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REGULATION OF PEA LEAF RIBULOSE-5-PHOSPHATE KINASE ACTIVITY

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SUMMARY

Ribulose-5-*P* kinase (ATP:D-ribulose-5-phosphate 1-phosphotransferase, EC 2.7.1.19) from the pea *(Pisum sativum)* plant has a high apparent affinity for both ATP (K_m = 0.069 mM) and ribulose-5-*P* (K_m = 0.17 mM). The enzyme is not inhibited by AMP or ADP and is unaffected by energy charge levels. The oxidative pentose phosphate pathway intermediate, 6-phosphogluconate, is an effective inhibitor. Apparently the residual dark activity of this light-activated reductive pentose phosphate pathway enzyme is reduced by the product of the dark-activated enzyme glucose-6-*P* dehydrogenase.

INTRODUCTION

Ribulose 5-phosphate is an intermediate in both the reductive and oxidative pentose phosphate pathways, being generated in the case of the photosynthetic pathway by the action of the enzymes ribose-5-*P* isomerase and ribulose-5-*P* 3-epimerase and, in the case of the oxidative pathway, by the action of the enzyme 6-phosphogluconate dehydrogenase. In the illuminated leaf the flow of carbon is through the isomerase and the epimerase to ribulose-5-*P* kinase (ATP:D-ribulose-5-phosphate 1-phosphotransferase, EC 2.7.1.19) and ribulose-1,5-P_2 carboxylase. Glucose-6-*P* dehydrogenase, which generates 6-phosphogluconate in the oxidative pathway, is inactivated by a light-dependent process[1,2]. In the non-illuminated leaf the flow of carbon is through the two dehydrogenases to the epimerase and the isomerase; the Calvin cycle enzyme ribulose-5-*P* kinase is apparently inactivated in the dark[3,4]. Clearly the activity of the kinase is important in determining the fate of ribulose-5-*P* in the green plant chloroplast.

Although ribulose-5-*P* kinase is known to be light activated, relatively high levels of activity are found in dark-treated plants, which suggests that other factors may also be involved in regulation of this enzyme. Recently 6-phosphogluconate has been shown to affect the activity of ribulose-1,5-P_2 carboxylase[5,6]; it seemed possible that this key oxidative pentose phosphate pathway intermediate might also modulate

the activity of the kinase. In the photosynthetic bacteria the kinase has been shown to respond to adenylate energy charge levels[7]. The purpose of the present experiments was to determine whether energy charge levels and metabolites such as 6-phosphogluconate would affect the activity of a higher plant ribulose-5-*P* kinase.

Pea *(Pisum sativum)* leaf ribulose-5-*P* kinase was found in the present experiments to be insensitive to AMP or ADP and totally unaffected by energy charge. Of a number of metabolic intermediates tested only 6-*P*-gluconate significantly affected the activity of the enzyme. It would appear that in darkness the enzyme is not only inactivated, but in addition is inhibited by the 6-phosphogluconate generated by the dark-activated enzyme glucose-6-*P* dehydrogenase.

MATERIALS AND METHODS

Preparation of enzyme

Ribulose-5-*P* kinase was purified from greenhouse-grown pea (*Pisum sativum*, var. Little Marvel) plants by a modification of the methods of Racker[8] and of Hurwitz *et al.*[9]. Fractionation with polyethyleneglycol replaced solvent fractionation and the final step in purification was elution of the enzyme from DEAE-Sephadex with NaCl in buffer. Details will be published elsewhere. Even with activities as high as 0.5 μmole ribulose-1,5-P_2 formed/min per ml insufficient protein was isolated to allow determination of protein and hence of specific activity, but only one protein band was observed when the isolated enzyme was subjected to gel electrophoresis (relative mobility 0.12 on 7–1/2% acrylamide gels). The enzyme was remarkably stable in 10 mM potassium phosphate buffer, 5 mM mercaptoethanol, activity being retained for several months at ice bath temperatures. Freezing totally eliminated enzyme activity.

Assays

Kinase activity was routinely measured using the coupled assay of Hurwitz *et al.*[9]. Change in absorbance at 340 nm was followed on a Gilford 2400 recording spectrophotometer. The cuvette contained 100 μmoles Tris–HCl, pH 7.8, 0.2 μmole NADH, 1 μmole ATP, 5 μmoles reduced glutathione, 10 μmoles $MgCl_2$, 0.5 μmole phosphoenolpyruvate, 0.4 μmole ribulose-5-*P*, about 20 μg lactate dehydrogenase and pyruvate kinase, and enzyme in a total volume of 1 ml. After initial screening experiments inhibitors were tested using a two-stage modification of this assay[7], and in the second stage of this modified assay, to eliminate the possibility that the coupling enzymes rather than the kinase were being affected. All enzyme assays were run at 25 °C.

In experiments involving adenine nucleotides an assay based on the alkalilability of the phosphate groups of ribulose-1,5-P_2 was used[9]. The assay mixture contained the same concentrations of Tris buffer, NADH, $MgCl_2$, glutathione and ribulose-5-*P* as were used in the coupled assay. In energy charge experiments ATP levels were between 0.2 and 0.7 mM and energy charge levels between 0.5 and 1.0. Samples (500 μl) were removed at timed intervals into 50 μl 10 M NaOH, and hydrolyzed (25 min at room temperature), after which the mixture was neutralized with 50 μl 5 M H_2SO_4 on ice. Liberated inorganic phosphate was measured using the modification of Taussky *et al.*[10] of the Sumner method. Initial velocity values were extrapolated from plots of $A_{660\text{ nm}}$ *versus* time.

Determination of kinetic constants

Six substrate levels, varied at even reciprocal intervals between 0.011 and 0.3 mM for ATP, ribulose-5-*P* concentration 0.4 mM, and between 0.055 and 1.2 mM for ribulose-5-*P*, ATP concentration 0.5 mM, were used. Values and standard error, V, K_m, and K_i were estimated as described previously[11].

Chemicals

Biochemicals were obtained from Sigma except for reduced glutathione, which was obtained from Calbiochem. Polyethyleneglycol, PEG-6000, was obtained from General Biochemicals Co. and used without further purification.

RESULTS AND DISCUSSION

Pea leaf ribulose-5-*P* kinase has pH dependence (Table I) essentially identical with that reported for the spinach enzyme[9], similar to that reported for the enzyme from *Thiobacillus neopolitanus*[12], and distinct from that reported for the *Chromatium* enzyme[7] (data not shown). The Michaelis constant for ribulose-5-*P*, 0.17 mM, is close to that reported for the spinach[9] and *Chromatium*[7] enzymes, 0.22 mM, and somewhat higher than that reported for the enzyme from *T. neopolitanus*[12], 0.024 mM. The Michaelis constant for ATP, 0.069 mM, is significantly lower than that reported for the spinach enzyme[9], 0.28 mM, and for the *T. neopolitanus* enzyme[12], 0.7 mM. In contrast sigmoid kinetics are observed with the *Chromatium*[7] enzyme and under some conditions with the *Thiobacillus*[12,13] enzymes when ATP is the variable substrate and, with the *Chromatium* enzyme when ribulose-5-*P* is varied, as well.

The following compounds have essentially no effect on the activity of the pea leaf kinase, inhibition being 10% or less: 100 mM potassium phosphate; 10 mM sucrose, glucose-6-*P*, fructose-6-*P*, fructose-1-*P*, fructose-1,6-P_2, ribose-5-*P*, phosphoglycolate, AMP and ADP; 2 mM phosphoenolpyruvate, 1 mM ribulose-1,5-P_2, 1 mM dihydroxyacetone-*P* and 1 mM glyceraldehyde-3-*P*. Effectively only 3-phosphoglycerate and 6-phosphogluconate were found to inhibit. The K_i for 3-phosphoglycerate is quite high, about 10 mM. The observed inhibition may be due to chelation of Mg^{2+} in the reaction mixture; it seems unlikely that phosphoglycerate affects the activity of the enzyme *in vivo*. 6-Phosphogluconate is competitive ($K_p > K_m$, $V_p = V$) with ribulose-5-*P*, K_i 0.75 ± 0.06 mM, and non-competitive ($V > V_p$, $K_p = K_m$) with ATP, K_i 1.7 ± 0.1 mM (Table I).

TABLE I

PROPERTIES OF PEA LEAF RIBULOSE-5-*P* KINASE

		K_m (mM)	K_i (mM)
Optimum pH	7.9		
Ribulose-5-*P*		0.17 ± 0.01	
ATP		0.069 ± 0.004	
6-Phosphogluconate			
competitive with ribulose-5-*P*			0.75 ± 0.06
non-competitive with ATP			1.7 ± 0.1

The bacterial kinases, in contrast, are affected by a number of metabolically important compounds including most of those listed above.

Although Johnson[14] has reported data which suggests that the kinase from spinach is sensitive to AMP and ADP, no evidence was obtained for inhibition of the pea leaf kinase by either of these compounds. Hart and Gibson[7] have reported that the activity of the enzyme from *Chromatium* is modulated by energy charge. No evidence for regulation of the activity of this higher plant enzyme by energy charge was obtained in the present experiments. Inclusion of AMP and adenylate kinase in the reaction mixture to give energy charge levels between 0.5 and 1.0 had no effect on the activity of the enzyme; the activity observed simply reflected the ATP levels. Energy charge does affect the activity of both chloroplastic and cytoplasmic pea leaf phosphoglyceric acid kinases[15]. It would appear that energy charge control of the Calvin cycle in *P. sativum* occurs at the level of phosphoglyceric acid kinase but not ribulose-5-*P* kinase.

Ribulose-5-*P* kinase in higher plants is activated by a light-dependent process[3,4] which may be enzyme modulated (see ref. 16). The oxidative pentose phosphate pathway enzyme glucose-6-*P* dehydrogenase is inactivated in the illuminated plant[1,2]. In either case substantial residual activity remains after inactivation. It seems highly significant that the product of the light-inactivated enzyme glucose-6-*P* dehydrogenase, 6-phosphogluconate* inhibits the activity of the light-activated kinase in addition to inhibiting the activity of ribulose-1,5-P_2 carboxylase[5,6]. The effect of 6-phosphogluconate on the carboxylase will be magnified by the lowering of ribulose-1,5-P_2 levels as a result of inhibition of ribulose-5-*P* kinase by this compound. Evidently dark-generated 6-phosphogluconate acts also to reenforce the dark inactivation of the kinase. Pelroy *et al.*[17] have reported that glucose-6-*P* dehydrogenase from the blue-green alga *Aphanocapsa* is inhibited by ribulose-1,5-P_2. If the pea leaf dehydrogenase is inhibited by ribulose-1,5-P_2 then these two enzymes, affected oppositely by light *in vivo*, will act reciprocally, with the activated enzyme catalyzing the formation of product which inhibits the residual activity of the inactivated enzyme. Inhibition of kinase activity will be reenforced by inhibition of carboxylase activity, effectively blocking two of the unique steps of the reductive pentose phosphate pathway.

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* Actually the product of the dehydrogenase is the lactone, but this compound is rapidly hydrolyzed both enzymatically and non-enzymatically to the free acid.

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