Effects of Adenine Nucleotides on Rice-Root Adenosine Triphosphate Sulphurylase Activity *in vitro*

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The presence of ATP sulphurylase activity in the 30000g supernatant fraction of rice-root homogenate has been demonstrated. Studies of the effects of adenosine and its nucleotides on the enzymic activity showed that AMP activated but that ADP and adenosine inhibited the enzyme.

Previous reports have shown the existence of ATP sulphurylase (ATP-sulphate adenylyltransferase, EC 2.7.7.4), an enzyme that catalyses the formation of adenosine 5'-sulphatophosphate from ATP and sulphate, in plant tissues (Balharry & Nicholas, 1970; Roy & Trudinger, 1970; Shaw & Anderson, 1972; Onajobi *et al.*, 1973). In some cases the properties of this enzyme were also studied; such properties include the effects of sulphate, ATP, MgCl₂ and thiols on the enzymic activity. In the present investigation the effects of adenosine and its nucleotides on ATP sulphurylase activity were studied. These studies were prompted by some earlier observations made with corn ATP sulphurylase, which showed that some extracts obtained from different corn-root preparations apparently possessed different concentrations of enzymes that hydrolyse ATP, with concomitant differences in the observed ATP sulphurylase activity. It was therefore decided to investigate whether any of the hydrolytic products of ATP had any effect on ATP sulphurylase activity.

Experimental

Materials. Rice seeds (*Oriza sativa*, variety 6) were obtained as a gift from Federal Agricultural Research Institute, Ibadan, Nigeria. Radioactive sulphate was from The Radiochemical Centre, Amersham, Bucks., U.K. Reagents for liquid-scintillation counting were obtained from Nuclear Enterprises, Edinburgh, U.K. Nucleotides were products of Sigma Chemical Co., St. Louis, Mo., U.S.A. All other chemicals were of analytical-reagent grade.

Methods. Rice seeds were washed and spread over two layers of gauze cloth on a growing rack placed in a plastic tray containing 2mM-CaSO₄ solution. The solution was continually aerated in a Gallenkamp cooled incubator (model IH270, without interior illumination) at 27–29°C and changed regularly. The roots were harvested for use after about 8 days.

A 30000g supernatant of the roots in 0.2M-Tris-HCl buffer containing 0.01M-cysteine and 0.01M-EDTA at pH8.0 (medium 1) was prepared by the method of Ellis (1969). The supernatant was dialysed overnight against 10-fold-diluted medium 1.

ATP sulphurylase activity in the root extract was assayed by measurement of the amount of [³⁵S]-sulphate incorporated into adenosine 5'-sulphatophosphate according to the following equation:

Root extract (0.5 ml containing 0.5–1 mg of protein) was incubated with 2 μmol of ATP, 1 μmol of Na₂³⁵SO₄ (1–4 μCi), 10 μmol of MgCl₂ and 50 μmol of Tris-HCl buffer at pH8.0 in a total volume of 1 ml in a shaking reaction incubator at 37°C for 15 min. The reaction was terminated by heating for 1 min in a boiling-water bath. The coagulated protein was centrifuged off and the supernatant was analysed by high-voltage paper electrophoresis and two-dimensional t.l.c.

Electrophoresis was conducted on Whatman 3MM paper in 0.05M-sodium citrate buffer, pH5.9, at 1200V in a Shandon Southern high-voltage electrophoretic apparatus (model L24) for 1.5 h. Standards of nucleotides were run on the paper concurrently with the incubation samples. After electrophoresis, the paper was dried in the oven and the locations of the nucleotides on the paper were viewed under u.v. light.

The radioactivity on the paper was assayed either by scanning in a gas-flow radioactive scanner or by cutting the paper into 1 cm strips and counting the radioactivity on each strip by transferring it into a vial containing 5 ml of a liquid scintillator, followed by assay of the radioactivity in a liquid-scintillation counter. The liquid scintillator used was either toluene containing 0.4% *p*-terphenyl or NE250 liquid scintillator obtained from Nuclear Enterprises.

Cellulose two-dimensional t.l.c. of the products of incubation was carried out with solvent systems (a) isobutyric acid-aq. NH₃ (sp.gr. 0.880)-water (57:4:39, by vol.) and (b) methanol-1M-ammonium acetate (7:3, v/v). This method gives a good separation of phosphate esters (Cole & Ross, 1966). Moreover, sulphate (detected by radiochromatography), adenosine sulphatophosphate, ATP, ADP and AMP were found to be clearly separated from one another

by this technique. R_F values in the solvent systems could not be calculated because the solvent was usually allowed to run for longer periods after reaching the top of the plate in order to obtain good separation of the nucleotides and sulphate. However, relative mobilities in solvent system (a) were: sulphate, 1; adenosine 5'-sulphatophosphate, 1.3; ATP, 1.35; ADP, 1.45; AMP, 1.6; adenosine, 1.9. In solvent system (b) the relative mobilities were: sulphate, 1; adenosine 5'-sulphatophosphate, 0.82; ATP, 0.14; ADP, 0.26; AMP, 0.55; adenosine, 1.62.

The protein content of the root extract was assayed by the biuret method (Dawson *et al.*, 1969).

Results and discussion

Formation of adenosine 5'-sulphatophosphate. When the 30000g supernatant from the rice-root homogenate was incubated for 15 min with $\text{Na}_2^{35}\text{SO}_4$ (4 μCi), ATP and MgCl_2 in the amounts indicated above, and the products of incubation were analysed by electrophoresis followed by scanning for radioactivity, two radioactive peaks were obtained. The faster-migrating peak (distance of migration 33–34 cm) contained most of the radioactivity and corresponded to sulphate, and the slower component ran between ADP and ATP, having the same mobility as standard adenosine 5'-sulphatophosphate. (The mobility of the latter relative to sulphate was 0.46–0.47.)

The region of radioactivity between ATP and ADP was cut off and eluted with water. A portion of the eluate was co-electrophoresed with standard non-radioactive adenosine 5'-sulphatophosphate. Results showed that the radioactive peak and the u.v. spot corresponding to adenosine 5'-sulphatophosphate coincided on the electrophoretogram, and no other radioactive spot could be detected when the paper was cut into sections and the radioactivity on each section determined by liquid-scintillation counting.

Another portion of the eluate was mixed with standard non-radioactive adenosine 5'-sulphatophosphate and analysed by cellulose two-dimensional t.l.c. as described above. Viewing under u.v. light and radioautography of the t.l.c. plate showed that there was only one radioactive spot, which was at the same position as the u.v. spot. This confirms the radioactive homogeneity of the adenosine 5'-sulphatophosphate as obtained on the paper electrophoretogram of ATP sulphurylase assay mixtures.

No adenosine 5'-sulphatophosphate was formed when the root extract was boiled before being added to enzyme assay mixtures.

When paper electrophoretograms from the enzyme assay mixtures were cut into sections and radioactivity on each section was analysed by liquid-scintillation counting, in most experiments radioactivity was associated only with the adenosine 5'-sulphatophosphate and sulphate spots. In a few

experiments, however, a small peak of radioactivity that ran just ahead of ATP (mobility relative to sulphate 0.59) was also detected. However, since this extra peak of radioactivity (which is termed 'substance X' in the present paper) was not consistently obtained, no attempt was made to characterize it further, although the radioactivity in this region was estimated in all the experiments.

The amount of radioactivity incorporated into adenosine 5'-sulphatophosphate increased linearly with the length of the incubation period from 5 min up to at least 20 min. A standard assay period of 15 min was therefore employed throughout. Moreover, the amount of radioactivity incorporated into adenosine 5'-sulphatophosphate increased proportionally with increasing amount of the 30000g supernatant in the assay mixture between 0.2 and 1 mg of protein.

Effects of ATP, ADP, AMP and adenosine on the formation of adenosine 5'-sulphatophosphate. ATP, a substrate of ATP sulphurylase, caused a decrease in the adenosine 5'-sulphatophosphate obtained when present in assay mixtures at concentrations higher than 2 mM (Table 1). Similar inhibition of ATP sulphurylases of *Penicillium chrysogenum* and yeast by high concentrations of ATP have been observed by Tweedie & Segel (1971) and by Hawes & Nicholas (1973) respectively.

ADP and, generally to a smaller extent, adenosine decreased the amount of radioactivity incorporated into adenosine 5'-sulphatophosphate (Figs. 1a and 1b).

The decreases in the formation of adenosine 5'-sulphatophosphate caused by high concentrations of ATP, and also by ADP and adenosine, may reflect an

Table 1. *Effects of various concentrations of ATP on ATP sulphurylase activity*

Each incubation mixture contained 1 μmol of $\text{Na}_2^{35}\text{SO}_4$ (about 1 μCi), 10 μmol of MgCl_2 , 0.5 ml of rice-root 30000g supernatant, 50 μmol of Tris-HCl buffer, pH 8.0, and the indicated amount of ATP in a total volume of 1 ml. The given radioactivity in adenosine 5'-sulphatophosphate is the amount obtained from a 15 min incubation mixture. Each assay mixture was analysed by high-voltage electrophoresis.

Concn. of ATP in assay mixture (mM)	Radioactivity in adenosine 5'-sulphatophosphate (d.p.m./mg of protein)	Inhibition (%)
1	7800	
2	47500	
5	25000	47.5
10	13000	73.0
20	2000	96.0

Fig. 1. Effects of various concentrations of ADP, adenosine and AMP on ATP sulphurylase activity at different ATP concentrations

Each incubation mixture contained the specified amount of ADP (a) or adenosine (b) or AMP (c) plus 2 μmol (▲), 5 μmol (●) or 10 μmol (■) of ATP; other contents and conditions were as given in Table 1.

inhibition of the enzyme ATP sulphurylase in the rice-root extract or an activation of the conversion of adenosine 5'-sulphatophosphate into some other product such as substance X without any effect on the ATP sulphurylase activity. However, an estimation of radioactivity associated with substance X showed that there was no increase in the formation of substance X when the amount of adenosine 5'-sulphatophosphate was decreased in any of the experiments. Moreover, no new radioactive peak could be detected in these experiments. Therefore the decrease in the radioactivity associated with adenosine 5'-sulphatophosphate reflects a true inhibition of the ATP sulphurylase activity in the rice-root extract.

The effects of different concentrations of AMP in incubations containing different amounts of ATP are shown in Fig. 1(c). This shows that AMP stimulated the production of adenosine 5'-sulphatophosphate, the activation being more significant at low concentrations of ATP. Possible formation of adenosine 5'-sulphatophosphate from AMP was ruled out by the observation that, when incubations were carried out with mixtures containing 5 μmol of AMP/ml, but no ATP, no adenosine 5'-sulphatophosphate was formed.

The activation of the rice-root ATP sulphurylase *in vitro* by AMP may be due to the possibility that it antagonizes an inhibitor present in the rice-root extract or that the activation reflects a true inherent property of the ATP sulphurylase. AMP is known to be an allosteric effector for a number of regulatory

enzymes (Atkinson, 1966). In some of these enzymes, e.g. yeast phosphofructokinase (EC 2.7.1.11) (Ramaiah *et al.*, 1964), AMP activates the enzyme, whereas ATP, a substrate of the enzyme, inhibits it at high concentrations, similar to the observations made with the rice-root ATP sulphurylase.

The inhibition by ATP at high concentrations may be due to binding to the enzyme at a regulatory site, possibly the AMP-binding site. The greater effectiveness of AMP in stimulating adenosine 5'-sulphatophosphate production at low concentrations than at high concentrations of ATP, as observed in the present work (Fig. 1c), is consistent with this view. If the activation of the ATP sulphurylase by AMP and its inhibition by high concentrations of ATP, ADP and possibly adenosine are true reflections of what happens *in vivo*, then it is possible that the relative concentrations of these compounds in the plant regulate the activity of ATP sulphurylase. Since this enzyme catalyses the first step of sulphate metabolism, a control of its activity would ultimately lead to the regulation of sulphate metabolism in the rice plant.

- Atkinson, D. E. (1966) *Annu. Rev. Biochem.* **35**, 85–124
 Balharry, G. J. E. & Nicholas, D. J. D. (1970) *Biochim. Biophys. Acta* **220**, 513–524
 Cole, C. V. & Ross, C. (1966) *Anal. Biochem.* **17**, 526–539
 Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969) *Data for Biochemical Research*, 2nd edn., p. 618, Oxford University Press, Oxford and New York

- Ellis, R. J. (1969) *Planta* **88**, 34–42
- Hawes, C. S. & Nicholas, D. J. D. (1973) *Biochem. J.* **133**, 541–550
- Onajobi, F. D., Cole, C. V. & Ross, C. (1973) *Plant Physiol.* **52**, 580–584
- Ramaiah, A., Hathaway, J. A. & Atkinson, D. E. (1964) *J. Biol. Chem.* **239**, 3619–3622
- Roy, A. B. & Trudinger, P. A. (1970) *The Biochemistry of Inorganic Compounds of Sulphur*, pp. 91–105 and 251–288, Cambridge University Press, Cambridge
- Shaw, W. H. & Anderson, J. W. (1972) *J. Biol. Chem.* **246**, 237–247
- Tweedie, J. W. & Segel, I. H. (1971) *J. Biol. Chem.* **246**, 2438–2446