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AN ALLOSTERIC α-GLUCAN PHOSPHORYLASE FROM BANANA FRUITS

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SUMMARY

α-Glucan phosphorylase (α-1,4-glucan orthophosphate glucosyl-transferase, EC 2.4.1.1) isolated from the pulp of mature banana fruit is an allosteric protein; its activity is inhibited by tyrosine ($I_{0.5}$ = 1.6 mM) and ATP ($I_{0.5}$ = 2.6 mM). The substrate saturation curve for Glc-1-*P* is changed from hyperbolic to sigmoidal in the presence of 3 mM tyrosine, which increases the $S_{0.5}$ value for Glc-1-*P* from 4.5 to 6 0 mM. The substrate saturation curve in the presence of ATP is biphasic. The inhibitions by tyrosine and ATP were cooperative in nature. Freezing and thawing of the enzyme preparation led to desensitization with respect to inhibition by tyrosine.

INTRODUCTION

α-Glucan phosphorylase (α-1,4-glucan orthophosphate glucosyltransferase, EC 2.4.1.1) isolated from muscle behaves as an allosteric enzyme[1–6]. The best-studied plant phosphorylase, from potato, shows no allosteric or regulatory properties[7,8]. One of the phosphorylase isoenzymes isolated from maize endosperm was claimed to be regulatory[9], since a sigmoid substrate saturation curve was obtained in the presence of ATP. In all known cases, the allosteric effector of phosphorylase has been a nucleotide[1–5], a hexose[6] or a hexose phosphate[4]. The present communication presents evidence for the first time for the allosteric control of α-1,4-glucan phosphorylase, isolated from a plant tissue, by an amino acid, tyrosine.

EXPERIMENTAL PROCEDURE

Enzyme assay

The enzyme activity was assayed in the direction of polysaccharide synthesis according to the method of Green and Stumpf[10], as modified by Khanna *et al.*[11]. Fluoride was added to inhibit any phosphatase activity. The reaction mixture contained the following in a volume of 0.75 ml: 2.5 μmoles Glc-1-*P* (disodium salt, pH adjusted to 6.0); 15 μmoles NaF; 1 mg soluble starch (BDH, AnalaR); 25 μmoles

citrate buffer (pH 6.0) and enzyme. The incubation was carried out at 30 °C for 30 min. When the purified enzyme was used in the assay, inactivation was induced by the addition of 0.4 ml of ammonium molybdate (2.5%, w/v). The orthophosphate liberated was determined colorimetrically by the method of Fiske and SubbaRow[12], as modified by LePage[13].

1 unit of the enzyme activity was defined as the liberation of 1 μmole orthophosphate in 30 min under the given experimental conditions. Specific activity was expressed as units/mg protein.

Protein determination

Protein was estimated by the method of Lowry *et al.*[14], as modified by Khanna *et al.*[15], using crystalline bovine serum albumin as a primary protein standard.

Purification of the enzyme

40 g pulp from raw banana fruits (*Musa paradisiaca* species) was ground in the chilled bowl of a Waring blendor for 1 min with 200 ml of 0.05 M Tris–HCl buffer (pH 7.2) containing freshly neutralized 0.02 M cysteine·HCl, 0.02 M neutralized EDTA and 1.0% (v/v) Triton X-100. The homogenate, after straining through two layers of muslin, was centrifuged at 1600 × *g* for 30 min. The supernatant was treated with solid $(NH_4)_2SO_4$ to 60% satn and the precipitate collected. The enzyme was extracted by grinding the precipitate in a chilled mortar with 25 ml 0.05 M Tris–HCl buffer (pH 7.2) and centrifuging the suspension at 1600 × *g* for 30 min. The residue was re-extracted with 25 ml buffer and the two extracts combined. The salt was removed by filtration through a column of Sephadex G-25 equilibrated with 0.005 M Tris–HCl buffer (pH 7.2). 30 ml of the active fraction was applied onto a DEAE-cellulose column (1.4 cm × 21 cm) pre-equilibrated with 0.005 M Tris–HCl buffer (pH 7.2). The column was washed with 50 ml of the above buffer. A part of the activity passed straight through the column. This form of the enzyme, with properties which will be presented in detail separately, will be referred to as phosphorylase A. The adsorbed proteins were fractionated by stepwise elution employing 50 ml portions of equilibrating buffer containing respectively 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl. The enzyme eluted in the buffer containing 0.3 M NaCl was desalted by filtration through Sephadex G-25, equilibrated with 0.005 M Tris–HCl buffer (pH 7.2) and was employed in the following studies. This will be designated as phosphorylase B.

When the supernatant from the 60% $(NH_4)_2SO_4$ saturation step was taken to 90% saturation with the salt, a protein fraction, which contained phosphorylase activity, was obtained as a precipitate. This form, with properties which will be reported separately, will be referred to as phosphorylase C.

RESULTS

Purification of the enzyme

The summary of a typical purification procedure for α-glucan phosphorylase from banana fruits is outlined in Table I.

The recovery of over 12-times the original activity in the $(NH_4)_2SO_4$ fraction indicated the removal of inhibitor(s). The enzyme was enriched 13-fold by the DEAE-cellulose chromatography step. The degree of enrichment (627-fold) of the final

TABLE I

PURIFICATION OF α-1,4-GLUCAN PHOSPHORYLASE FROM BANANA FRUITS

Only 30 ml of the $(NH_4)_2SO_4$ fraction was applied to the DEAE-cellulose column; however, the values for the fraction eluted from the column are calculated for 100 ml.

Fraction	*Volume (ml)*	*Total activity (units)*	*Total protein (mg)*	*Spec. act. (units/mg protein)*	*Purification*
1600 × *g* supernatant of pulp homogenate	200	184	164.0	1.1	
$(NH_4)_2SO_4$ precipitate and extraction	100	2300	44.0	52.2	47
DEAE-cellulose eluate	150	518	0.75	690	627

preparation was an apparent figure in view of the likely inhibition which occurred in the crude tissue extract. The specific activity of the enzyme, 690, was about 75% of that of the potato enzyme[16]. The preparation was free from phosphoglucomutase, non-specific phosphatase, ATPase and branching enzyme, all tested at pH 6.0 under the conditions of the phosphorylase assay. The enzyme exhibited optimum activity at pH 6.0 in the presence of citrate buffer. Perfect linearity of enzyme activity with time for at least 35 min was observed when 0.5 μg enzyme protein was employed in the assay system. The incubation period of the standard assay was then fixed as 30 min. Keeping this period as constant, the amount of protein in the enzyme aliquot was varied in the range 0.25–2.0 μg; again a linearity was observed. A test of linearity with respect to time was not conducted with 2.0 μg protein or at any level other than with 0.5 μg in the assay system.

Effects of amino acids, hexose, glycolytic intermediates and adenine nucleotides

The enzyme was inhibited by L-tyrosine, DL-phenylalanine and DL-tryptophan; the inhibition produced was 47, 29 and 38%, respectively, at 1 mM concentration; increasing the concentration of the amino acids to 5 mM resulted in 90–97% inhibition (Table II). The activity of potato phosphorylase was reported to remain

TABLE II

EFFECTS OF AROMATIC AMINO ACIDS AND ADENINE NUCLEOTIDES ON BANANA PHOSPHORYLASE ACTIVITY

Assay under standard conditions.

Supplement	*Concn (mM)*	*Activity (units/ml)*
Nil		3.40
L-Tyrosine	1	1.80
	5	0.11
DL-Phenylalanine	1	2.40
	5	0.18
DL-Tryptophan	1	2.10
	5	0.15
ATP	1	2.70
	5	0.34
AMP	1	3.20
	5	2.80

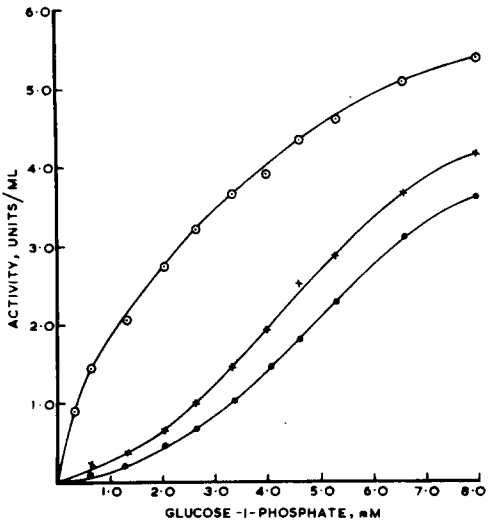

Fig. 1. Substrate saturation curve in the absence and presence of tyrosine: ○—○, no tyrosine; ×—×, 2 mM tyrosine; ●—●, 3 mM tyrosine.

unaltered, or to be slightly elevated, in the presence of 1 mM tyrosine[17]. Other amino acids, tested at 1–5 mM, did not influence the banana enzyme activity. D-Glucose and glycolytic intermediates, Glc-6-*P*, Fru-1,6-P_2 and 3-phosphoglycerate also did not increase nor decrease the velocity of the reaction. ATP was a powerful inhibitor. 5′-AMP, tested in the range of 1–5 mM, did not activate the reaction, but produced 20% inhibition at 5 mM.

Non-utilization of tyrosine in the reaction

A hypothesis that tyrosine functioned as a negative effector for banana phosphorylase necessitated experimental demonstration of its non-utilization in the reaction. In an experiment in which tyrosine added to the assay system was measured at the start of the reaction and after its termination with trichloroacetic acid (the precipitated protein was removed by centrifugation and tyrosine measured in the clear supernatant using Folin–Ciocalteu reagent) the concentration of tyrosine was found not to change.

Kinetics of phosphorylase inhibition by L-*tyrosine*

Substrate saturation in the absence and the presence of tyrosine. Fig. 1 shows the Glc-1-*P* saturation curve in the absence and the presence of tyrosine. The enzyme exhibited a hyperbolic substrate saturation curve, indicating the absence of interaction between substrate molecules. However, in the presence of tyrosine the curve changed to a sigmoid shape. The sigmoid inflexion increased with increasing concentration of the inhibitor. The interaction coefficient, or apparent order of reaction (n value), was determined from the Hill equation[18]:

$$\log v/V - v = n \log [S] - \log K$$

where v is the velocity of the enzymatic reaction at a given concentration of S (sub-

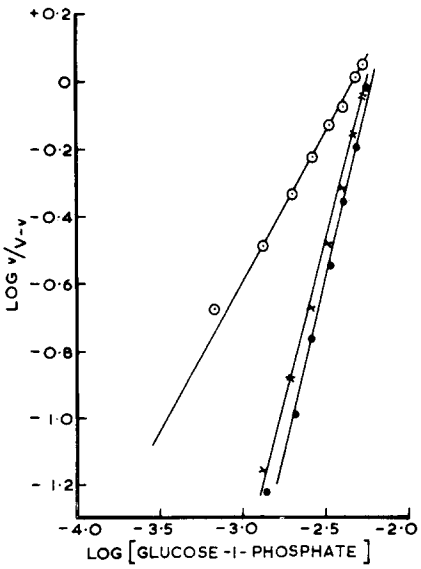

Fig. 2. Hill plot for tyrosine: ○—○, no tyrosine; ×—×, 2 mM tyrosine; ●—●, 3 mM tyrosine

strate, activator or inhibitor); V is the maximum velocity and K is the product of n dissociation constants of n binding sites.

The n value of the enzyme for Glc-1-P increased from 0.9 to 1.9 in the presence of 2 mM tyrosine, and further increased to 2.2 at 3 mM tyrosine (Fig. 2). The $S_{0.5}$ value, or the concentration of substrate giving half maximal velocity, was calculated from the substrate concentration when log $v/V-v = 0$. The presence of tyrosine increased the $S_{0.5}$ value for Glc-1-P from 4.5 to 5.5 and 6.0 mM in the presence of 2 and 3 mM tyrosine, respectively.

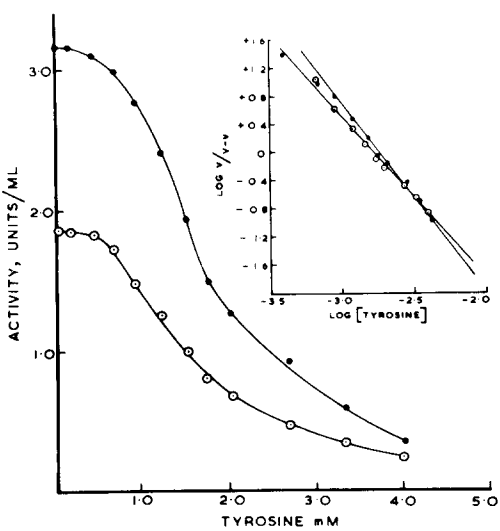

Fig. 3. Effect of tyrosine concentration on phosphorylase activity at two concentrations of Glc-1-P: ○—○, 1.65 mM; ●—●, 3.3 mM. Inset shows the Hill plot.

Cooperativity in tyrosine binding. Initial velocity measurements were performed at a constant starch concentration (1 mg/assay system) in the presence of various concentrations of tyrosine and two concentrations of Glc-1-*P* (1.65 and 3.3 mM). The plots of enzyme activity and tyrosine concentration gave sigmoid curves (Fig. 3). The concentration producing 50% inhibition ($I_{0.5}$) was 1.6 mM. Hill plots for the data (inset of Fig. 3) indicated *n* values of 2.3 and 2.7 in the presence of 1.65 and 3.3 mM Glc-1-*P*, respectively.

Since Glc-1-*P* and tyrosine are quite dissimilar in structure, these data can best be interpreted by postulating that tyrosine is an allosteric inhibitor, exerting its effect by modifying the conformation of the enzyme[19].

Desensitization of the enzyme

The data from a typical experiment on the effect of freezing and thawing on the enzyme activity and on its inhibition by 2 mM tyrosine are recorded in Table III.

TABLE III

EFFECTS OF FREEZING AND THAWING ON ENZYME ACTIVITY AND INHIBITION BY TYROSINE

The enzyme was assayed under standard conditions. The inhibition by tyrosine was tested at 2 mM. The enzyme stored at −20 °C was thawed every 2 h.

Storage time (h)	*Enzyme stored at 0–4 °C*		*Enzyme stored frozen at −20 °C and thawed*	
	Activity (units)	*Inhibition by tyrosine (%)*	*Activity (units)*	*Inhibition by tyrosine (%)*
0	3.70	84	3.70	84
2	3.70	84	3.68	83
4	3.68	80	3.52	70
6	3.68	73	3.46	45
8	3.60	70	3.32	20

Freezing and thawing did not significantly affect the catalytic activity of the enzyme, but inhibition by tyrosine was reduced from 84% at the commencement to 20% at the end of 8 h of storage at −20 °C with four thawings in between. In contrast, the inhibition by tyrosine was only slightly lowered when a control sample of enzyme was stored at 0–4 °C for 8 h.

Kinetics of the phosphorylase inhibition by ATP

Substrate saturation in the absence and the presence of ATP. When the concentration of ATP was held constant (3 mM) and the concentration of Glc-1-*P* varied, a biphasic curve was obtained when the activity was plotted against substrate concentration (Fig. 4).

Cooperativity in ATP binding. At 3.3 mM Glc-1-*P*, when the concentration of ATP was varied and the results plotted as percentage inhibition against the concentration of ATP, the resulting curve appeared to be sigmoid in nature (Fig. 5). The Hill plot of the data indicated an *n* value of 2.1 (inset Fig. 5). The $I_{0.5}$ value for ATP was 2.6 mM.

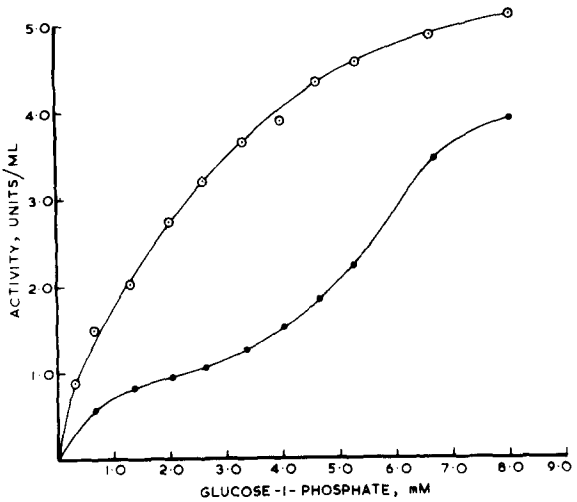

Fig. 4. Substrate saturation curve in absence and presence of ATP: ○—○, no ATP; ●—●, 3 mM ATP.

DISCUSSION

No claim is made that the phosphorylase enzyme used in the present studies was purified to homogeneity. Results with the animal enzyme show that phosphorylase activity in an assay system without primer is markedly accelerated by the presence of the branching enzyme (*cf.* Gibson *et al.*[20]); the latter enzyme functions by providing increased numbers of end groups which, in turn, can accept the glucose residues transferred by phosphorylase. An apparent value for phosphorylase activity would, therefore, be obtained. If the plant enzymes system functioned in an analogous manner, the kinetic properties of the preparation containing both enzymes would not be the same as those when phosphorylase alone was present. However, in the authors' experiments, no orthophosphate liberation occurred when the enzyme preparation was incubated with 3.3 or 80 mM Glc-1-*P* for 60 min in the absence of primer and in

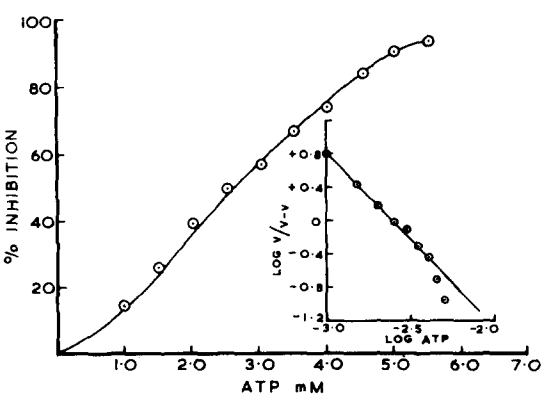

Fig. 5. Effect of ATP concentration on phosphorylase activity. Inset shows the Hill plot.

the presence and absence of AMP. No branching enzyme activity could be detected in the enzyme preparation when assayed according to Larner[21] using potato amylopectin or amylose. Again, the assays in the present experiments were conducted in the presence of an excess of primer, so that acceptor groups for phosphorylase were not limiting at any stage.

The content of Glc-1-*P* in the banana fruit was not determined in the present experiment. Calculation and extrapolation of the top curve of Fig. 1 shows that a saturating level of Glc-1-*P* is achieved at about 15 mM. This appears to be too unphysiological a concentration to be used in tests on enzyme inhibitors. The authors have, therefore, chosen 3.3 mM Glc-1-*P* (75% of K_m), a non-saturating concentration, which approaches the anticipated physiological concentration.

α-1,4-Glucan phosphorylase may be considered to be an allosteric enzyme on the basis of both the sigmoid substrate saturation curve in the presence of the modulator, tyrosine, and the desensitization on freezing and thawing.

The addition of tyrosine caused the curves for rate with respect to substrate concentration to change from hyperbolic to sigmoidal. It thus belongs to the modulator-dependent cooperative system[22]. The increase of the *n* value from 0.9 to 2.2 in the presence of 3 mM tyrosine indicates that the inhibitor caused increased interaction of the substrate binding sites. Loss of inhibition by tyrosine, without affecting α-glucan phosphorylase activity, on freezing and thawing the enzyme preparation indicates an allosteric site distinct from the catalytic site.

ATP also produced inhibition of α-glucan phosphorylase activity. Whereas the plot of percentage inhibition of enzyme activity against ATP concentration yielded a sigmoid curve, the plot for activity against substrate concentration at a fixed ATP concentration yielded a biphasic curve. It may be mentioned that, according to one of the theoretical protein subunit models discussed by Koshland *et al.*[23], the combination of certain kinetic parameters could give a biphasic shape to the substrate-binding curve.

α-Glucan phosphorylase is the first enzyme acting in the non-hydrolytic degradation of starch. The catabolism of starch yields phosphoenolpyruvate and erythrose 4-phosphate by the glycolytic and pentose phosphate pathways, respectively. The two intermediates are involved in the biosynthesis of aromatic amino acids in plant tissues[24] along the pathway established for microorganisms[25]. Thus glucan phosphorylase may be considered as the first enzyme in a long and highly branched chain of reactions leading to the biosynthesis of aromatic amino acids from starch. Tyrosine, an end product of metabolism, presumably modulates the enzyme to regulate the flow of starch towards the synthesis of aromatic amino acids. It is significant, in this context, that the other two forms of α-glucan phosphorylase, A and C, isolated from banana fruit are not inhibited by aromatic amino acids.

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