Adenosine Diphosphate Sulphurylase Activity in Leaf Tissue

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1. A new method is described for the assay of ADP sulphurylase. The method involves sulphate-dependent $[^{32}\text{P}]\text{P}_i$ -ADP exchange; the method is simpler, more sensitive and more direct than the method involving adenosine 5'-sulphatophosphate-dependent uptake of P_i . 2. ADP sulphurylase activity was demonstrated in crude extracts of leaf tissue from a range of plants. Crude spinach extract catalysed the sulphate-dependent synthesis of $[^{32}\text{P}]\text{ADP}$ from $[^{32}\text{P}]\text{P}_i$; spinach extracts did not catalyse sulphate-dependent AMP- P_i , ADP- PP_i or ATP- P_i exchange under standard assay conditions. ADP sulphurylase activity in spinach leaf tissue was associated with chloroplasts and was liberated by sonication. 3. Some elementary kinetics of crude spinach leaf and purified yeast ADP sulphurylases in the standard assay are described; addition of Ba^{2+} was necessary to minimize endogenous P_i -ADP exchange of the yeast enzyme and crude extracts of winter-grown spinach. 4. Spinach leaf ADP sulphurylase was activated by Ba^{2+} and Ca^{2+} ; Mg^{2+} was ineffective. The yeast enzyme was also activated by Ba^{2+} . The activity of both enzymes decreased with increasing ionic strength. 5. Purified yeast and spinach leaf ADP sulphurylases were sensitive to thiol-group reagents and fluoride. The pH optimum was 8. ATP inhibited sulphate-dependent P_i -ADP exchange. Neither selenate nor molybdate inhibited sulphate-dependent P_i -ADP exchange and crude spinach extracts did not catalyse selenate-dependent P_i -ADP exchange. 6. The presence of ADP sulphurylase activity jeopardizes the enzymic synthesis of adenosine 5'-sulphatophosphate from ATP and sulphate with purified ATP sulphurylase and pyrophosphatase.

ADP sulphurylase (ADP-sulphate adenylyltransferase, EC 2.7.7.5) catalyses the reaction:

The enzyme was first described in yeast and partially purified by Robbins & Lipmann (1958); ADP sulphurylase was also reported in the chemoautotroph *Thiobacillus thioparus* (Peck, 1960) and in the dissimilatory sulphate-reducing bacterium *Desulfovibrio desulfuricans* (Peck, 1962). The enzyme has been previously described only in micro-organisms.

The standard free-energy change of the reaction catalysed by ADP sulphurylase has not been reported but it is probably similar to the free-energy change of the reaction catalysed by ATP sulphurylase (+46 kJ/mol), since both enzymes catalyse the hydrolysis of a P-O-P bond with the concomitant formation of adenosine 5'-sulphatophosphate (Roy & Trudinger, 1970). ATP sulphurylase (ATP-sulphate adenylyltransferase, EC 2.7.7.4) catalyses the reaction:

The unfavourable free-energy change for the synthesis of adenosine 5'-sulphatophosphate is presumed

to be coupled *in vivo* to the pyrophosphatase-catalysed hydrolysis of PP_i , resulting in a more favourable yield of adenosine 5'-sulphatophosphate (Roy & Trudinger, 1970). Since the reaction catalysed by ADP sulphurylase cannot be coupled to pyrophosphatase the presence of ADP sulphurylase activity would jeopardize the synthesis of adenosine 5'-sulphatophosphate from sulphate and ATP in a coupled enzyme system containing ATP sulphurylase and pyrophosphatase.

While performing experiments to investigate the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate, we conducted an incubation (incubation A) with ATP, $[^{35}\text{S}]\text{sulphate}$, Mg^{2+} , purified ATP sulphurylase and pyrophosphatase to synthesize adenosine 5'- $[^{35}\text{S}]\text{sulphatophosphate}$ and P_i . During a second incubation (incubation C), with spinach crude

extract, adenosine 5'-[³⁵S]sulphatophosphate disappeared and ADP was formed, suggesting the operation of ADP sulphurylase activity. This paper describes a new and sensitive method for measuring ADP sulphurylase activity involving sulphate-dependent [³²P]P_i-ADP exchange and reports the occurrence of ADP sulphurylase in crude extracts of spinach leaf tissue and in some other plants. Some properties of the crude enzyme from spinach leaf and purified yeast ADP sulphurylase are described.

Experimental

Chemicals

ADP and ATP (sodium salts) were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; ADP was free from ATP when analysed by paper chromatography in solvent 2 (see below). [³²P]P_i was supplied as a solution in HCl by the Australian Atomic Energy Commission, Lucas Heights, N.S.W., Australia. The HCl was removed by evaporation and the specific radioactivity adjusted to 0.5 Ci/mol. All other chemicals were obtained from the sources described by Shaw & Anderson (1972).

Methods

Preparation of enzymes. ATP sulphurylase and Mg²⁺-dependent alkaline pyrophosphatase were purified from spinach as described by Shaw & Anderson (1972); the specific activities of purified ATP sulphurylase and purified pyrophosphatase were 102 and 9 units/mg of protein respectively, the definition of enzyme units described by Shaw & Anderson (1972) being used. ADP sulphurylase and adenosine 5'-sulphatophosphate kinase (EC 2.7.1.25) activities were absent from both of these purified enzymes. A sample of partially purified ADP sulphurylase was isolated from baker's yeast as described by Robbins & Lipmann (1958).

ADP sulphurylase was extracted from de-ribbed leaf tissue with 2 ml of extraction medium/g fresh wt. in a Waring Blendor; the extraction medium contained 100 mM-Tris-HCl buffer, pH 8.0, and 20 mM-potassium thioglycollate, pH 8.0. The extract was squeezed through muslin and the supernatant solution was recovered by centrifugation at 30000g for 30 min. The supernatant solution, referred to as the crude extract, was used as the source of ADP sulphurylase. Crude solutions were not dialysed, since dialysis destroyed ADP sulphurylase activity.

Preparation of chloroplasts. Washed and unwashed chloroplasts were prepared from spinach leaf tissue as described by Spencer & Wildman (1964), except that the extraction medium contained MgCl₂ (1 mM) in lieu of magnesium acetate and both Ficoll and dextran were omitted. Washed chloroplasts, suspended in extraction medium, were fragmented by two 20 s

applications of ultrasonic irradiation (20–25 kHz) at 2°C with an MSE Ultrasonic Disintegrator; this treatment produced a high degree of fragmentation. Chloroplast fragments and chloroplast supernatant solution were recovered by centrifugation at 12000g for 20 min.

Assay of enzyme activities. ADP sulphurylase activity was measured by two methods.

Method 1. This is essentially a combination of incubations A and B to synthesize adenosine 5'-sulphatophosphate and P_i in a coupled enzyme system as described by Shaw & Anderson (1972), followed by an assay incubation (incubation C) in which spinach crude extract was added and the incorporation of P_i during incubation C was monitored as described by Robbins & Lipmann (1958); molybdate was added to initiate incubation B, catalysing the conversion of residual ATP into AMP and P_i by ATP sulphurylase and pyrophosphatase (Wilson & Bandurski, 1958). Incubation A was done at 35°C and contained 10.2 units of ATP sulphurylase, 9 units of pyrophosphatase, 100 μmol of Tris-HCl buffer, pH 8, 40 μmol of K₂SO₄, 2 μmol of Na₂K₂ATP and 10 μmol of MgCl₂ in a final volume of 1 ml; the presence of adenosine 5'-sulphatophosphate was demonstrated at the conclusion of incubation A by the methods described by Shaw & Anderson (1972). After 60 min, 10 μmol of Na₂MoO₄ was added to initiate the second phase to remove ATP (incubation B); the incubation mixtures contained sufficient ATP sulphurylase and pyrophosphatase to convert 2 μmol of ATP quantitatively into AMP and P_i in 10 min. At 70 min from the start, 0.2–0.4 ml of crude leaf extract was added to initiate incubation C (final volume 2.5 ml). Immediately incubation C was initiated, a sample (1 ml) was withdrawn, deproteinized with 2.3 ml of perchloric acid (10%, w/v) and the supernatant solution was assayed for P_i by the method described by Anderson & Rowan (1966) for measuring P_i in the presence of thioglycollate. Incubation C was continued for 30 min, when another 1 ml sample was removed and assayed for P_i. ADP sulphurylase activity, assayed in this way (method 1), is expressed as the depletion of P_i in nmol/min per ml during incubation C.

Method 2. This method for measuring ADP sulphurylase activity involves sulphate-dependent [³²P]P_i-ADP exchange; the method is analogous to the [³²P]PP_i-ATP-exchange method for the estimation of ATP sulphurylase (Shaw & Anderson, 1971). Reactions were conducted at 35°C for 15 min and standard assay mixtures contained 40 μmol of K₂SO₄, 2 μmol of ADP, 2 μmol of [³²P]P_i (1 μCi), 50 μmol of Tris-HCl buffer, pH 8, 3 μmol of BaCl₂ and crude extract; control incubation mixtures contained 120 μmol of KCl in lieu of K₂SO₄. The amount of crude extract used was 50–300 μl for spinach grown in the winter and 5–50 μl for spinach grown in the summer. Reactions were terminated with 2 ml of

trichloroacetic acid solution (7.5%, w/v) and 4ml of 100mM-NaH₂PO₄ was added. Activated charcoal (50mg) suspended in 0.5ml of 0.1M-NaH₂PO₄ containing 1% (w/v) H₃PO₄ was added; [³²P]ADP was adsorbed on charcoal and [³²P]P_i was removed by filtering and washing with 50mM-sodium acetate buffer, pH4, as described by Anderson (1968); the adsorption of ADP by charcoal was always 98–100% efficient. The [³²P]ADP adsorbed on the charcoal was counted at infinite thickness in a gas-flow planchette counter. P_i–ADP exchange was calculated for assays with and without sulphate by the method of Davie *et al.* (1956). ADP sulphurylase activities, assayed by method 2, are expressed as sulphate-dependent P_i–ADP exchange in nmol/min (ADP sulphurylase units); specific activities are expressed as units/mg of protein.

Assays designed to demonstrate the synthesis of adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate from [³⁵S]sulphate contained the coupled enzyme system for the synthesis of adenosine 5'-sulphatophosphate (Shaw & Anderson, 1972) and crude extract of spinach prepared as described for ADP sulphurylase as a possible source of adenosine 5'-sulphatophosphate kinase; spinach extract was omitted from control assays. ³⁵S-labelled and unlabelled nucleotides were adsorbed on charcoal and monitored by paper chromatography in solvent 1 as described by Shaw & Anderson (1972).

ATP sulphurylase and pyrophosphatase were assayed as described by Shaw & Anderson (1972), except that extracts containing thioglycollate were assayed for pyrophosphatase, the P_i produced being measured by the method of Anderson & Rowan (1966).

Chromatography. The ³²P-labelled product adsorbed on charcoal in ADP sulphurylase assays by using method 2, and the ³⁵S-labelled product adsorbed on charcoal in assays designed to demonstrate the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate, were eluted from charcoal and chromatographed on paper as described by Shaw & Anderson (1972). The following solvents were used: 1, propan-1-ol–aq. NH₃ (sp.gr. 0.88)–water (6:3:1, by vol.); 2, isobutyric acid–aq. NH₃ (sp.gr. 0.88)–water (66:1:33, by vol.). Radiochromatograms were monitored for radioactivity with a gas-flow radiochromatogram scanner.

Determination of protein. Protein was measured as described by Shaw & Anderson (1971).

Results

Preliminary studies

Experiments were designed to investigate the synthesis of adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate by crude extracts of winter-grown spinach. Incubation mixtures contained [³⁵S]sulphate,

ATP, MgCl₂, purified ATP sulphurylase and pyrophosphatase to synthesize adenosine 5'-[³⁵S]sulphatophosphate and crude extract as a possible source of adenosine 5'-sulphatophosphate kinase. In the presence of crude extract neither adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate nor adenosine 5'-[³⁵S]sulphatophosphate was synthesized, but ADP was formed. In the absence of crude extract, however, adenosine 5'-[³⁵S]sulphatophosphate was formed but neither ADP nor adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate was synthesized. Since crude spinach extracts contained negligible adenosine triphosphatase activity this suggests that spinach extract contains an enzyme which catalyses the formation of ADP from adenosine 5'-sulphatophosphate.

ADP sulphurylase activity

Demonstration of activity in crude extracts of spinach by assay method 1. Addition of crude extract (0.2ml) to incubation C caused disappearance of P_i; the uptake of P_i during incubation C was dependent on the synthesis of adenosine 5'-sulphatophosphate during incubation A (Table 1). This result is consistent with the operation of ADP sulphurylase activity in crude extracts of spinach. Additional enzyme caused no further increase in incorporation of P_i, suggesting that a quantitative conversion of adenosine 5'-sulphatophosphate into ADP was achieved with 0.2ml of crude extract. Addition of fluoride during incubation C had no effect on P_i incorporation. Omission of molybdate from incubation B caused no net change in the concentration of P_i, presumably because the production of P_i by the coupled enzyme system for the synthesis of adenosine 5'-sulphatophosphate continued during incubation C and jeopardized P_i incorporation by ADP sulphurylase.

Studies of activity in crude extracts of spinach by assay method 2. Preliminary studies with method 2 were conducted in the absence of BaCl₂ and KCl was omitted from the control incubations. Under these conditions P_i–ADP exchange was consistently greater in the controls than in incubations containing sulphate, for both crude spinach extract and purified yeast ADP sulphurylase. The endogenous P_i–ADP exchange, however, was decreased to less than the exchange in incubations containing sulphate by addition of KCl (equivalent in ionic strength to the substrate concentration of K₂SO₄) to control incubations (Table 2). Endogenous P_i–ADP exchange was decreased by further increasing the ionic strength but sulphate-dependent P_i–ADP exchange was also decreased (Table 3). Accordingly, 120 μmol of KCl was included in the control incubations in all subsequent experiments.

When KCl was included in control incubations, sulphate-dependent P_i–ADP exchange relative to the

Table 1. *Factors influencing the uptake of P_i during the incubation C in assay method 1*

Adenosine 5'-sulphatophosphate was synthesized in 60min during incubation A as described in the text. Molybdate ($10\mu\text{mol}$) was added 10min before commencing incubation C to convert quantitatively any residual ATP remaining from the primary incubation into AMP and P_i . The concentration of protein in the crude extract of spinach used in incubation C was 1.14mg/ml . The volume of incubation C was 2.5ml .

Incubation treatments			Uptake of P_i (nmol/min per ml)
A	B	C	
Adenosine 5'-sulphatophosphate synthesis	Molybdate	No additions	0.1
Adenosine 5'-sulphatophosphate synthesis	Molybdate	0.2ml of spinach extract	5.6
Adenosine 5'-sulphatophosphate synthesis	Molybdate	0.4ml of spinach extract	5.4
Adenosine 5'-sulphatophosphate synthesis	Molybdate	0.2ml of spinach extract $10\mu\text{mol}$ of NaF	6.3
Adenosine 5'-sulphatophosphate synthesis	No addition	0.4ml of spinach extract	0
No additions	Molybdate	0.2ml of spinach extract $15\mu\text{mol}$ of P_i	0.02
No additions	Molybdate	0.2ml of spinach extract $10\mu\text{mol}$ of PP_i $20\mu\text{mol}$ of NaF	0.01

Table 2. *Effect of K_2SO_4 and KCl on P_i -ADP exchange in crude extracts of spinach and purified ADP sulphurylase from yeast in the absence of $BaCl_2$*

Assays were conducted by using method 2. Reaction mixtures were as described in the text except that KCl was omitted from some control incubations as indicated. Spinach crude extract was prepared from winter-grown plants.

		P_i -ADP exchange (nmol/min per mg of protein)			
		Expt. 1		Expt. 2	
Source of enzyme	Concn. of enzyme in assay (mg of protein/ml)	No additions	With K_2SO_4 ($40\mu\text{mol}$)	With KCl ($120\mu\text{mol}$)	With K_2SO_4 ($40\mu\text{mol}$)
Spinach	0.108	0.68	0.59	0.49	0.54
	0.324	2.07	1.94	1.81	1.89
Yeast	0.037	0.81	0.72	0.61	0.69
	0.114	2.41	2.03	1.79	1.88

Table 3. *Effect of ionic strength on endogenous and sulphate-dependent P_i -ADP exchange of crude extracts of spinach and purified yeast ADP sulphurylase in the absence of $BaCl_2$*

The ionic strength refers to the components KCl and K_2SO_4 only. P_i -ADP exchange was measured by method 2 as described in the text except that $BaCl_2$ was omitted and incubations with and without sulphate were adjusted to the specified ionic strength with KCl. Spinach crude extract was prepared from winter-grown plants.

P_i -ADP exchange (nmol/min per mg of protein)				
Ionic strength	Spinach		Yeast	
	Endogenous exchange	Sulphate-dependent exchange	Endogenous exchange	Sulphate-dependent exchange
0.12	2.04	0.72	2.36	0.85
0.36	1.17	0.44	1.42	0.56
0.48	0.85	0.36	1.08	0.47
0.72	0.71	0.33	0.91	0.44
1.02	0.53	0.09	0.69	0.16

Table 4. *Effect of concentration of BaCl₂ on endogenous and sulphate-dependent P_i-ADP exchange of crude extracts of spinach and purified ADP sulphurylase from yeast with and without ADP*

P_i-ADP exchange was measured by method 2; incubation mixtures were as described in the text for method 2 except that the specified amounts of BaCl₂ and ADP were used. At high concentrations of BaCl₂ (greater than 6 mM) P_i-ADP exchange could not be determined since a ³²P-labelled compound (possibly a barium phosphate compound) was formed in the absence of enzyme (see the text). Spinach crude extract was prepared from winter-grown plants.

Source of enzyme	Concn. of BaCl ₂ in assay (mM)	ADP-P _i exchange activity (nmol/min per mg of protein)					
		Without ADP		With 0.5 μmol of ADP		With 2.0 μmol of ADP	
		Endogenous	Sulphate-dependent	Endogenous	Sulphate-dependent	Endogenous	Sulphate-dependent
Spinach	0	0.02	0	1.93	0.01	2.13	0.17
	0.3	0.01	0	1.61	0.08	1.92	0.23
	1.0	0.01	0	0.24	2.01	0.44	2.39
	3.0	0.01	0	0.06	3.72	0.11	4.96
Yeast	0	0.08	0	2.29	0.02	2.34	0.20
	0.3	0.05	0	1.94	0.18	2.21	0.59
	1.0	0	0	0.34	2.52	0.74	3.13
	3.0	0	0	0.11	3.91	0.17	5.36

Table 5. *Effect of some common inhibitors on the activity of spinach leaf ADP sulphurylase and purified yeast ADP sulphurylase*

ADP sulphurylase activity was measured by method 2; crude extracts prepared from winter-grown plants were used as the source of the spinach enzyme. The absolute activities of the spinach and yeast enzymes in standard assays in the absence of inhibitor were 3.82 and 4.34 units/mg of protein respectively. Results are expressed as a percentage of the activity measured by the standard assay for the respective enzyme.

Addition	Activity (%)	
	Yeast	Spinach
Iodoacetamide (10mM)	42	2
N-Ethylmaleimide (10mM)	0	0
p-Chloromercuribenzoate (8 μM)	20	0
Potassium fluoride (10mM)	0	0
Phenylhydrazine (10mM)	60	54

endogenous exchange was low for both the purified yeast and crude spinach enzymes (Tables 2 and 3). Since high endogenous concentrations of sulphate might contribute to the high endogenous P_i-ADP exchange the effect of Ba²⁺ (supplied as BaCl₂) was studied (Table 4). At low concentrations of BaCl₂ (less than 0.3 mM) and in the absence of Ba²⁺ endogenous P_i-ADP exchange was high and sulphate-dependent exchange was low, confirming the results

described in Tables 2 and 3. When 1–3 μmol of BaCl₂ was added, however, the endogenous P_i-ADP exchange was decreased and the sulphate-dependent P_i-ADP exchange was 10- to 50-fold the endogenous exchange; the optimum concentration of BaCl₂ was 3 mM. At higher concentrations of BaCl₂ (greater than 6 mM) a ³²P-labelled compound (possibly a barium phosphate complex) was formed in the absence of enzyme; this label was adsorbed on charcoal and was only partially removed by extensive washing with 50mM-sodium acetate buffer, pH4. The adsorption of ³²P-labelled material by charcoal in ADP sulphurylase assays containing 3mM-BaCl₂ was, however, unrelated to the formation of the ³²P-labelled product in the absence of enzyme at higher concentration of BaCl₂, since the formation of ³²P-labelled product in enzyme assays containing 3mM-BaCl₂ and 40mM-K₂SO₄ was sensitive to thiol-group reagents (Table 5) and the ³²P-labelled product adsorbed on charcoal was identified as [³²P]ADP (Fig. 1). [³²P]ADP was not formed in the presence of BaCl₂ in enzyme assays containing 120mM-KCl in lieu of K₂SO₄.

There are several problems associated with the use of BaCl₂ in assays by method 2 since the concentration of free Ba²⁺ is presumably higher in control assays than in assays containing 40mM-K₂SO₄. Pre-incubation of spinach leaf crude extracts at 2°C for 30min in the presence of 3mM-BaCl₂ but in the absence of sulphate did not inhibit ADP sulphurylase. Another possible problem is that Ba²⁺, in the absence of sulphate, might precipitate [³²P]P_i or ADP or both. In reaction mixtures containing 2 μmol of

Fig. 1. Radiochromatogram trace of the ^{32}P -labelled product synthesized by ADP sulphurylase assay method 2

The ^{32}P -labelled product was separated from $[\text{}^{32}\text{P}]\text{P}_i$ and applied to chromatograms as described in the text. The chromatogram was developed in solvent 2 for 24 h; marker spots of ATP, ADP, AMP and P_i are shown. The radioactivity with the R_f of ADP was completely absent in control assays containing KCl in lieu of K_2SO_4 .

Fig. 2. Effect of concentration of ADP on sulphate-dependent P_i -ADP-exchange activity of crude extracts of spinach leaf tissue and purified yeast ADP sulphurylase

Sulphate-dependent P_i -ADP exchange was measured by method 2; the incubation mixtures were as described in the text except that the specified amount of ADP was used. ●, Spinach leaf extract prepared from winter-grown plants; ▲, purified yeast ADP sulphurylase.

ADP, $2\mu\text{mol}$ of P_i and only $1\mu\text{mol}$ of BaCl_2 , the endogenous P_i -ADP exchange was low (Table 4); similar results were obtained in an independent experiment with $0.5\mu\text{mol}$ of BaCl_2 . Assuming that the removal of endogenous sulphate by Ba^{2+} was nil, then the decrease in the concentration of free P_i or ADP due to precipitation as the barium salts would be insufficient to account for the low endogenous P_i -ADP exchange at 0.5mM or 1.0mM - BaCl_2 as calculated from the affinity of spinach leaf ADP sulphurylase for ADP and P_i (Figs. 2 and 3). The concentration of BaCl_2 required to cause minimum endogenous P_i -ADP exchange increased with the volume of enzyme extract used in the assay (Fig. 4). Addition of CaCl_2 in lieu of BaCl_2 did not decrease endogenous P_i -ADP exchange (Fig. 4). We conclude that the lower P_i -ADP exchange in control assays containing BaCl_2 with crude extracts of winter-grown plants is due to precipitation of endogenous sulphate introduced with the enzyme sample.

The sum of the sulphate-dependent P_i -ADP exchange plus endogenous exchange (total exchange) in the presence of 3mM - BaCl_2 was approximately two-fold the total exchange in the absence of BaCl_2 for both the spinach and yeast enzymes (Table 4 and Fig. 4). Addition of CaCl_2 in lieu of BaCl_2 also increased total exchange (Fig. 4); addition of MgCl_2 (1 – 20mM) in lieu of BaCl_2 did not affect either total exchange or sulphate-dependent P_i -ADP exchange. One explanation for the higher total exchange in the presence of BaCl_2 or CaCl_2 could be activation of ADP sulphurylase by Ca^{2+} or Ba^{2+} . Since BaCl_2 both decreased endogenous exchange and increased

total exchange, 3 mM-BaCl₂ was included in all subsequent assays.

Studies of activity in crude extracts of summer-grown spinach by assay method 2. Crude extracts of summer-grown spinach catalysed sulphate-dependent P_i-ADP exchange only when small volumes of extract were used (5–50 µl). The endogenous exchange was not decreased by Ba²⁺ (Table 6), as described for crude extracts of winter-grown spinach (Table 4), although Ba²⁺ increased sulphate-dependent P_i-ADP exchange. The endogenous exchange of crude extracts of summer-grown plants, however, was not inhibited by fluoride or *N*-ethylmaleimide (Table 6).

Properties of spinach-leaf ADP sulphurylase

Some properties of the enzyme from winter-grown spinach were examined by using method 2. The enzyme was sensitive to heat; pretreatment at 100°C for 3 min abolished sulphate-dependent P_i-ADP exchange activity. Activity was also abolished when extracts were dialysed against extraction medium for 12 h. Sulphate-dependent P_i-ADP exchange was proportional to enzyme concentration up to 0.52 mg of protein/ml of standard incubation mixture. The pH optimum of the enzyme was 8.0 (Fig. 5).

Fig. 3. Effect of concentration of P_i on sulphate-dependent P_i-ADP-exchange activity of crude extracts of spinach leaf

Sulphate-dependent P_i-ADP exchange was measured by method 2; the incubation mixtures were as described in the text except that the specified amount of [³²P]P_i was used. The specific radioactivity of the [³²P]P_i was the same for each concentration of P_i used. Crude extract was prepared from winter-grown plants.

Both endogenous and sulphate-dependent exchange were negligible in the absence of ADP (Table 4). Sulphate-dependent P_i-ADP exchange of crude spinach extract and purified yeast enzyme increased

Fig. 4. Effect of enzyme concentration on endogenous and total P_i-ADP exchange in crude spinach extracts prepared from winter-grown plants in the presence and absence of BaCl₂ and CaCl₂

P_i-ADP exchange was measured by method 2; total exchange is the sum of sulphate-dependent exchange and endogenous exchange. Incubation mixtures were as described in the text except that the standard amount of BaCl₂ was replaced with the amounts of BaCl₂ and CaCl₂ specified. The concentration of protein in the crude extracts used to study the effects of BaCl₂ and CaCl₂ respectively were 0.96 mg/ml and 1.08 mg/ml. (a) Effect of BaCl₂; (b) effect of CaCl₂. ○, Endogenous exchange without BaCl₂ or CaCl₂; □, △, endogenous exchange with 1 mM- and 3 mM-BaCl₂ or CaCl₂ respectively; ●, total exchange without BaCl₂ or CaCl₂; ■, ▲, total exchange with 1 mM- and 3 mM-BaCl₂ or CaCl₂ respectively.

Table 6. *Effect of Ba²⁺, N-ethylmaleimide and KF on endogenous and sulphate-dependent P_i-ADP exchange of crude extracts prepared from summer-grown spinach*

P_i-ADP exchange was measured by method 2; incubation mixtures were as described in the text except that the amount of BaCl₂ included in the assay was 5 mM and KF (10 mM) and N-ethylmaleimide were included as specified.

Addition	P _i -ADP exchange (nmol/min per mg of protein)			
	Without Ba ²⁺		With Ba ²⁺ (5 mM)	
	Endogenous exchange	Sulphate-dependent exchange	Endogenous exchange	Sulphate-dependent exchange
Nil	1.35	1.98	1.23	4.08
KF (10 mM)	1.51	0.20	1.51	0.60
N-Ethylmaleimide (10 mM)	1.25	0.42	1.80	0.73

Fig. 5. *Effect of pH on ADP sulphurylase activity of crude extracts from spinach leaf tissue*

ADP sulphurylase activity was measured by method 2; the incubation mixtures were as described in the text except that Tris-HCl buffer, pH 8, was replaced by the buffers specified. The buffers were prepared as described by Colowick & Kaplan (1955) and 0.5 ml of the buffer was used in each assay. ▲, Tris-maleic acid-KOH buffer; ●, Tris-HCl buffer. Crude extract was prepared from winter-grown plants.

with concentration of ADP up to 1.5 mM; higher concentrations of ADP (1.5–5 mM) caused no further increase in activity (Fig. 2). Sulphate-dependent P_i-ADP exchange of crude spinach extracts increased with concentration of P_i up to 1 mM (Fig. 3). Neither crude extracts of spinach nor purified yeast enzyme catalysed [³²P]P_i exchange when ADP was replaced

Fig. 6. *Effect of concentration of ATP on sulphate-dependent P_i-ADP exchange of crude spinach extracts*

Sulphate-dependent P_i-ADP exchange was measured by method 2 except that reaction mixtures also contained the amounts of ATP specified. The crude spinach extract was prepared from winter-grown plants; the extract did not catalyse [³²P]P_i-ATP exchange.

with ATP, but ATP inhibited sulphate-dependent P_i-ATP exchange (Fig. 6). Neither the spinach nor the yeast enzymes catalysed the incorporation of PP_i into ADP in a standard assay in which [³²P]P_i was replaced with [³²P]PP_i; Mg²⁺-dependent alkaline pyrophosphatase, which was present in the crude spinach extracts, was inoperative since Mg²⁺ was not included in the standard assay, and the enzyme is inhibited by Ba²⁺ (Naganna & Menon, 1948).

It was not possible to derive an accurate value for the affinity of spinach leaf ADP sulphurylase for sulphate because the enzyme was not purified; further, the free sulphate concentration in individual assays was uncertain because reaction mixtures contained endogenous sulphate, 3 mM-BaCl₂ and exogenous

sulphate. Notwithstanding these limitations, the concentration of sulphate required for half-maximum activity was approx. 2–3 mM (Fig. 7). Selenate (1–40 mM) was not an alternative substrate of sulphate for either the yeast or spinach enzymes.

Subcellular distribution of ADP sulphurylase activity and occurrence in the plant kingdom

Chloroplasts and the crude supernatant solution remaining after pelleting the chloroplasts were prepared from spinach and monitored for ADP sulphurylase, ATP sulphurylase and Mg^{2+} -dependent alkaline pyrophosphatase activities; Balharry & Nicholas (1970) and El-Badry & Bassham (1970) have reported that the two last-named enzymes in spinach leaf tissue are localized in chloroplasts. ADP sulphurylase and ATP sulphurylase activities were associated with intact chloroplasts (Table 7); pyrophosphatase activity of intact chloroplasts was low though high activity was recovered in the crude supernatant fraction. Sonication of washed chloroplasts, however, released all three enzymes; ADP sulphurylase, ATP sulphurylase and pyrophosphatase activities were not associated with chloroplast fragments. Since the pyrophosphatase activity of disrupted chloroplasts exceeded the activity of intact chloroplasts, we presume that the chloroplast membrane(s) are impermeable to PP_i . The localization of ATP sulphurylase in chloroplasts confirms the results of Balharry & Nicholas (1970). Sulphate-dependent P_i -ADP exchange was demonstrated in crude extracts of leaf tissue from widely distributed members of the plant kingdom (Table 8); the ADP sulphurylase activity was 40–60% of the ATP sulphurylase activity in all species examined by using the P_i -ADP-exchange and PP_i -ATP-exchange techniques respectively.

Discussion

The failure to detect the synthesis of adenosine 5'-sulphatophosphate in incubation mixtures containing an adenosine 5'-sulphatophosphate-synthesizing system and spinach crude extract cannot be attributed to hydrolysis of adenosine 5'-sulphatophosphate by sulphatase or phosphatase activities since ADP was synthesized. Although P_i was not added to the reaction mixtures employed in these experiments, P_i was synthesized by the action of ATP sulphurylase and pyrophosphatase during the secondary incubation. It is therefore possible that the failure to detect adenosine 5'-sulphatophosphate and the occurrence of ADP synthesis could be due to the operation of ADP sulphurylase activity:

Fig. 7. Effect of concentration of sulphate on sulphate-dependent P_i -ADP-exchange activity of crude extracts of spinach and purified ADP sulphurylase from yeast

Sulphate-dependent P_i -ADP exchange was measured by method 2; the incubation mixtures were as described in the text except that K_2SO_4 was replaced with the amounts specified. The amount of free SO_4^{2-} in the reaction mixtures at any concentration of K_2SO_4 is uncertain, since reaction mixtures contained 3 mM-BaCl₂ and endogenous sulphate in addition to K_2SO_4 . The large increase in sulphate-dependent P_i -ADP exchange at low concentrations of K_2SO_4 , however, suggests that the concentration of free SO_4^{2-} is approximately equal to the amount of K_2SO_4 added. ●, Spinach leaf extract prepared from winter-grown plants; ▲, purified ADP sulphurylase from yeast.

The assay of ADP sulphurylase by method 1 thus has several practical and theoretical disadvantages. ATP sulphurylase and pyrophosphatase must be purified and freed from ADP sulphurylase activity. Large amounts of these enzymes must be used to ensure a yield of adenosine 5'-sulphatophosphate that is sufficient for the incorporation of measurable amounts of P_i during the tertiary incubation; large amounts of the enzymes are also necessary to ensure quantitative catalysis of residual ATP to AMP and P_i during the secondary incubation, unless purified adenosine triphosphatase could

Table 7. Subcellular distribution of ADP sulphurylase, ATP sulphurylase and Mg²⁺-dependent alkaline pyrophosphatase in spinach leaf tissue

Unwashed chloroplasts and the crude supernatant solution were prepared from winter-grown plants as described in the text; the coarse particulate material (cell walls, nuclei etc.), which was removed by centrifugation before isolating the unwashed chloroplasts, was not analysed for enzyme activities. The unwashed chloroplasts were washed with extraction medium and the washed chloroplasts were disrupted by sonication as described in the text. ADP sulphurylase activity was measured by method 2 (standard assay) except that the assays were conducted in extraction medium. ATP sulphurylase and pyrophosphatase activities were measured by the method of Shaw & Anderson (1972) except that ATP sulphurylase was measured in the presence of 10mM-NaF. All enzyme activities are expressed as units/g fresh wt. of leaf tissue.

Treatment	Fraction	ATP sulphurylase (units)	ADP sulphurylase (unit)	Pyrophosphatase (units)
Extraction	P1 Unwashed chloroplasts	2.24	0.34	0.14
	S1 Crude supernatant solution	0.28	0.02	1.06
Wash P1	P2 Washed chloroplasts	5.27	0.47	0.15
	S2 Chloroplast washings	0.48	0.21	0.06
Sonicate P2	P3 Chloroplast fragments	0.14	0.05	0.09
	S3 Chloroplast supernatant solution	4.86	0.35	1.83

Table 8. Activity of ADP sulphurylase and ATP sulphurylase in crude extracts of leaf tissue from selected plant species

ADP sulphurylase activity was measured in undialysed extracts prepared from winter-grown plants by method 2; ATP sulphurylase was measured in dialysed extracts as described by Shaw & Anderson (1972), except that assays contained 10mM-NaF.

Species	ADP sulphurylase (units/mg of protein)	ATP sulphurylase (units/mg of protein)
<i>Spinacea oleracea</i> (spinach)	3.51	6.81
<i>Zea mays</i> (maize)	2.72	4.39
<i>Vicia faba</i> (broad bean)	3.47	6.73
<i>Brassica oleracea</i> (cabbage)	3.21	6.32
<i>Lycopersicum esculentum</i> (tomato)	3.12	5.67
<i>Eucalyptus ovata</i> (swamp gum)	2.06	5.19
<i>Drosera whittakeri</i> (sundew)	3.07	5.03
<i>Dicksonia antarctica</i> (soft tree fern)	2.16	4.92

be added. Even if the difficulties associated with the use of the adenosine 5'-sulphatophosphate-generating system were overcome by the use of defined concentrations of adenosine 5'-sulphatophosphate substrate, the method only demonstrates adenosine 5'-sulphatophosphate-dependent uptake of P_i and not the formation of ADP. Notwithstanding these limitations, ADP sulphurylase activity was demonstrated in crude extracts of spinach by method 1 (Table 1).

Assay of ADP sulphurylase by method 2 is a more rigorous test for ADP sulphurylase activity and is more sensitive and technically simpler than either the method of Robbins & Lipmann (1958) or method 1. Neither adenosine 5'-sulphatophosphate nor an adenosine 5'-sulphatophosphate-synthesizing system is required, presumably because the sub-

strates ADP, P_i and sulphate used in method 2 come to chemical equilibrium with adenosine 5'-sulphatophosphate very rapidly. Under the conditions of the standard assay, sulphate-dependent incorporation of P_i into ADP was demonstrated (Fig. 1), confirming the validity of method 2 as an assay for ADP sulphurylase activity. The validity of method 2, however, only applies if the concentration of BaCl₂ does not exceed 6mM, because at higher concentrations of BaCl₂ a ³²P-labelled compound was formed in the absence of enzyme and was adsorbed on charcoal. Sulphate-dependent P_i-ADP exchange in crude extracts of winter-grown spinach relative to endogenous exchange was low in the absence of BaCl₂ (Table 4). The low endogenous activity in the presence of BaCl₂ was not due to Ba²⁺-inactivation of the enzyme nor to precipitation of the substrates P_i and

ADP. Calcium chloride did not lower the endogenous exchange (Fig. 4b). The solubility product of CaSO_4 (6.1×10^{-5}) is greater than the solubility product of BaSO_4 (1.0×10^{-10}) (Kolthoff & Sandell, 1952). The decreased endogenous activity in the presence of Ba^{2+} (but not of Ca^{2+}) is consistent with the precipitation of endogenous sulphate by Ba^{2+} . We conclude that the decreased endogenous exchange of extracts of winter-grown spinach in the presence of Ba^{2+} was due to the precipitation of endogenous sulphate. Dialysis could not be used to remove endogenous sulphate since dialysis destroyed ADP sulphurylase activity; this would explain why Shaw & Anderson (1972) failed to detect sulphate-dependent P_i -ADP-exchange activity in dialysed crude extracts of spinach. Ba^{2+} did not decrease endogenous exchange of crude extracts of summer-grown spinach (Table 6). The endogenous exchange was not inhibited by fluoride or *N*-ethylmaleimide, which are inhibitors of ADP sulphurylase (Table 5). This suggests that, unlike the endogenous exchange of crude extracts of winter-grown spinach, the high endogenous exchange of crude extracts of summer-grown spinach was not due to endogenous sulphate acting as a substrate of ADP sulphurylase, but rather due to some other P_i -ADP-exchange reaction that is insensitive to fluoride and *N*-ethylmaleimide.

Both BaCl_2 and CaCl_2 caused an increase in total P_i -ADP exchange (Table 4 and Fig. 4). The increased activity could be due to activation or because either Ba^{2+} or Ca^{2+} (but not Mg^{2+}) are essential cofactors for the enzyme. Since the affinity of ADP sulphurylase for sulphate was low (Fig. 7), a large excess of K_2SO_4 ($40 \mu\text{mol}$) relative to BaCl_2 ($3 \mu\text{mol}$) ensured an almost saturating concentration of sulphate for measuring sulphate-dependent P_i -ADP exchange.

The sulphate-dependent P_i -ADP-exchange reaction catalysed by crude spinach extracts is distinct from the reaction described by Grunberg-Manago *et al.* (1966) in yeast:

This reaction is not sulphate-dependent although the enzyme catalysed an analogous reaction in which $[^{35}\text{S}]\text{sulphate}$ and adenosine 5'- $[^{35}\text{S}]\text{sulphatophosphate}$ could be substituted for $[^{32}\text{P}]\text{P}_i$ and $[^{32}\text{P}]\text{ADP}$ respectively. Further, the enzyme described by Grunberg-Manago *et al.* (1966) catalysed exchange between P_i and a wide range of ribonucleotide diphosphates and deoxyribonucleotide diphosphates; the spinach enzyme did not catalyse a sulphate-dependent P_i -GDP exchange.

Although ADP sulphurylase has been described in yeast (Robbins & Lipmann, 1958), *T. thioparus* (Peck, 1960) and *D. desulfuricans* (Peck, 1962), very few of its properties have been reported; the yeast enzyme does not require Mg^{2+} as a cofactor and

molybdate is not an alternative substrate of the enzyme from *D. desulfuricans*. Studies of the spinach enzyme by method 2 confirm and extend reported properties of ADP sulphurylase in micro-organisms; the elementary kinetics of the ADP sulphurylases from spinach and yeast (Figs. 2, 3 and 7) have not been reported previously.

ADP sulphurylase can be readily distinguished from ATP sulphurylase in spinach. ADP sulphurylase catalyses sulphate-dependent P_i -ADP exchange but does not catalyse selenate-dependent P_i -ADP exchange; molybdate does not inhibit sulphate-dependent P_i -ADP exchange. ADP sulphurylase does not require Mg^{2+} but is activated by Ca^{2+} and Ba^{2+} ; the enzyme is sensitive to thiol-group reagents (Table 5). ATP sulphurylase, however, catalyses both sulphate- and selenate-dependent PP_i -ATP exchange and molybdate inhibits sulphate-dependent PP_i -ATP exchange. ATP sulphurylase is inactive in the absence of Mg^{2+} and is insensitive to thiol-group reagents (Shaw & Anderson, 1972). Both ATP sulphurylase and ADP sulphurylase are associated with chloroplasts in spinach leaf tissue and are released into solution when the chloroplasts are sonicated (Table 7). Both enzymes are widely distributed throughout the plant kingdom (Adams & Rinne, 1969; Table 8).

The physiological role of ADP sulphurylase is uncertain (Roy & Trudinger, 1970), but Peck (1960, 1962) has reported that the enzyme is not associated with the synthesis of adenosine 5'-sulphatophosphate in *T. thioparus* and *D. desulfuricans*. Asahi (1964) and Balharry & Nicholas (1970) have reported that chloroplasts are probably the main sites of synthesis of adenosine 5'-sulphatophosphate in leaves. Peck (1962) has suggested that since the free-energy change of the reaction catalysed by ADP sulphurylase is probably approx. $+50 \text{ kJ/mol}$ and since the reaction catalysed by ADP sulphurylase cannot be coupled to pyrophosphatase to augment the yield of adenosine 5'-sulphatophosphate then the function of ADP sulphurylase in *D. desulfuricans* might be the phosphorylation of adenosine 5'-sulphatophosphate. The association of ADP sulphurylase with the adenosine 5'-sulphatophosphate-synthesizing enzymes ATP sulphurylase and pyrophosphatase in chloroplasts (Table 6) is consistent with Peck's (1962) hypothesis and the inhibition of ADP sulphurylase by ATP (Fig. 7) could be important in regulating the steady-state concentration of adenosine 5'-sulphatophosphate in chloroplasts. A clear understanding of the pathway of adenosine 5'-sulphatophosphate metabolism in leaf tissue, together with the free-energy change of the individual reactions, should greatly assist in establishing the function of ADP sulphurylase.

Variability in ADP sulphurylase activity might explain the conflicting reports on the synthesis of

adenosine 3'-phosphate 5'-sulphatophosphate from sulphate and ATP by unfractionated plant extracts (Ellis, 1969) and chloroplasts (Asahi, 1964; Mercer & Thomas, 1969); some plant extracts might contain sufficient ADP sulphurylase activity to prevent the net synthesis of adenosine 5'-sulphatophosphate by ATP sulphurylase and pyrophosphatase. Balharry & Nicholas (1970) studied the metabolism of adenosine 5'-sulphatophosphate by fragmented chloroplasts and observed the formation of sulphate, but not adenosine 3'-phosphate 5'-sulphatophosphate. Balharry & Nicholas (1970) attributed the formation of sulphate to ATP sulphurylase activity since ATP inhibited the formation of sulphate. The results of Balharry & Nicholas (1970), however, could also be explained by ADP sulphurylase activity since disrupted chloroplasts contain ADP sulphurylase (Table 7) and ATP inhibits ADP sulphurylase (Fig. 6). Nevertheless, if the fragmented chloroplasts used by Balharry & Nicholas (1970) did contain adenosine 5'-sulphatophosphate kinase activity, neither ATP sulphurylase nor ADP sulphurylase activity would explain why adenosine 3'-phosphate 5'-sulphatophosphate was not synthesized, since only 16–37% of the adenosine 5'-sulphatophosphate was metabolized (Balharry & Nicholas, 1970).

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