

Assimilatory Sulfate Reduction by Chloroplasts: The Regulatory Influence of Adenosine-mono- and Adenosine-diphosphate

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The first three enzymatic steps of assimilatory sulfate reduction in chloroplasts of higher plants have been investigated with emphasis on the influence of adenosine-mono- and -diphosphate upon the formation of APS, PAPS and bound sulfite.

The data show that the activation process is governed by the energy charge of the chloroplast. The regulatory step is localized at the ATP-sulfurylase reaction. It was found that this enzyme is inhibited by low concentrations of AMP and ADP, with apparent $K_{iAMP}=1.8$ mM and $K_{iADP}=0.5$ mM for the chloroplast preparations. The isolated purified ATP-sulfurylase is inhibited by the nucleotides accordingly, with $K_{iAMP}=0.2$ mM and $K_{iADP}=0.4$ mM. The results are interpreted as a regulatory mechanism for the complete process of assimilatory sulfate reduction in the chloroplast.

Introduction

Plants like many microorganisms reduce inorganic sulfate to meet their nutritional sulfur requirements. Most of the sulfate appears to be reduced by the chloroplast (see ref. 1 for review).

Inorganic sulfate has to be activated prior to the reduction. Two enzymes carry out the biosynthesis of the energetically favoured “activated sulfate” PAPS. Both enzymes, ATP-sulfurylase (EC 2.7.7.4) and APS-kinase (EC 2.7.1.25) have been detected in plants. While the action of the ATP-sulfurylase has amply been demonstrated^{2, 3} biosynthesis of PAPS^{4–6} or the activity of APS-kinase in leaf extracts⁷ or isolated chloroplasts^{7, 8} have been established only recently. In the past, enzymatic degradation of PAPS by hydrolytic enzymes (sulfhydrolases or nucleotidases^{7, 9–11}) or rapid metabolic turnover (sulfotransferases⁸) have been made responsible for the difficulties with the determination of the reaction product of the APS-kinase. Adenosines, like 3'AMP, 5'AMP and ADP, or pyro- and orthophosphate have previously been used in assay mixtures with crude extracts from plants or with isolated chloroplasts with the intention to prevent degradation of PAPS by these hydrolytic enzymes^{6, 7, 9} or generally to improve the recovery

of the sulfonucleotide. However, some of the previous data obtained in the presence of these nucleotides were divergent, and we were unable to detect their stimulatory effect upon the formation of sulfur metabolites from sulfate in the chloroplast^{8, 12}.

Recently, evidence has been presented that the action of ATP-sulfurylase of *Penicillium chrysogenum*¹³ and of *Anabaena cylindrica*¹⁴ is inhibited by AMP and by ADP. This inhibition would severely affect sulfate reduction because the enzyme furnishes the sulfate reduction with the activated sulfate precursor APS. In this paper the process of assimilatory sulfate reduction in the chloroplast of higher plants has been investigated in order to understand the effect of the nucleotides upon the primary steps of sulfate assimilation. It is shown that in the chloroplast the sequence of enzymatic steps leading from sulfate to the reduced sulfide sulfur is regulated by the energy charge of the chloroplast. The regulatory step was traced back to the ATP-sulfurylase. Data obtained with isolated chloroplasts were confirmed by results obtained with the purified enzyme.

Materials and Methods

a) Assay of sulfate activation and -transfer in chloroplasts

Chloroplasts were prepared from freshly harvested spinach leaves or peas as described earlier¹⁵. They

Abbreviations: APS, Adenosine-5'-sulfatophosphate; PAP, 3'-phosphoadenosine-5'-phosphate; PAPS, adenosine-3'-phosphate-5'-sulfatophosphate.



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were washed in 100 mM Tris-Cl buffer (pH 8.4) containing 5 mM MgCl_2 . Two ml of buffer were used for 1 mg chlorophyll. The wash procedure was carried out by gently stirring the chloroplasts under an atmosphere of nitrogen to obviate oxydation. After collection of the chloroplasts in a Servall RC 2 centrifuge at $26000 \times g$ for 15 min the pellet was resuspended in the same Tris-buffer. The chlorophyll content was then adjusted to 1 mg/ml. If not specified otherwise these "washed chloroplasts" were used for the assay of sulfate activation (formation of APS and PAPS) and transfer of the activated sulfate group (formation of exchangeable sulfite). For the kinetic experiment osmotically "ruptured chloroplasts" were used which have been prepared as before but without the final centrifugation. These chloroplasts have been assayed with a NADPH_2 regenerating system with glucose-6-phosphate as reductant as described earlier¹⁵. The enzymatic activities of the chloroplasts were monitored by the use of ^{35}S -labelled sulfate. The amount of each compound was determined after an incubation period of 15 min at 25 °C in the dark under nitrogen. A typical assay contained: 50 mM Tris-Cl buffer (pH 8.4) 10 mM MgCl_2 , 5 mM $[\text{S}^{35}]\text{SO}_4$ (specific activity 10 $\mu\text{Ci}/3 \mu\text{M}$), 2 mM reduced glutathion in experiments designated for the assay of exchangeable sulfite and chloroplasts with a chlorophyll content of 500 μg . The reaction mixtures were separated by ion chromatography. APS was eluted from a column packed with DEAE-cellulose (ϕ 1 cm \times 10 cm length, Whatman precision columns). Elution was performed with a linear gradient of NH_4HCO_3 from 20 mM to 200 mM with a total volume of 150 ml, generally following the procedure of Adams *et al.*¹⁶. PAPS was determined on the same chromatographic system. The sulfonucleotide was eluted from this column at 340 mM NH_4HCO_3 with a linear gradient of NH_4HCO_3 ranging from 20 mM to 400 mM — total volume of 200 ml. PAPS was identified fluorimetrically or by TLC techniques as described previously⁸. Exchangeable sulfite and sulfide formed from sulfate were measured after isotope exchange as free $[\text{S}^{35}]\text{sulfite}$ or $[\text{S}^{35}]\text{sulfide}$. Both were separated as described earlier¹⁷ using a continuous flow analyzer system for detection.

b) ATP-sulfurylase

ATP-sulfurylase was purified from spinach leaf homogenate by the procedure of Shaw and Anderson³. The specific activity of the enzyme was increased by a factor of 132 with respect to the crude dialysed extract. The enzyme (40 μg of protein) was assayed as the molybdate-dependent fission of ATP as introduced by Robbins⁸. The purified enzyme was found to be devoid of Mg -dependent PPase,

negligible amounts of rather unspecific ATP-hydrolysis were observed and corrected if necessary. The action of the ATP-sulfurylase was manifested in the forward reaction by tracer measurements, employing ^{14}C -labelled ATP or ^{35}S -labelled sulfate as substrates.

c) Inorganic pyrophosphatase

Inorganic pyrophosphatase was isolated from the same leaf material and purified as described earlier¹⁹. If necessary it was replaced by commercial yeast enzyme purchased from Boehringer.

d) Determination of orthophosphate, ATP, chlorophyll and protein

Orthophosphate was measured as the molybdenum complex according to Fiske and SubbaRow²⁰. ATP was determined enzymatically and chlorophyll as described by Arnon²¹, protein concentrations were estimated by the phenol method of Lowry *et al.*²².

Results

Assimilatory sulfate reduction as carried out by osmotically ruptured chloroplasts exhibit a rather unusual reaction kinetic (Fig. 1). In the presence of excess phosphorylating and reducing power (ATP and HADPH_2) as well as Mg^{2+} the formation

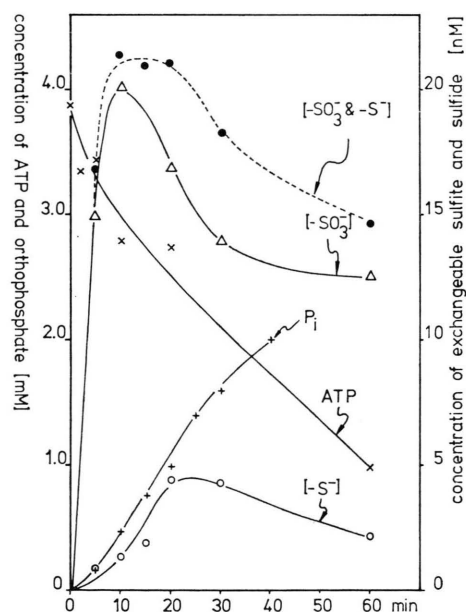


Fig. 1. Kinetic of assimilatory sulfate reduction in chloroplasts and simultaneous recording of ATP-hydrolysis and orthophosphate liberation. (●) Sum of bound sulfite and sulfide (Δ) bound sulfite, (○) bound sulfide, (×) ATP recovered and (+) orthophosphate. Conditions see Materials and Methods.

of the two key intermediates bound sulfite and bound sulfite ceases after a period of 20 min without maintaining a steady state level (dashed curve). Under the experimental condition both metabolites should keep their apparent pools at a constant level, if one assumes that bound sulfite is formed continuously and reduced to bound sulfide. This also demands a constant generation of activated sulfates via the enzymes ATP-sulfurylase and APS-kinase. Both reactions consume one mole of ATP each. However, the simultaneous recording of the ATP consumption reveals a discrepancy between the ATP hydrolysed and metabolised via the activated sulfates into bound sulfite and sulfide. The linear stoichiometry between orthophosphate liberated and remaining ATP indicates the action of an ATP-ase rather than the consumption of ATP by the ATP-sulfurylase and APS-kinase. The ATP-ase activity would lead to the liberation of substantial amounts ADP and thereby effectively alter the energy charge.

The effect of a different energy charge upon sulfate activation is shown in Fig. 2, where increasing amounts of ADP have been added to the assay mixture from the beginning of the experiment. The concentration of ATP has remained unaltered. As

shown in the figure, the sulfate activation and -transfer are severely affected by the added ADP. The concentrations of ADP were in a range as found in the previous experiment (0 to 5 mM). Maximal inhibition was observed above 2 mM of ADP. As shown in Fig. 1 this concentration of ADP is formed by the chloroplasts already after 30 to 40 min. A 50% inhibition of APS and PAPS formation per assay is observed below 1 mM ADP which the chloroplasts would have released from ATP after 20 min. The highest inhibition of the formation of sulfur metabolites was found at 10 mM of ADP where APS remains at 35% of the control, PAPS at 30% and the bound sulfite at 55%.

A quite similar inhibition of the accumulation of these metabolites has been observed when ADP is replaced by AMP. As shown in Fig. 3 a 50% decrease of the sulfonucleotides was obtained with 3 mM AMP with APS being slightly more affected at higher concentrations of the inhibitor than PAPS. No saturation with the inhibitor was observed up to 10 mM of AMP and, in comparison to the data shown in Fig. 2, ADP appears to be a more effective inhibitor of the sulfate activation than AMP in isolated chloroplasts.

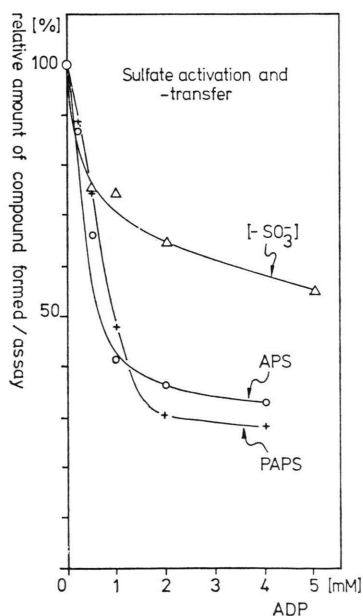


Fig. 2. Inhibition by ADP of sulfate activation and formation of the bound sulfite via transferase in isolated chloroplasts. Control APS=37.5 nmol per assay, PAPS=5.6 nmol per assay, bound sulfite=12 nmol per assay. Conditions see Materials and Methods.

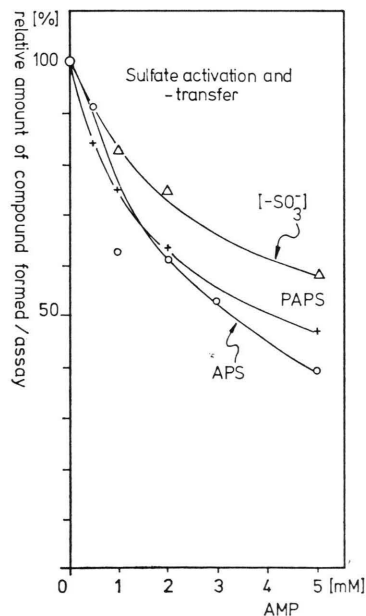


Fig. 3. Inhibition by AMP of sulfate activation and formation of the bound sulfite via transferase in isolated chloroplasts. Control APS=44.1 nmol per assay, PAPS=10.8 nmol per assay, bound sulfite=26.3 nmol per assay. Conditions see Materials and Methods.

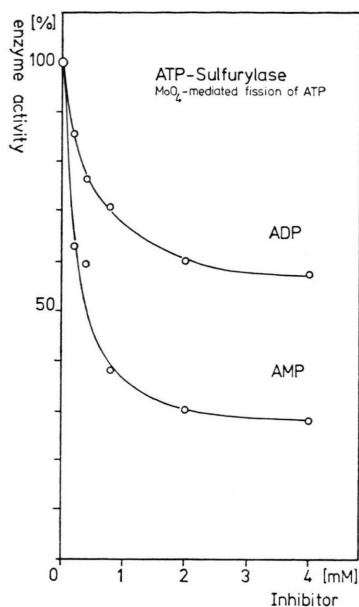


Fig. 4. Inhibition by AMP and ADP of ATP-sulfurylase from spinach leaf material. 1 mg of enzyme hydrolysed 0.79 μ mol of ATP/min, the assay contained 40 μ g in a final volume of 0.5 ml, ATP was 5.0 mM at the beginning of the experiment. Other conditions see Materials and Methods.

The results obtained with chloroplasts which still hold the complete sequence of enzymes necessary for the assimilatory process point towards a selective inhibition of the entry step, e.g. the ATP-sulfurylase. We therefore isolated and purified the enzyme using the molybdate assay¹⁸ instead of the sulfate dependent ATP-PP_i exchange². As depicted in Fig. 4 the isolated and purified enzyme showed that AMP and ADP both act as inhibitors, but in contrast to the inhibition observed in the chloroplasts, AMP is far more effective as inhibitor with the enzyme. An inhibition of 50% was obtained below 1 mM AMP the same concentration of ADP leads to an inhibition of 25%. At higher concentrations of inhibitor AMP inhibited the ATP-sulfurylase up to 70% (4 mM AMP) but ADP did not exceed 40% (even up to 10 mM, not shown in the figure).

The comparison of the apparent inhibitor constants (Table I) showed that higher concentrations of the nucleotides are needed with the chloroplasts than with the isolated enzyme.

The inhibition of ATP-sulfurylase by AMP or ADP may reflect a mechanism by which *in vivo* the energy consuming sulfate reduction is blocked when

Table I. Apparent K_i -values^a.

Metabolite formed from sulfate	Chloroplast			ATP-sulfurylase
	APS	PAPS	-SO ₃	APS
Inhibitor:	1.8	1.3	1.6	0.2
AMP	0.5	0.5	1.0	0.4
ADP				

mM, ^avalues corrected for non-inhibited enzymatic activity, determined from Figs 2–4. Chloroplasts assayed in the forward reaction of assimilatory sulfate reduction with ³⁵S-labelled sulfate, ATP-sulfurylase according to Robbins¹⁸, other conditions see Materials and Methods.

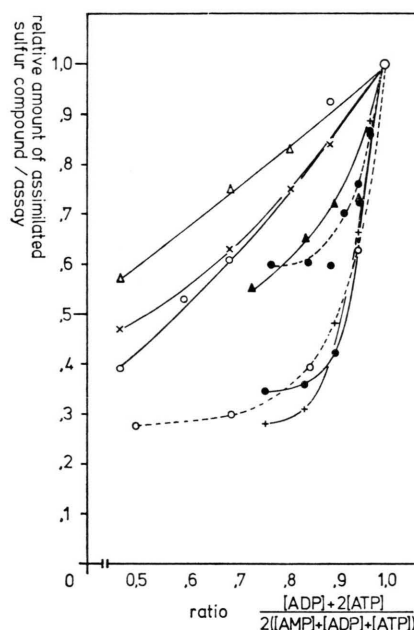


Fig. 5. Regulation of the assimilatory sulfate reduction by the energy charge of the chloroplasts (solid curves) in comparison to the isolated purified ATP-sulfurylase (dashed curves). The energy charge calculated as the ratio:

$$\frac{[\text{ADP}] + 2[\text{ATP}]}{2 \times ([\text{AMP}] + [\text{ADP}] + [\text{ATP}])}$$

at the start of the experiment. Mg²⁺-concentration was 12.5 mM throughout the experiments, different ionic species of Mg-nucleotide complexes have been neglected. Values taken from Figs 2–4. Symbols: (Δ) bound sulfite in the presence of AMP, (▲) in the presence of ADP, (○) APS in the presence of AMP, (●) in the presence of ADP, (×) PAPS in the presence of AMP, (+) in the presence of ADP as inhibitors.

the energy charge of the chloroplast is low. Fig. 5 shows that in the chloroplast or with the isolated enzyme the accumulation of APS or PAPS is de-

celerated abruptly when the energy charge is lowered. Accordingly, the amount of bound sulfite is diminished but it appeared less affected than the amount of sulfonucleotides. This finding may indicate that the sulfotransferase has a high affinity for the active sulfate and remains still active at very low concentrations of its substrate.

Discussion

Chloroplasts have been shown to be the major site of assimilatory sulfate reduction by higher plants. In the light the chloroplasts produce the phosphorylating and reducing power by the electron transport system of the thylakoid lamellae. As an organelle enclosed by a selectively permeable double membrane it maintains its adenylate pool at a constant level (see ref.²³ for review). The energy charge built up inside the chloroplast therefore governs assimilatory processes which consume ATP. The results presented in this paper demonstrate that the ATP consuming sulfate assimilation is submitted to this regulation. It is shown that the first enzymatic reaction of the sulfate assimilation is inhibited by AMP and ADP. This inhibitory effect was observed with isolated chloroplasts as a system containing all the enzymes of assimilatory sulfate reduction as well as with the isolated enzyme ATP-sulfurylase. The inhibition by AMP and ADP, however, is not uniform with both — enzyme and chloroplasts. It was found that the sulfate activation in chloroplasts is inhibited by ADP very effectively whilst AMP has a greater inhibitory effect upon the isolated enzyme.

It has previously been shown that purified ATP-sulfurylase from spinach leaf extract is inhibited competitively by ADP when assayed as the sulfate dependent ATP — PP_i exchange^{3, 24}. The same characteristics and the inhibition by AMP were found for the enzyme from *Anabaena cylindrica*¹⁴ and *Penicillium chrysogenum*¹³. Previous publications established the biosynthesis of PAPS in plants^{4, 8, 5, 25, 6}. The recovery of this compound was improved by the addition of ortho- or pyrophosphate¹¹, 5'AMP⁹ and 3'AMP^{6, 24} to the assay mixtures besides newly developed sensitive methods of detection^{26, 8, 6}. The effect of these nucleotides was variable with the source of enzyme(s) and type of reaction investigated. Data from extracts of *Anabaena cylindrica*¹⁴ or chloroplast preparations⁷ in-

dicated that PAPS biosynthesis was enhanced either in the presence of 3'AMP or 5'AMP. In all cases the positive effect of the nucleotides was attributed to the inhibition of a nucleotidase-type enzyme^{9, 6} or a specific 3'nucleotidase. In deed, leaf material did contain a 3'nucleotidase, but in the same publication it was shown that this enzyme is virtually absent from chloroplasts. Tsang *et al.*¹⁰ purified a 3'nucleotidase which degrades PAPS to APS without attacking other 3'nucleotides such as 3'AMP or 3':5'AMP. So far, this enzyme has only been detected in extracts from *Chlorella pyrenoidosa*.

Another explanation for the protective effect of adenine nucleotides is presented in this paper. Due to the regulation by AMP and ADP of the ATP-sulfurylase in the chloroplast a low rate of APS formation is maintained. This low rate would help to avoid a substrate inhibition of the APS-kinase which has been proposed for the enzyme from yeast²⁷ and from *Micrococcus denitrificans*²⁶. In addition, it was to be considered that high concentrations of AMP or ADP inhibit the adenylate-kinase and an ATP-ase always present in the chloroplasts. Both types of inhibition then would improve the stability of ATP rather than protect PAPS from degradation. When chloroplasts were assayed for the formation of the bound sulfite from sulfate the sequence of enzymatic steps (including sulfate activation and sulfate transfer) was severely affected by AMP. The apparent K_{iAMP} for this reaction sequence was found to be higher (1.8 mM) than the apparent K_{iAMP} for the isolated ATP-sulfurylase (0.2 mM). This difference may be due to unspecific binding of AMP by the chloroplast thylakoid lamellae system.

The inhibitor constants for the reaction sequence from sulfate to bound sulfite were approximately the same as found for the reaction of the APS-sulfotransferase (K_{iAMP} of 1.3 mM, ref.¹⁷). The APS-sulfotransferase which transfers the sulfogroup from APS onto an acceptor thiol^{28–30} has also been proposed to regulate assimilatory sulfate reduction in plants and in green algae. However, from data presented here, it is more likely that the assimilation of sulfate is regulated at the "entry step" of the sulfur pathway, *e.g.* at the ATP-sulfurylase, because the K_{iAMP} of this enzyme is considerably lower than the K_{iAMP} of the APS-sulfotransferase.

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