Effects of Adenine Nucleotides and Phosphate on Adenosine Triphosphate Sulphurylase from *Anabaena cylindrica*

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Production of adenosine 5'-[³⁵S]sulphatophosphate by a partially purified ATP sulphurylase from *Anabaena cylindrica* was inhibited by AMP, ADP and P_i. Decreases in enzyme activity in the presence of these inhibitors were reversed by increasing the concentrations of ATP. The adenine nucleotides inhibited the enzyme competitively with respect to ATP. In the presence of P_i, ATP showed a positive co-operative effect on enzyme activity. The inhibition by P_i was enhanced by increasing concentrations of Mg²⁺. The effects of the adenine nucleotides and the interaction of P_i and Mg²⁺ on ATP sulphurylase activity are discussed in relation to the regulation of sulphate assimilation via the energy metabolism of the alga.

It is well established that the assimilation of sulphate involves its activation before it is reduced and incorporated into cell sulphur compounds. The activation of sulphate is achieved through two ATP-dependent reactions (Roy & Trudinger, 1970; Schiff & Hodson, 1973; Siegel, 1975). In the first reaction, which is catalysed by ATP sulphurylase (ATP-sulphate adenylyltransferase, EC 2.7.7.4), adenosine 5'-sulphatophosphate is produced. This nucleotide is then further converted into adenosine 3'-phosphate 5'-sulphatophosphate by adenosine 5'-sulphatophosphate kinase (ATP-adenylyl sulphate 3'-phosphotransferase, EC 2.7.1.25). In non-photosynthetic organisms, adenosine 3'-phosphate 5'-sulphatophosphate serves as the substrate for the production of sulphide (Tsang & Schiff, 1975). Since photosynthetic organisms contain an active adenosine 5'-sulphatophosphate sulphotransferase, it has been proposed that adenosine 5'-sulphatophosphate is metabolized to sulphide without the intermediate involvement of adenosine 3'-phosphate 5'-sulphatophosphate (Schmidt, 1973, 1975a,b; Tsang & Schiff, 1975). However, production of the latter sulphur nucleotide has been demonstrated in photosynthetic organisms as well (Hodson *et al.*, 1968; Hodson & Schiff, 1969; Schmidt, 1972; Sawhney & Nicholas, 1976b). Because the first step in the assimilation of sulphate is ATP-dependent, it is likely that a mechanism exists which regulates this enzyme via the energy status of the organism. In the present paper, some effects of adenine nucleotides and phosphate on a partially purified ATP sulphurylase from *Anabaena cylindrica* are described. The results are discussed in relation to a possible regulation of the enzyme activity by the energy metabolism of the alga.

Experimental

Materials

A culture of the blue-green alga *Anabaena cylindrica* (Lemmerman, 1403/2A) was obtained from the Culture Collection of Algae and Protozoa, The Botany School, Cambridge, U.K. Carrier-free Na₂³⁵SO₄ was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Adenosine 5'-[³⁵S]sulphatophosphate was synthesized enzymically from Na₂³⁵SO₃ and AMP by using extracts (S₁₀) of *Thiobacillus denitrificans*, as described by Adams *et al.* (1971). Yeast inorganic pyrophosphatase (type III), hexokinase, glucose 6-phosphate dehydrogenase and the various nucleotides were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation of cell-free extracts and partial purification of ATP sulphurylase

Anabaena cylindrica was cultured as described by Brownell & Nicholas (1967). Unless otherwise stated, all operations during the preparation of cell extracts and enzyme purification were carried out at 4°C. The cells, harvested by centrifuging at 3000g for 10min, were washed twice with 50ml of 0.1M-Tris/HCl buffer (pH7.5). The washed cells (about 30g) were suspended in 2 vol. of the washing buffer and then broken in an Aminco French pressure cell at 16000lb/in² (110 MPa). The homogenate was centrifuged at 3000g for 10min. The supernatant (S₃) was maintained at 53°C for 20min and stored overnight at -15°C. The preparation was then thawed and centrifuged at 30000g for 30min. The supernatant fraction (S₃₀) was passed through a column (1.5 cm × 25 cm) of Sephadex G-25 which had been previously

equilibrated with 0.1 M-Tris/HCl buffer (pH 7.5). The effluent was concentrated to a final volume of 4–6 ml in an Amicon ultrafiltration cell (Amicon Corp., Lexington, MA, U.S.A.) with a PM 10 membrane filter. The concentrated extract was loaded on a Sephadex G-100 column (3.5 cm × 70 cm) and eluted with 0.1 M-Tris/HCl (pH 7.5) and collected in 5 ml fractions at a flow rate of about 20 ml/h. Only those fractions of ATP sulphurylase which were completely free of adenylate kinase activity were pooled and then concentrated to about 10 ml in the Amicon ultrafiltration cell.

Assays

ATP sulphurylase. During purification, the enzyme activity in various fractions was assayed by following the rate of ATP production from adenosine 5'-sulphatophosphate and pyrophosphate by using the luciferin/luciferase bioluminescence technique as described by Balharry & Nicholas (1971). In all other experiments reported in the present paper, ATP sulphurylase was assayed by determining the amounts of adenosine 5'-[³⁵S]sulphatophosphate produced from ATP and Na₂³⁵SO₄. The incubation mixture (final volume 1 ml) contained: Tris/HCl (pH 7.5), 60 μmol; MgCl₂, 10 μmol; ATP, 6 μmol; K₂SO₄, 0.2 μmol; carrier-free Na₂³⁵SO₄, 1.2 μCi; yeast inorganic pyrophosphatase, 0.4 unit, and an appropriate amount (0.2–0.4 ml, containing 200–350 μg of protein) of the enzyme preparation. The reaction was started by adding ATP and, after a 40 min incubation at 30°C, stopped with 1 ml of ice-cold 95% (v/v) ethanol. The ³⁵S-labelled compounds in the reaction mixture, separated on 3MM Whatman paper by high-voltage electrophoresis in 0.1 M-sodium citrate buffer (pH 5.0) at 1.5 kV for 45 min, were identified and radioassayed as described previously (Sawhney & Nicholas, 1976b). One unit of enzyme activity represents 1 nmol of adenosine 5'-[³⁵S]sulphatophosphate produced in the reaction mixture in 60 min. When examining the effect of nucleotides and phosphate (KH₂PO₄) on the enzyme activity, solutions of these compounds were titrated to pH 7.5 with NaHCO₃ before adding them to the reaction mixtures.

Adenylate kinase. Enzyme activity was assayed by determining the amount of ATP produced from ADP, by coupling it to the reduction of NADP⁺ in the presence of hexokinase and glucose 6-phosphate dehydrogenase, as described by Kitchin & Watts (1974).

5'-Nucleotidase. The activity was determined by measuring the amount of P_i released from ATP by the procedure of Chen *et al.* (1956).

Protein content. Protein was precipitated in a final concentration of 5% (w/v) trichloroacetic acid, washed and dissolved in 0.1 M-NaOH, and then determined by the method of Lowry *et al.* (1951), with

bovine serum albumin (fraction V; Calbiochem, Sydney, N.S.W., Australia) as a standard.

Results

Enzyme preparation

The partially purified ATP sulphurylase from *Anabaena cylindrica* had about tenfold higher specific activity than that of the crude extract (S₃). This preparation was completely free of the enzymes which hydrolyse adenosine 5'-sulphatophosphate and adenosine 3'-phosphate 5'-sulphatophosphate (Sawhney & Nicholas, 1976a) and also of adenylate kinase and 5'-nucleotidase activities. In preliminary experiments, an appropriate amount of the enzyme preparation for ATP sulphurylase assay was determined, so that the production of adenosine 5'-sulphatophosphate was proportional to the time of incubation up to 60 min. In all the subsequent experiments, incubations were carried out for 40 min.

Effect of adenine nucleotides

Inhibition of ATP sulphurylase activity by different concentrations of AMP is shown in Fig. 1. Inclusion of 2 mM-AMP in the incubation mixture diminished the production of adenosine 5'-[³⁵S]sulphatophosphate by more than half ($K_i = 1.6$ mM). Although ADP also decreased the enzyme activity, as much as 10 mM was required to produce a similar effect (Fig. 1). Inhibition of enzyme activity by ADP

Fig. 1. Effect of AMP and ADP on ATP sulphurylase activity

The enzyme activity was assayed as described in the Experimental section, except that the reaction mixture contained either AMP (●) or ADP (▲). In the control tubes (without nucleotides) for the experiments with AMP and ADP, 4.73 and 5.68 nmol of adenosine 5'-[³⁵S]sulphatophosphate respectively were produced in the reaction mixture after 60 min.

showed a sigmoidal response in the presence of different concentrations of the nucleotide. The results in Table 1 show that, of the various adenine derivatives examined, the enzyme was affected most by 5'-AMP and ADP, whereas 3'-AMP was without effect. A comparison of different 5'-nucleoside monophosphates indicates that the effect was quite specific for AMP (Table 2). The enzyme was inhibited to a lesser extent by TMP, whereas UMP at 2 mM enhanced enzyme activity by about 36%. The inhibition by AMP and ADP was additive, as shown in Table 3.

Table 1. *Effect of various adenine derivatives on ATP sulphurylase activity*

The enzyme activity was assayed as described in the Experimental section. Neutralized solutions of the nucleotides were added to the reaction mixture.

Additions to the reaction mixture	Concn. of nucleotide ...	ATP sulphurylase activity			
		2 mM		4 mM	
		A*	B†	A*	B†
None (control)		4.68	100	4.68	100
ADP		4.17	89	2.78	59
5'-AMP		2.59	55	1.38	29
3'-AMP		4.68	100	3.70	80
Adenosine		5.09	108	3.83	82
Adenine		4.74	101	3.72	80

* nmol of adenosine 5'-[³⁵S]sulphatophosphate produced in reaction mixture in 60 min.

† Activity as % of control.

Table 2. *Effect of various nucleoside monophosphates on enzyme activity*

The assay procedure is described in the Experimental section. The reaction mixtures contained either 1 or 2 mM of the nucleotides as indicated.

Additions to the reaction mixture	Concn. of nucleotide ...	ATP sulphurylase activity			
		1 mM		2 mM	
		A*	B†	A*	B†
None		6.24	100	6.24	100
5'-AMP		4.60	74	3.45	55
GMP		6.78	108	6.19	99
TMP		5.30	85	4.40	71
UMP		6.94	111	8.46	135
CMP		5.83	93	5.70	91
IMP		5.19	83	6.15	98

* nmol of adenosine 5'-[³⁵S]sulphatophosphate produced in reaction mixture in 60 min.

† Activity as % of control.

Table 3. *Combined inhibitory effects of AMP and ADP on enzyme activity*

Experimental procedures were as described in the Experimental section. Neutralized solutions of the nucleotides were added to the reaction mixture. One unit of enzyme activity represents 1 nmol of adenosine 5'-[³⁵S]sulphatophosphate produced in the reaction mixture in 60 min.

Additions to the reaction mixture (mM)		Enzyme activity (units)	Inhibition (%)
AMP	ADP		
0	0	8.52	—
1	0	6.71	21
2	0	5.86	31
0	2	7.56	11
0	5	5.92	31
1	2	5.52	35
1	5	4.45	48
2	2	4.55	47
2	5	3.66	57

Effect of P_i

The data in Fig. 2 show that P_i also markedly decreased the enzyme activity. The curve for the inhibitory effects of different concentrations of P_i was sigmoidal. The inclusion of 3 mM-P_i suppressed adenosine 5'-[³⁵S]sulphatophosphate formation by about half.

The equilibrium of the ATP sulphurylase reaction is extremely unfavourable for the synthesis of adenosine 5'-sulphatophosphate. This thermodynamic constraint is overcome by continuous removal of the other product of the enzyme reaction, namely PP_i, by the action of exogenously added inorganic pyrophosphatase (Siegel, 1975). The observed restricted production of adenosine 5'-[³⁵S]sulphatophosphate, in the presence of P_i, could have resulted from the inhibitory effect of this anion on inorganic pyrophosphatase. However, this possibility was discounted, since a fourfold excess of inorganic pyrophosphatase over that normally used in the reaction mixture did not reverse the small inhibition (about 20%) produced by 2 mM-P_i.

Effect of Mg²⁺ on inhibition by P_i and AMP

It was shown earlier that relative concentrations of ATP and Mg²⁺ have a profound effect on ATP sulphurylase activity (Sawhney & Nicholas, 1976b). Although the enzyme was not affected by about a twofold excess of Mg²⁺ over ATP, a similar excess of ATP over Mg²⁺ was severely inhibitory. In the present study, Mg²⁺ was used in small excess over ATP (10 mM-Mg²⁺ and 6 mM-ATP) in the incubation mixtures. Since P_i reacts chemically with Mg²⁺, it

could indirectly inhibit the enzyme because of a relative excess of ATP over Mg^{2+} . Should the effect of P_i result from such a reaction, then increasing concentrations of Mg^{2+} would be expected to relieve the inhibition. The results in Fig. 3 show that higher concentrations of Mg^{2+} did in fact enhance the inhibitory effect of P_i . Thus 6mm- P_i with 4mm- Mg^{2+} had no effect on ATP sulphurylase, whereas increasing Mg^{2+} to 6 and 10mm decreased the enzyme activity by 40 and 80% respectively. At these higher

concentrations, Mg^{2+} alone did not have any significant effect on adenosine 5'-sulphatophosphate production. It should also be noted that with increas-

Fig. 2. Effect of various concentrations of P_i on enzyme activity

Details of the assay procedure are described in the Experimental section. A neutralized solution of KH_2PO_4 was added to obtain the desired concentrations of phosphate in the reaction mixtures. Control tubes (without phosphate) produced 9.13nmol of adenosine 5'-[^{35}S]sulphatophosphate in 60min.

Fig. 3. Effect of phosphate on enzyme activity in the presence of various concentrations of Mg^{2+}

Details of the experimental procedures are given in the Experimental section. Concentration of Mg^{2+} in the reaction mixtures was, however, varied as shown. The various concentrations of phosphate used were: $\bullet$, none; Δ , 1 mM; $\square$, 2 mM; $\circ$, 4 mM; $\blacktriangle$, 6 mM; $\blacksquare$, 8 mM. The enzyme activities in controls (without phosphate) containing 2, 4, 6, 8 and 10mm- Mg^{2+} were 1.64, 3.87, 6.39, 6.59 and 6.28 nmol of adenosine 5'-[^{35}S]sulphatophosphate produced, respectively, in the incubation mixture in 60min. Values for the percentage inhibition of activity at various concentrations of phosphate were determined in relation to the activity of the respective control.

Table 4. Effect of Mg^{2+} concentration on inhibition of enzyme activity by AMP

The enzyme activity was determined as described in the Experimental section. Reaction mixtures contained the specified concentrations of Mg^{2+} and AMP.

Concn. of AMP (mM)	Concn. of Mg^{2+} ...	ATP sulphurylase activity							
		2 mm		4 mm		8 mm		12 mm	
		A*	B†	A*	B†	A*	B†	A*	B†
0		1.25	100	4.19	100	5.76	100	5.57	100
0.5		1.48	117	3.45	82	4.87	85	4.40	79
1.0		1.21	97	2.90	69	4.09	71	3.80	68
2.0		0.96	77	1.93	46	2.92	50	2.55	46

* nmol of adenosine 5'-[^{35}S]sulphatophosphate produced in reaction mixture in 60min.

† Activity as % of control.

Fig. 4. Double-reciprocal plots of the inhibition of enzyme activity by AMP at various concentrations of ATP. Enzyme activity was determined as described in the Experimental section, except that concentration of ATP was varied as shown in the Figure and the reaction mixture contained the following concentrations of AMP: ○, none; △, 1 mM; □, 2 mM; ●, 3 mM. v is expressed as nmol of adenosine 5'-[35 S]sulphatophosphate produced in 60 min.

Fig. 5. Double-reciprocal plot of the effect of ADP on enzyme activity with various concentrations of ATP. Experimental details are as described in Fig. 4, except that in place of AMP the following concentrations of ADP were used: ●, none; ▲, 2 mM; ■, 4 mM. v is expressed as nmol of adenosine 5'-[35 S]sulphatophosphate produced in 60 min.

ing concentrations of Mg^{2+} , the enzyme activity was affected at relatively lower concentrations of P_i .

An experiment was then done to examine whether, as with P_i , the inhibitory effect of AMP was also

Fig. 6. Double-reciprocal plot of the inhibition of enzyme by P_i in the presence of different concentrations of ATP. The experimental details are given in Fig. 2, except that the concentration of ATP was varied as shown in the Figure and the reaction mixture contained the following concentrations of P_i : ○, none; △, 1 mM; □, 2 mM; ●, 3 mM. v is expressed as nmol of adenosine 5'-[35 S]sulphatophosphate produced in 60 min.

dependent on the concentration of Mg^{2+} (Table 4). In the presence of 4 mM- Mg^{2+} , the addition of AMP resulted in a more marked inhibition of the enzyme than at the lower concentration of 2 mM- Mg^{2+} . However, unlike the effect of P_i , further increases in concentration of Mg^{2+} beyond 4 mM did not exert any additional inhibitory effect.

Mechanism of inhibition of enzyme by AMP, ADP and P_i

The inhibition of the enzyme by AMP, ADP and P_i was reversed by increasing amounts of ATP. Double-reciprocal plots of the effects of various concentrations of ATP, at fixed concentration of the inhibitors, indicate that the inhibitions by both AMP and ADP were competitive with respect to ATP (Figs. 4 and 5). However, with P_i as an inhibitor, the curves obtained were concave upwards (Fig. 6). This suggests that, in the presence of P_i , ATP exerts a positive co-operative effect on the enzyme. Replotting the same data as in Fig. 7 shows that the reversal of the phosphate

Fig. 7. Effect of different concentrations of ATP on the inhibition of enzyme activity by P_i .

The data of Fig. 6 are replotted as activity in the presence of fixed concentrations of P_i at various concentrations of ATP. Activities are calculated as a percentage of the control at each concentration of ATP, in the absence of P_i .

inhibition of the enzyme was not a linear function of the concentration of ATP added, but the recovery of the activity was proportionally greater at higher concentrations of ATP than at the lower ones.

Discussion

Hitherto, regulation of sulphate assimilation has been studied in relation to the requirements for reduced sulphur compounds by the organism. Thus repression of the enzymes (Dreyfuss & Pardee, 1966; Wheldrake, 1967; Brunold & Erismann, 1975), as well as feedback inhibition of ATP sulphurylase (DeVito & Dreyfuss, 1964; Levi & Wolf, 1969; Tweedie & Segel, 1971; Hawes & Nicholas, 1973) by the intermediates and the products of the pathway, has been investigated. The present study on the effects of adenine nucleotides and phosphate on ATP sulphurylase indicates that assimilation of sulphate may also be regulated by the energy status of an organism. Such a control mechanism is to be expected, since two molecules of ATP are consumed for activation of sulphate, via adenosine 5'-sulphatophosphate, to adenosine 3'-phosphate 5'-sulphatophosphate.

Inhibition of partially purified ATP sulphurylase from *Anabaena cylindrica* by ADP confirms the report by Onajobi (1975) on its effect on enzyme from rice roots. However, inhibition of the enzyme from

the alga by AMP is in marked contrast with the stimulation of enzyme activity by this nucleotide in crude extracts of rice roots (Onajobi, 1975). The reason for this difference is not clear, but the presence of enzymes such as 5'-nucleotidase, adenylate kinase and adenosine 5'-sulphatophosphate-degrading enzymes in unpurified extracts would seriously interfere with studies on the effects of nucleotides on ATP sulphurylase activity.

Formation of adenosine 5'-sulphatophosphate by the enzyme from the alga was inhibited competitively, with respect to ATP, by AMP and ADP. In addition, P_i also restricted ATP sulphurylase activity. The decrease in enzyme activity with increasing concentrations of P_i gave a sigmoidal plot. The inhibition by P_i was also relieved by increasing the concentration of ATP. However, the double-reciprocal plot of the reversal of P_i inhibition by ATP indicated a positive co-operative action of ATP on the enzyme activity. This implies that binding of a few initial ATP molecules promoted subsequent catalytic interaction of this nucleotide with the enzyme. Hence a certain threshold concentration of ATP was required to overcome the inhibition by P_i .

These results can be explained in terms of regulation of ATP sulphurylase activity, and consequently of sulphate assimilation, by the energy metabolism of the cell. Any constraint in the generation of cellular ATP would require the organism to decelerate consumption of ATP in reactions of secondary metabolic importance in order to conserve this nucleotide for vital cell processes. Failure to regenerate ATP would result in an increase in the intracellular concentrations of ADP, AMP and P_i . Since ATP sulphurylase is sensitive to AMP and P_i , these metabolites would exert an inhibitory effect on the enzyme activity, thus decreasing the demand for ATP for sulphate assimilation.

It is significant that the inhibition by P_i was determined by the concentration of Mg^{2+} (Fig. 3). Thus P_i , even at fairly high concentrations, would be ineffective as long as the concentration of free Mg^{2+} was low. However, an increase in free Mg^{2+} , which occurs when adenylate charge declines (Blair, 1970), would enhance the inhibition by P_i . Our results with ATP sulphurylase support the proposal by Blair (1970) that fluctuations in the concentration of free Mg^{2+} are important in regulating enzyme activities. Our observations suggest that this phenomenon should be investigated in other energy-regulated processes also.

Inhibition of adenosine 5'-sulphatophosphate sulphotransferase by AMP (Schmidt, 1975c) has led to the proposal that the assimilation of sulphate might be regulated through modulation of this enzyme activity (Schmidt, 1976). Our results indicate that ATP sulphurylase is controlled by concentrations of adenine nucleotides, P_i and free Mg^{2+} , as

would be expected under conditions of restricted energy metabolism. Regulation of sulphate assimilation through adenosine 5'-sulphatophosphate sulphotransferase can be achieved if its inhibition results in the accumulation of adenosine 5'-sulphatophosphate in concentrations high enough to act as an inhibitor of ATP sulphurylase (Tweedie & Segel, 1971; Hawes & Nicholas, 1973). Until then ATP would continue to be consumed for the production of adenosine 5'-sulphatophosphate and also adenosine 3'-phosphate 5'-sulphatophosphate. The accumulation of adenosine 5'-sulphatophosphate might be restricted because of other adenosine 5'-sulphatophosphate-metabolizing enzymes, namely adenosine 5'-sulphatophosphate kinase and adenosine 5'-sulphatophosphate-hydrolysing enzymes (Sawhney & Nicholas, 1976a; Tsang & Schiff, 1976), which could deplete this metabolite. The presence of these enzymes would consequently render the control through adenosine 5'-sulphatophosphate sulphotransferase less effective. The comparable K_i values of AMP for adenosine 5'-sulphatophosphate sulphotransferase (Schmidt, 1975c) and ATP sulphurylase from *Anabaena cylindrica* indicate that the latter enzyme might serve as the primary site for energy-dependent regulation of sulphate assimilation. The control through modulating activity of the first enzyme of the pathway would have greater advantage, since it would not only effectively restrict the initial ATP-utilizing reactions of sulphate activation to produce adenosine 5'-sulphatophosphate and adenosine 3'-phosphate 5'-sulphatophosphate, but would also prevent unnecessary accumulation of intermediates of the pathway.

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