPS; the K_m for this nucleotide was determined to be 8.3 μM. In crude extracts monothiols like glutathione, mercaptoethanol, or cysteine catalyzed a rate of about 80% of the rate obtained with dithioerythritol; however after purification of the PAPS-sulfotransferase the monothiol activity decreased to about 20% of the rate obtained with dithiols. Addition of heated, crude extract partly restored the monothiol activity. This PAPS-sulfotransferase was inhibited by 5′-ADP and 5′-AMP, and especially by adenosine-5′-phosphosulfate (APS). Practically no inhibition was found with 2′-AMP, 3′-AMP, c-AMP, 3′-5′-ADP and 2′-5′-ADP. The inhibitor constants have been determined to be 430 μM for 5′-AMP; 290 μM for 5′-ADP; and 0.3 μM for APS. These inhibitions seem to be competetive to PAPS. The role of these inhibitors for a possible regulation of assimilatory sulfate reduction are discussed.

Introduction

PAPS-dependent sulfotransferases have been detected recently in Cyanobacteria [1], and an enzyme system from *Synechococcus* has been analyzed in some detail. These data have shown, that the *Synechococcus* 6301 sulfotransferase is stimulated by thioredoxin and is inhibited by 3′-5′-ADP, one end-product of the PAPS-sulfotransferase activity, whereas other adenine-containing nucleotides like AMP, ADP, or APS did not influence this reaction [2]. During an investigation of sulfotransferase activities in Cyanobacteria we noticed, however, that PAPS-sulfotransferases can be found which are not dependent on thioredoxin, which are inhibited by 5′-AMP and 5′-ADP, thus showing a remarkable difference to the thioredoxin-dependent sulfotransferase of *Synechococcus* [3]. *Cyanophora paradoxa* was chosen for the characterization of such a different PAPS-sulfotransferase, since the enzyme of this organism was quite stable and therefore suitable for characterization.

Abbreviations: APS, adenosine-5′-phosphosulfate; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; 5′-AMP, adenosine-5′-monophosphate; 5′-ADP, adenosine-5′-diphosphate; 3′-5′-ADP, 3′-phosphoadenosine-5′-phosphate; 2′-5′-ADP, 2′-phosphoadenosine-5′-phosphate; c-AMP, adenosine-3′-5′-monophosphate; DTE, dithioerythritol.

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0341-0382 / 79 / 0300-0222 $ 01.00/0

Materials and Methods

a) Organism: Cyanophora paradoxa cyanelle extract was a generous gift of the group of Prof. Dr. Stanier, Institut Pasteur, Paris. The extract was prepared as described by Herdmann and Stanier [4].

b) Protein purification: Crude cyanelle extract was supplied to a DEAE-cellulose column (2 × 10 cm) equilibrated with 0.02 M Tris-HCl buffer pH 8.0 containing 10 mM mercaptoethanol. The column was developed with a linear gradient of 0 to 0.5 M NaCl using the buffer mentioned above (200 ml for each reservoir). Active fractions were pooled, concentrated and separated on a Sephadex-G-100 column equilibrated with the same buffer with addition of KCl (0.1 M). Active fractions were pooled and used for the experiments without further treatment.

c) Determination of PAPS-sulfotransferase activity: This activity was determined as acid-volatile radioactivity derived from [^{35}S]PAPS as described earlier [5] using dithioerythritol as thiol.

d) Determination of radioactivity: Radioactivity was measured according to Patterson and Greene [6] in a Beckmann LS-100 scintillation counter.

e) Preparation of sulfonucleotides: PAPS and APS were prepared enzymatically with the *Chlorella* system from sulfate and ATP according to the method of Hodson and Schiff [7] modified as described by Schmidt [5].

f) Protein determinations: Protein was determined as described previously [8].

g) Chemicals: [^{35}S]sulfate was obtained from Buchler (Braunschweig, W.-Germany); AMP, ADP, 3-AMP, 2′-AMP, c-AMP was purchased from Boehringer (Mannheim, W.-Germany); 2′-5′-ADP and 3′-5′-ADP was obtained from Sigma (München, W.-Germany); Sephadex-G-100 was obtained from Pharmacia (Freiburg, W.-Germany); DEAE-cellulose was ordered by Serva (Heidelberg, W.-Germany). All other chemicals not mentioned were purchased from Merck (Darmstadt, W.-Germany).

Results

Cyanelle extracts of *Cyanophora paradoxa* contained sulfotransferase activity, which was purified as described in Materials and Methods. The so far purified sulfotransferase was used for the experiments described in this paper.

a) Nucleotide specificity and K_m-determination for PAPS

The sulfotransferase isolated from *Cyanophora* was found to be specific for PAPS; the K_m for this sulfonucleotide is determined to be 8.3 μM $\pm$ 3 μM, using different enzyme batches and different PAPS-preparations; an example is shown in Fig. 1. For routine assays 50 μM PAPS was used. This sulfotransferase showed no activity with APS, however, the sulfotransferase activity was influenced by APS and other adenine-containing nucleotides as is shown in Table I. From these data it is evident, that 2′-

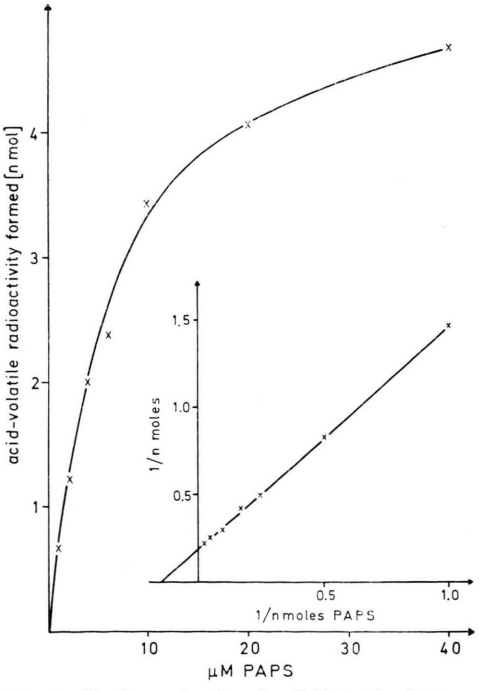

Fig. 1. K_m-determination for PAPS. Conditions as in Fig. 2 without addition of 5′-AMP and PAPS varied as indicated.

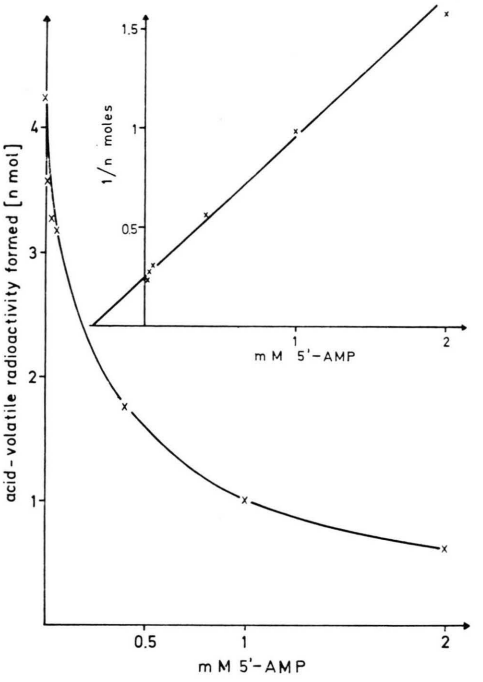

Fig. 2. K_i-determination for the 5′-AMP inhibition. Conditions: Tris-HCl pH 8.5: 100; $MgCl_2$: 10; Na_2SO_4: 500; DTE: 10; PAPS: 0.01 (1 nmol=3.000 cpm); 5′-AMP as indicated; enzyme: 0.08 mg in a total volume of 1 ml. Incubation for 1 h under N_2.

Table I. Inhibition of the PAPS-sulfotransferase activity by various adenine-containing nucleotides.

	nmol Acid-volatile radioactivity formed	% of control
none	23.4	100
+2′-AMP	20.9	86
+3′-AMP	21.9	90
+5′-AMP	2.7	11
+c-AMP	22.5	93
+3′-5′-ADP	24.3	100
+2′-5′-ADP	24.2	99
+5′-ADP	2.1	9
+[^{35}S]APS	1.9	8

Conditions: Tris-HCl pH 8.5: 100; $MgCl_2$: 10; DTE: 10; Na_2SO_4: 500; PAPS: 0.05 (1 nmol=770 cpm); nucleotides when indicated: 2 except [^{35}S]APS, here 50 nmol were added (1 nmol=830 cpm); enzyme: 0.24 mg in a total volume of 1 ml. Incubation for 1 hour under N_2.

AMP, 3′-AMP, c-AMP, 2′-5′-ADP, and 3′-5′-ADP do not influence this activity significant, however, 5′-AMP and 5′-ADP severely inhibit this enzymatic activity. To our surprise addition of labelled APS also inhibited this sulfotransferase activity, although APS was added only in a concentration of 0.05 mM compared to the other adenine-containing nucleotides, which were added to a final concentration of 2 mM (see Table I). The nature of the APS, 5′-ADP, and 5′-ADP inhibitions were studied in some detail.

1) Inhibition by 5′-AMP: The data of Fig. 2 show a decrease of the PAPS-sulfotransferase activity when increasing amounts of 5′-AMP were added. From these data the K_i for the 5′-AMP inhibition was determined to be 430 μM (see insert of Fig. 2). K_m-determinations in the presence and absence of 5′-AMP suggest, that this inhibition seems to be competetive, since the data of Fig. 3 clearly show, that the K_m is varied whereas V_{max} is not altered. This suggests, that 5′-AMP and PAPS are competing for the same binding site.

2) Inhibition by 5′-ADP: Practically the same experiments reported for the inhibition of 5′-AMP

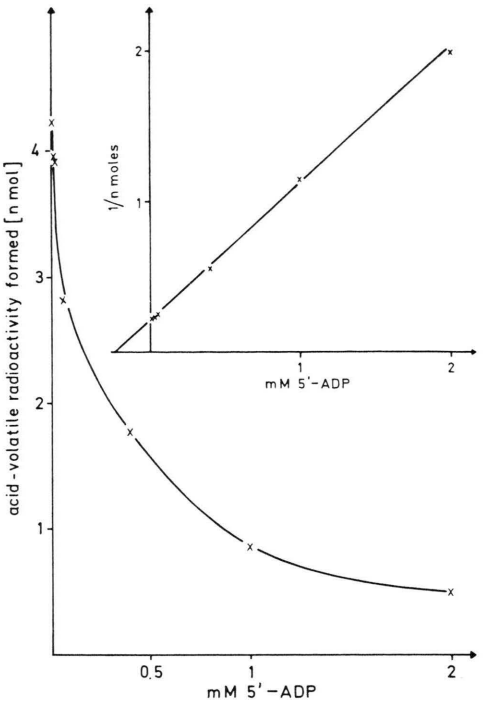

Fig. 4. K_i-determination for the 5′-ADP inhibition. Conditions as in Fig. 2, however 5′-ADP was varied as indicated instead of 5′-AMP.

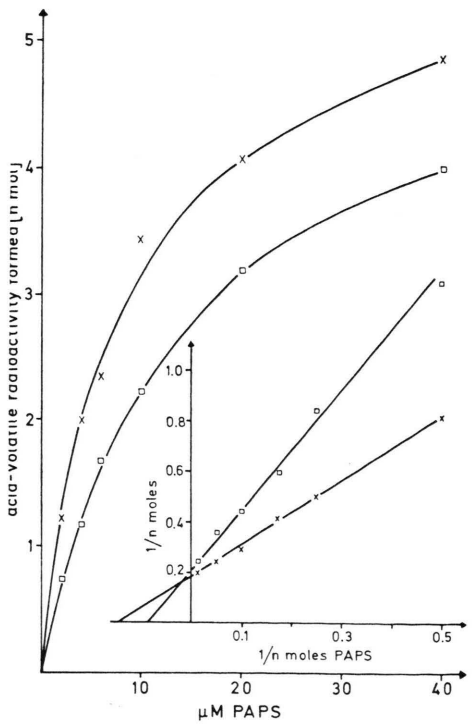

Fig. 3. Competetive inhibition of the PAPS-sulfotransferase by 5′-AMP. Conditions as in Fig. 2, however 5′-AMP was kept constant at 50 μM and PAPS was varied as indicated. $\times-\times$, without 5′-AMP; $\square-\square$, 5′-AMP added.

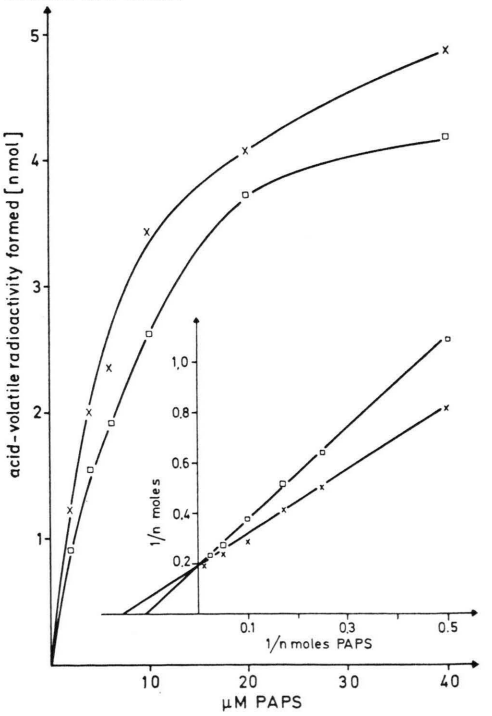

Fig. 5. Competetive inhibition of the PAPS-sulfotransferase by 5′-ADP. Conditions as in Fig. 2, however 50 μM 5′-ADP was added and the PAPS-concentration varied as indicated. $\times-\times$, without 5′-ADP; $\square-\square$, 5′-ADP added.

were conducted using 5′-ADP. These data are shown in Figs 4 and 5. Again it is evident, that 5′-ADP inhibits the PAPS-sulfotransferase and the K_i for the 5′-ADP inhibition was determined to be 290 μM (see insert to Fig. 4). Also in the case of 5′-ADP this inhibition was found to be competetive, which is evident from the data of Fig. 5.

3) Inhibition by APS: From the data of Table I it was evident, that the sulfonucleotide APS severely inhibited the PAPS-sulfotransferase of *Cyanophora paradoxa*. From these data it was evident, that the sulfotransferase is specific for PAPS, since labelled APS was not metabolized at all; however this APS inhibited the sulfotransferase already at very low concentrations. From the data of Fig. 6 the K_i for this APS-inhibition was determined to be 0.3 μM, which is about 1000-fold lower than the K_i obtained for either 5′-ADP or 5′-AMP measured under identical conditions (note that the APS-concentration in Fig. 6 is given in μM and not in mM). To establish the nature of this APS-inhibition, K_m-determinations for PAPS in the presence of 0.5 μM and 1.0 μM APS were measured. The data of Fig. 7 show that the inhibition of APS can be overcome by higher PAPS-concentrations, showing that APS is not bound irreversible to the enzyme. When these data are plotted according to Lineweaver and Burk as is shown in Fig. 8, it is clearly seen, that the APS-inhibition is competetive for the substrate binding site, however catalysis of sulfate transfer seems to be dependent of the phosphate group in the 3′-position of the sulfonucleotide, since labelled APS can not be used as a substrate for sulfate transfer with this sulfotransferase (see also Table I).

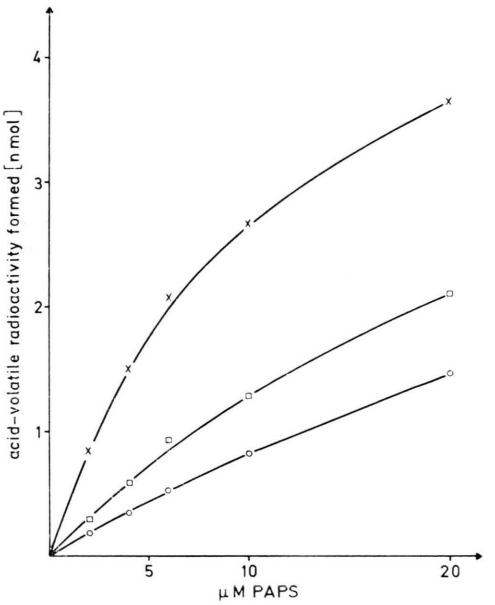

Fig. 7. Inhibition of the PAPS-sulfotransferase in the presence of APS. Conditions as in Fig. 2 with addition of APS instead of 5′-AMP. ×—×, activity without APS; ———, activity with 0.5 μM APS; ○—○, activity with 1 μM APS.

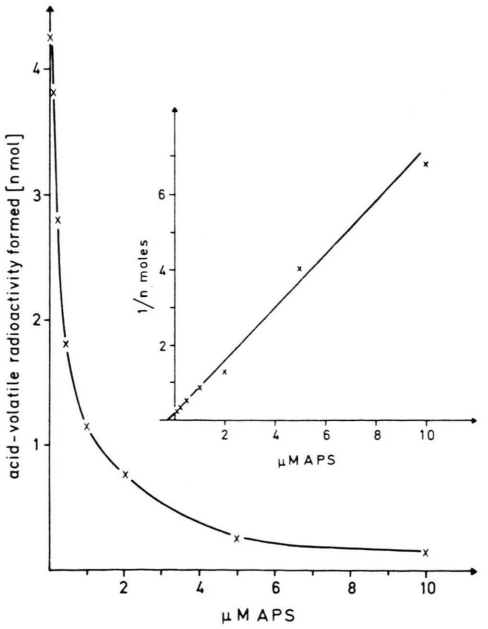

Fig. 6. K_i-determination for APS. Conditions as in Fig. 2, however APS was added and varied as indicated.

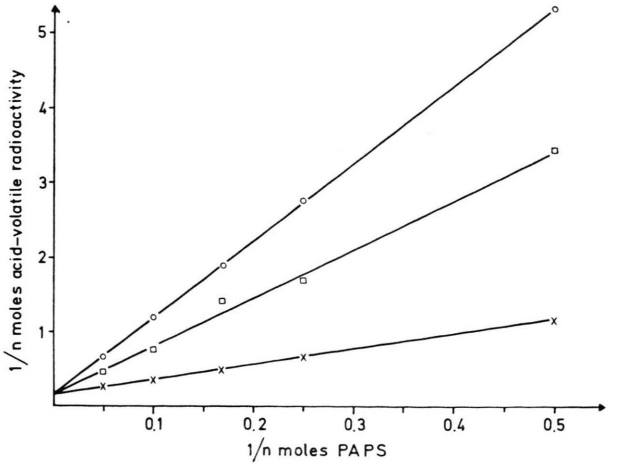

Fig. 8. Competetive inhibition of the PAPS-sulfotransferase by APS. Conditions as in Fig. 7.

b) Thiol specificity of the sulfotransferase activity

The PAPS-sulfotransferase of *Cyanophora paradoxa* is active with different thiols, which is evident from the data given in Table II. Good activity was obtained with the dithiols dithioerythritol and 2′-3′-dimercaptopropanol, whereas other thiols like mercaptoethanol, glutathione, or cysteine (monothiols) stimulated only to about 1/4 of the rate obtained with dithioerythritol when analyzed under identical conditions. Thioredoxin, isolated from *Synechococcus*, stimulated somewhat, however, the activity of this PAPS-sulfotransferase is not dependent on the addition of thioredoxin, since the activation with thioredoxin was found to be 1.6 fold. It should be noticed that the assay was performed in 0.5 M Na_2SO_4; this was necessary to obtain good activity; omittence of Na_2SO_4 resulted in a loss of 84% activity. It was noticed with the purified extract, that the efficiency with monothiols was about 20 to 30% compared to the rate with DTE whereas in crude extracts the rate with monothiols was about 80% of the rate obtained with dithiols. As can be seen from the data of Table III, it is evident, that heated extract contains material, which indeed stimulated PAPS-sulfotransferase activity from monothiols. With 0.4 ml of heated extract the activity of this sulfotransferase can be raised to about 80%, which was obtained with DTE in crude extracts. This heated extract by itself had no sulfotransferase activity. It should be noticed, however, that this heated extract stimulated also the PAPS-sulfotransferase in the presence of DTE to about the same extent as thioredoxin from *Synechococcus* when analyzed in combination with the *Cyanophora* PAPS-sulfotransferase (compare the data with thioredoxin in Table II). The heated extract of *Cyanophora* did not stimulate the thioredoxin-dependent PAPS-sulfotransferase of *Synechococcus* 6301 using either monothiols or dithiols. Therefore the stimulation of the heated extract is not due to thioredoxin, since the *Synechococcus* PAPS-sulfotransferase will accept thioredoxins from different sources [9]. Purification of the material stimulating the PAPS-sulfotransferase of *Cyanophora* has failed so far, thus the nature of this substance(s) is not known, however our observations demonstrate that it is not identical with thioredoxin.

Table II. Activity of the PAPS-sulfotransferase with different thiols.

	nmol Acid-volatile radioactivity formed	% of control
without thiol	0.14	4
+DTE	3.17	100
+mercaptoethanol	0.78	24
+2′-3′-dimercaptopropanol	4.53	142
+cysteine	1.07	33
+glutathione	0.72	22
+DTE−Na_2SO_4	0.50	16
+DTE+thioredoxin	5.18	163

Conditions: Tris-HCl pH 8.5: 100; $MgCl_2$: 10; Na_2SO_4: 500; thiol: 10 when indicated; PAPS: 0.05 (1 nmol = 646 cpm); thioredoxin: 0.1 ml (A_{278}=1.23) from *Synechococcus;* enzyme: 0.08 mg in a total volume of 1 ml. Incubation for 1 hour under N_2.

Table III. Stimulation of PAPS-reduction by heated supernatant.

	nmol acid-volatile radioactivity formed	% of control
enzyme+DTE	4.29	100
enzyme+ME	1.21	28
enzyme+ME+0.1 ml heated extr.	2.0	47
enzyme+ME+0.2 ml heated extr.	2.5	58
enzyme+ME+0.4 ml heated extr.	3.31	77
enzyme+DTE+0.4 ml heated extr.	5.47	183
0.4 ml heated extr.+ME	0.09	2
0.4 ml heated extr.+DTE	0.1	2

Conditions: Tris-HCl pH 8.5: 100; $MgCl_2$: 10; thiol as indicated: 10; Na_2SO_4: 500; PAPS: 0.05 (1 nmol=770 cpm); heated extract: 6.8 mg/ml; enzyme: 0.11 mg in a total volume of 1 ml. Incubation for 1 hour under N_2.

Discussion

Assimilatory sulfate reduction in photosynthetic organisms needs activated sulfate for further reduction. Whereas in higher plants and green algae APS was found to be the sulfate donor involved [8, 10, 11], it was noticed recently that within the group of Cyanobacteria different systems with either APS or PAPS are operative, and we have characterized a PAPS-dependent sulfotransferase from *Synechococcus* 6301, which was inhibited by 3′-5′-ADP; however not by 5′-AMP or 5′-ADP [2]. This enzyme needed further a heat-stable factor which was purified and identified as a thioredoxin [9]. Different thioredoxins from *Escherichia coli*, *Scenedesmus*, and spinach were able to replace the *Synechococcus* thioredoxin [9]. During our investigation of sulfotransferases in Cyanobacteria a different PAPS-sulfotransferase was recognized, which was inhibited by 5′-AMP and

5′-ADP, whereas 3′-5′-ADP had no inhibitory effect. It was further noticed that this different PAPS-sulfotransferase did not need thioredoxin for activity [3]. For stability reasons the PAPS-sulfotransferase of *Cyanophora paradoxa* was selected to analyze this thioredoxin-independent PAPS-sulfotransferase in some detail.

The Cyanophora PAPS-sulfotransferase had a relatively good K_m for PAPS, which was found to be $8.3 \pm 3\ \mu M$. This K_m is in the same order as the K_m for the PAPS-sulfotransferase of *Synechococcus* 6301, which was found to be $20\ \mu M$ with a partly purified enzyme preparation [2]. Since 5′-AMP inhibited APS-sulfotransferases from spinach, *Chlorella* and *Rhodospirillum* [12, 13] and the PAPS-sulfotransferase of *Synechococcus* 6301 on the other hand was not influenced by 5′-AMP, however was severely inhibited by 3′-5′-ADP, the PAPS-sulfotransferase of *Cyanophora paradoxa* was analyzed in the presence of different adenine-containing nucleotides. It is evident from the data presented in this paper, that only 5′-AMP and 5′-ADP showed a strong inhibition; other adenine-containing nucleotides as for instance 3′-5′-ADP, 2′-5′-ADP, c-AMP, or 2′-AMP were practically ineffective. Analysis of the nature of the 5′-AMP and 5′-ADP inhibitions revealed inhibitor constants for 5′-AMP to be $430\ \mu M$ and for 5′-ADP to be $290\ \mu M$. It is shown that these inhibitions seem to be competetive to PAPS, which can be deduced from Figs 3 and 5. We had shown previously that APS-sulfotransferase are inhibited by 5′-AMP and we suggested a regulation of the sulfate reducing pathway by the "energy state" of the cell [12]. This principle has been extended to regulations by ATP-sulfurylases from spinach and *Anabena* [14, 15], and it is shown by this investigation that it can be extended also to organisms, having this type of PAPS-dependent sulfotransferases. However, besides inhibition with 5′-AMP we find effective inhibition also by 5′-ADP.

The PAPS-sulfotransferase of *Cyanophora paradoxa* is also inhibited by APS. This inhibition was detected, when the sulfonucleotide specificity of this sulfotransferase was analyzed. There was no activity with APS, however, addition of non-labelled APS added to labelled PAPS drastically reduced enzymatic activity. To our surprise addition of labelled APS also drastically reduced the PAPS-sulfotransferase activity (Table I). This demonstrates, that reduced activity from labelled PAPS in the presence of non-labelled APS can not always be interpreted as an indication of an active APS-sulfotransferase, since – as is shown in this case – APS might inhibit PAPS-sulfotransferase activity. This inhibition of the *Cyanophora* PAPS-sulfotransferase by APS is very pronounced beeing about 1000-fold better that the inhibitions of 5′-AMP and 5′-ADP as shown above. The inhibitor constant for APS was determined to be $0.3\ \mu M$, when PAPS was used in $10\ \mu M$ concentration. Analysis with two different APS-concentrations demonstrates, that this inhibition is competetive to PAPS. Therefore the PAPS-sulfotransferase of *Cyanophora* seems to have a very strong affinity to APS, which is even better than the affinity for PAPS, which can be deduced for the K_i for APS of $0.3\ \mu M$ and the K_m for PAPS of $8.3\ \mu M$! APS, however, is the wrong substrate, which can not be used for catalysis of the sulfotransferase reaction. This demonstrates, that the phosphate group in the 3′-position of the sulfonucleotide seems to hold PAPS in a defined position which is necessary for catalysis. Since the thioredoxin-dependent PAPS-sulfotransferase is not inhibited by APS, when analyzed under simular conditions, it can be deduced that in different organisms different parts of the PAPS-molecule are needed as recognition site.

This APS-inhibition of a PAPS-sulfotransferase is not restricted to *Cyanophora paradoxa;* we have found such inhibitions also in *Fischerella* 7115 and in the red alga *Porphyridium.*

The inhibition of a PAPS-sulfotransferase by APS suggests, that 3′-phosphatases and APS-sulfhydrolases might play a role in the regulation of assimilatory sulfate reduction. It is known, that PAPS can be dephosphorylated to APS, and in the green alga *Chlorella* at least two different phosphatases are described, which have the capacity to dephosphorylate PAPS to APS [16 – 18]. On the other hand APS-degrading activities can be found, which hydrolyse APS to AMP and sulfate [16, 18]. It is possible therefore that assimilatory sulfate reduction in Cyanophora paradoxa is effectively controlled by the APS-pool, and that the interaction of APS-kinase, 3′-phosphatases and APS-sulfhydrolases control the threshold of this sulfonucleotide.

The PAPS-sulfotransferase of *Cyanophora* is stimulated by heated extract, as is evident from the data of Table III. The heated extract does not contain thioredoxin, since this heated extract can not stimulate the thioredoxin-dependent PAPS-sulfo-

transferase of *Synechococcus* 6301 in the presence of dithioerythritol. Secondly this extract increases PAPS-sulfotransferase of *Cyanophora* with monothiols, whereas these thiols can not be used to stimulate the thioredoxin-dependent PAPS-sulfotransferase of *Synechococcus* in the presence of either thioredoxin or heated *Cyanophora* extract. The PAPS-sulfotransferase of *Cyanophora* is active without additional proteins if dithioerythritol is used, however, addition of either *Synechococcus* thioredoxin or heated extract stimulated this activity about 2-fold suggesting that the heated extract contains activity which fits the enzyme better than the added thiol, which is very pronounced when monothiols like glutathione, mercaptoethanol or cysteine are used.

Attempts to purify this activity from *Cyanophora* using DEAE-cellulose or Sephadex chromatography have failed so far. Thus, the nature of this stimulating activity is not known yet; however our data obtained with *Synechococcus* clearly show that it is not identical with thioredoxin.

So far we have found 4 different sulfotransferases in Cyanobacteria. The first enzyme is a PAPS-sulfotransferase specific for PAPS and this enzyme is inhibited by 3′-5′-ADP and to some extent by 2′-5′-ADP, however it is not inhibited by 5′-AMP, 5′-ADP or APS. Such an enzyme was found in *Synechococcus* 6301 [2]. The second type is a PAPS-sulfotransferase, which is described in this paper. This enzyme is specific for PAPS and it is inhibited by 5′-AMP, and 5′-ADP, and exceptionally by APS! Other adenine-containing nucleotides as 2′-AMP, 3′-AMP, and 3′-5′-ADP do not inhibit this enzyme activity.

Thirdly we can describe an enzyme specific for APS, which is stimulated by theoredoxin and which is inhibited by 5′-AMP, however no inhibition was found using 2′-AMP, 3′-AMP or 3′-5′-ADP. This type of enzyme has been found in *Synechococcus* 6312, *Chroococcidiopsis* 7203, and in *Pseudanabena* 6901. A fourth type of a sulfotransferase has been found in the *Cyanobacterium Plectonema*. Here the sulfotransferase is found to be specific for APS, however otherwise has similar properties as the PAPS-enzyme of *Cyanophora:* it is not dependent on thioredoxin for activity, although addition of thioredoxin wil stimulate about 1.6-fold, and this enzyme is inhibited by 5′-AMP [3]. Such an APS-dependent enzyme has also been found in *Rhodospirillum rubrum* [13].

These data clearly indicate, that within the *Cyanobacteria* sulfotransferases with different sulfonucleotide specificity, cofactor requirement, and regulatory properties can be demonstrated.

These studies were supported by a grant from Deutsche Forschungsgemeinschaft. We thank Prof. Stanier and his group (Pasteur Institut, Paris) for frozen *Cyanophora paradoxa* extract and the encouragement to study sulfur metabolism in Cyanobacteria.

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