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MONOVALENT-STIMULATED ADENOSINE TRIPHOSPHATASE AS INFLUENCED BY NITRATE REDUCTASE IN WHEAT ROOTS

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SUMMARY

Adenosine triphosphatase (ATPase) was isolated and studied in cell free preparation from 10 days old wheat roots. The enzyme showed single peak at acidic pH of 6.0. Monovalent ions, Na^+ and K^+, when included in the assay medium, significantly stimulated the activity of the enzyme (47% - 54%), stimulation with K^+ was slightly more than that with Na^+. When taken alone, Mg^{2+} ions did not stimulate the enzyme activity but interestingly registered a marginal decrease. Further inclusion of Na^+ and K^+ along with Mg^{2+}, however, brought the enzyme activity near to the basal level. NO_3^- and Cl^- tested did not influence the activity of the enzyme. After placing the whole wheat plants, with roots dipped in nitrate solution (contained in darkened beaker), in light for 4 hr, nitrate reductase (NR) and (Na^+ - K^+) - stimulated ATPase were determined in cell free preparation. The activity of former increased significantly (45%), while latter exhibited inhibition (49%). Influence of NR on the level of Na^+ and K^+ - stimulated ATPase activity is discussed.

Key words : **(Na^+ - K^+) -stimulated ATPase, nitrate reductase, *Triticum aestivum*.**

INTRODUCTION

The membrane associated enzyme that utilizes adenosine triphosphate (ATP) to energize ion transport and plays an important role in physiological function is referred to as ATP phosphohydrolase or simply as adenosine triphosphatase (ATPase). The molecular mechanism by which roots selectively concentrate inorganic nutrients from the soil solution is not fully understood, although much progress has been made in this regard (Lüttge and Higinbotham 1979, Lauchli 1984). Cells of the root cortex can accumulate certain ions to concentrations that are many times greater than those found in the soil solution. This process, however, requires the expenditure of energy, which is provided in the form of ATP (Spanswick 1981). There are now sufficient data available which indicate link between membrane bound ATPase activity, an active protein, and ion transport in higher plant cells (Sze 1985). Similarly, Leonard and Hodges (1973) have shown that ion stimulated activity of ATPase associated with the plasma membrane is highly correlated with ion absorption by roots. There is a considerable evidence that cation transport is driven by ATP (Lüttge and Higinbotham 1979). The tonoplast of carrot root cells has been shown to employ ATP or ~P (Lüttge 1971) for transport of Na^+, K^+ in plant systems. Conflicting reports also exist in the literature about the dependency of plant ATPases on various cations. Mg^{2+} dependent ATPase in wheat leaves plasma membrane fraction was inhibited by Ca^{2+} (Terry *et al.* 1989), whereas H^+-ATPase had high activity in the absence of a divalent cation (Pomeroy and McMurchie 1982).

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Membrane-bound ATPase is a latent enzyme and is activated by membrane energization; the mechanism of the activation process, however, is still not clear (Strotmann and Bickel-Sandkotter 1984). The uptake of nitrate by plants takes place against an electrochemical gradient (Heimer and Filner 1971, Higinbotham 1973, Glass *et al.* 2002). Nitrate after uptake by plants is transported to photosynthetic tissue for rapid assimilation (Beevers and Hageman 1980). The nitrate reductase (NR) activity itself requires input of reducing equivalents, therefore, a competition for NADH is expected in roots during reduction of nitrate. Nitrate reductase is a soluble enzyme whereas ATPase related to ion transport is thought to be a membrane bound enzyme. In view of these observations it would be interesting to study the level of ATPase activity present in wheat root and its relationship with root NR activity. The present study reports the ATPase in cell free extract from wheat root as influenced by monovalent ions and NR.

MATERIALS AND METHODS

Wheat (*Triticum aestivum* var. NP 875) seeds were sterilized by using mercuric chloride and seedlings were grown in earthen or plastic pots containing sterilized sand under normal sunlight conditions. Whenever required they were irrigated with 15 mM KNO_3. Chemicals used were of AR or an equivalent purity grade, and glass double distilled water was used.

Extraction of enzyme: Roots from 10 days old seedlings were removed, washed thoroughly with tap water and finally with distilled water and blotted partially dry in filter paper folds and kept in cold before use. Samples were cut with stainless steel scissors, ground in a cold pre-chilled porcelain mortar with a pestle in a medium containing : Tris-HCl 25 mM, cysteine hydrochloride 5 mM, and ethylene-diaminetetraacetic acid (EDTA) 5 mM at pH 7.1 or as indicated. Fresh buffer was added from time to time during 5-10 min period of grinding. The extract was passed through four layers of muslin cloth and centrifuged at 9000 × g for 15 min. The pellet was discarded, supernatant decanted carefully into a clean, cold test tube and used as source of enzyme (cell free preparation). In order to document the level of Na^+ and K^+ –stimulated enzyme in the various fractions of the cell free extract, the 3000 × g, 10 min supernatant was divided into two parts. One part was centrifuged at 9000 × g, 15 min and the other part was centrifuged at 15000 × g, 15 min. The pellet of 15000 × g was also collected and suspended in Tris-MES buffer (pH 6.5) to equivalent of 400% (w/v, pellet derived from 4 g fresh tissue equivalent per ml). In experiments when nitrate reductase activity was not to be assayed, the enzyme preparation was dialyzed against the buffer for 2 hr. Change in the extraction media, pH and centrifugal field were as indicated in Tables. All the operations were carried out in cold (0° - 4°C). The protein content was estimated following Lowry *et al.* (1951) using bovine albumin as standard.

Estimation of ATPase: Enzyme activity was determined by the method of Hanson *et al.* (1965) with some modifications (Sitaramamma and Singh 1990). The reaction mixture contained 40 mM histidine-HCl buffer, pH 6.5, ATP 2 mM, suitable amount of enzyme preparation and water to a final volume of 1 ml. When required Mg^{2+} (2 mM) or K^+ (100 mM) or Na^+ (100 mM) or Na^+ plus K^+ (50 : 50 mM ratio) or specified amounts of other salts were included in the reaction mixture against suitable control. In some experiments to test the effect of ions, Tris-ATP replaced ATP salt as substrate in order to eliminate interference due to associated ions with the substrate. The basal activity measured in the absence of cations, was subtracted from the final values (Fakhrai and Hall 1984).

Estimation of nitrate reductase: Nitrate reductase assay, *in vivo* and *in vitro* were done according to Jaworski (1971) and Hageman and Hucklesby (1971), respectively. For *in vivo* assay 0.2 g root tissue was placed in open culture tubes (2.4 × 9.5 cm) containing 5 ml solution of 0.1 M Na-Pi buffer, pH 7.2, 10 mM KNO_3 and 4% (v/v) n-propanol. The tubes were vaccum infiltrated, stoppered and kept at 30°C in dark. After 30 min, nitrite formed was extracted and determined (Sawhney *et al.* 1978). The *in vitro* assay system in 2 ml contained, 0.1 M Na-Pi buffer, pH 7.2, 10 mM KNO_3, 0.34 mM NADH and enzyme preparation. The reaction was started by the addition of NADH. After 30 min incubation at 30°C the reaction was stopped by the addition of 0.1 ml of 1 M zinc acetate and 1.9 ml of 70% (v/v) ethanol. In control the NADH was added after zinc acetate and ethanol treatments. The precipitated material was removed by centrifugation and nitrite formed was determined as above.

ATPase activity in relation to nitrate reductase: Ten days old wheat seedlings were gently uprooted from the earthen pots, the roots were washed thoroughly under tap water, rinsed in distilled water twice and were divided into two lots. Roots from one lot were subjected to extraction of enzyme for zero time control, while the roots from the other lot were extracted after the plants had been kept in light with roots dipped in 15 mM KNO_3 solution for 4 hours. Nitrate reductase and (Na^+ - K^+)-stimulated ATPase assays were done as described above from 9000 × g, supernatant. Enzyme activity is expressed as nmol of nitrite formed or µmol orthophosphate released per hour per gram fresh tissue equivalent and wherever applicable per mg protein, under the standard assay conditions. Each experiment was repeated at least thrice, using different tissue samples and results are expressed as average values. Different batch of seedlings were used for different set of experiments.

RESULTS

Effect of pH on extraction and assay of Na^+ and K^+-stimulated ATPase : In order to find out the optimum pH of the extraction medium, the wheat root tissue was extracted in Tris-Histidine-HCl buffer with pH range of 5.5-8.5. Highest activity was obtained at acidic pH of 6.0, which decreased gradually to pH 8.5 (Table 1). Similarly pH 6.0 of the assay medium exhibited the highest activity of the enzyme, ATPase.

Table 1. Effect of pH on extraction and assay of Na^+ and K^+ stimulated ATPase in wheat root.

pH	ATPase µmol Pi released $h^{-1}g^{-1}fw$	
	Extraction medium	Assay medium
5.5	50	40
6.0	98	104
6.5	88	92
7.0	70	76
7.5	64	67
8.0	54	37
8.5	48	23

Tris-Histidine-HCl buffer (25 mM) was used for the extraction and assay of the enzyme. For extraction, assay pH was maintained at 6.5, similarly for assay, extraction pH was maintained at 6.5. Na^+ and K^+ were included in the assay at 50:50 mM ratio. The basal activity was 20 to 32. Other details are as in the text.

Distribution of Na^+ and K^+- stimulated ATPase : Results in Table 2 showed the level of (Na^+ + K^+) -, (Na^+ + K^+ + Mg^{2+}) – and Mg^{2+}-stimulated ATPase in various fractions of wheat root preparation. (Na^+ - K^+) –stimulated ATPase was maximum in 15000 × g supernatant followed by 9000 × g supernatant. Inclusion of Mg^{2+} along with Na^+ and K^+ (50 : 50 mM, ratio) did not enhance (Na^+ - K^+) - stimulated ATPase. Most of the data presented is with soluble

Table 2. Distribution of Na^+ and K^+ stimulated ATPase from wheat root.

Fraction	Protein mg g^{-1} fw	ATPase µmol Pi released $h^{-1}mg^{-1}$ protein		
		$Na^+ + K^+$	$Na^+ + K^+ + Mg^{2+}$	Mg^{2+}
3000 × g Supernatant	4.90	4.5 (22)	5.5 (27)	5.0 (25)
9000 × g Supernatant	0.64	28.6 (18)	26.0 (17)	14.0 (9)
15000 × g Supernatant	0.58	32.4 (19)	30.0 (17)	16.2 (9)
Pellet	0.34	16.6 (6)	54.0 (18)	62.4 (21)

Extraction and assay of the enzyme was at pH 6.0 using Tris-MES buffer (25 mM). DL-Dithiothreitol (2 mM) replaced cysteine-HCl and EDTA Na_2 was omitted from the extraction medium. Tris-ATP was used as substrate. 3000 × g supernatant of 25% (w/v) root extract was divided into two parts, one was centrifuged at 9000 × g, 15 min and the other part was centrifuged at 15000 × g, 15 min. The 15000 × g pellet was carefully suspended in extraction buffer (4 g fresh tissue equivalent per ml). Values in parenthesis are per g fresh tissue equivalent. Whole homogenate contained 6.12 mg protein g^{-1} fresh tissue equivalent.

fraction at 15000 × g, except the one wherein both nitrate reductase and ATPase were studied (9000 × g).

Effect of mono- and divalent ions on ATPase activity : The enzyme extraction and assay media were devoid of any metal ions (cationic or anionic). Tris-ATP replaced ATP salt as substrate. Both Na^+ and K^+ ions stimulated the enzyme activity, the stimulation was 29% to 47% with Na^+ and 34% to 54% with K^+ (Table 3). In order to find out the synergistic effect of metal ions, ATPase was studied in presence of mono- and/or divalent ions (Table 4). The maximum stimulation was observed with K^+ (44%) followed by Na^+ (28%). However, the enzyme did not respond to Mg^{2+} (2 mM) stimulation when used alone. Incorporation of Mg^{2+} along with K^+ or Na^+ in the assay medium slightly increased the enzyme activity, which was more or less same as the activity in the absence of any ion. Cl^- and NO_3^- did not influence the enzyme activity. Like that from rice leaf the enzyme in wheat root is insensitive to NO_3^- (Sitaramamma and Singh 1990). NO_3^--sensitive proton pump ATPase has been used as a marker enzyme for tonoplast (Hansen 1980, Sze 1984).

ATPase activity in relation to nitrate reductase : Nitrate reductase activity was stimulated by 45% on fresh weight basis and 61% on protein basis after keeping the plants in nitrate solution under sunlight for four hour (Table 5), which was significant at 5% level. ATPase activity (in presence of 50 mM each of Na^+ and K^+) was significantly inhibited (49%) unlike in rice leaves (Sitaramamma and Singh 1990).

DISCUSSION

In order to study the influence of NR on level of (Na^+-K^+) –stimulated ATPase activity it was necessary to optimize the conditions of extraction and assay of ATPase

Table 3. Effect of monovalent cations on the ATPase activity in wheat root.

Concentrations (mM)	ATPase µmol Pi released $h^{-1}g^{-1}$fw	
	Na^+	K^+
Nil	29.6	35.5
10	38.3	51.6
20	43.5	53.3
40	38.3	52.5
80	38.6	49.5
100	42.8	54.6

Tris-MES buffer at pH 6.0 was used for extraction and assay media. DL-Dithiothreitol (2 mM) replaced cysteine-HCl and EDTA Na_2 was omitted from the extraction medium. Tris-ATP was used as substrate. Other details are as in the text.

Table 4. Effect of Na^+, K^+, Mg^{2+}, Cl^- and NO_3^- on the ATPase activity in wheat root.

Additions (mM)	ATPase µmol Pi released $h^{-1}g^{-1}$fw
Nil (basal)	32.8
Na^+ (100)	42.1
K^+ (100)	47.1
Mg^{2+} (2)	26.1
Na^+ (100) + Mg^{2+} (2)	34.2
K^+ (100) + Mg^{2+} (2)	36.4
Cl^- (50)	35.0
NO_3^- (10)	31.3

Both extraction and assay of the enzyme was at pH 6.0 using Tris-MES buffer (25 mM). DL-Dithiothreitol was used in place of cysteine-HCl and EDTA Na_2 was omitted from the extraction medium. Tris-ATP was used as substrate. Other details are as in the text.

Table 5. Nitrate reductase and ATPase activities upon supply of nitrate to whole plant.

Time (h)	Nitrate reductase nmol NO_2^- formed h^{-1}		ATPase µmol Pi released h^{-1}	
	g^{-1} fw	mg^{-1} protein	g^{-1} fw	mg^{-1} protein
0	11±0.025	23±0.08	53.8±6.1	108.8±37.1
4	16±0.017	37±0.14	27.6±3.5	62.8±19.7

Experimental details are as indicated in materials and methods. Values are mean of four individual experiments±S.D. Zero time and four hour data were analyzed by Students *t* test (paired test).

from wheat root. Wheat roots exhibited maximum ATPase activity when extraction and assay media were at slightly acidic pH (pH 6.0) and had only one peak as reported for rice leaves (Sitaramamma and Singh 1990), unlike that of sugar beet leaf homogenate (Karlsson and Kylin 1974).

All enzyme preparations had certain amount of basal activity, i.e. the activity when the assay medium was devoid of cations and this activity was reduced considerably after dialysis. Both K^+ and Na^+ increased significantly the enzyme activity (29-54%), however, stimulation was more with K^+ (Table 4). Similar results have been reported in soybean root (Travis and Booz 1979). Mg^{2+} ions of their own when used alone showed very little activity in 9000 × g and 15000 × g supernatant fractions, being less than one-fourth of that at 15000 × g pellet (Table 2), which showed that the enzyme preparation substantially lacked Mg^{2+} -stimulated ATPase (Singh and Krishnan 1977, Chanson *et al.* 1984). The results in Table 2, thus, showed that the enzyme preparation from wheat root largely contained (Na^+ - K^+)-stimulated ATPase rather than particulate enzyme, as the latter is greatly stimulated by Mg^{2+} ions. Monovalent ion (especially Na^+ and K^+)-stimulated ATPase, is known to be associated with plasma membrane (Hodges and Leonard 1974, Leonard 1984), as a large intrinsic membrane protein or protein complex (Leonard 1984), the activity of which is correlated with the transport of these ions (Kylin *et al.* 1972).

NO_3^- uptake takes place in the roots from the soil by plants against an electrochemical gradient (Higinbotham 1973), and its absorption is an active process utilizing respiratory energy (Heimer and Filner 1971, Hansen 1980). Nitrate reductase is the first enzyme in the reduction of NO_3^- and is considered to be the rate limiting step. Moreover, it has been shown that uptake and assimilation of nitrogen increases the hydrolysis of ATP in pea roots and this requirement of excess of ATP alleviates the ADP restriction on glycolysis and oxidative phosphorylation (Stepan-Sarkissian and Fowler 1978). In roots, besides energy requirement for uptake of nutrients from the soil and biosynthetic reactions, ATP is also required for nitrate assimilation process in the absence of chloroplasts through shunt pathway by making use of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase reactions (Prakash *et al.* 1984).

Nitrate reductase activity is continuously adjusted in response to internal signals including the availability of nitrate and carbohydrate and the accumulation of the products of nitrate assimilation (Scheible *et al.* 1997). In soybean, decrease in leaf nitrate concentration has been reported in association with decrease in leaf nitrate reductase activity (Thibodeau and Jaworski 1975). Similarly, in wheat root like that in rice leaf (Sitaramamma and Singh 1990), there is significant increase (45%) in nitrate reductase level in plants kept in nitrate solution for 4 hr (Table 5), indicating thereby that the enhanced level of enzyme activity is because of the increase in availability of NO_3^- from the medium. However, during this period no increase in (Na^+ - K^+)-stimulated ATPase activity could be observed and in fact there was a significant decrease in (Na^+ - K^+)-stimulated ATPase level. This is an interesting result which shows negative association between NO_3^- flux into the wheat root cell and the level of (Na^+ - K^+) -stimulated ATPase as has been reported between K^+-ATPase and K^+-influx for plasma membrane fraction from primary roots of corn (Leonard and Hotchkiss 1976). Negative relationship observed could be due to increased level of nitrate in the cytosol in wheat roots, which is known to bring changes in enzymatic properties of ATPase (Ratajczak *et al.* 1998). Nitrate uptake from the medium, being an active process would generate ADP at the expense of ATP, the ADP is known to form a complex with enzyme ATPase thus, leading to its inhibition (Boyer 1997, McCarty *et al.* 2000).

The study, therefore, showed that the enzyme preparation from wheat root largely contained (Na^+ - K^+)-stimulated ATPase rather than a particulate enzyme, as it is not stimulated by Mg^{2+} ions. The activity of such a monovalent stimulated ATPase is correlated with the transport of these ions. Since nitrate reductase activity is continuously adjusted in response to the availability of nitrate and carbohydrates (Scheible *et al.* 1997), the significant increase in nitrate reductase (Table 5) clearly demonstrates the uptake of nitrate from the medium by roots. However, a significant decrease in (Na^+ - K^+) -stimulated ATPase level in the same preparation indicate a negative cooperation between NO_3^- flux into the wheat root cell and the level of (Na^+ - K^+)-stimulated ATPase. The plausible explanation for the observed negative cooperation could be due to increased level of nitrate in

the cytosol, which is known to bring changes in enzymatic properties of ATPase (Ratajczak *et al.* 1998) or the ADP generated at the expense of ATP hydrolysis may form a complex with the enzyme ATPase resulting in its inhibition (Boyer 1997, McCarty *et al.* 2000). Further experiments are desired to support the explanation advanced regarding negative-cooperativity between nitrate reductase and (Na^+ - K^+)-stimulated ATPase in wheat root.

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