Genet. Res., Camb. (1985), **45**, *pp.* 239–249
With 6 text-figures
Printed in Great Britain

Effect of phosphate levels on the synthesis of acid phosphatases (EC 3.1.3.2) in *Neurospora crassa*

BY SERGIO A. RODRIGUES AND ANTONIO ROSSI*

Departamento de Química, FFCLRP-USP 14.100 Ribeirão Preto, SP, Brazil

(*Received 30 November 1984 and in revised form 17 January 1985*)

SUMMARY

When grown on high-phosphate medium, the wild-type strain 74A of *N. crassa* synthesized two acid phosphatases, as shown by DEAE – cellulose chromatography. These purified enzymes showed heterogeneity on PAGE, low specific activities towards PNP-P, molecular weight values of at least 300000, no deviation from Michaelian behaviour, and great stability in 50 mM sodium acetate buffer, at pH 5·4, when kept at 54 °C. These acid phosphatases were synthesized in reduced amounts or not at all when the mould was grown under conditions of phosphate starvation, indicating that the level of phosphate also regulates the synthesis of the high molecular weight enzyme forms. When grown on high phosphate medium, the *pho-3* mutant strain also synthesized two acid phosphatases, whose purified enzymes showed no pronounced differences when compared to those synthesized by the wild-type strain in terms of electrophoretic analysis, specific activities towards PNP-P, molecular weight values, and Michaelian behaviour. However, one enzyme form had a higher K_m value and a lower heat stability than the corresponding enzyme of the wild-type strain. Even though the *pho-3* locus might not be responsible for an alteration in the primary structure of the repressible acid phosphatase, it seems clear that the enzymes synthesized by the mould grown on low- or high-phosphate medium must share some structural features. Thus, the drastic differences observed in the molecular properties of the enzymes synthesized by the mould grown under conditions of phosphate starvation as opposed to phosphate repression might be due to an effect exerted by the level of inorganic phosphate in regulating the translation, post-translational modifications and/or excretion, but not necessarily the gene-directed synthesis of distinct mRNAs.

1. INTRODUCTION

During phosphate starvation or growth on limiting amounts of inorganic phosphate, the mould *Neurospora crassa* derepresses the synthesis of an acid phosphatase and a number of related enzymes which are reported to be controlled at the transcriptional level (Metzenberg, 1979) by the same regulatory circuit. This regulation model, developed from genetic studies, postulates that the first factor involved in the repression of the gene-directed synthesis of mRNA, mediated by

* To whom correspondence should be addressed.

at least three genes, is the high phosphate level or a co-repressor derived from it (Littlewood, Chia & Metzenberg, 1975; Metzenberg & Chia, 1979; Free & Metzenberg, 1982). Furthermore, it has also been reported that, for a specified carbon source, the production and secretion of the repressible acid and alkaline phosphatases are dependent on the pH of the growth medium (Nahas, Terenzi & Rossi, 1982; Nahas & Rossi, 1984). Since the repressible acid phosphatase is the only enzyme that expresses phosphodiesterase activity (Nyc, 1967), all of the acid phosphatase activity detected when the mould is grown on high phosphate medium (Kuo & Blumenthal, 1961) is considered to be constitutively synthesized. Thus, the expression of the different structural genes coding for the constitutive and repressible acid phosphatases must be under the control of different mechanisms.

In this paper we report that the wild-type strain 74A of *N. crassa* synthesizes two acid phosphatases when grown on saturating concentrations of inorganic phosphate. These two enzymes were synthesized in reduced amounts or not at all when the mould was grown under conditions of phosphate starvation. The *pho-3* mutant strain, which has been shown to carry a mutation in the structural gene for the repressible acid phosphatase (Nelson, Lehman & Metzenberg, 1976), also showed a similar pattern of acid phosphatase production under conditions of phosphate repression except that one enzyme form had an increased K_m for the hydrolysis of PNP-P and reduced heat stability when these properties were compared to those of the enzyme synthesized by the wild-type strain.

2. MATERIAL AND METHODS

(i) *Chemicals*

Disodium 4-nitrophenyl orthophosphate (PNP-P) and bis-(4-nitrophenyl) phosphate free acid (bis-PNP-P) were purchased from Merck and Sigma, respectively. All other chemicals were of analytical reagent grade.

(ii) *Strains and culture conditions*

The wild-type strain St. L. 74A and the *pho-3A* mutant (FGSC 3051) used in this study were obtained from the Fungal Genetic Stock Center, Humboldt University, Arcata, California. Stock cultures were maintained on slants of semi-solid Vogel's (1956) medium. Conidia from each strain were grown for 72 h, at 30 °C and without shaking, on high or low phosphate medium (respectively 5 mM or 50 μM phosphate) adjusted to pH 5·6, supplemented with 44 mM sucrose as carbon source and prepared as previously described (Nahas *et al.* 1982; Crocken & Nyc, 1963).

(iii) *Preparation of cell extracts and assay procedures.*

The harvested mycelium was extracted with sand and 50 μM sodium acetate buffer, pH 5·0 (7·5 ml buffer (g mycelium)$^{-1}$). Extracts were centrifuged for 20 min at 20000 ***g***, at 4 °C, and the supernatant was used for enzyme assay (crude extract). Under these extraction conditions, constitutive alkaline phosphatase was practically destroyed (Nyc, 1967). In addition, under the assay conditions used, it could not have been detected because it is inactive at pH 5·4 (Kuo & Blumenthal,

1961). The acid phosphatase assay was carried out as previously described (Nahas & Rossi, 1984), using 2 ml 6 mM PNP-P or 4 mM bis-PNP-P as substrates. One unit of phosphatase activity was defined as 1 μM substrate hydrolysed min^{-1}. Specific activities were expressed as units $(mg\ protein)^{-1}$. Protein was measured by the Lowry method, as modified by Hartree (Hartree, 1972), using bovine serum albumin as standard. Disc electrophoresis was carried out on 7·5% acrylamide gel as previously described (Davis, 1964). Molecular weights were measured by gel filtration (Andrews, 1964) using a Sephadex G-200 column (1·5 × 134 cm), equilibrated and eluted with 50 mM sodium acetate buffer, pH 5·0, at a flow rate of 10·5 ml h^{-1} (3 ml fractions). Yeast alcohol dehydrogenase, bovine serum albumin and α-chymotrypsinogen A were used as protein standards. The relative heat stability was determined by incubating the enzyme at 54 °C, in a final volume of 3 ml 50 mM sodium acetate buffer, pH 5·4. At appropriate times, samples were taken to measure the residual phosphatase activity. Maximum velocity (V_{max}) and Michaelis constant (K_m) were determined as Lineweaver and Burk plots (Lineweaver & Burk, 1934). The apparent inhibitor constant for P_i was determined as described by Dixon (1953) and the value obtained was compared to that calculated as $K_i = K_m\ (i)/Km_{app}\text{-}K_m$. Interaction constants for substrate (n) and P_i (n'), which equals the apparent number of P_i bound per enzyme molecule (Atkinson, Hathaway & Smith, 1965), were determined by the Hill procedure as described by Koshland (Koshland, 1970). All kinetic constants given in this paper were obtained from linear-square analysis.

(iv) *Enzyme fractionation*

All purification steps were carried out at 0–4 °C. PNP-P and bis-PNP-P phosphatase activities were determined at each stage of purification. The supernatant of the centrifuged extract was fractionated by ammonium sulphate precipitation and the phosphatase activity was recovered in the 35–97% ammonium sulphate saturation fraction. The precipitate was suspended in a minimal volume of 10 mM-Tris. HCl buffer, pH 7·4, and dialysed for 14 h (with 3 changes of the buffer) against 8 l of this buffer. This dialysed fraction was centrifuged for 20 min at 20000 ***g*** and the supernatant was applied to a column (2·1 × 40 cm) of DEAE-cellulose (Kuo & Blumenthal, 1961), previously equilibrated with the buffer used for dialysis. The unabsorbed proteins were eluted with 800 ml of the equilibrating buffer, at a flow rate of about 100 ml h^{-1}, with 50 ml fractions being collected. The absorbed proteins were then eluted with a gradient of acetate buffer concentration, where the mixing chamber contained 1 litre of 10 mM sodium acetate buffer, at pH 5·4, and the reservoir bottle contained 1 litre of 300 mM acetate buffer at the same pH. Elution was performed at a flow rate of about 70 ml h^{-1}, with 6 ml fractions being collected.

In an independent experiment (3 replicates), the precipitate recovered in the 35–97% ammonium sulphate saturation fraction was suspended in a minimal volume of 50 mM sodium acetate buffer, pH 5·0, and then dialysed for 18 h (with 3 changes of buffer) against 10 l of this buffer. This dialysed fraction was centrifuged for 20 min at 20000 ***g*** and the supernatant was applied to a Sephadex G-100 column (2·1 cm × 138 cm) previously equilibrated with buffer used for

dialysis (Nyc, 1967). Elution was performed with this buffer at a flow rate of 10 ml per hour with 3 ml fractions being collected.

3. RESULTS AND DISCUSSION

When grown on high phosphate medium, the wild-type strain 74A of *Neurospora crassa* synthesized two acid phosphatases as shown by the profile of elution from the DEAE-cellulose column with a gradient of acetate buffer concentration (Fig. 1). These two enzymes showed heterogeneity on 7·5 % PAGE at pH 8·3, very

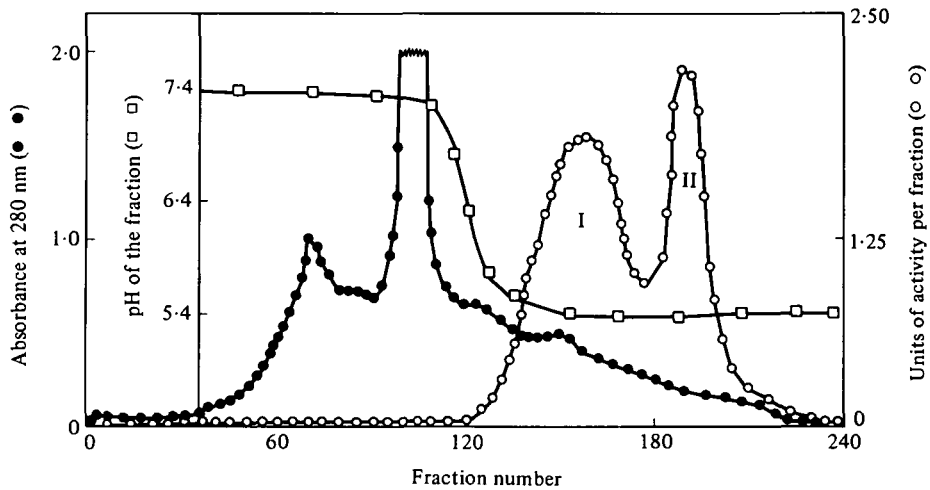

Fig. 1. Chromatography on DEAE-cellulose of acid phosphatases synthesized by the wild-type strain 74A of *N. crassa* (155 units) grown for 72 h by standing in high phosphate medium at 30 °C. The enzymes, purified through ammonium sulphate fractionation, were applied to the column (146 units) and the unabsorbed proteins were eluted with 800 ml of the equilibrating buffer (not represented in the figure). The column was then eluted with a gradient of acetate buffer concentration as described in Material and Methods (flow rate of 60 ml h^{-1} with 6 ml fraction being collected). ●, absorbance at 280 nm and ○, enzyme activity over PNP-P.

low specific activities over PNP-P (about 1·1 and 1·6 units . mg^{-1} for enzymes I and II, respectively) as compared to that described earlier (Kuo & Blumenthal, 1961), and Molecular Weight values of at least 300000 when determined by exclusion chromatography on Sephadex G-200 columns (Nyc, 1967). The double reciprocal plots of initial velocities, at pH 5·4, as a function of PNP-P concentration gave straight lines and Hill coefficients equal to one for both enzymes, indicating no deviation from Michaelian behaviour (Levitzki & Koshland, 1969). The K_m values for this substrate were $4{\cdot}7 \times 10^{-4}$ M ($n = 0{\cdot}97$) and $4{\cdot}2 \times 10^{-4}$ M ($n = 0{\cdot}98$) for enzymes I and II, respectively. Furthermore, these two enzymes showed great stability in 50 mM sodium acetate buffer, at pH 5·4, when heated at 54 °C, despite the fact that the time course for the inactivation of enzyme II did not follow simple first-order kinetics (Fig. 2). The unexpected K_m values and heat stability for these

enzymes could be explained by the low degree of enzyme homogeneity or by the growth of the mould under different physiological conditions (Kuo & Blumenthal, 1961). Activity corresponding to repressible acid phosphatase (mycelial bound and with activity on bis-PNP-P) was eluted from the DEAE-cellulose column with the equilibrating buffer in contrast to the two enzymes synthesized by the mould

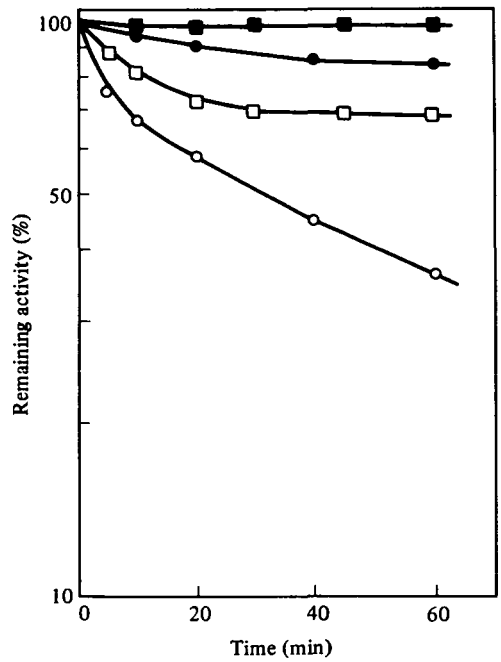

Fig. 2. Thermal stability of acid phosphatases synthesized by the wild-type and *pho-3* mutant strains grown for 72 h by standing in high phosphate medium at 30 °C. Each enzyme fraction, partially purified through chromatography on DEAE-cellulose (see Fig. 1), was incubated at 54 °C, pH 5·4, and the remaining enzyme activity was assayed with PNP-P as substrate at 37 °C. ■, enzyme I synthesized by the wild-type strain; ●, enzyme I′ synthesized by the *pho-3* mutant strain; □, enzyme II synthesized by the wild-type strain and ○, enzyme II′ synthesized by the *pho-3* mutant strain.

grown on high-phosphate medium (for details, see Material and Methods). Thus, if enzymes I and II were constitutive, i.e. if they were also synthesized by the mould when grown on limiting amounts of inorganic phosphate, their elution with a gradient of acetate buffer concentration should have a profile similar to that shown in Fig. 1. Fraction I, however, was practically suppressed (Fig. 3) and a new fraction was identified (I_A). Furthermore, the recovery in units of the fraction II was significantly lower (about three-fold less) than that observed for the enzyme fraction II (Fig. 1) synthesized by the mould grown on high phosphate medium. Because the enzyme fraction II_A showed lowered heat stability (Fig. 4), we can consider it to be a new molecular specie or to have undergone structural modifications during synthesis when the mould was grown on low-phosphate medium. It can also be seen that the heat stability of fraction I_A was similar to

https://doi.org/10.1017/S0016672300022230 Published online by Cambridge University Press

that of fraction I and that the fraction eluted from the column with the equilibrating buffer was heat-activated under the experimental conditions used.

The acid phosphatases considered to be constitutive and repressible can also be fractionated by molecular exclusion chromatography (Nyc, 1967). As expected (not shown), acid phosphatases I and II were eluted as a single fraction (approximately 80–90 % of the enzyme activity units added to the column were recovered in the eluate) and the molecular weight of the repressible enzyme was about 47000, as determined by exclusion chromatography on Sephadex G-200 (Nyc, 1967). It

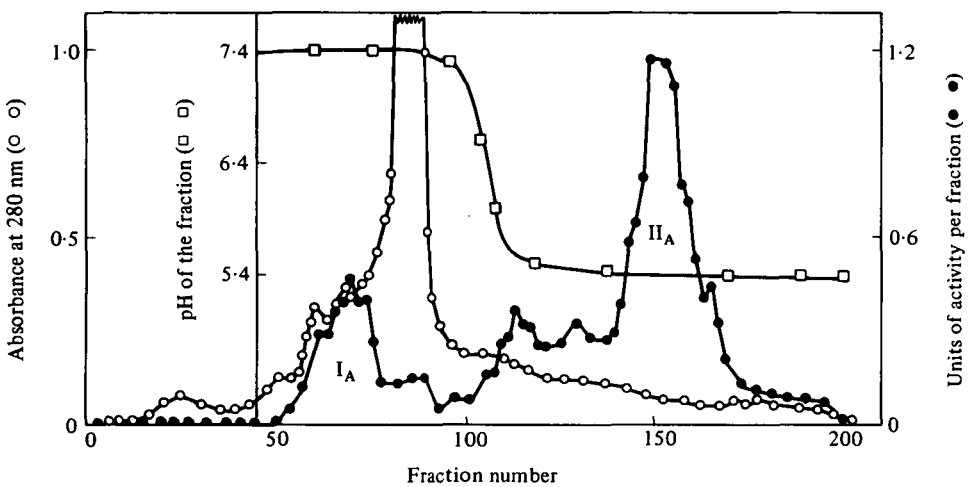

Fig 3. Chromatography on DEAE-cellulose of acid phosphatases synthesized by the wild-type strain 74A of *N. crassa* (78 units) grown for 72 h by standing in low phosphate medium at 30 °C. The enzymes, purified through ammonium sulphate fractionation, were applied to the column (58 units) and the unabsorbed proteins and the repressible acid phosphatase were eluted with 800 ml of the equilibrating buffer (not represented in the figure). The column was then eluted with a gradient of acetate buffer concentration as described in Material and Methods (flow rate of 64 ml h^{-1} with 7 ml fractions collected). Enzyme activity in each fraction was assayed with PNP-P and bis-PNP-P as substrates. Only the enzyme fraction eluted with the equilibrating buffer shows bis-PNP-Pase activity. ○, absorbance at 280 nm and ●, enzyme activity over PNP-P.

seems that fraction 1 (Fig. 5) corresponds to the acid phosphatases synthesized by the mould when grown on high-phosphate medium, because, in addition to having a high molecular weight, they do not hydrolyse bis-PNP-P. However, recovery of fraction 1, measured in units, was about 8 % of the total amount submitted to chromatography. If the enzyme fractions retained by DEAE-cellulose (Fig. 3) consisted exclusively of high-molecular-weight molecules, this value should have been at least twice as high. In addition, 30 % heat activation was observed for this fraction (Fig. 6), indicating that, although its molecular weight is high, it is eluted from the DEAE-cellulose column with the equilibrating buffer (Fig. 3). Fraction 2 practically showed the same heat lability (Fig. 6) when residual phosphatase activity, was measured on PNP-P (24 min half-life) or on bis-PNP-P

(19 min half-life). It seems evident that the deviation from 1st order kinetics shown in the crude extract for PNP-P hydrolysis (Fig. 6) is due to molecular forms that do not hydrolyse bis-PNP-P. The hydrolytic activity of fraction 2 on both PNP-P and bis-PNP-P was unaffected by 10 mM-$CaCl_2$ or 5 mM-$MgCl_2$, even though the hydrolysis of both substrates was inhibited by $CuSO_4$ and Pi. This inhibitory effect was greater when hydrolytic activity was measured on PNP-P. This enzyme

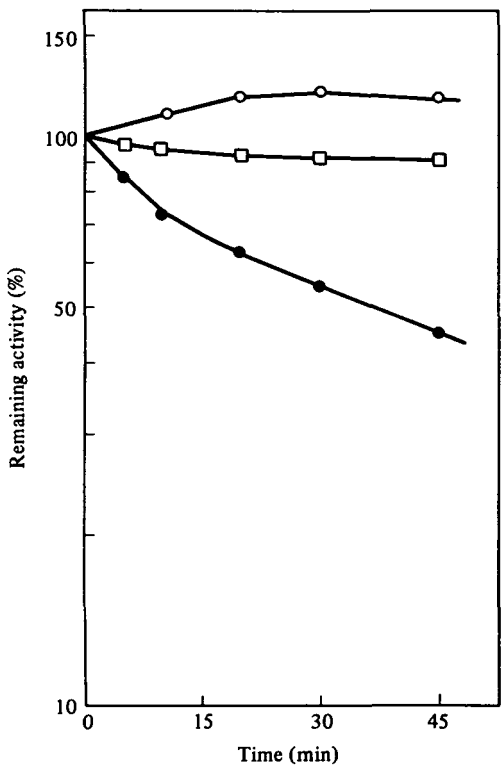

Fig 4. Thermal stability of acid phosphatases synthesized by the wild-type strain grown for 72 h by standing in low phosphate medium at 30 °C. Each enzyme fraction, partially purified through chromatography on DEAE-cellulose, was incubated at 54 °C, pH 5·4, and the remaining enzyme activity was assayed with PNP-P as substrate at 37 °C. ○, enzyme fraction eluted with the equilibrating buffer; □, enzyme I_A and ●, enzyme II_A. For details see Material and Methods and Fig. 3.

fraction showed no deviation from Michaelian behaviour (Levitzki & Koshland, 1969) for PNP-P or bis-PNP-P hydrolysis, with the respective K_m values being $1{\cdot}6 \times 10^{-4}$ M ($n = 1{\cdot}04$) and $1{\cdot}3 \times 10^{-3}$ M ($n = 0{\cdot}96$) when determined at pH 5·4. The same K_i value at pH 5·4 ($3{\cdot}0 \times 10^{-4}$ M) was obtained for the P_i inhibition of the hydrolytic activity toward PNP-P or bis-PNP-P. The same interaction constant value was also obtained for inorganic phosphate when the inhibition was measured toward 2 mM PNP-P ($n' = 1{\cdot}04$) or 2·5 mM bis-PNP-P ($n' = 1{\cdot}02$) at pH 5·4. These results indicate that the hydrolysis of PNP-P and bis-PNP-P can be catalysed by the same active site of the enzyme.

https://doi.org/10.1017/S0016672300022230 Published online by Cambridge University Press

These results clearly indicate that in cultures supplemented with limiting inorganic phosphate concentrations the mould does not synthesize, or only synthesizes reduced amounts of, the high-molecular weight enzyme forms. Under these culture conditions, in addition to stimulating the synthesis of reasonable amounts of a low-molecular-weight enzyme, the molecular changes at the translational and/or post-translational level needed for enzyme excretion lead to a

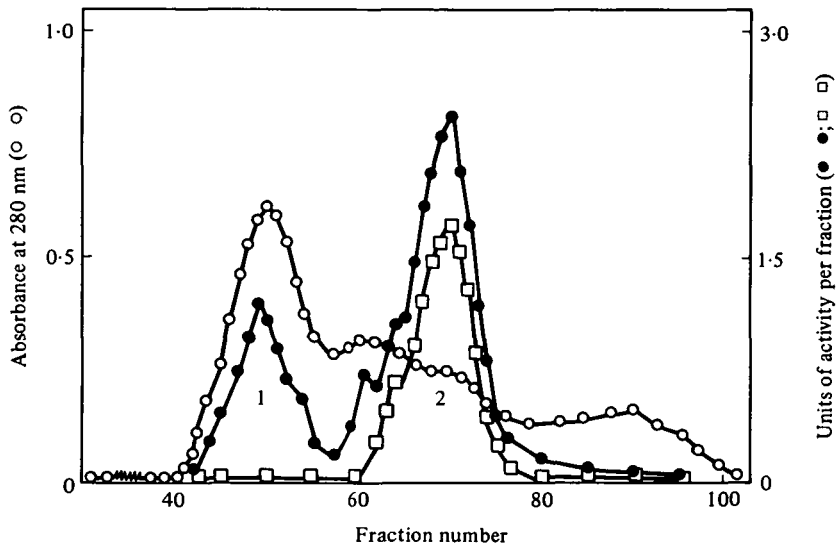

Fig. 5. Chromatography on Sephadex G-100 of acid phosphatases synthesized by the wild-type strain 74A of *N. crassa* (55 units) grown for 72 h by standing in low phosphate medium at 30 °C. The enzymes, purified through ammonium sulphate fractionation, were applied to the column (40 units) and the proteins eluted with 50 mM sodium acetate buffer, pH 5·0 (flow rate of 10 ml per hour with 3 ml fractions being collected). ○, absorbance at 280 nm; ●, enzyme activity towards PNP-P and □, enzyme activity towards bis-PNP-P.

homodimeric form (Jacobs, Nyc & Brown, 1971). We may postulate that, although regulated by the extracellular P_i level, the synthesis of the stored or excreted enzyme forms (which may represent distinct primary structures) may be turned on through independent regulatory circuits (Metzenberg, 1979; Oshima, 1982). However, results obtained with the mutant strain *pho-3* seem to indicate a different pathway. This strain has been characterized as carrying a mutation at the locus that specifies the primary structure of the acid phosphatase considered repressible (Nelson *et al.* 1976), since it synthesizes small amounts of an enzyme that, in addition to having an increased Michaelis constant for bis-PNP-P hydrolysis, is more heat labile than the enzyme synthesized by the wild type strain. When grown on high-phosphate medium, the mutant strain *pho-3* also synthesized two acid phosphatases which could be easily identified by elution on DEAE-cellulose with gradient of acetate buffer concentration, whose profile was identical to that observed for the wild-type strain (Fig. 1). These enzyme fractions, designated I′

and II′, showed no pronounced differences when compared to the enzymes synthesized by the wild-type strain in terms of electrophoretic analysis, specific activity towards PNP-P and molecular weight. No deviation from Michaelian behaviour was observed for PNP-P hydrolysis (Levitzki & Koshland, 1969), and the K_m values obtained for this substrate ($3{\cdot}7 \times 10^{-4}$ M ($n = 0{\cdot}96$) and $8{\cdot}3 \times 10^{-4}$ M

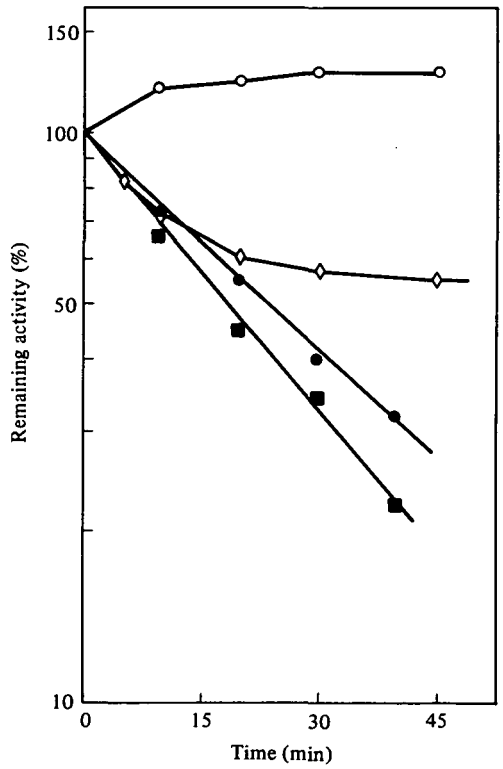

Fig. 6. Thermal stability of acid phosphatases synthesized by the wild-type strain grown for 72 h by standing in low phosphate medium at 30 °C. Each enzyme fraction, crude extract or partially purified through chromatography on Sephadex G-100 (Fig. 5), was incubated at 54 °C, pH 5·4, and the remaining enzyme activity was assayed, unless specified, with PNP-P as substrate at 37 °C. ○, enzyme 1; ◇, crude extract; ●, enzyme 2 and ■, enzyme 2 assayed using bis-PNP-P.

($n = 1{\cdot}02$) for enzymes I′ and II′, respectively) seem to indicate that the value for enzyme II′ is enhanced when compared to that shown by the corresponding enzyme fraction synthesized by the wild-type strain. The most striking data, however, were those observed for the heat stability of these enzymes (Fig. 2). If an alteration in the heat stability of a protein clearly indicates a change in its molecular structure, this does not necessarily signify that this alteration occurs at the amino acid sequence level. If alteration in amino acid sequence were obligatorily implied, the locus *nuc-1* could not have a role in regulating the synthesis of the phosphorus family enzymes at the transcriptional level as well as in ribosomal RNA synthesis

https://doi.org/10.1017/S0016672300022230 Published online by Cambridge University Press

(Stellwag & Metzenberg, 1984) because the *nuc-1* mutant strain synthesizes a more heat-labile nuclease than that synthesized by the wild-type strain (Hasunuma & Ishikawa, 1972). In any case, the results reported here do not support the hypothesis that the acid phosphatases synthesized by the mould grown on high phosphate medium are molecular entities totally different from the enzyme synthesized by the mould grown on low phosphate medium because, even though they may not have the same amino acid sequence, they must share some structural features. Thus, it is possible but speculative that the wild-type products of the loci *nuc-2*, *preg*, *nuc-1* and the other *nuc* genes reported (Forsthoefel & Mishra, 1983; Mishra & Forsthoefel, 1983) might have a function in the control of translation, post-translational modification and/or excretion of acid aphosphatase and related enzymes synthesized by the mould *N. crassa* grown on low-phosphate medium. In this case, the amount of P_i available may not be responsible for shutting off or turning on the gene-directed synthesis of distinct mRNAs. Even the *pho-3* gene product might be involved in regulating the translation, post translational modification and/or excretion of the acid phosphatase, since the mutant strain grown on low phosphate medium showed, besides a reduced excretion of this enzyme (Nelson *et al.* 1976), an elution profile from the DEAE-cellulose column identical to that observed for the wild-type strain (Fig. 3), except that a low level of activity towards bis-PNP-P was detected in the enzyme fraction eluted with the equilibrating buffer.

This work was supported by grants from the CNPq and FAPESP. S.A.R. was supported by a research fellowship from FAPESP. We are grateful to Newton R. Alves for the technical help, N. M. Martinez-Rossi for helpful discussions and Mrs Elettra Greene for revising this manuscript.

REFERENCES

ANDREWS, P. (1964). Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochemical Journal* **91**, 222–233.

ATKINSON, D. E., HATHAWAY, J. A. & SMITH, E. C. (1965). Kinetics of regulatory enzymes. *The Journal of Biological Chemistry* **240**, 2682–2690.

CROCKEN, B. & NYC, J. F. (1963). Utilization of L-α-glycero-phosphorylcholine by a lecithin-deficient strain of *Neurospora crassa*. *Canadian Journal of Microbiology* **9**, 689–696.

DAVIS, B. J. (1964). Disc-electrophoresis. II. Methods and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**, 404–427.

DIXON, M. (1953). The determination of enzyme inhibitor constants. *Biochemical Journal* **55**, 170–171.

FORSTHOEFEL, A. M. & MISHRA, N. C. (1983). Biochemical genetics of Neurospora nuclease I. Isolation and characterization of nuclease (*nuc*) mutants. *Genetical Research, Cambridge* **41**, 271–286.

FREE, S. J. & METZENBERG, R. L. (1982). The synthesis of alkaline phosphatase in *Neurospora crassa*. *Biochemical Genetics* **20**, 883–889.

HARTREE, E. F. (1972). Determination of protein: a modification of Lowry method that gives a linear photometric response. Analytical Biochemistry **48**, 422–427.

HASUNUMA, K. & ISHIKAWA, T. (1972). Properties of two nuclease genes in *Neurospora crassa*. *Genetics* **70**, 371–384.

JACOBS, M. M., NYC, J. F. & BROWN, D. M. (1971). Isolation and chemical properties of a repressible acid phosphatase in *Neurospora crassa*. *The Journal of Biological Chemistry* **246**, 1419–1425.

KOSHLAND, D. E., JR (1970). The molecular basis for enzyme regulation. In *The Enzymes* (ed. Paul D. Boyer), pp. 341–396. New York: Academic Press.

KUO, M.-M. & BLUMENTHAL, H. J. (1961). An alkaline phosphomonoesterase from *Neurospora crassa*. *Biochimica et Biophysica Acta* **54**, 101–109.

KUO, M.-M. & BLUMENTHAL, H. J. (1961). Purification and properties of an acid phosphomonosterase from *Neurospora crassa*. *Biochimica et Biophysica Acta* **52**, 13–29.

LEVITZKI, A. & KOSHLAND, D. E. JR. (1969). Negative cooperativity in regulatory enzymes. *Proceedings of the New York Academy of Sciences* **62**, 1121–1128.

LINEWEAVER, H. & BURK, D. (1934). The determination of enzyme dissociation constants. *Journal of American Chemical Society* **56**, 658–666.

LITTLEWOOD, B. S., CHIA, W. & METZENBERG, R. L. (1975). Genetic control of phosphate-metabolizing enzymes in *Neurospora crassa:* Relationships among regulatory mutations. *Genetics* **79**, 419–434.

METZENBERG, R. L. (1979). Implications of some genetic control mechanisms in *Neurospora*. *Microbiological Reviews* **43**, 361–383.

METZENBERG, R. L. & CHIA, W. (1979). Genetic control of phosphorus assimilation in *Neurospora crassa:* dose-dependent dominance and recessiveness in constitutive mutants. *Genetics* **93**, 625–643.

MISHRA, N. C. & FORSTHOEFEL, A. M. (1983). Biochemical genetics of *Neurospora* nuclease II. Mutagen sensitivity and other characteristics of the nuclease mutants. *Genetical Research, Cambridge* **41**, 287–297.

NAHAS, E. & ROSSI, A. (1984). Properties of a repressible alkaline phosphatase secreted by the wild-type strain 74A of *Neurospora crassa*. *Phytochemistry* **23**, 507–510.

NAHAS, E., TERENZI, H. F. & ROSSI, A. (1982). Effect of carbon source and pH on the production and secretion of acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) in *Neurospora crassa*. *Journal of General Microbiology* **128**, 2017–2021.

NELSON, R. E., LEHMAN, J. F. & METZENBERG, R. L. (1976). Regulation of phosphate metabolism in *Neurospora crassa:* identification of the structural gene for repressible acid phosphatase. *Genetics* **84**, 183–192.

NYC, J. F. (1967). A repressible acid phosphatase in *Neurospora crassa*. *Biochemical and Biophysical Research Communications* **27**, 183–188.

OSHIMA, Y. (1982). Regulatory circuits for gene expression: the metabolism of galactose and phosphate. In the molecular biology of the yeast *Saccharomyces cerevisae* ed. J. N. Stratherm, E. W. Jones and J. R. Broach), pp. 159–180. New York: Cold Spring Harbor Laboratory.

STELLWAG, E. J. & METZENBERG, R. L. (1984). Ribosomal RNA synthesis during phosphorus starvation in wild type *Neurospora* and in phosphorus regulatory mutants. *Molecular and General Genetics* **194**, 105–110.

VOGEL, H. J. (1956). A convenient growth medium for *Neurospora*. *Microbial Genetics Bulletin* **13**, 42–43.