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KINETICS OF STARCH PHOSPHORYLASE FROM YOUNG BANANA LEAVES

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Key Word Index—*Musa paradisiaca*, Musaceae, banana; starch phosphorylase, kinetics, inhibition; biphasicity

Abstract—At pH 6 0, the initial velocities of the reaction between glucose 1-phosphate and amylose, glucose 1-phosphate and amylopectin, glucose 1-phosphate and glycogen, and glucose 1-phosphate and starch in the absence of orthophosphate, using purified starch phosphorylase from young banana leaves are characteristic of a sequential reaction mechanism. K_m values for starch, amylose, amylopectin, glycogen, glucose 1-phosphate and orthophosphate are reported. ATP and UDP-glucose are competitive inhibitors of the enzyme with respect to glucose 1-phosphate Tyrosine is a non-competitive inhibitor of the enzyme with respect to glucose 1-phosphate Kinetic studies at pH 7 0 also show characteristics of a sequential reaction mechanism. The aromatic amino acids L-phenylalanine, L-tyrosine and DL-tryptophan inhibit the phosphorylase reaction at pH 7.0. Starch phosphorylase shows biphasic kinetic behaviour with more than one Hill coefficient as calculated from initial velocity data, which becomes more prominent in the presence of aromatic amino acids.

INTRODUCTION

Several reports are available on the kinetics of glycogen phosphorylase (EC. 2.4.1,1) [1–7], but detailed kinetic studies on plant leaf phosphorylases (EC. 2 4.1 1) are lacking. Kinetic studies on glycogen phosphorylase from animals have revealed the allosteric nature of the enzyme [8–15]. In the case of plant tissues, starch phosphorylase obeys the classical Michaelis–Menten equation [16–21], but there are reports of allosteric behaviour [22, 23]. Singh and Sanwal [23] have shown that one of the forms of starch phosphorylase from banana fruits is allosterically regulated by the aromatic amino acid, L-tyrosine Earlier, we have shown the absence of pyridoxal 5′-phosphate in banana leaf starch phosphorylase [24]. Therefore, it was of interest to compare the kinetic behaviour of the enzyme from banana leaf with other α-glucan phosphorylases known to contain pyridoxal 5′-phosphate as a prosthetic group. The present work deals with the kinetic studies of starch phosphorylase from young banana leaves by means of initial velocity methods. An interesting finding is the presence of biphasic kinetics at pH 7 0 which is more prominent in the presence of aromatic amino acids.

RESULTS

The enzyme shows optimum activity at pH 6.0 in the direction of polysaccharide synthesis and at pH 7.5 in the direction of polysaccharide degradation [24]. Double reciprocal plots of a representative initial velocity study at pH 6 0 in the direction of polysaccharide synthesis are shown in Fig 1, using starch as a primer. The same

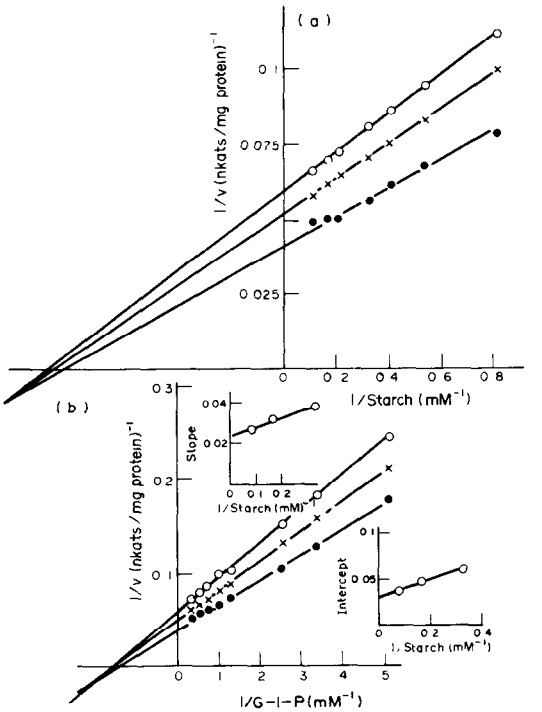

Fig 1 Lineweaver–Burk plots of: (a) Velocity of phosphorylase as a function of [starch] at three levels of [G-1-P] ○, 1 mM G-1-P, ×, 2 mM G-1-P; ●, 5 mM G-1-P, (b) Velocity of phosphorylase as a function of [G-1-P] at three levels of [starch], ○, 3 1 mM starch; ×, 6 2 mM starch; ●, 12 4 mM starch Lower inset shows a replot of vertical intercepts vs reciprocal of [starch] Upper inset shows a replot of slopes vs reciprocal of [starch] The phosphorylase reaction was carried out in the direction of polysaccharide synthesis in the absence of Pi

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pattern of lines was obtained when amylose, amylopectin or glycogen was used as a primer (data not shown). The intersection of the lines at a point to the left of the vertical axis is characteristic of a sequential reaction mechanism in which both the reactants must be added to the enzyme before any product(s) is released

This reaction mechanism is represented by the rate equation [25]

$$v = \frac{V_1}{1 + K_a/A + K_b/B + (K_{ia}K_b)/AB}$$

where A and B are polysaccharide and glucose 1-phosphate (G-1-P), respectively K_a and K_b are the Michaelis constants for polysaccharide and G-1-P, respectively The Michaelis constants are the dissociation constants of the appropriate ligand from a ternary complex K_{ia} is the dissociation constant of the enzyme–starch binary complex, v and V_1 are the initial velocity and the maximal velocity of the reaction. For the degradation reaction, a similar equation is obtained in which P_i representing inorganic phosphate (Pi) replaces B

Reciprocal plots were all linear indicating a lack of homotropic co-operativity between polysaccharide sites and G-1-P sites Various Cleland's constants derived from the secondary plots of Fig 1B for phosphorylase are presented in Table 1 K_{ib} was calculated according to the following equation

$$K_{ia}\ K_b = K_a\ K_{ib}$$

The K_m values of the enzyme in the direction of polysaccharide synthesis at pH 6 0 for various primers and G-1-P are given in Table 2 At pH 7 5, initial rate reciprocal plots for the phosphorolysis of starch were linear (data not shown) K_m values for Pi were 13 5, 12 9, and 11 4 mM at 0 6, 3 1 and 6.2 mM starch, respectively Various Cleland's coefficients for the reaction are reported in Table 3

Inhibition experiments at pH 6.0

G-1-P saturation curves at pH 6 0 in the presence as well as in the absence of ATP were linear for phosphorylase

Table 1 Cleland's kinetic coefficients of phosphorylase in the direction of polysaccharide synthesis using starch as a primer at pH 6 0

Kinetic coefficient	Value
K_a	3 21 mM
K_b	0 74 mM
K_{ia}	3 99 mM
K_{ib}	0 92 mM
V_1	32 1 nkat/mg protein

K_a and K_b are the Michaelis constants for starch and G-1-P, respectively K_{ia} and K_{ib} are the dissociation constants for the enzyme–starch binary complex and the enzyme-G-1-P binary complex, respectively. V_1 is the maximal velocity of the reaction

Table 2 Michaelis constants of phosphorylase in the direction of polysaccharide synthesis using various primers at pH 6 0

Primer	[Primer] (mM)	[G-1-P] (mM)	K_m Primer (mM)	K_m G-1-P (mM)
Starch	Var*	1 0	1 1	
	Var	2 0	1.2	
	Var	5 0	1 3	
	3 1	Var		0 63
	6 2	Var		0 65
	12 4	Var		0 67
Amylose	Var	1 0	2 4	
	Var	2 0	2 6	
	Var	5 0	2 8	
	3 1	Var		0 47
	6 2	Var		0 48
	12 4	Var		0 50
Amylopectin	Var	1 0	3 6	
	Var	5 0	4 4	
	3 1	Var		0 83
	12 4	Var		0 91
Glycogen	Var	1 0	3 7	
	Var	5 0	3 8	
	3 1	Var		0 71
	12 4	Var		0 83

* Var, variable primer or G-1-P concentration

Table 3 Cleland's kinetic coefficients of phosphorylase in the direction of phosphorolysis using starch as a primer at pH 7 5

Kinetic coefficient	Value
K_a	0 36 mM
K_p	11 94 mM
K_{ia}	0 50 mM
K_{ip}	16 58 mM
V_1	8 53 nkat/mg protein

K_a and K_p are the Michaelis constants for the starch and Pi, respectively K_{ia} and K_{ip} are the dissociation constants for the enzyme-starch binary complex and the enzyme–Pi binary complex, respectively V_1 is the maximal velocity

The K_m value of phosphorylase for G-1-P increased from 0.66 to 0 87 mM in the presence of 0 3 mM ATP without any change in V_{max}, thereby establishing a competitive type of inhibition. The K_i value for ATP was 1 mM as determined by Dixon plot

Like ATP, the G-1-P saturation curve at pH 6 0 in the presence of UDP-glucose (10 mM) was linear in nature The inhibition was of a competitive type with respect to G-1-P One mM UDP-glucose caused no inhibition The

K_i value for UDP-glucose was 12.7 mM as calculated from a Lineweaver–Burk plot The K_m value of phosphorylase for G-1-P increased from 0 66 to 1.18 mM in the presence of 10 mM UDP-glucose without any change in V_{max}

Like ATP and UDP-glucose, the G-1-P saturation curve at pH 6.0 in the presence of L-tyrosine was also hyperbolic in nature The V_{max} of phosphorylase decreased from 26 3 to 14 3 nkat/mg protein in the presence of 5 mM tyrosine without any change in K_m, thereby evidencing non-competitive type of enzyme inhibition The K_i for L-tyrosine was 5 4 mM, as determined by a Dixon plot At 1 0 mM tyrosine, there was no significant inhibition.

Initial velocity experiments at pH 7.0

The results of a representative initial velocity study in the direction of polysaccharide synthesis at pH 7 0 in the form of reciprocal plots are shown in Fig. 2 using different concentrations of G-1-P keeping the concentration of starch constant The Lineweaver–Burk plots show biphasic characteristics The intersection of the lines at a point to the left of the vertical axis (using straight line regions at high or low G-1-P conc) is characteristic of a sequential reaction mechanism. A secondary plot, shown as upper inset of Fig 2, does not give a straight horizontal line and indicates the absence of a ping-pong mechanism Various Cleland's constants were calculated from Fig. 2 and its lower inset at the higher concentration range of G-1-P (0.25–10 mM). The values of K_a, K_b, K_{ia}, K_{ib} and V_1 were found to be 4.63, 5 71, 1 04, 1 28 mM and 23 81 nkat/mg protein, respectively (cf Table 1)

Inhibition experiments at pH 7 0

Representative substrate saturation curves in which starch was varied keeping the concentration of G-1-P constant (5 mM) are shown in Fig. 3A (in presence of phenylalanine), 3B (in presence of tyrosine) and 3C (in presence of tryptophan). There is marked deviation from the normal hyperbolic behaviour in the presence of aromatic amino acid. Lineweaver–Burk plots of Fig 3 also show biphasicity Hill plots show two or more Hill coefficients, one being less than one, the other being more than one (data not shown).

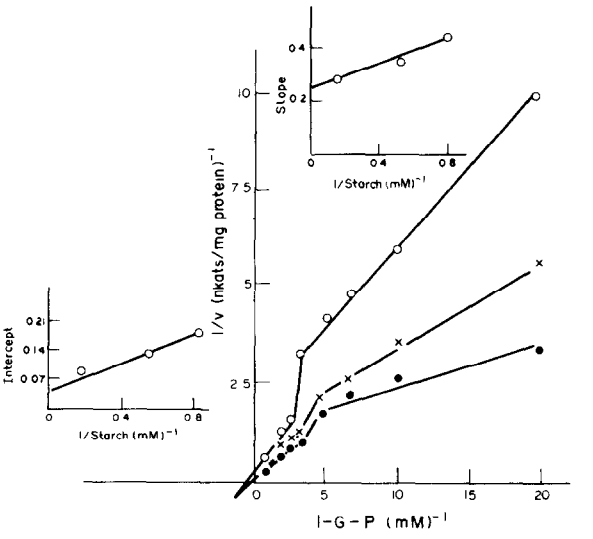

Fig 2 Lineweaver–Burk plot of velocity of phosphorylase at pH 7 0 as a function of [G-1-P] at three levels of [starch] ○, 1 2 mM starch, ×, 1 9 mM starch, ●, 6 2 mM starch Lower inset shows a replot of vertical intercepts of the main plot vs reciprocal of [starch] Upper inset shows a replot of slopes of the lines drawn using low [G-1-P] (0 2–0 05 mM) vs reciprocal of [starch] The phosphorylase reaction was carried out in the direction of polysaccharide synthesis in the absence of Pi

Similarly, in another set of experiments, G-1-P was varied keeping the concentration of starch constant (18 6 mM)· Fig. 4A (in presence of phenylalanine), 4B (in presence of tyrosine) and 4C (in presence of tryptophan) They show biphasic kinetic behaviour as observed in Fig. 3 Again biphasicity is prominent in the presence of aromatic amino acid. Lineweaver–Burk plots are also

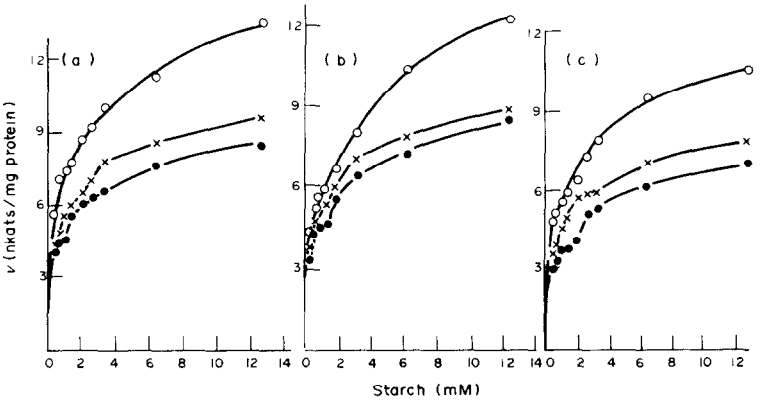

Fig 3 (a) Velocity of phosphorylase as a function of [starch] at fixed [G-1-P] (5 mM) in the absence as well as presence of phenylalanine ○, 0 mM, ×, 0 3 mM, ●, 0 6 mM phenylalanine (b) Velocity of phosphorylase as a function of [starch] at fixed [G-1-P] (5 mM) in the absence as well as presence of tyrosine ○, 0 mM, ×, 0 3 mM, ●, 0 6 mM tyrosine (c) Velocity of phosphorylase as a function of [starch] at fixed [G-1-P] (5 mM) in absence as well as presence of tryptophan ○, 0 mM, ×, 0 3 mM; ●, 0 6 mM tryptophan The phosphorylase reaction was carried out in the direction of polysaccharide synthesis at pH 7 0 in the absence of Pi

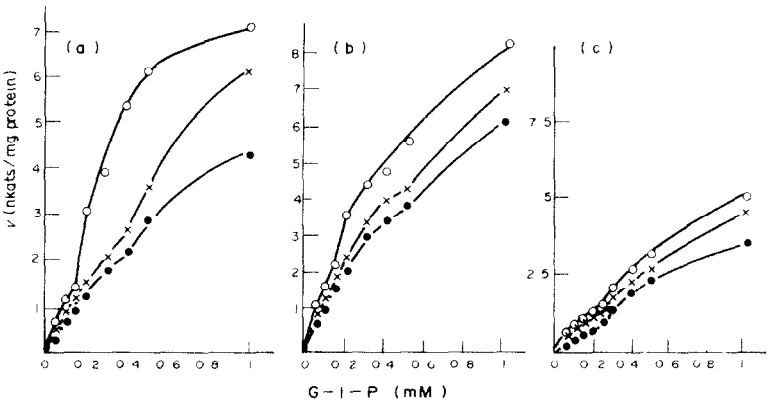

Fig 4 (a) Velocity of phosphorylase as a function of [G-1-P] at a fixed [starch] (18 6 mM) in the absence as well as presence of phenylalanine ○, 0 mM, ×, 0 3 mM, ●, 0 6 mM phenylalanine (b) Velocity of phosphorylase as a function of [G-1-P] at a fixed [starch] (18 6 mM) in the absence as well as presence of tyrosine ○, 0 mM, ×, 0 3 mM, ●, 0 6 mM tyrosine (c) Velocity of phosphorylase as a function of [G-1-P] at a fixed [starch] (18 6 mM) in the absence as well as presence of tryptophan ○, 0 mM, ×, 0 3 mM, ●, 0 6 mM tryptophan The phosphorylase reaction was carried out in the direction of polysaccharide synthesis at pH 7 0 in the absence of Pi

biphasic in nature and Hill plots show two or more Hill coefficients, one being less than one and the other more than one (data not shown)

DISCUSSION

Banana leaf starch phosphorylase exhibits a sequential mechanism, as has been reported for other α-1,4-glucan phosphorylases [19, 26, 27] The K_m values of the enzyme are in the same range as those reported earlier for phosphorylases from other sources [1, 17–22] In the present study, the K_m at pH 6 0 in the direction of polysaccharide synthesis indicates highest affinity of the enzyme for starch followed by amylose, glycogen and amylopectin when the primer was the varying substrate keeping G-1-P constant On increasing the concentration of the fixed substrate, the K_m value of the enzyme for the varying substrate increased showing decrease in the enzyme affinity for the varying substrate In the direction of polysaccharide degradation, there is a decrease in the K_m of the enzyme on increasing the concentration of the fixed substrate (starch) showing an increase in the affinity of enzyme for the varying substrate These data show that the Pi (or G-1-P)-binding site and the polysaccharide-binding site are not completely independent, an observation similar to that made for potato phosphorylase [27] The dissociation constants are about 20–25% larger than the Michaelis constants in the direction of polysaccharide synthesis, whereas they are about 40% larger in the direction of degradation At pH 7 0, in the direction of polysaccharide synthesis, the dissociation constants are about four-fold smaller than the Michaelis constants Gold *et al* [27] also reported that differences between Michaelis and dissociation constants are less than a factor of two in potato phosphorylase

Singh and Sanwal [23] have shown that one of the forms of starch phosphorylase from banana fruit pulp is inhibited by L-tyrosine and ATP allosterically at pH 6 0. In the present case, the enzyme is inhibited by L-tyrosine and ATP at pH 6 0 but with normal Michaelis kinetics Here, the concentration of tyrosine required for 50% inhibition is 3 4-fold higher than banana fruit phosphorylase, whereas, the concentration of ATP required for 50% inhibition is 2 6-fold lower than banana fruit phosphorylase Soman and Philip [28] have shown that rabbit muscle phosphorylase *b* is inhibited by a wide variety of soluble aromatic compounds, showing the possibility of a separate binding site for phenolic compounds. Aromatic amino acids are the feedback inhibitor of starch phosphorylase For the biosynthesis of aromatic amino acids, erythrose 4-phosphate and phosphoenolpyruvate are provided from starch catabolism for which starch phosphorylase is the first enzyme

The phenomenon of a biphasic curve is not rare Levitzki and Koshland [29] ascribed it to the protein exhibiting positive and negative cooperativity towards the same ligand in a single saturation curve Plots of initial velocity or degree of saturation vs ligand concentration are sigmoidal at higher ligand concentrations The transition from hyperbolic behaviour to a sigmoidal curve is manifested by the appearance of an intermediary plateau region It can be shown mathematically that such behaviour can occur only in an oligomeric protein that possesses three or more interacting sites [29] There are many reports of biphasic kinetics in enzymes [30–38]

It is pertinent to note that like banana leaf phosphorylase, mitochondrial malic enzyme from rat skeletal muscle shows normal Michaelis kinetics at pH 7 0, whereas, it shows sigmoidal kinetics at pH 7 5 and 8 0 [39] It is possible, on changing the pH, certain groups present in the active centre change their ionization state It is also possible that at pH 6 0, the allosteric site of the enzyme is desensitized whereas at pH 7 0 which is more close to the physiological pH of the system, the enzyme shows regulatory properties indicating the regulatory nature of the enzyme *in vivo* In the absence of cooperativity also, the enzyme may show biphasic kinetics for the following reason(s): (i) the presence of more than one kinetically active form of the enzyme Although the possibility of more than one enzyme form is remote, the homogeneity of the enzyme was confirmed by (a) PAGE at various pHs and various gel concentrations, (b) identity of the

protein band with starch phosphorylase activity, (c) SDS-PAGE, (d) no change in sp activity on affinity chromatography or crystallization of the enzyme, (e) immunodiffusion and (f) immunoelectrophoresis [24] (ii) The presence of any other enzyme in the purified preparation which binds G-1-P, starch and/or aromatic amino acids. (iii) Non-identical regulatory sites which bind the ligand with different affinities (iv) Identical regulatory subunits which are geometrically arranged within the molecule, giving rise to sites of different affinities for a ligand (v) Binding of the ligand on more than one site in one subunit with different affinity

It is possible that some impurity(ies) in the substrates or inhibitor is responsible for the biphasic curve Although the possibility seems remote since on using the same substrates and inhibitors at pH 6 0, the enzyme did not reveal biphasic kinetics In the case of banana phosphorylase, the possibility of two enzymic forms under the experimental conditions cannot be completely ruled out. There is dissociation of the enzyme into two halves on maturity under *in vivo* condition [40], although all efforts to interconvert the two forms of banana leaf phosphorylase have failed (unpublished work)

In the present study, the experiments have been repeated a number of times and always the same type of pattern is found From the overall study, it seems that the kinetics of banana leaf phosphorylase at the optimum pH is similar with other phosphorylases studied However, the kinetics at pH 7 0 is somewhat different compared to other plant phosphorylases There is deviation from normal hyperbolic behaviour The plots showed biphasicity which is more prominent in the presence of aromatic amino acids

EXPERIMENTAL

Chemicals Soluble starch (Analar grade), L-phenylalanine, L-tyrosine, DL-tryptophan and glycogen from BDH, U K , amylose from Koch Light Laboratory Ltd, U K , UDP-glucose and glucose-6-phosphate dehydrogenase (grade II) from Sigma, U S A , Phosphoglucomutase from Boehringer Mannheim, F R G , ATP from E Merck, F R G and amylopectin from Biochemical Units, V P Chest Institute, Delhi, India G-1-P from E Merck was purified according to the procedure of ref [41] using a charcoal column All the other chemicals used, were of Analytical grade

Enzyme preparation Starch phosphorylase from young banana (*Musa paradisiaca*) leaves was purified to homogeneity using the techniques of $(NH_4)_2SO_4$ fractionation, DEAE-cellulose chromatography and filtration through Sephadex G-100 and Sephadex G-200, as described earlier [24]

Enzyme assays

In the direction of polysaccharide synthesis The initial velocity of starch phosphorylase in the direction of polysaccharide synthesis in the presence and absence of inhibitors was determined at 30° by the colorimetric assay as described earlier [24] The reaction was always started by the addition of G-1-P There was no effect of preincubation of the enzyme with starch and/or other ligand There was also no change in activity when the reaction was started by the addition of the enzyme The reaction was stopped by the addition of ammonium molybdate (0 24 ml, 2 5%) instead of TCA There was only slight turbidity after stopping the reaction which did not interfere with colour formation. The phosphate colour was developed using total aliquot in a total 3 ml vol instead of 10 ml for Pi estimation according to the method of ref [42] The assay system contained a fixed amount of enzyme preparation (5–10 μg enzyme protein) and varied amounts of substrates and inhibitors in 0 02 M Tris–maleate buffer The [primers] has been defined in mM (as glucosyl residues) instead of g/l One g/l starch or any other primer has been taken to be equivalent to 6.2 mM

In the direction of polysaccharide degradation The enzyme assay in the direction of polysaccharide degradation direction was the same as described earlier [24]

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