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Comparative Enzymology of the Adenosine Triphosphate Sulphurylases from Leaf Tissue of Selenium-Accumulator and Non-Accumulator Plants

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1. ATP sulphurylases were partially purified (20–40-fold) from leaf tissue of *Astragalus bisulcatus*, *Astragalus racemosus* (selenium-accumulator species) and *Astragalus hamosus* and *Astragalus sinicus* (non-accumulator species). Activity was measured by sulphate-dependent PP_i–ATP exchange. The enzymes were separated from pyrophosphatase and adenosine triphosphatase activities. The properties of the *Astragalus* ATP sulphurylases were similar to the spinach enzyme. 2. The ATP sulphurylases from both selenium-accumulator and non-accumulator species catalysed selenate-dependent PP_i–ATP exchange; selenate competed with sulphate. The ratio of V(selenate)/V(sulphate) and K_m(selenate)/K_m(sulphate) was approximately the same for the enzyme from each species. 3. Sulphate-dependent PP_i–ATP exchange was inhibited by ADP, chlorate and nitrate. The kinetics of the inhibition for each enzyme were consistent with an ordered reaction mechanism, in which ATP is the first substrate to react with the enzyme and PP_i is the first product released. 4. Synthesis of adenosine 5′-[^{35}S]sulphatophosphate from [^{35}S]sulphate was demonstrated by coupling the *Astragalus* ATP sulphurylases with Mg^{2+}-dependent pyrophosphatase; the reaction was inhibited by selenate. An analogous reaction using [^{75}Se]selenate as substrate could not be demonstrated.

ATP sulphurylase (ATP–sulphate adenylyltransferase, EC 2.7.7.4) is widely distributed in plants and the enzyme has been purified from spinach leaf (Balharry & Nicholas, 1970; Shaw & Anderson, 1972). The spinach enzyme catalyses both sulphate- and selenate-dependent PP_i–ATP exchange. The K_m for selenate is much smaller (Shaw & Anderson, 1972). This suggests that selenium could enter metabolism in place of sulphur, giving rise to selenium analogues, such as selenomethionine (Peterson & Butler, 1962; Ferrari & Renosto, 1972).

Some soils (e.g. north-west Queensland, Australia, and Wyoming, U.S.A.) contain relatively high concentrations of selenium. These soils characteristically support plant species which are not only insensitive to selenium, but also accumulate selenium to such high concentrations that they are toxic to livestock (Rosenfeld & Beath, 1964). Typical species accumulating selenium are *Astragalus racemosus* and *Astragalus bisulcatus*, and these characteristically accumulate selenium as non-protein seleno amino acids, e.g. *Se*-methylselenocysteine. Non-accumulating species include *Astragalus canadensis* and *Astragalus succulentus*, which incorporate selenium into *Se*-methylselenomethionine.

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Wilson & Bandurski (1958) reported that yeast ATP sulphurylase catalysed the synthesis of adenosine 5′-selenophosphate from selenate. Yeast adenosine 5′-sulphatophosphate kinase (ATP–adenylyl sulphate 3′-phosphotransferase, EC 2.7.1.25), however, did not catalyse the synthesis of adenosine 3′-phosphate 5′-selenophosphate from adenosine 5′-selenophosphate (Wilson & Bandurski, 1958). This indicates that at least one part of the assimilatory sulphate pathway is closed to selenate in yeast and suggests that it is unlikely that selenium is metabolized in competition with sulphur by all stages of the assimilatory sulphate pathway in other organisms, including selenium-accumulator and non-accumulator plants. Spinach leaf ATP sulphurylase catalyses selenate-dependent PP_i–ATP exchange (Shaw & Anderson, 1972), but it has not been established whether selenate is an alternative substrate of the ATP sulphurylases of selenium-accumulator plants. It has also not been established whether ATP sulphurylase from a plant source catalyses the synthesis of adenosine 5′-selenophosphate. The present paper describes the partial purification and substrate specificity of the ATP sulphurylases from two selenium-accumulator species of *Astragalus* (*A. bisulcatus* and *A. racemosus*) and two non-accumulator species of the same genus (*Astragalus hamosus* and *Astragalus sinicus*).

Experimental

Materials

Plant material. Seeds of *A. bisulcatus* and *A. racemosus* (selenium accumulators) were collected from seleniferous areas of the U.S.A. in 1956 and supplied by the Division of Biochemistry, University of Wyoming, Laramie, Wyo., U.S.A.; further seeds of *A. racemosus* were supplied by Professor L. Fowden, University College, London, U.K. Seeds of *A. hamosus* and *A. sinicus* (non-accumulators) were obtained from the Division of Plant Industry, C.S.I.R.O., Canberra, A.C.T., Australia. Seeds of accumulator species were treated with 18M-H_2SO_4 for 10min before planting to render the hard seed coats permeable to water and then raised in a glasshouse. Seeds from mature specimens of non-accumulator species raised in the glasshouse were in turn used to raise additional mature plants as sources of leaf tissue, but leaf tissue of accumulator species was obtained from plants raised from the supplied seed.

Chemicals. [^{75}Se]Selenate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other compounds were obtained from the sources described by Shaw & Anderson (1972, 1974).

Methods

Extraction and purification of ATP sulphurylases. ATP sulphurylase activity was extracted from leaf tissue as described by Shaw & Anderson (1972), except that a top-drive blender (Sorvall Omni-Mixer) was used. The procedures for purifying the ATP sulphurylases from three of the four *Astragalus* species were essentially the same as described for spinach by Shaw & Anderson (1972) except for minor modifications for each enzyme source.

ATP sulphurylase activity in undialysed crude extracts of *A. racemosus* was fractionated by adding 0.22g of solid $(NH_4)_2SO_4$/ml of crude solution. Precipitated protein was discarded and additional $(NH_4)_2SO_4$ (0.095g/ml) was added. Precipitated protein (containing ATP sulphurylase activity) was recovered and dissolved in 20mM-Tris–HCl buffer, pH8, containing 20mM-K_2SO_4 (medium 1). The enzyme solution was dialysed against medium 1 and passed through a Sephadex G-200 column (60cm×2.5cm) equilibrated with medium 1. Fractions containing ATP sulphurylase activity were pooled and applied to a DEAE-cellulose column (25cm×2cm) equilibrated with medium 1. After addition of the sample, the column was eluted with 50ml of 20mM-Tris–HCl buffer, pH8 (medium 2), containing 30mM-K_2SO_4 and then developed with a linear gradient of K_2SO_4 (30–200mM). Fractions containing enzyme activity were pooled and stored at −15°C until required for use. When required, the enzyme solution was thawed, dialysed against medium 1 and the chromatography on DEAE-cellulose was repeated as described above to remove insoluble protein. ATP sulphurylase activity was concentrated as described for the spinach enzyme (Shaw & Anderson (1972) except that the DEAE-cellulose column was equilibrated with medium 1. The column was prewashed with medium 1 (50ml) and eluted with either 0.4M-K_2SO_4 or 1.0M-KCl in medium 2.

Crude extracts of *A. bisulcatus*, *A. hamosus* and *A. sinicus* were fractionated with $(NH_4)_2SO_4$ as described for *A. racemosus* except that the first and second additions of $(NH_4)_2SO_4$ were 0.023g/ml and 0.085g/ml for *A. bisulcatus*, 0.22g/ml and 0.090g/ml for *A. hamosus* and 0.209g/ml and 0.065g/ml for *A. sinicus* respectively. The remainder of the purification procedures for the enzymes from *A. bisulcatus* and *A. hamosus* were as described for *A. racemosus*, except that after addition of the enzyme solution to the DEAE-cellulose column, the column was eluted with medium 2 containing 40mM-K_2SO_4 (50ml) and then developed with a linear gradient of K_2SO_4 (40–240mM) in medium 2.

Gel filtration on Sephadex G-200 followed by ion-exchange chromatography of the $(NH_4)_2SO_4$ fraction, which contained ATP sulphurylase activity from *A. sinicus*, did not effect any significant increase in specific activity of ATP sulphurylase nor remove pigmented material. Therefore the $(NH_4)_2SO_4$ fraction from *A. sinicus* was extensively dialysed against medium 1 and applied to a DEAE-Sephadex column (20cm×2.5cm) equilibrated with medium 1. The column was developed with a linear gradient of K_2SO_4 (20–400mM) in medium 2. Active fractions were concentrated on DEAE-cellulose as described for the concentration of the enzyme from *A. racemosus*. The enzyme was stored at −15°C. When required, the thawed enzyme was dialysed against medium 1 and subjected to gel filtration of a Sephadex G-200 column (25cm×2cm) equilibrated with medium 1 to remove insoluble and pigmented material. Spinach leaf ATP sulphurylase was purified from spinach as described by Shaw & Anderson (1972).

Enzyme preparations used for monitoring ATP sulphurylase activity during purification and purified enzymes stored or stabilized in either K_2SO_4 or high concentrations of KCl (1M) were dialysed against medium 2 containing 100mM-KCl before use.

Preparation of pyrophosphatase. Mg^{2+}-dependent pyrophosphatase was purified from spinach leaf tissue as described by Shaw & Anderson (1972).

Assay of enzyme activities. Purified ATP sulphurylases, free from pyrophosphatase and ATPase (adenosine triphosphatase) activities, were assayed in the absence of fluoride as described by Shaw & Anderson

(1972). All other extracts were assayed for ATP sulphurylase activity in the presence of 10mM-NaF to inhibit pyrophosphatase activity. ATP sulphurylase activity is expressed as sulphate- (or selenate-) dependent PP_i–ATP exchange in nmol of PP_i exchanged/min (ATP sulphurylase units). Pyrophosphatase activity was measured as described by Shaw & Anderson (1972). ATPase activity was measured as described for pyrophosphatase except that incubation mixtures contained 2mM-Na_2K_2ATP in lieu of PP_i. Pyrophosphatase and ATPase activities are expressed as μmol of P_i/min (units). The coupled enzyme system for the synthesis of adenosine 5′-sulphatophosphate involving ATP sulphurylase and pyrophosphatase was as described by Shaw & Anderson (1972) except that only 1–5 units of the purified *Astragalus* ATP sulphurylases were used.

Determination of ^{75}Se-labelled compounds on paper chromatograms. Chromatograms were cut into 0.5cm sections and analysed for radioactivity with a Nuclear–Chicago 956 scintillation detector operated at 805V coupled to a Nuclear–Chicago 8727 pulse/height analyser calibrated to detect photoemissions of 0.4 to 0.5MeV.

Determination of protein. Protein was determined as described by Shaw & Anderson (1972).

Results

Purification of ATP sulphurylases

Crude extracts of leaf tissue from all four species of *Astragalus* contained pyrophosphatase and ATPase activities (Table 1), which interfere with the determination of ATP sulphurylase by the sulphate-dependent PP_i–ATP exchange technique (Shaw & Anderson, 1971). The purification procedures for the four enzymes completely removed both pyrophosphatase and ATPase activities. The specific activity of the purified enzymes was independent of enzyme concentration up to 0.1mg of protein/assay. However, the purification procedures only effected a small increase in the specific activities of the ATP sulphurylases (20–40-fold).

Properties of the PP_i–ATP exchange reactions catalysed by the ATP sulphurylases from Astragalus species

The purified ATP sulphurylases from all four species of *Astragalus* were completely inactivated when dialysed against 20mM-Tris–HCl buffer, pH8 (medium 2). The enzymes could, however, be stabilized

Table 1. *Typical purification of the ATP sulphurylases from leaf tissue of Astragalus spp. and their separation from pyrophosphatase and ATPase activities*

All enzyme activities were measured by the standard assays described in the text except that ATP sulphurylase of all fractions was assayed in the presence of 10mM-NaF. The purification treatments for the ATP sulphurylase from each species are also described in the text. The weight of leaf tissue used for preparing the ATP sulphurylases from *A. racemosus*, *A. bisulcatus*, *A. sinicus* and *A. hamosus* was 26g, 58g, 51g and 46g respectively. Enzyme units are defined in the text.

Species	Treatment	Protein (mg)	Enzyme activity (units/mg of protein)		
			ATP sulphurylase	Pyrophosphatase	ATPase
A. racemosus	Dialysed crude extract	312	0.98	1.5	0.16
	$(NH_4)_2SO_4$ fraction	139	1.21	0.45	0.01
	Sephadex G-200	78	2.81	0.49	0.01
	DEAE-cellulose (first elution)	21	10.9	0.09	0
	DEAE-cellulose (second elution)	7	26.0	0	0
A. bisulcatus	Dialysed crude extract	865	1.0	0.60	0.03
	$(NH_4)_2SO_4$ fraction	315	1.14	0.36	0.01
	Sephadex G-200	138	3.5	0.26	0
	DEAE-cellulose (first elution)	32	14.0	0.04	0
	DEAE-cellulose (second elution)	14	31.0	0	0
A. sinicus	Dialysed crude extract	491	0.84	2.52	0.71
	$(NH_4)_2SO_4$ fraction	208	1.98	0.83	0.09
	DEAE-Sephadex	131	2.31	0.02	0.06
	Sephadex G-200	7	33.7	0	0
A. hamosus	Dialysed crude extract	1151	0.70	0.82	0.11
	$(NH_4)_2SO_4$ fraction	527	0.87	0.87	0.02
	Sephadex G-200	194	2.3	0.92	0
	DEAE-cellulose (first elution)	82	3.7	0.18	0
	DEAE-cellulose (second elution)	19	14.1	0	0

Table 2. *Stabilization of purified ATP sulphurylases from A. sinicus and A. hamosus during dialysis by inorganic salts*

ATP sulphurylases from *A. sinicus* and *A. hamosus* were purified as described in the text, except that active fractions from a Sephadex G-200 column were used as the source of purified enzyme for *A. sinicus* and active fractions from the second DEAE-cellulose column were used as the source of the enzyme for *A. hamosus*. The enzymes were dialysed for 24h at 2°C against medium 2 containing the inorganic salts listed below. ATP sulphurylase activity was measured by the standard PP_i–ATP exchange assay, except that the inorganic salt in the dialysing medium was introduced with the enzyme sample (0.2ml). When the salt in the dialysing medium contained sulphate as the anion, the amount of sulphate in the incubation mixture was adjusted to 40mM by addition of K_2SO_4.

Addition to medium 2		ATP sulphurylase activity (units/mg of protein)	
Salt	Concn. (mM)	*A. sinicus*	*A. hamosus*
K_2SO_4	20	13.9	13.2
K_2SO_4	10	13.8	13.0
K_2SO_4	5	13.24	11.0
KCl	100	13.5	12.8
KCl	40	10.6	11.6
KCl	10	7.5	9.6
No additions	—	0	2

by low concentrations of K_2SO_4 or higher concentrations of KCl (Table 2). The pH optimum of the purified ATP sulphurylases from the four species of *Astragalus* was pH9. Tris (0.03M)–maleic acid (0.03M)–KOH buffer (pH6–8.5) was inhibitory to the activity of these enzymes. All four purified ATP sulphurylases were virtually insensitive to thiol-group reagents. The following reagents caused less than 15% inhibition: *p*-chloromercuribenzoate (2–8μM), iodoacetamide (0.25–25mM) and *N*-ethylmaleimide (0.25–2.5mM). The purified enzymes from all four species were virtually inactive in the absence of Mg^{2+}. The activity in the absence of Mg^{2+} (approx. 4% of the activity with 10nM-$MgCl_2$) could be accounted for by contamination of the ATP with Mg^{2+} (5μM in the incubation mixture).

The K_m of the four *Astragalus* ATP sulphurylases for sulphate was essentially the same (Table 3). All four enzymes catalysed selenate-dependent PP_i–ATP exchange; the K_m for selenate was essentially the same for the four *Astragalus* enzymes. The K_m of the enzymes for selenate was approx. 3–4-fold greater than for sulphate, but V(selenate) was 3–5-fold less than V(sulphate) for each enzyme. The ^{32}P-labelled products synthesized by all four purified enzymes in standard assays containing either sulphate or selenate were identified by paper chromatography as [^{32}P]-ATP; [^{32}P]ADP and unlabelled AMP were not

Table 3. *Summary of some kinetic properties of the purified ATP sulphurylases from four species of Astragalus*

Reaction rates, v, were measured by substrate-dependent PP_i–ATP exchange at ten concentrations of substrate S. Incubations were conducted at pH9 in the absence of fluoride. Values for K_m and V were calculated as described by Shaw & Anderson (1972).

Species	K_m(sulphate) (mM)	$\frac{K_m(\text{selenate})}{K_m(\text{sulphate})}$	$\frac{V(\text{selenate})}{V(\text{sulphate})}$
A. racemosus	3.1	0.26	0.25
A. bisulcatus	2.8	0.29	0.23
A. sinicus	3.0	0.33	0.27
A. hamosus	3.0	0.27	0.27

detected. The PP_i–ATP exchange of standard incubation mixtures (containing sulphate) catalysed by all four purified ATP sulphurylases was inhibited by addition of selenate. The kinetics of the inhibition of PP_i–ATP exchange by selenate were similar to that described for spinach leaf ATP sulphurylase (Shaw & Anderson, 1972) and were consistent with the kinetics of two substrates competing for one enzyme (Pocklington & Jeffery, 1969).

The PP_i–ATP exchange reaction catalysed by the four *Astragalus* enzymes was inhibited by chlorate, nitrate and ADP; mean values for the inhibition of activity by 1mM-$KClO_3$, 1mM-ADP and 20mM-KNO_3 were 63, 66 and 43% respectively. The kinetics of the inhibition of sulphate-dependent PP_i–ATP exchange by chlorate and ADP were studied in factorial experiments with sulphate and ATP as described by Shaw & Anderson (1974). The inhibition by chlorate was uncompetitive with respect to ATP, but competitive with respect to sulphate. The inhibition of exchange by ADP, however, was competitive with respect to ATP and non-competitive with respect to sulphate.

Synthesis of adenosine 5′-sulphatophosphate by the ATP sulphurylase–pyrophosphatase coupled enzyme system

The enzymes from all four species of *Astragalus* catalysed the synthesis of adenosine 5′-sulphatophosphate from 40mM-sulphate by the coupled enzyme system. Adenosine 5′-sulphatophosphate was not synthesized in the absence of Mg^{2+} nor in the absence of spinach pyrophosphatase. The accumulation of adenosine 5′-sulphatophosphate increased with time, but the rate of synthesis decreased with time and ceased after 1h. Selenate (20mM) inhibited the synthesis of adenosine 5′-sulphatophosphate by approx. 20% in 1h in coupled enzyme assays when either *Astragalus* or spinach ATP sulphurylases were used. The synthesis of adenosine 5′-[^{35}S]sulphatophosphate was confirmed by electrophoresis and

paper chromatography as described by Shaw & Anderson (1974).

Purified ATP sulphurylases from *A. bisulcatus*, *A. hamosus* and spinach did not catalyse the synthesis of adenosine 5′-[^{75}Se]selenophosphate in coupled enzyme systems when [^{75}Se]selenate was used in lieu of [^{35}S]sulphate. These experiments were conducted over a wide range of reaction conditions including 5–40mM-$K_2{}^{75}SeO_4$ (up to 0.5Ci/mol), 1–10mM-ATP, 1–5 units of purified ATP sulphurylase from *A. hamosus* and *A. bisulcatus*, 5–50 units of spinach ATP sulphurylase, 0–4 units of spinach leaf pyrophosphatase and reaction times from 5 to 60min. Several methods were used to terminate the reactions, since Wilson & Bandurski (1958) have suggested that adenosine 5′-selenophosphate is unstable: the methods included: (*a*) boiling for 2min; (*b*) the addition of 2ml of trichloroacetic acid (7.5%, w/v); (*c*) cooling rapidly in a salt–ice bath.

Discussion

The purification procedures described for the *Astragalus* enzymes are based on the method for purifying the spinach leaf ATP sulphurylase (Shaw & Anderson, 1972); the spinach enzyme was purified 1000-fold, but the specific activity of the *Astragalus* enzymes were increased only 20–40-fold (Table 1). The *Astragalus* leaf ATP sulphurylases, however, were completely separated from Mg^{2+}-dependent pyrophosphatase and ATPase activities (Table 1) and the endogenous PP_i–ATP exchange of the purified *Astragalus* enzymes was negligible.

The *Astragalus* ATP sulphurylases were inhibited by chlorate, ADP and nitrate and the inhibition patterns were qualitatively similar to those reported for spinach ATP sulphurylase (Shaw & Anderson, 1974), suggesting that the *Astragalus* enzymes also have ordered reaction mechanisms and that ATP is the first substrate to react with the enzymes. The stabilization of *Astragalus* ATP sulphurylases by sulphate is not understood. Since the instability of some enzymes is due to the instability of one or more substrate-binding sites, then the stabilization of ATP sulphurylase by sulphate suggests that the sulphate-binding site is labile and is protected by binding of sulphate. However, ATP did not stabilize the enzyme. Since the enzyme was stabilized by 100mM-KCl as well as by sulphate (Table 2) this suggests that ionic strength might be important, though at equivalent ionic strengths K_2SO_4 was more effective than KCl and 25mM-$MgCl_2$ failed to stabilize the enzyme during gel filtration on Sephadex G-200.

The four *Astragalus* ATP sulphurylases have very similar properties; the enzymes cannot be distinguished by their pH optima, K_m values for sulphate or selenate (Table 3), sensitivity to inhibitors, or by their reaction mechanisms. The properties of the ATP sulphurylases from *Astragalus* are very similar to the enzyme from *Penicillium chrysogenum* and spinach (Tweedie & Segel, 1971; Shaw & Anderson, 1972); the only major difference is that the purified *Astragalus* enzymes were unstable in the absence of sulphate whereas the *Penicillium* and spinach enzymes were stable. The *Astragalus* enzymes catalysed the synthesis of adenosine 5′-sulphatophosphate in the coupled enzyme system, and were virtually insensitive to thiol reagents.

The kinetics of sulphate/selenate competition studies of the ATP sulphurylases from spinach and selenium-accumulator and non-accumulator species of *Astragalus* demonstrate that sulphate and selenate compete for the same enzyme. Taken alone, these results suggest that under the appropriate experimental conditions plant ATP sulphurylases catalyse the synthesis of adenosine 5′-selenophosphate in a manner analogous to the synthesis of adenosine 5′-sulphatophosphate. However, we were unable to demonstrate the formation of adenosine 5′-selenophosphate or AMP in either single or coupled enzyme experiments as reported by Wilson & Bandurski (1958). Selenate, however, decreased the synthesis of adenosine 5′-sulphatophosphate in coupled enzyme systems, as has been described by Ellis (1969) and Varma & Nicholas (1971).

The reaction mechanism for selenate-dependent PP_i–ATP exchange is assumed to be similar to that proposed for sulphate-dependent exchange of the spinach enzyme (Shaw & Anderson, 1974). A consequence of this mechanism is that sulphate- and selenate-dependent exchange need only entail the first four partial reactions and need not entail the presence of free adenosine 5′-sulphatophosphate or adenosine 5′-selenophosphate. One explanation why the synthesis of adenosine 5′-sulphatophosphate, but not of adenosine 5′-selenophosphate, was detected in the coupled enzyme experiments could be due to a large difference in the value of the equilibrium constant for the fifth partial reaction for the sulphur and selenium analogues.

The foregoing discussion suggests that it is unlikely that the selenium of selenate enters metabolism as an analogue of sulphate via the reaction catalysed by ATP sulphurylase in either selenium-accumulator or non-accumulator plants. Since plants synthesize compounds containing selenium in valency state −2 this suggests either that some other form of inorganic selenium other than selenate is metabolized by later stages of the assimilatory sulphate pathway or, alternatively, that selenium enters metabolism by an independent pathway.

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