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KINETIC PROPERTIES OF ACID PHOSPHATASE FROM SCUTELLA OF GERMINATING MAIZE SEEDS

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Key Word Index—*Zea mays*; Gramineae; maize; scutellum; germination; acid phosphatase; negative cooperativity.

Abstract—Acid phosphatase purified from maize scutellum showed a kinetic transition from negative cooperativity at pH 5.4, to Michaelian behavior at pH 6.7. The negative cooperativity phenomenon was also abolished by increasing Pi concentration, by succinylation of the enzyme molecule or by lowering the assay pH. Our results suggest that a mechanism, which involves interaction between catalytic sites, could not be the source of negative cooperativity at pH 5.4, since the enzyme is monomeric and probably has only a single catalytic site.

INTRODUCTION

Acid phosphatase (EC 3.1.3.2) purified from maize scutellum showed a pH-dependence on the *Km* values for the hydrolysis of *p*-nitrophenylphosphate and glucose-6-phosphate[1]. Acid phosphatases of other species have shown a similar pH-dependence[2–5], which could be due either to ionization of the substrate[4] or to ionization of the enzyme molecule leading to the interconvention between two forms resulting in different *Km* values for the substrates[5]. In order to study the relationship between kinetic and structural properties of acid phosphatase from maize scutella, we reinvestigated the kinetic properties of the enzyme. Our results suggest that, at pH 5.4, the kinetics deviate significantly from Michaelis–Menten behavior, which provides evidence for a kinetic transition from negative cooperativity (at pH 5.4) to Michaelian behavior (at pH 6.7).

RESULTS AND DISCUSSION

Figure 1 shows the double-reciprocal plots of initial velocities, at pH 5.4, as a function of the *p*-nitrophenylphosphate concentration. It can be observed that the double-reciprocal plots did not give a straight line and are characterized by a Hill coefficient[6] lower than one (Table 1), which (in the phenomenological sense) is compatible with the

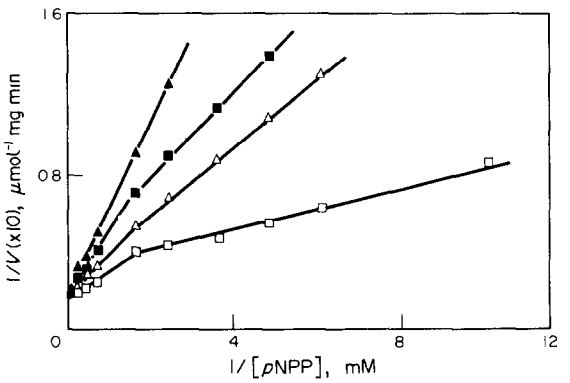

Fig. 1. Double-reciprocal plots of velocity of maize acid phosphatase as a function of *p*-nitrophenylphosphate concentration at pH 5.4. Each tube contained 390 ng of native enzyme. □, △, ■, ▲ represent acid phosphatase velocity without and with 0.2, 0.5 and 0.7 mM Pi, respectively.

Table 1. Summary of some kinetic constants for the enzymatic hydrolysis of *p*-nitrophenylphosphate at pH 5.4 and at 37°

	Km(mM)		v_{max}(μmol mgE min)		K_{cat}/Km(mM sec)		
Pi(mM)	High	Low	High	Low	High	Low	*n*
—	0.9	0.3	65	32	46.7	70.0	0.74
0.2	1.3	0.5	63	35	31.8	44.2	0.80
0.5	2.1	0.9	64	31	20.0	21.9	0.85
0.7	4.2	—	65	—	10.1	—	1.09

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negative cooperativity phenomenon[7]. It can also be observed that increasing concentration of Pi (a competitive inhibitor) progressively abolishes the negative cooperativity until, at 0.7 mM Pi the double-reciprocal plot presents a Michaelian behavior (Fig. 1, Table 1).

Figure 2 shows the double-reciprocal plots of initial velocities at pH 6.7 as a function of the *p*-nitrophenylphosphate concentration. It can be observed that, either in the absence or in the presence of increasing concentration of Pi, the double-reciprocal plots give a straight line and are characterized by a Hill coefficient equal to one (Table 2), which is compatible with Michaelian behavior[7]. These results suggest a kinetic transition from negative cooperativity (at pH 5.4) to Michaelian behavior (at pH 6.7), which could not be explained by site–site interactions from subunits, since we have shown that acid phosphatase from maize scutellum is monomeric[1,8]. A similar kinetic transition from negative cooperativity (at pH 5.4) to Michaelian behavior (at pH 6.7) was also observed for the enzymatic hydrolysis of glucose-6-phosphate (not shown). Furthermore, the MW of the enzyme, determined by molecular exclusion chromatography at pH 5.4, also showed a value of *ca* 65 000.

The plots of residual enzyme activities, at pH 5.4 and 6.7, in a fixed concentration of *p*-nitrophenylphosphate (2.37 mM), against Pi concentration is shown in Fig. 3(a). From these data, a plot of log $(v_o - v_i)/v_i$ vs log Pi concentration (where v_o and v_i are, respectively, the reaction velocities without and with Pi) give straight lines with slopes of 1.0 (Fig. 3b). The same slope was obtained when *p*-nitrophenylphosphate 0.25 mM was assayed against Pi concentration (not shown). The value of these slopes equals the apparent number of Pi bound per enzyme molecule[9].

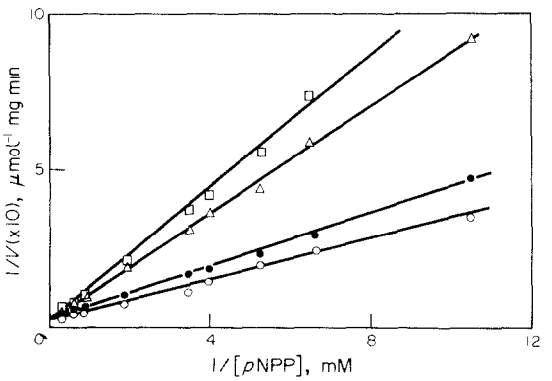

Fig. 2. Double-reciprocal plots of velocity of maize acid phosphatase as a function of *p*-nitrophenylphosphate concentration at pH 6.7. Each tube contained 390 ng of native enzyme. ○, ●, △, □, represent acid phosphatase velocity without and with 0.1, 0.2 and 0.4 mM Pi, respectively.

Taken together, the results presented above suggest that the mechanism which involves interaction between catalytic sites could not be the source of the negative cooperativity phenomenon, since this acid phosphatase is monomeric and probably has only a single catalytic site.

The negative cooperativity is also abolished by lowering the assay pH (at pH 4.8, the *Km* value and Hill coefficient are 1.5×10^{-4} M and 1.0, respectively) and by succinylation of the enzyme molecule (at pH 5.4, the *Km* value and Hill coefficient are 5.0×10^{-4} M and 1.0, respectively).

These results suggest that the source of negative cooperativity at pH 5.4 could be the ionization of the enzyme molecule, which leads to an equilibrium between two forms, resulting in different affinities for *p*-nitrophenylphosphate. These two supposed forms of the enzyme seem to have the same affinity for Pi, since the *Ki* values at pH 5.4 (high, 4.7×10^{-4} M; low, 2.5×10^{-4} M) are quite similar to those obtained at pH 6.7 (4×10^{-4} M). Thus, it could be supposed that the suppression of negative cooperativity by Pi is due to total displacement of the protonated form of the

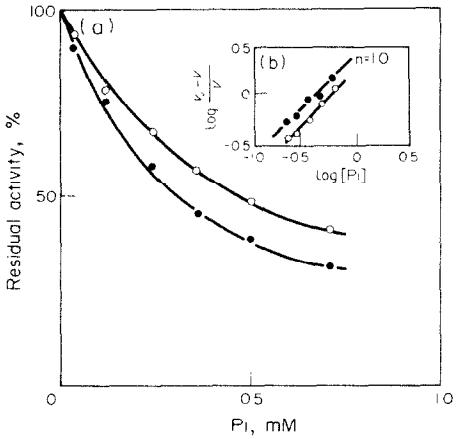

Fig. 3. (a) Plots of % residual enzyme activity vs Pi concentration at pH 5.4 (○) and pH 6.7 (●). (b) Hill plot of log $(v_o - v_i)/v_i$ vs log Pi concentration, where v_o and v_i are the velocities of the enzyme catalysed reaction without and with inhibitior, respectively. ○, ● represent the velocities measured at pH 5.4 and 6.7, respectively. For assay conditions, see Experimental.

Table 2. Summary of some kinetic constants for the enzymatic hydrolysis of *p*-nitrophenylphosphate at pH 6.7 and at 37°

Pi(mM)	*Km*(mM)	v_{max}(μmol mgE min)	K_{cat}/Km(mM sec)	*n*
—	1.6	40	16.4	0.96
0.1	2.6	45	11.4	0.96
0.2	3.6	43	7.8	1.04
0.4	6.1	45	4.8	0.97

enzyme at Pi concentrations greater than 0.6 mM (see Tables 1 and 2). This idea is also supported by the facts that the succinylated enzyme did not show negative cooperativity and that the *Ki* value for Pi at pH 5.4 is quite similar to that found for the native enzyme at pH 6.7. However, we cannot say at present that the equilibrium between protonation–deprotonation of free amino groups of the enzyme molecule is directly responsible for the appearence of negative phenomenon at pH 5.4.

It has already been pointed out that a monomeric enzyme, which exhibits one catalytic site, can possess a non-Michaelian behavior[10–13]. Although the proposition of enzyme ionization, at pH 5.4, implies the existence of a pre-equilibrium between two enzyme forms, this pre-equilibrium can be reduced, in the phenomenological sense, to the proposed "mnemonical" model[13].

EXPERIMENTAL

The following reagents were purchased from the sources indicated: glucose-6-phosphate (monosodium salt), BSA, ovalbumin and trypsinogen were from Sigma. *p*-Nitrophenylphosphate was from Merck. All other chemicals were reagent grade.

Enzyme preparation. Acid phosphatase from germinating maize scutellum was purified as described in ref. [1].

Assay procedures. The enzyme assays were carried out in 0.1 NaOAc buffer (pH 5.4), using 2 ml of 6 mM *p*-nitrophenylphosphate as substrate at 37°. The reaction was stopped by addition of 1 ml of 1 M NaOH, and *p*-nitrophenol *A* was measured at 405 nm ($E = 17{,}800$/mol cm). Glucose-6-phosphate hydrolysis was carried out in 0.1 M NaOAc buffer (pH 5.4) using 2 ml of 30 mM glucose-6-phosphate as substrate and by measuring the Pi liberated by the method of ref. [14]. Incubation was carried out at 37° for 15–30 min and the reaction was terminated by adding 1 ml cold 10% TCA. When the assays were run at pH 6.7, the buffer used was 0.1 M Na maleate. All enzyme activities were measured in duplicate for at least two time intervals. One unit of phosphatase activity is defined as 1 μmol substrate hydrolysed/min. Protein was measured by the method of ref. [15], using BSA as a standard. Succinylation of the enzyme molecule was performed as described in ref. [8]. MW was measured by gel filtration, using a Sephadex G-200 column (2 × 120 cm) as described in ref. [16], equilibrated with 10 mM NaOAc buffer (pH 5.4) containing 10 mM EDTA, at a flow rate of 15 ml/hr (3 ml fractions). BSA, ovalbumin and trypsinogen were used as protein standards.

Kinetic studies. Initial velocities were plotted according to the method described in ref. [17]. Values of apparent inhibition constants (*Ki*) were determined from Dixon plots of $1/v$ vs inhibitor concentration[18] or calculated as $Ki = Km[i]/Km_{app} - Km$. For these plots enzyme was assayed by using fixed substrate concentrations in the range of 0.1 to 2.37 mM and 4 different inhibitor concentrations in the range 0–0.7 mM. The *Ki* values obtained from Dixon plots were in agreement with those values obtained from calculations. Interaction constant (n) was determined by the method of ref. [6]. Kinetic constants given in this paper were obtained from linear-square analysis. When biphasic kinetic was observed, linear-square analysis was performed for each component of the curve (Table 1, low and high *Km* values).

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