Biochimica et Biophysica Acta, 522 (1978) 122–129

BBA 68309

SOME KINETIC ASPECTS OF THE MECHANISM OF HYDROLYSIS OF PHOSPHORIC ACID ESTERS BY NONSPECIFIC ACID PHOSPHATASE FROM *SCHIZOSACCHAROMYCES POMBE*

GIUSEPPINA DIBENEDETTO and UMBERTO MURA

International Institute of Genetics and Biophysics, C.N.R. Napoli and Laboratory of Biochemistry, University of Pisa, (Italy)

(Received May 4th, 1977)

Summary

1. The kinetics of the hydrolysis of nitrophenylphosphate by nonspecific acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2.) from *Schizosaccharomices pombe* was studied.

2. The kinetic parameters, K_m and V, were determined as well as the inhibition constants, K_i, for the inhibitors, phosphate and fluoride, as a function of pH.

3. The results, interpreted according to the theories of Dixon and Waley indicated the presence of three ionizable groups on the enzyme itself and one on the enzyme-substrate complex.

4. A model of the hydrolysis of phosphoric acid monoesters by the *S. pombe* acid phosphatase is proposed based on the ionization state of the reactants and on the results of the inhibition by the competitive inhibitors.

Introduction

In the course of studies on the regulatory mechanism of nonspecific acid phosphatase (orthophosphoric acid-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) formation in a eukarotic microorganism, the yeast *Schizosaccharomyces pombe* [1,2], we purified the acid phosphatase and we investigated the chemical and physical features of the enzyme molecule [3]. In order to obtain information about the mechanism of hydrolysis of phosphoric acid esters by the *S. pombe* acid phosphatase so as to compare the data with those of the *Saccharomyces cerevisiae* enzyme [4,5] we carried out a kinetic study of the *S. pombe* acid phosphatase. The variations of pK_m and log V as a function of pH according to the theories of Dixon [6,7] and Waley [8] were determined. From the shape of the graphs thus obtained, conclusions may be drawn about the pK of ionizing group essential for enzymatic activity. To con-

firm the results, the variations of pK_i for phosphate, fluoride and sulphate as inhibitors of acid phosphatase and the % inhibition as a function of pH were calculated. It was concluded that in contrast to the *S. cerevisiae* phosphatase, the activity of the *S. pombe* acid phosphatase depends on at least three ionizable groups. This paper discusses the kinetic data obtained so far and presents a hypothesis for the interpretation of enzymic hydrolysis of phosphoric acid monoesters.

Materials and Methods

Chemicals

All chemicals were reagent grade and were purchased from BDH. Standard solutions of pH 2.0, 4.0 (citrate · HCl) and 7.0 (phosphate) were obtained from Merck. Purification procedure.

Purification procedure

The enzyme was purified from cells grown in a low-phosphate minimal medium [1] following the procedure described previously [3]. All experiments were carried out with the pure enzyme in 0.2 M sodium acetate buffer at pH 4.6. Protein concentration was determined spectrophometrically using an absorbance index $A_{280}^{1\%}$ of 13.2 [3].

Activity assays

The nonspecific acid phosphatase was assayed manually as described previously [1] by a modification of the method of Torriani [9]. One unit of acid phosphatase activity represents the amount of enzyme catalyzing the release of 1 μmol of nitrophenol/min at 30° C.

The kinetic parameters were all obtained by determining the pH curve of the enzyme at several substrate concentrations in the absence or presence of inhibitors by an automatic technique previously described. The apparatus for the determination of the pH curve has been reported by Bauer et al. [1]. The pH gradient was obtained by mixing barbital/acetate solution 1/35 M at pH 9.6 with barbital/acetate/HCl solution 1/35 M at pH 1.5 in a suitable vessel equipped with a glass electrode connected to a measuring unit and a recorder. The buffer fractionated with air was mixed with the substrate (nitrophenylphosphate dissolved in water) and, later on, with the enzyme. The insertion of suitable mixing coils ensured a thorough mixing after each addition. The reaction mixture then entered a delayed coil immersed in a bath at 30° C for 5 min. It was then mixed with 0.5 M sodium hydroxide to stop the reaction and develop the yellow color of the nitrophenol formed. The absorbance of the solution was read at 400 nm in a colorimeter equipped with a flow cell and recorded. Suitable signals correlated the time of the recording of the activity with the pH of the gradient, thus it was possible to obtain the final pH curve. The incubation volume determined by the true flow rates (ml/min) was 3.343 ml (2.78 ml of which was buffer), 0.161 ml substrate, 0.244 ml enzyme and 0.157 ml water or inhibitor solution. The final concentration of the buffer was 24.8 mM and the effective concentrations of the substrate in the incubation mixture corresponding to each pH curve were respectively 0.114, 0.227, 0.454, 0.908, 1.362 and 1.816 mM.

In order to correct for alterations in the original pH of the buffer, as measured by the automatic method before the additions of substrate enzyme and inhibitors, the pH of complete incubation mixtures containing enzymes and substrate and inhibitor obtained manually was measured with a Metrohm pH meter every 0.5 unit and the values obtained are the ones reported. For pH values within the 0.5 pH unit intervals the correction was made by interpolation.

The kinetical parameters, K_m (Michaelis constant) and V (Maximum velocity) were determined from the Lineweaver-Burk plot [11] with the six substrate concentrations in the entire pH range of the enzyme (approx. pH 2—7).

The K_i for phosphate, sulphate and fluoride have been calculated in the entire pH range of the enzyme by the Dixon plot [12] using two substrate concentrations 0.434 and 1.303 mM (effective concentrations in the incubation mixture) and two inhibitor concentrations: 2.08—4.16 mM for phosphate, 4.16—10.30 mM for sulphate and 2.08—4.16 mM for fluoride.

Results

The kinetics of hydrolysis of sodium nitrophenylphosphate was studied at 30°C between pH 2.5 and 6.0. The concentration of the substrate was varied from 1.14 to $18.16 \cdot 10^{-4}$ M. From the profiles of the pH vs. activity curves it appears that the pH optimum of the enzyme was dependent on the substrate concentration as shown in Fig. 1. It can be seen that on lowering the substrate concentration the pH optimum is progressively shifted from pH 2.90

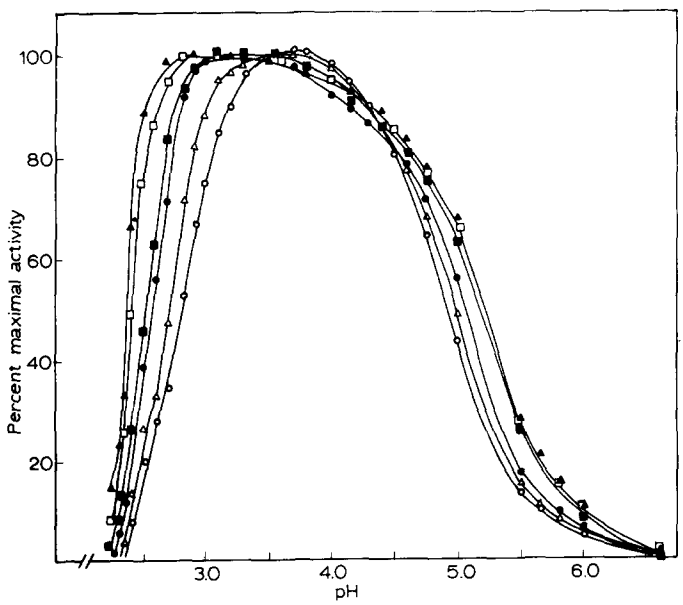


Fig. 1. Percent pH curves at different substrate concentrations. The pH vs. activity curves were obtained automatically as described in Materials and Methods. The effective concentrations of the substrate in the incubation mixture corresponding to each pH curve were respectively 0.114 mM (○), 0.227 mM (△), 0.454 mM (●), 0.908 mM (■), 1.362 mM (□), 1.816 mM (▲).

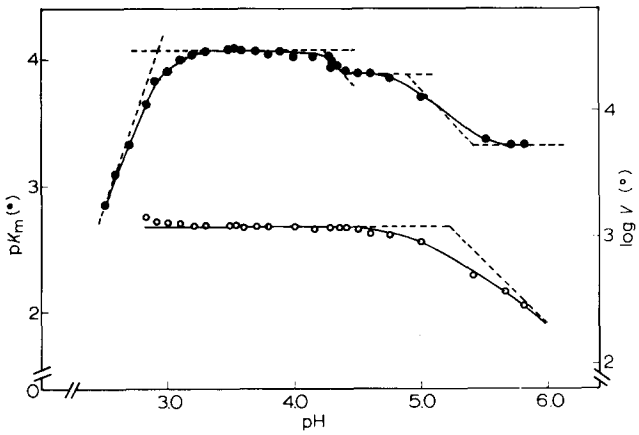


Fig. 2. Diagrams according to Dixon (•) and Waley (○) for the hydrolysis of nitrophenylphosphate with purified yeast acid phosphatase.

at the highest concentration of the substrate to pH 3.80 at the lowest, and the width of the pH vs. activity curves is reduced.

An ample series of values for K_m and V in the entire pH range of enzyme activity with a total of 29 values of pH were determined. From the above data, treating the variations of pK_m with pH according to the method of Dixon and of log V with pH according to the method of Waley, the pK of ionization was determined for the ionizable groups of the different components of the reaction system whose state of ionization affects the velocity of the enzymic reaction. From the diagrams obtained, (Fig 2), it was deduced that the free enzyme ionizes at pH 2.94, 4.24, 4.90; the enzyme · substrate complex at pH 5.24—5.40 and at pH 4.35. However, the last ionization of the complex was not observed, as expected, in the log V plot. A tentative interpretation is offered below.

The dependence of pK_i on the pH obtained with either phosphate or fluoride as inhibitors were similar. Diagrammatic profiles, as shown in Fig. 3,

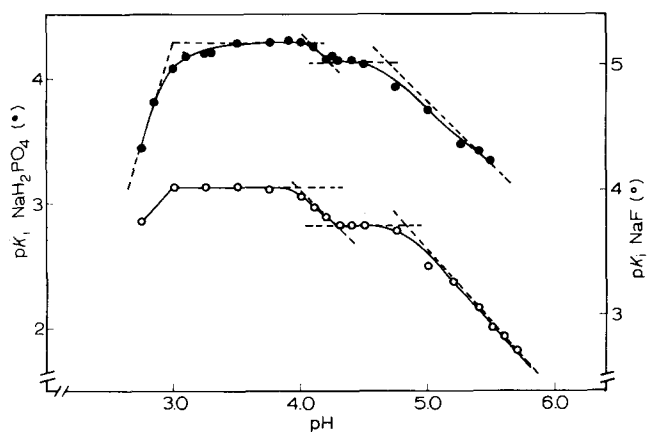


Fig. 3. Dependence of pK_i on the pH with either phosphate (•) or fluoride (○) as inhibitors.

TABLE I

pK values for the active ionizable groups of the enzyme and of the complex enzyme-substrate for the hydrolysis of nitrophenylphosphate by yeast acid phosphatase.

Diagrams	pK_1	pK_2	pK_3	pK_4	pK_5
pK_m	2.94	4.24	4.40	4.90	5.40
log V					5.24
pK_i ($H_2PO_4^-$)	3	4.06	4.23	4.70	
pK_i (F^-)	3	3.98	4.24	4.84	

closely matched that reported for pK_m except for the last inflexion point (at pH 5.40) which is lacking in the pK_i plots. The ionization values deduced from latter plots are in good agreement with those obtained from the pK_m plots as shown in Table I summarizing the overall pK values. It should be observed that the inflexion points are not affected by differences in the inhibition mechanism, which seems formally different for phosphate and fluoride. Indeed by comparing (according to Chou [13]) the experimental curves of % inhibition as a function of pH with the theoretical curves obtained by inserting the experimental K_m, V and K_i values into the appropriate equations for each inhibition model, it was possible to obtain further insight into the inhibition mecahnism. Fig. 4 indicates that phosphate acts according to the competitive mechanism; while fluoride inhibition is best fitting with the mixed type as already reported in the literature for other phosphatases [14].

Indications on the hydrolysis mechanism of the *S. pombe* acid phosphatase are given by the profiles of the % inhibition curve as a function of pH for two

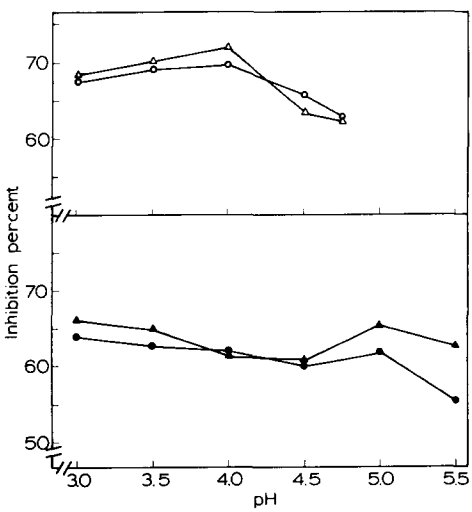
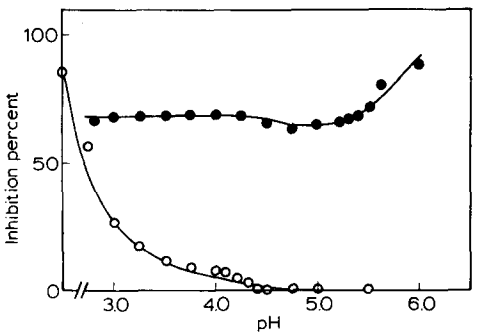


Fig. 4. Comparison of the experimental curves of % inhibition with the theoretical curves obtained as described in Results. Part A: experimental (○) and theoretical (△) curves for competitive inhibition for phosphate. Part B: experimental (●) and theorical curve for inhibition of the mixed type (▲) for fluoride.



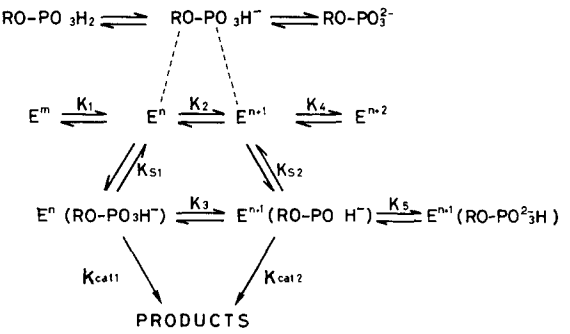


Fig. 5. Percent inhibition curves as a function of pH for phosphate and sulphate. The experimental conditions are described in Materials and Methods. The effective substrate concentration was 0.454 mM for both inhibitors, the phosphate (•) concentration 2.08 mM and the sulphate (○) concentration 10.30 mM.

Fig. 6. Diagram as proable mechanism of the hydrolysis of phosphoric acid monoesters catalysed by acid yeast phosphatase, with due limitations of the chemical nature of the active site of the enzyme. *m* and *n* are the negative charges on the enzyme, with $n > m$.

competitive inhibitors, phosphate and sulphate, studied with nitrophenylphosphate as substrate. The two compounds possess different pK_2 (7.21 [15] and 1.99 [16] respectively) and show quite different % inhibition as a function of pH (Fig. 5). The inhibition of the phosphatase activity by the sulphate in the form of SO_4^{2-} in the entire enzyme activity range, drammatically decreased in the pH range of ionization of the second ionizable group on the enzyme. In contrast the inhibition by phosphate in the form of $H_2PO_4^-$ was constant up to the pH range of the ionization corresponding to pK_5 which, as discussed below, can be attributed to the second dissociation of the substrate in the form of enzyme-substrate complex and increased thereafter.

Discussion

The dependence of the pH optimum of non-specific acid phosphatase (also reported by Bauer et al. [17] using intact cells of *S. pombe*) on the substrate concentration, can hardly be attributed, in our case, to the presence of multiple isoenzymes [3] or different enzyme forms [18]. It is likely therefore that the interpretation proposed by Bauer et al. [17] is relevant. For a given substrate the fact that K_m decreases to a minimum and increases thereafter with increasing pH while V approaches a limiting value in the same pH range can explain the change in pH optimum with substrate concentration, taking into account that the reaction velocity (v_0) tends to V at highest substrate concentration and depends on V/K_m at the lowest.

The kinetic parameters of the enzyme, log V and pK_m for nitrophenylphosphate as well as pK_i for the inhibitors phosphate and fluoride as function of pH indicate three stages of ionization of the free enzyme: $pK_1 = 2.94—3$; $pK_2 = 3.98—4.24$; $pK_4 = 4.70—4.90$; and two pertaining to the enzyme · substrate complex: $pK_3 = 4.23—4.40$; $pK_5 = 5.24—5.40$. A tentative interpretation of the kinetic data is summarized in Fig. 6. The first inflexion point, corresponding to pK_1, can be associated with an activation of the enzyme in which more than

one dissociation is apparently implicated. This interpretation is implied by the slopes of either pK_m or pK_i as a function of pH [6,7].

Based on the theory of Dixon, pK_2 and pK_3 would be interpreted as equilibrium constant relating to a dissociable group present on the free enzyme, or inhibitor or substrate, and the dissociation of the same group in the enzyme-ligand (ES or EI) complex, respectively. However, whilst both pK_2 and pK_3 are visible either in plots of pK_m and pK_i vs. pH, an indication of the involvement of a dissociable group on the enzyme, no inflexion is evident at a pH corresponding to pK_3 in the plot of log V vs. pH. This fact is difficult to accomodate with Dixon's theory, at least without considering the possibility that the acid-base equilibrium of the enzyme-substrate complex influences exclusively the dissociation to form enzyme and substrate, and not the formation of the product. An alternative interpretation of these data would be to assume that an ionization on the enzyme leads to the interconversion between two enzyme forms, with different but nonetheless measurable activities. In this way both the inflexions corresponding to pK_2 and pK_3 could be explained by a single ionization process (Mura, U. and Bauer, C., unpublished results). The inflexion characterized by pK_4 in the pH-dependence of pK_m and pK_i may be attributed to the free enzyme passing in this instance to an inactive form. The sharp decrease of the affinity of the enzyme for the substrate or the inhibitor can be attributed either to a conformational change of the enzyme or to electrostatic repulsion between the substrate (or inhibitor) and the newly dissociated group on the enzyme. The hypothesis of the influence of electrostatic repulsion between the components the reaction system (E, S and I) is supported by the above reported curves of inhibition percent by sulphate and phosphate. In fact the inhibition by SO_4^{2-} ions disappears in the pH range of $E^n \rightleftharpoons E^{n+1}$ ionization (where the ionic form of the substrate does not change), whereas the inhibition by $H_2PO_4^-$ ions increases in the pH range of the second ionization of the substrate (where almost all the enzyme is in the E^{n+2} form).

As far as the chemical nature of the ionizable groups is concerned, all the pK values are compatible with the dissociation of carboxyl groups. The precise attribution of the pK values to α-carboxyl terminal groups or to aspartyl-β- and glutamyl-γ-carboxyl residues was not attempted owing the sizable differences between the pK values for the same group as determined for the free aminoacid or in a protein molecule. It should be noted nonetheless, that the protein part of the enzyme is remarkably rich in aspartic acid and the glycidic moiety is lacking in ionizable groups [3].

The inflexion-labeled pK_s can be attributed to a second dissociation of the substrate in the form of enzyme-substrate complex. Besides the good agreement with literature values [19] for the second dissociation of the free nitrophenylphosphate, (pK = 5.83), the observed inflexion on the plot of pK_m vs. pH corresponds well with that in the analogous graph for V, but it is not found in graphs of pK_i for phosphate or fluoride against pH. The lowering of the observed pK of dissociation for substrate once inserted in the enzyme-substrate complex is in accordance with a partial neutralization of the phosphate group that very probably accompanies the formation of the ES complex itself. Furthermore, the fall in maximal rate, and therefore in the catalytic activity in the strictest sense, related with the second dissociation of the substrate in the ES

complex, is in agreement with indications that the mechanism of hydrolysis of phosphoric acid esters by acid phosphatase is analogous to that of the non-enzymatic hydrolysis of phosphate monoesters which proceeds by means of an elimination-addition mechanism [14].

It should finally be observed that the variations of pK_m and log V_m as a function of pH (according to the procedure of Dixon and Waley) reported for unspecific acid phosphatase from *S. cerevisiae* in the pH range 3.5—5 did not provide evidence of ionizable groups involved in the phosphatase activity. The present knowledge of structural properties and catalytic mechanism of yeast acid phosphatases does not allow to relate this discrepancy to differences in the molecular properties or in catalytic mechanism.

References

1 Dibenedetto, G. (1972) Biochim. Biophys. Acta 286, 363—374
2 Dibenedetto, G. (1975) Atti Assoc. Genet. Ital. 16, 133—135
3 Dibenedetto, G. and Cozzani, I. (1975) Biochemistry 14, 2847—2852
4 Boer, P. and Steyn-Parvé, E.P. (1969) Biochim. Biophys. Acta 171, 360—362
5 Albers, H. Büsing, G. and Schudt, G. (1966) Enzymologia 30, 149—178
6 Dixon, M. (1953) Biochemical J. 55, 161—170
7 Dixon, M. and Webb, E.C. (1964) Enzymes, 2nd edn., pp. 116—144, Longmans Green Co., London
8 Waley, S.G. (1953) Biochim. Biophys. Acta 10, 27—34
9 Torriani, A. (1960) Biochim. Biophys. Acta 38, 460—479
10 Bauer, C., Cinci, A., Bronzetti, G. and Loprieno, N. (1974) Biochem. Genet. 11, 1—14
11 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658—666
12 Dixon, M. (1953) Biochem. J. 55, 170—171
13 Ting-Chao Chou (1974) Mol. Pharmacol 10, 235—147
14 Hollander, V.P. (1971) in The Enzymes (Boyer, P.D., ed.), Vol. IV, 3rd edn., pp. 450—498, Academic Press, N.Y.
15 Butler, J.N. (1964) Ionic Equilibrium, p. 465, Addison Wesley Publishing Co. Inc., London
16 Butler, J.N. (1964) Ionic Equilibrium, p. 113, Addison Wesley Publishing Co. Inc., London
17 Bauer, C., Cinci, A. and Petruzziello, M. (1973) Chim. Ind. 55, 372—373
18 Dibenedetto, G. and Cozzani, I. (1975) Abstr. 10th FEBS Meet. Paris, 814
19 Alvarez, E.F. (1962) Biochim. Biophys. Acta 59, 663—672