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DISSOCIATION AND CATALYTIC ACTIVITY OF PHOSPHATE-REPRESSIBLE ALKALINE PHOSPHATASE FROM *NEUROSPORA CRASSA*

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Key Word Index—*Neurospora crassa*; pH regulation, enzyme secretion, alkaline phosphatase, cooperativity

Abstract—The dimeric form of the mycelial alkaline phosphatase from *Neurospora crassa* synthesized under limiting Pi conditions and at pH 5.4 dissociates into monomers after incubation in 0.2 M Tris–HCl for 40 min at 50°, pH 9. The monomeric enzyme is active and shows a non-Michaelian behaviour for the hydrolysis of *p*-nitrophenylphosphate (PNP-P) at pH 9 ($k_{0.5} = 1.4 \times 10^{-3}$ M, $h_1 = 0.59$ and $h_2 = 2.25$). These two Hill coefficients suggest the existence of an equilibrium between the two monomeric forms of the enzyme, one of which shows positive co-operativity behaviour, the other negative co-operativity for hydrolysis of PNP-P at pH 9. The mycelial enzyme synthesized by the fungus grown under limiting amounts of Pi and at pH 7.8 is predominantly monomeric at alkaline pH and 4°. This monomeric enzyme shows positive co-operativity for the hydrolysis of PNP-P at pH 9 ($k_{0.5} = 9.3 \times 10^{-4}$ M, $h = 2.16$). Negative co-operativity ($k_{0.5} = 2.2 \times 10^{-4}$M, $h = 0.58$) is only observed after incubation of the enzyme for 40 min at 50°, pH 9.

INTRODUCTION

Neurospora crassa synthesizes in its enzymatically active form an alkaline phosphatase and other phosphorus-repressible enzymes and permeases in response to signals of the absence of inorganic phosphate in the medium (reviewed in ref. [1]). In addition to phosphate starvation, the pH of the growth medium controls the secretion of this enzyme, that is, alkaline phosphatase is retained by the mycelium or is secreted when the pH of the medium is 5.4 or 8, respectively [2]. This regulatory mechanism, which ensures that extracellular enzymes are secreted only at pH values at which they can function effectively, is dependent on the glycosylation of the enzyme molecule [3]. It was also shown that, besides being excreted at alkaline pH, alkaline phosphatase is produced by sucrose-grown mycelium at a rate at least four times higher at pH 8 than that at pH 5.4 [2]. Because the *pho*-2 mutant, which represents this structural locus [4, 5], does not produce and secrete this enzyme at any pH, we considered these activities expressed at different pH to be the product of the same gene [2]. However, this does not mean *a priori* that the number of copies of the enzyme molecule synthesized at alkaline pH is larger than the number of copies synthesized at acid pH and retained by the mycelium. Even though the structural and steady state kinetics analysis of alkaline phosphatase is not sufficient *per se* to elucidate the mechanism of alkaline phosphatase excretion, this type of study may reveal a few important aspects. With this in mind we purified alkaline phosphatase from mycelium grown on medium supplemented with sucrose and buffered at pH 7.8 and compared its properties with those of the enzyme purified from mycelium grown at pH 5.4. Our results show that the dimeric form of the alkaline phosphatase synthesized at pH 5.4 dissociates into monomers after incubation at 50° for 40 min, pH 9. This dissociation is accompanied by a pronounced increase in the catalytic activity of alkaline phosphatase. The mycelial enzyme synthesized at pH 7.8 is predominantly monomeric at alkaline pH and at 4°.

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RESULTS AND DISCUSSION

The purification procedure described in this paper provided optimal conditions for the purification of mycelial alkaline phosphatase synthesized by the wild-type strain 74A of *N. crassa* when the fungus was grown at pH 5.4 or 7.8. All enzyme preparations (at least five independent preparations from each growth pH) appeared homogeneous by 7.5% PAGE at pH 8.3, with the protein band being superimposable on alkaline phosphatase activity (all of these enzyme preparations also showed essentially the same electrophoretic mobility). The electrophoretic analysis performed after each step of the purification procedure also showed that the protein population synthesized at pH 5.4 is not the same as that synthesized at pH 7.8, clearly showing that the effect of pH on protein synthesis is not limited to acid and alkaline phosphatases. Overall the enzyme obtained from mycelium grown at pH 5.4 and 7.8 was purified 18-fold with a yield of 12.5% (sp. act. 18 units/mg) and 17-fold with a yield of 13% (sp. act. 76 units/mg), respectively.

When the crude extract from mycelium grown at pH 5.4 under limiting phosphate conditions is incubated in 0.2 M Tris-HCl buffer at 50°, pH 9, alkaline phosphatase activity increases slowly and progressively, reaching 100% after 40 min of incubation (Fig. 1). This activation is faster and higher (*ca* 500% after 40 min) when the

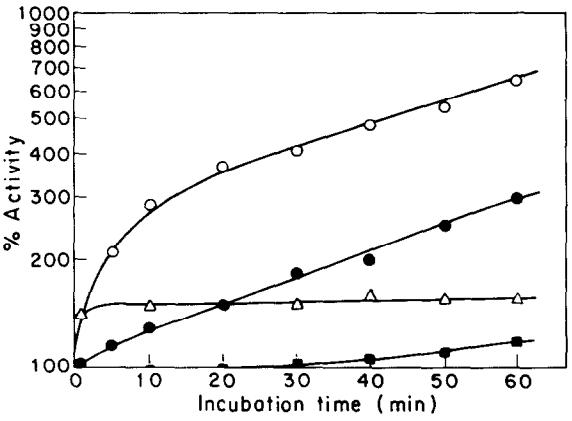

Fig. 1. Thermal activation of repressible intracellular alkaline phosphatase at 50° and pH 9. ●, ■ Represent the crude extract from mycelium grown at pH 5.4 and pH 7.8, respectively; ○, △ represent the purified enzyme obtained from mycelium grown at pH 5.4 and pH 7.8, respectively. For details see Experimental.

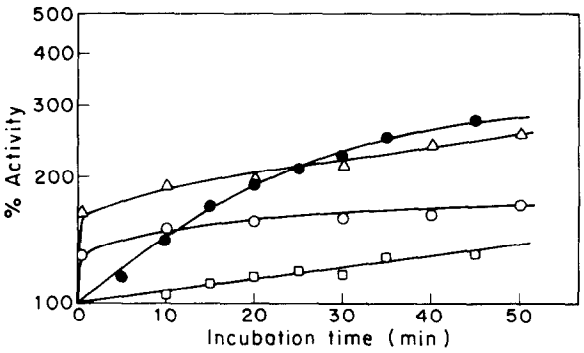

Fig. 2. Thermal activation of repressible alkaline phosphatase at 50° and at pH 9.0. □, ● Represent the enzyme purified from mycelium grown at pH 5.4, each tube containing 10 and 35 μg of protein, respectively. ○, △ represent the enzyme purified from mycelium grown at pH 7.8, each tube containing 10 and 24 μg of protein, respectively.

electrophoretically homogeneous enzyme is incubated under the same experimental conditions. Two distinct phases can be observed, the first taking the form of a burst and the second apparently linear and much slower. After *ca* 2 hr of incubation, enzyme inactivation starts and a further incubation of at least 4 hr is needed for the remaining activity to reach the initial value (data not shown). Heat activation is also undetectable (Fig. 2) when the crude extract obtained from mycelium grown at pH 7.8 under non-saturating phosphate conditions is incubated at 50°, pH 9 (Fig. 1). Only a small level of activation (*ca* 50%) is observed when the purified enzyme is incubated even when higher concentrations are assayed Fig. 2).

The neutral sugar content of the alkaline phosphatase purified from mycelium grown on sucrose buffered at pH 7.8 was *ca* 21%, a value similar to those previously reported for the enzymes secreted by the wild type strain grown on acetate [3] and by 'slime' cells [6].

The M_r of the alkaline phosphatase synthesized at pH 5.4 was *ca* 165 000 as determined by exclusion chromatography at pH 5 or at pH 9 before preincubation of the purified enzyme at pH 9 and *ca* 81 000 as determined after incubation of the purified enzyme for 40 min at 50° at pH 9. This result clearly shows that the monomeric form of the alkaline phosphatase synthesized at pH 5.4 is active and that its dissociation leads to a marked increase in catalytic activity. It is also clear that the dimeric form is stable when chromatographed on Sephadex G-200, pH 9 and at 4°. The M_r of the alkaline phosphatase synthesized at pH 7.8 was *ca* 82 000 as determined at pH 9 without any preincubation of the purified enzyme. The dimeric form is obtained only if the purification steps are carried out at acidic pH. Even so, spontaneous dissociation occurs at pH 5.4 when the enzyme is stored overnight at 4°. As previously shown [3], the dimeric form of alkaline phosphatase excreted into the culture medium is stable at pH 5 and at 4°. However, monomerization occurs spontaneously at pH 9. These results indicate that the transport of the enzyme synthesized by the mycelium grown at pH 9 through the plasma membrane may be facilitated by

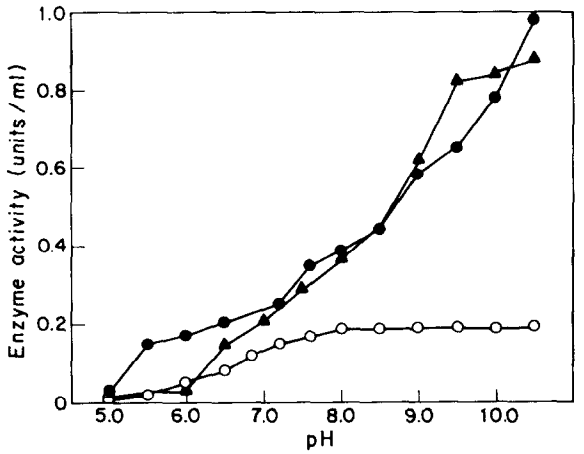

Fig. 3. pH Activity profile (PNP-P as substrate) of repressible alkaline phosphatase. ○, ● Represent the enzyme purified from mycelium grown at pH 5.4 before and after incubation at pH 9 and at 50°, respectively. ▲ Represents the enzyme purified from mycelium grown at pH 7.8 without preincubation at pH 9 and at 50°.

monomerization. Furthermore, catalytic activity of the monomeric form accounts for the increase in enzyme activity detected in the crude extract of fungal mycelium grown on medium supplemented with sucrose as a carbon source and buffered at pH 7.8 [2], suggesting in turn that the number of copies of the enzyme molecule synthesized is the same regardless of pH of the growth medium.

Dissociation of the alkaline phosphatase molecule alters the pH activity profile of the enzyme, showing no apparent optimum in the alkaline pH range (Fig. 3). Because the mycelial enzyme synthesized at pH 7.8 showed no reassociation when the M_r was determined at pH 5, it seems clear that the steady increase in activity even up to pH 10.5 is characteristic of the monomer. Thus, during the assay at 37°, pH 9, and at very low concentration, the mycelial alkaline phosphatase excreted into the culture medium appears to dissociate

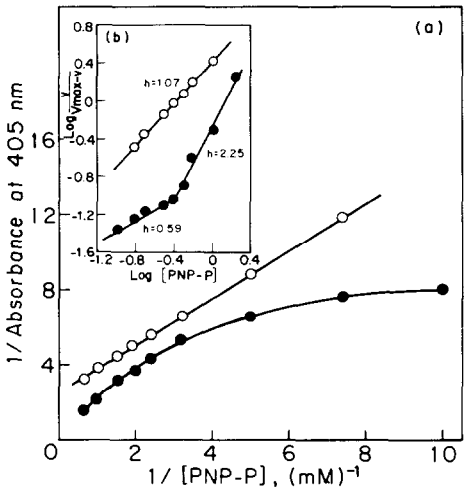

Fig. 4. (a) Double-reciprocal plots of velocity of repressible alkaline phosphatase purified from mycelium grown at pH 5.4, as a function of PNP-P concentration at pH 9. ○, ● Represent the enzyme before and after incubation at pH 9 and at 50°, respectively. (b) Hill plots of log $v/(V_{max}-v)$ vs log [PNP-P] concentration. ○, ● represent the enzyme before and after incubation at pH 9 and at 50°, respectively.

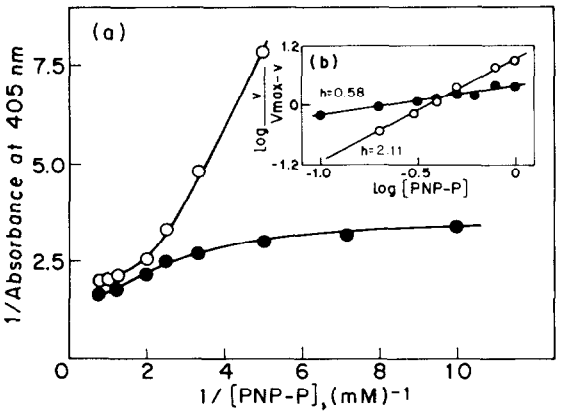

Fig. 5. (a) Double-reciprocal plots of velocity of repressible alkaline phosphatase purified from mycelium grown at pH 7.8, as a function of PNP-P concentration at pH 9. ○, ● Represent the enzyme before and after incubation at pH 9 and at 50°, respectively. (b) Hill plots of log $v/(V_{max}-v)$ vs log [PNP-P] concentration. ○, ● Represent the enzyme before and after incubation at pH 9 and at 50°, respectively.

promptly [3]. Dissociation of the enzyme also changes its steady state kinetic properties (Fig. 4). Alkaline phosphatase synthesized at pH 5.4 is of the Michaelian type when assayed in the dimeric form ($K_m = 3.4 \times 10^{-4}$ M; $h = 1.07$) and deviates from Michaelian behaviour when assayed after dissociation ($K_{0.5} = 1.4 \times 10^{-3}$ M; $h_1 = 0.59$ and $h_2 = 2.25$). This kinetic behaviour, which is possible in monomeric enzymes [7, 8], seems to reflect the existence of two monomeric forms, one of which shows negative co-operativity and the other positive co-operativity, or the existence of a kinetic transition from negative co-operativity to positive co-operativity as a function of increasing substrate concentration. When the steady state kinetic behaviour of mycelial alkaline phosphatase synthesized at pH 7.8 is analysed, it can be seen that the enzyme, freshly prepared and probably in the monomeric state during the enzymatic assay at pH 9 and at 37°, shows positive co-operativity ($K_{0.5} = 9.3 \times 10^{-4}$ M; $h = 2.16$) (Fig. 5). Negative co-operativity ($K_{0.5} = 2.2 \times 10^{-4}$ M; $h = 0.58$) is only observed after incubation at 50° for 40 min, pH 9. These results are essentially identical to those obtained for the enzyme synthesized at pH 5.4 after incubation at pH 9 (Fig. 4), suggesting the existence of an equilibrium between forms when the activity of the enzyme synthesized at pH 5.4 is monomerized and assayed at pH 9. Indeed, the heat activation consists of two phases (Fig. 1), the first (burst) possibly corresponding to the dissociation of alkaline phosphatase synthesized at pH 5.4, and the second (apparently linear) possibly corresponding to a conformational variation of this monomeric form during prolonged incubation at 50° and pH 9.

EXPERIMENTAL

Strain and growth conditions. The wild-type strain St. L. 74A was obtained from the Fungal Genetic Stock Center, University of Kansas Medical Center, Kansas city, Kansas. Stock cultures were maintained on slants of Vogel's medium [9]. Conidial suspension (0.5 ml) of this strain containing *ca* 10^8 cells/ml was grown for 72 hr at 30° without shaking on 50 ml of low-phosphate medium supplemented with 44 mM sucrose as carbon source, adjusted to pH 5.4 (non-buffered) or pH 7.8 (buffered with 50 mM Tris–HCl) [2], and prepd as described in ref. [10].

Assay procedures. Mycelium was harvested by filtration and extracted with sand and 50 mM NaOAc buffer, pH 5 (crude extract), as described in ref. [11]. Under these extraction conditions, constitutive alkaline phosphatase is essentially destroyed [12]. Enzyme assays as described in ref. [3], were carried out in 0.3 M glycine buffer (pH 9), containing 1 mM EDTA, using 2 ml 6 mM *p*-nitrophenylphosphate (PNP-P). One unit of acid phosphatase is defined as 1 μmol substrate hydrolysed/min. Sp. acts were expressed as units/mg. The buffers used to cover the required pH range were 0.1 M HOAc–NaOH (pH 5–6), 0.1 M maleate (pH 6–7.2), 0.1 M Tris–HCl (pH 7.2–9) and 0.1 M glycine–NaOH (pH 9–10.5), all containing 1 mM EDTA. Protein was measured by a modification of the Lowry method as described in ref. [13] using bovine serum albumin as std. Neutral sugars were measured by the method described in ref. [14] with glucose as std. M_r was determined by gel filtration as described in ref. [15] using a Sephadex G-200 column (1.2 × 110 cm), equilibrated and eluted with 10 mM NaOAc buffer (pH 5) containing 0.1 M NaCl at a flow rate of 8.8 ml/hr (2.4 ml frs) or with 10 mM Tris–HCl buffer (pH 9) containing 0.1 M NaCl, at a flow rate of 9 ml/hr (2.6 ml frs). Yeast alcohol dehydrogenase, bovine serum albumin, ovalbumin and α-chymotrypsinogen A were used as protein stds. Disc electrophoresis was carried out on 7.5% acrylamide gel in Tris–glycine buffer (0.05 and 0.3 M, respectively), pH 8.3, by the Davis procedure as described in ref. [3]. Enzyme activity bands were developed by the method of ref. [16] as described in ref. [3].

Kinetic studies. Maximum velocities (V_{max}) and Michaelis constants (K_m) were determined by plotting initial velocities as described in ref. [17]. The interaction constant for the substrate (h) was determined by the Hill procedure as described in ref. [18]. All kinetic constants were obtained by linear-square analysis,

calculated from the data obtained in three independent experiments.

Thermal activation. An appropriate amount of alkaline phosphatase was incubated at 50° in a final vol. of 3 ml of 0.2 M Tris–HCl buffer, pH 9 as described in ref. [19]. At appropriate times, samples were taken to measure the enzyme activity as described under assay procedures.

Enzyme purification. All purification steps were carried out at 4°. Electrophoretic analysis on 7.5% acrylamide gel was carried out after each step as described under assay procedures. Mycelium extract (crude extract) of the wild-type strain grown at pH 5.4 or pH 7.8 was fractionated by $(NH_4)_2SO_4$ pptn and the alkaline phosphatase activity recovered in the 60–95% salt satn was suspended in a small vol. of 10 mM NaOAc, pH 5.2, and dialysed for 16 hr against 8 l of the same buffer (3 changes). Dialysed enzyme was centrifuged and the supernatant applied to a column (2.2 × 30 cm) of DEAE-cellulose previously equilibrated with the buffer used for dialysis. Enzyme elution was performed with the same buffer at a flow rate of 35 ml/hr (3.5 ml frs). Frs containing alkaline phosphatase activity were pooled, concd by ultrafiltration and solid NaCl at a final concn of 0.1 M added to this enzyme fr. The fr. was then chromatographed on a Sephadex G-200 column (2.2 × 116 cm), previously equilibrated with 10 mM NaOAc, pH 5.2, containing 0.1 M NaCl, at a flow rate of 16 ml/hr (3.5-ml frs). The fr. containing alkaline phosphatase was concd by ultrafiltration, dialysed or not against a desired buffer, and stored at 4°.

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