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THE OCCURRENCE OF ATP-ADENYLSULPHATE 3′-PHOSPHOTRANSFERASE IN THE CHLOROPLASTS OF HIGHER PLANTS

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Abstract—The presence of the enzyme ATP-adenylsulphate-3′-phosphotransferase (EC 2.7.1.25) has been demonstrated in the chloroplasts of French bean (*Phaseolus vulgaris*) and maize (*Zea mays*) leaves.

INTRODUCTION

THE FORMATION of "active sulphate" (3′-phosphoadenosine 5′-phosphosulphate; PAPS) from inorganic sulphate and adenosine 5′-triphosphate (ATP) in bacteria, fungi, algae and animals requires the activity of three enzymes, ATP sulphurylase (ATP-sulphate adenyltransferase, E.C. 2.7.7.4), APS kinase (ATP-adenylsulphate 3′-phosphotransferase, E.C. 2.7.1.25) and pyrophosphatase (pyrophosphate phosphohydrolase, E.C. 3.6.1.1) which catalyse reactions (1), (2) and (3) respectively.[1]

$$ATP + SO_4^{2-} \rightleftharpoons APS \text{ (adenosine 5′-phosphate)} + PP_i \quad (1)$$

$$APS + ATP \rightleftharpoons PAPS + ADP \quad (2)$$

$$PP_i \rightleftharpoons 2P_i \quad (3)$$

The presence of ATP sulphurylase in higher plant tissues has been demonstrated.[2] However, APS kinase has not been demonstrated in higher plants although there is strong presumptive evidence that it is present.[2] The work described in this paper demonstrates the presence of APS kinase in the chloroplasts of a monocotyledonous plant, maize (*Zea mays*), and a dicotyledonous plant, French bean (*Phaseolus vulgaris*).

RESULTS AND DISCUSSION

(*a*) *Incorporation of* $^{35}SO_4^{2-}$ *into APS and PAPS by Maize and Bean Chloroplast Fragments*

Chloroplasts were isolated from maize and bean leaves and disrupted by ultrasonication. Aliquots (0·5 ml) of the suspension of chloroplast fragments were incubated for 90 min in darkness at 30° with 5 μmoles ATP, 5 μmoles $MgCl_2$, 100 μc $^{35}SO_4^{2-}$, 1 unit of pyrophosphatase and 25 μmoles Tris buffer pH 7·5. The nucleotides were then extracted and separated by paper chromatography. The zones co-chromatographing with APS and PAPS were eluted and assayed for radioactivity (see Fig. 1). Significant amounts of radioactivity were found in both zones (see Table 1). Control incubations omitting pyrophosphatase and the chloroplast

[1] R. S. BANDURSKI, in *Plant Biochemistry* (edited by J. BONNER and J. E. VARNER), p. 473, Academic Press, New York and London (1965).

[2] T. ASAHI, *Biochim. Biophys. Acta* **82**, 58 (1964).

fragments respectively produced no radioactive APS or PAPS. Subsequent experimentation showed that incorporation of $^{35}SO_4^{2-}$ into APS and PAPS increased with time over a period of 3 hr when 5 μmoles ATP were used. When 10 μmoles ATP were used larger amounts of PAPS were produced as evidenced by the greater incorporation of ^{35}S into this compound; however, maximal incorporation occurred after incubation for 2 hr (see Fig. 2).

The fact that both APS and PAPS were labelled in these experiments strongly suggests that both ATP sulphurylase and APS kinase were present in the chloroplast fragment preparation. However, in order to demonstrate the APS kinase activity, it was necessary to

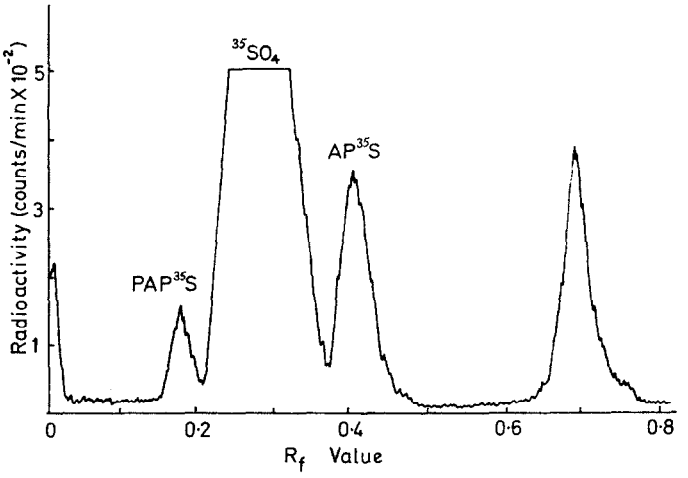

FIG. 1. RADIOSCAN OF A PAPER CHROMATOGRAM ON WHICH THE NUCLEOTIDES ISOLATED AFTER $^{35}SO_4^{2-}$ HAD BEEN INCUBATED WITH A PREPARATION OF FRENCH BEAN CHLOROPLAST FRAGMENTS HAVE BEEN SEPARATED.

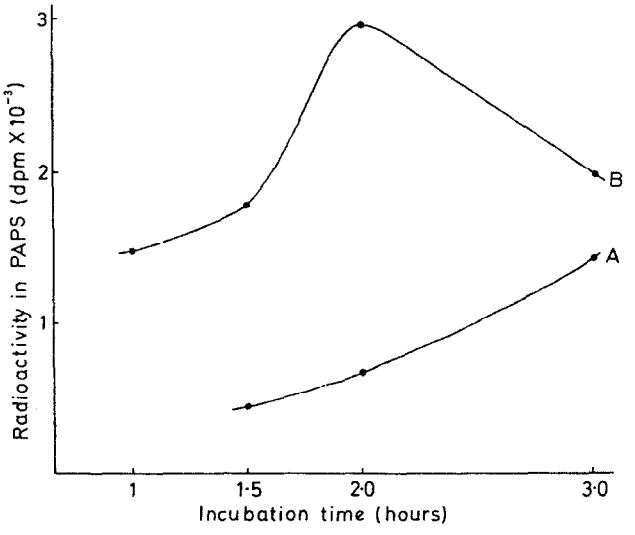

FIG. 2. EFFECT OF INCUBATION TIME ON THE INCORPORATION OF $^{35}SO_4$ INTO 3′-PHOSPHOADENOSINE 5′-PHOSPHOSULPHATE IN THE PRESENCE OF 5 μmoles ATP (CURVE A) AND 10 μmoles ATP (CURVE B).

add pyrophosphatase to the incubation mixture; this removes the pyrophosphate produced in reaction (1) and helps to overcome its unfavourable equilibrium (K for reaction (1) when catalysed by the yeast enzyme is about 10^{-8} at 37° and pH 8).

The paper chromatograms of the nucleotide extract from the incubation mixtures revealed a radioactive zone of R_f 0·7 (see Fig. 1). This zone was eluted and hydrolysed with 6 N HCl. The hydrolysis mixture was neutralized and subjected to the paper chromatographic system used for separating the nucleotides. The chromatogram was scanned for radioactivity and found to have one radioactive zone which co-chromatographed with $^{35}SO_4^{2-}$. Since the compound literates sulphate on acid hydrolysis it is a sulphate ester rather than a sulphonate. When the chromatogram was sprayed with the aniline phthalate reagent a brown zone which fluoresced yellow under u.v. light was visualized suggesting the presence of a reducing sugar. It is tentatively suggested that this labelled compound is a sugar sulphate

TABLE 1. INCORPORATION OF $^{35}SO_4^{2-}$ INTO ADENOSINE 5′-PHOSPHOSULPHATE AND 3′-PHOSPHOADENOSINE 5′-PHOSPHOSULPHATE BY MAIZE AND BEAN CHLOROPLAST FRAGMENTS

Source of chloroplast fragments	Incubation mixture	Radioactivity (dis./min) APS	PAPS
Maize	Complete*	2,035	470
	Minus chloroplast prepn.	0	0
	Minus pyrophosphatase	0	0
Bean	Complete*	10,875	1,755
	Minus chloroplast prepn.	0	0
	Minus pyrophosphatase	0	0

* 0·5 ml chloroplast fragment preparation plus 0·5 ml aqueous solution containing 25 μmoles Tris buffer, pH 7·5, 5 μmoles ATP, 5 μmoles $MgCl_2$, 100 μc $^{35}SO_4^{2-}$ and 1 unit pyrophosphatase.

(b) Effect of Molybdate, Chromate and Tungstate on the Incorporation of $^{35}SO_4$ into PAPS by Bean Chloroplast Fragments

Bandurski *et al.*[3] showed that ATP sulphurylase is relatively nonspecific with respect to the anion which participates in reaction (1); all the group VI anions will substitute for SO_4^{2-}. However, when molybdate, chromate or tungstate are incubated with ATP and ATP sulphurylase, AMP and pyrophosphate result. It is assumed the ATP sulphurylase catalyses the formation of the adenyl-anion anhydride but that the latter is very short-lived and breaks down to yield AMP and the anion. These anions are therefore competitive inhibitors of ATP sulphurylase and will thus inhibit the incorporation of $^{35}SO_4$ into APS and hence into PAPS.

The effect of molybdate, chromate and tungstate, each at three different concentrations (3×10^{-4} M, 3×10^{-5} M and 3×10^{-6} M), on the ability of a bean chloroplast fragment preparation to incorporate $^{35}SO_4^{2-}$ into PAPS was examined. Aliquots (0·5 ml) of a suspension of bean chloroplast fragments were incubated for 90 min in darkness at 30° with 10 μmoles ATP, 5 μmoles $MgCl_2$, 100 μc $^{35}SO_4^{2-}$, 1 unit of pyrophosphatase, 25 μmoles Tris

[3] R. S. BANDURSKI, L. G. WILSON and T. ASAHI, *J. Am. Chem. Soc.* **78**, 6408 (1956).

buffer, pH 7·5, and each of the inhibitors at the concentrations given previously. The incorporation of $^{35}SO_4^{2-}$ into PAPS in the presence of the inhibitors at these concentrations relative to the "no inhibitor" control is shown in Table 2. All of these group VI anions inhibit the incorporation of label from $^{35}SO_4$ into the PAPS and in each case the degree of inhibition increases with increasing concentration of anion. These results are consistent with the presence of the "sulphate-activating" system in bean chloroplasts.

TABLE 2. EFFECT OF MOLYBDATE, CHROMATE AND TUNGSTATE ON THE INCORPORATION OF $^{35}SO_4^{2-}$ INTO 3′-PHOSPHOADENOSINE 5′-PHOSPHOSULPHATE BY BEAN CHLOROPLAST FRAGMENTS

Inhibitor	Concentration (molarity)	Total activity of PAPS (dis/min)	Percentage inhibition
None	—	13,340	0
Molybdate	3×10^{-4}	2,770	79·3
	3×10^{-5}	6,130	54·0
	3×10^{-6}	8,200	38·5
Chromate	3×10^{-4}	1,100	91·7
	3×10^{-5}	5,870	56·0
	3×10^{-6}	11,460	14·1
Tungstate	3×10^{-4}	5,570	58·2
	3×10^{-5}	10,260	23·1
	3×10^{-6}	13,280	0·5

(c) *The Conversion of* [^{35}S]*APS into* [^{35}S]*PAPS by Maize and Bean Chloroplast Fragments*

APS labelled with ^{35}S was synthesized and purified by the method of Wilson *et al.*[4] from [^{35}S]sulphur trioxide–pyridine complex and adenosine 5′-phosphate (AMP). Aliquots (0·5 ml) of both maize and bean chloroplast fragment preparations were incubated for 60 min in darkness at 30° with [^{35}S]APS (67,500 disintegrations/min), 10 μmoles ATP, 5 μmoles $MgCl_2$, 25 μmoles Tris buffer, pH 7·5, and sufficient sodium molybdate to bring the molybdate concentration of the incubation mixture to 3×10^{-3} M. The molybdate was included in the incubation mixture to prevent any free $^{35}SO_4^{2-}$ present in the [^{35}S]APS preparation from being utilized; pyrophosphatase was omitted from the incubation mixture for the same reason. Thus any label found in the PAPS in these experiments could only have been derived directly from the [^{35}S]APS.

The nucleotides were extracted from the incubation mixture, separated and assayed in the usual way. The PAPS from the experiment with maize chloroplast fragments contained 1685 disintegrations/min representing a 2·49 per cent conversion of [^{35}S]APS into [^{35}S]PAPS. The PAPS from the experiment with bean chloroplast fragments contained 1563 disintegrations/min representing a 2·32 per cent conversion of [^{35}S]APS into [^{35}S]PAPS. Control incubations with boiled chloroplast fragment preparations showed no conversion of [^{35}S]APS into [^{35}S]PAPS. These results show that the two preparations of chloroplast fragments catalyse the conversion of APS into PAPS and suggest that APS kinase is present in them.

EXPERIMENTAL

Biological Material

Maize (*Zea mays* var. South African White Horse Tooth) seeds were purchased from Gunsons (Seeds) Ltd., London, E.C.3. French bean (*Phaseolus vulgaris* var. Lightning) seeds were purchased from Carter's

[4] L. G. WILSON, T. ASAHI and R. S. BANDURSKI, *J. Biol. Chem.* **236**, 1822 (1961).

Seeds Ltd., London, S.W.20. The seeds were soaked in water for 18 hr prior to planting in vermiculite. Maize seedlings were grown for 21 days and French bean seedlings for 10 days in continuous light at a temperature of 28° after which healthy leaves were harvested for chloroplast preparation.

Preparation of Chloroplast Fragments

Crude chloroplast preparations were made from 200 g batches of leaf tissue by the method of Stumpf and James[5] and purified by the density gradient centrifugation technique of James and Das[6]. The purified chloroplast pellets were resuspended in the minimal volume of deionized water and fragmented by ultrasonic irradiation at 2° using an MSE-Mullard Ultrasonic Disintegrator resonating at 16–24 kc/s. Two 15-sec bursts of ultrasonics separated by an interval of 30 sec were found sufficient to produce a high degree of fragmentation. The resulting suspension of chloroplast fragments was used immediately for incubation.

Incubation of Chloroplast Fragments

Aliquots (0·5 ml) of the suspension of chloroplast fragments were used in all incubations and were mixed with 0·5 ml of a solution containing all other reagents; the total volume of all incubation mixtures was therefore 1 ml.

In the experiments described in section (*a*) the chloroplast fragments were incubated with 25 μmoles Tris buffer, pH 7·5, 5 or 10 μmoles ATP, 5 μmoles $MgCl_2$, 100 μc $^{35}SO_4^{2-}$ and 1 unit of pyrophosphatase. The $^{35}SO_4$ was supplied by the Radiochemical Centre, Amersham, as an aqueous solution (pH 6–8; carrier free) of unspecified specific activity. Pyrophosphatase isolated from yeast was obtained from Koch Lights; 1 unit is capable of liberating 1 μmole orthophosphate per min from sodium pyrophosphate at 25° and pH 7·2.

In the experiments described in section (*b*) the chloroplast fragments were incubated with 25 μmoles Tris buffer, pH 7·5, 10 μmoles ATP, 5 μmoles $MgCl_2$, 100 μc $^{35}SO_4^{2-}$, 1 unit of pyrophosphatase and 0·3, 0·03 or 0·003 μmoles of sodium molybdate, sodium chromate or sodium tungstate.

In the experiments described in section (*c*) the chloroplast fragments were incubated with 25 μmoles Tris buffer, pH 7·5, 10 μmoles ATP, 5 μmoles $MgCl_2$, 3 μmoles sodium molybdate and [^{35}S]APS (67,500 disintegrations/min). All incubations were carried out in the absence of light at 30°. They were terminated by immersing in boiling water for 2 min.

Recovery of Nucleotides

The nucleotides (APS and PAPS) were recovered from the incubation mixture by a modification of the method of Asahi.[2] The boiled incubation mixture was centrifuged to remove the precipitate formed by boiling and the supernatant passed down a 0·5 cm i.d. column containing a mixture of 50 mg acid-washed Norit A charcoal and 80 mg Hyflo-super cel. The column was washed with 5 ml 0·05 M sodium acetate and 5 ml deionized water to remove the majority of any free $^{35}SO_4^{2-}$ present. The nucleotides were then eluted with 20 ml 50% (v/v) aqueous ethanol containing 2% (v/v) 0·880 ammonia. The eluate was evaporated to dryness under reduced pressure at 25° and redissolved in 1 ml 50% (v/v) aqueous ethanol.

Chromatographic Separation of the Nucleotides

The method of Asahi[2] was used. Aliquots of the 50% (v/v) aqueous ethanolic solution of the nucleotides were applied to Whatman No. 1 chromatography paper along with appropriate markers. The chromatogram was developed in an ascending fashion for 12 hr using a mixture of *n*-propanol, ammonia and water (6:3:1, v/v). The R_fs of APS, PAPS and SO_4^{2-} on this system are 0·42, 0·18 and 0·28. The position of the radioactive zones on the chromatogram was determined with a Nuclear Chicago Actigraph II chromatogram scanner. The radioactive zones were then cut out and eluted with 3 × 3 ml 50% (v/v) aqueous ethanol containing 2% (v/v) 0·880 ammonia. The eluate from each zone was then evaporated to dryness under reduced pressure at 25° and redissolved in 50% (v/v) aqueous methanol. Aliquots were then dispersed in 10 ml Bray's solution[7] and assayed for radioactivity using a Packard Tricarb Liquid Scintillation Spectrometer Series 314E.

Preparation of [^{35}S]*Adenosine 5′-Phosphosulphate*

[^{35}S]labelled APS was synthesized and purified by the method of Wilson *et al.*[4] The [^{35}S]sulphur trioxide-pyridine complex was purchased from the Radiochemical Centre, Amersham.

[5] P. K. Stumpf and A. T. James, *Biochim. Biophys. Acta* **70**, 20 (1963).
[6] W. O. James and V. S. R. Das, *New Phytologist* **56**, 325 (1957).
[7] G. A. Bray, *Anal. Biochem.* **1**, 279 (1960).