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Pseudomonas aeruginosa acid phosphatase

Activation by divalent cations and inhibition by aluminium ion

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In *Pseudomonas aeruginosa*, the effect of different cations on the acid phosphatase activity was studied in order to acquire more information related to a previously proposed mechanism, involving the coordinated action of this enzyme with phospholipase C. Although the natural substrate of this enzyme is phosphorylcholine, in order to avoid the possible interaction of its positive charge and those of the different cations with the enzyme molecule, the artificial substrate *p*-nitrophenylphosphate was utilized. Kinetic studies of the activation of acid phosphatase (phosphorycholine phosphatase) mediated by divalent cations Mg^{2+}, Zn^{2+} and Cu^{2+} revealed that all these ions bind to the enzyme in a compulsory order (ordered bireactant system). The K_m values obtained for *p*-NPP in the presence of Mg^{2+}, Zn^{2+} and Cu^{2+} were 1.4 mM, 1.0 mM and 3.5 mM, respectively. The K_A values for the same ions were 1.25 mM, 0.05 mM and 0.03 mM, respectively. The V_{max} obtained in the presence of Cu^{2+} was about twofold higher than that obtained in the presence of Mg^{2+} or Zn^{2+}. The inhibition observed with Al^{3+} seems to be a multi-site inhibition. The K'_{app} and *n* values, from the Hill plot, were about 0.25 mM and 4.0 mM, respectively, which were independent of the metal ion utilized as activator. It is proposed that the acid phosphatase may excert its action under physiological conditions, depending on the availability of either one of these metal ions.

Acid phosphatase; Phosphorylcholine phosphatase; *Pseudonomas aeruginosa*; Choline; Infection

1. INTRODUCTION

Earlier, we have reported an increase in cholinesterase activity in *Pseudomonas aeruginosa* in addition to acid phosphatase [1,2] and phospholipase C activities [3] in the presence of high P_i concentration, when several choline derivatives were used as the sole source of carbon, nitrogen or carbon and nitrogen.

Our observations on the properties of these enzymes have recently led us to propose that they, acting coordinately, may be considered as a group of factors contributing to the corneal infection [4].

Studies on the kinetic properties of these enzymes confirmed our proposal. Acid phosphatase was inhibited, in vitro, by different cationic organic compounds [1,2]. As all of these chemicals had a positively charged nitrogen atom, and the higher effect against the phosphatase was observed with compounds containing an *N*-trimethyl moiety, the presence of an anionic site in the enzyme with affinity for methyl groups was proposed [5]. These results led us to suggest that the acid phosphatase may be considered as a phosphorylcholine phosphatase [6,7]. As *P. aeruginosa* also produces phospholipase C in addition to the acid phosphatase under the same culture conditions [3], we thought that the coordinate action of phospholipase C and acid phosphatase may produce the breakdown of the choline containing phospholipids of the host cell [3,4].

To accounting for these observations it was necessary to have a better knowledge of the factors affecting the activity of these enzymes in order to understand the establishment and continuation of the infection produced by *P. aeruginosa*.

As *P. aeruginosa* acid phosphatase was magnesium ion dependent and was inhibited by cationic organic compounds [5], and phospholipase C was inhibited by Zn^{2+} ions [8], the attention was focused on some inorganic cations as effectors of the *P. aeruginosa* acid phosphatase. Results below describe the activating effect of the divalent cations Mg^{2+}, Zn^{2+}, Cu^{2+} and the inhibitory effect of the Al^{3+} on this enzyme.

2. MATERIALS AND METHODS

2.1. *Organism and growth conditions*

Pseudomonas aeruginosa (NCTC, Fildes III, 1924, UK) was grown aerobically at 37°C in a basal salt medium as previously described [1-3]. 20 mM choline was utilized as the sole carbon and nitrogen source.

2.2. *Enzyme preparation and assay*

Whole cells, crude periplasmic extracts [1,2] or partially purified enzyme from these extracts [5] were utilized as the source of the acid phosphatase. The enzyme activity was assayed with the sodium salt of *p*-nitrophenyl phosphate [1,2] or phosphorylcholine [3,4] as the

Abbreviation: *p*-NPP, *p*-nitrophenylphosphate

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substrate. Saturation curves with different cations were performed in the presence of EDTA. Free metallic ion concentrations were calculated using an iterative computer program kindly supplied by Dr. J. Kleineke, Abteilung Klinische Biochemie, Zentrum Innere Medizin der Universität Göttingen, Germany, previously utilized in [9]. The stability constants used for the cation–EDTA complex were described earlier [10].

Kinetic results were analyzed as described in [11,12]. The constant values for the substrate (S) and the divalent cations (A) were calculated from primary and secondary plots utilizing the equation $v = V_{max}/\{1 + K_m/[S] + K_A \cdot K_n/[A] \cdot [S]\}$ as described in [11], where the activator binds to the free enzyme in a compulsory order ordered bireactant system) by assuming rapid equilibrium or steady-state kinetics. Results of the enzyme inhibition by Al^{3+} ion were analyzed by utilizing the Hill equation: $\log v_i/v_o - v_i = -n \cdot \log [I] + \log K'$.

3. RESULTS AND DISCUSSION

In our previous papers, Mg^{2+} was utilized as the co-factor to measure the *P. aeruginosa* acid phosphatase activity [1–7]. Meanwhile, it was also observed that besides Mg^{2+}, this enzyme was activated by other divalent cations when *p*-NPP was used as the substrate and whole cells were used as the enzyme source [13]. In the absence of divalent ions the activity was about 5 U/ml·OD, whereas in the presence of 2 mM Mg^{2+}, Zn^{2+} or Cu^{2+} ions, it was increased to 110, 160, or 184 U/ml·OD, respectively. On the other hand, when divalent metal ions were replaced by 2 mM trivalent metal ion like Al^{3+} or added along with Mg^{2+}, the enzyme activity could not be seen.

As shown in Table I, results with purified enzyme indicated that Zn^{2+} and Cu^{2+} were better activators than the Mg^{2+} ion and Al^{3+} was an inhibitor of the enzyme, independent on the type of substrate utilized, i.e. *p*-NPP or phosphorylcholine. Although the activation by divalent cations or inhibition by the Al^{3+} ion could be observed with both substrates, it was preferred to perform the kinetic study with the artificial substrate *p*-NPP. Because the possible interference that might be produced by the interaction of the positive charges of the metallic ions and the charge of phosphorylcholine (the *N*-trimethylammonium moiety) with the enzyme molecule could be avoided.

Figs. 1A and B show double reciprocal plots from saturation curves of *P. aeruginosa* acid phosphatase by *p*-NPP or Zn^{2+} in the presence of different concentrations of Zn^{2+} or *p*-NPP, respectively. Similar results were obtained with Mg^{2+} or Cu^{2+} (data not shown).

Kinetic constants of this enzyme using *p*-NPP as the substrate in the presence of different metal ions are shown in Table II. The K_m values revealed that the enzyme had more affinity for *p*-NPP in the presence of Zn^{2+}. Although the Cu^{2+} ion was less effective to increase the affinity of the enzyme for *p*-NPP, but according to the K_A values, the higher affinity of the enzyme was for Cu^{2+}. The V_{max} obtained in the presence of Cu^{2+} ions was about twofold higher than that observed with Mg^{2+} or Zn^{2+}. Moreover, the data revealed that the higher catalytic efficiency V_{max}/K_m) was obtained when Zn^{2+} was present in the assay mixture.

Table I also shows the apparent *n* (Hill coefficient) and *K'* values obtained for the aluminium ion in the presence of different divalent cations. The inhibition of the acid phosphatase activity produced by Al^{3+} apparently, was not dependent on the nature of the divalent cation, since practically identical K' values were obtained. The very high *n* values might be interpreted as binding sites of the enzyme for the Al^{3+} cation. However, it might not correspond to a cooperative effect, but a multi-site inhibition, which could also be expressed in terms of the Hill equation [11 (page 470)]. This observation could be explained considering that the enzyme molecule contains, at least, one anionic site for the substrate and another for the metal ion.

From these data it is difficult to decide whether the enzyme has some special preference for any one of these activating cations to act in its natural environment. However, it is clear that the acid phosphatase may excert its action under physiological conditions, depending on the availability of either of these metal ions.

According to our previous results [3,4,7], where it was proposed that the coordinated action of phospholipase C and acid phosphatase may contribute to the breakdown of the membrane phospholipids, we feel that the above results permit us to show the importance of this enzyme in the infection which is produced by *P. aeruginosa*. Thus, the attack of phospholipase C on the membrane phospholipids containing choline, would result in the formation of phosphorylcholine which would be free to diffuse through the hydrophilic channels formed by the porin proteins, and reach the periplasmic space. Through a similar mechanism, the metallic ions reach the same space, where they find the acid phosphatase and stimulate its activity. As a consequence of this stimulation, phosphorylcholine may be easily hydrolyzed to render choline and P_i. Considering the K_A value for the Zn^{2+} ion, this enzyme can excert its action at a concentration twentyfold lower than that neccesary to produce the inhibition of phospholipase C. In this way, it is clear that both enzymes can act under conditions that

Table I

Relative activity of the *P. aeruginosa* acid phosphatase with respect to that obtained with Mg^{2+} ions

	Whole cells	Purified enzyme	
Substrate	*p*-NPP	*p*-NPP	Phosphoryl-choline
Zn^{2+}/Mg^{2+}	1.44	1.80	1.15
Cu^{2+}/Mg^{2+}	1.66	1.95	1.25
Al^{3+}/Mg^{2+}	0.10	0.05	0.06

Enzyme activity was measured in 70 mM sodium acetate buffer, pH 5.0, with 10 mM *p*-NPP or 0.25 mM phosphorycholine in the presence of 2 mM divalent cations or 0.5 mM Al^{3+}. Data are average from three independent experiments.

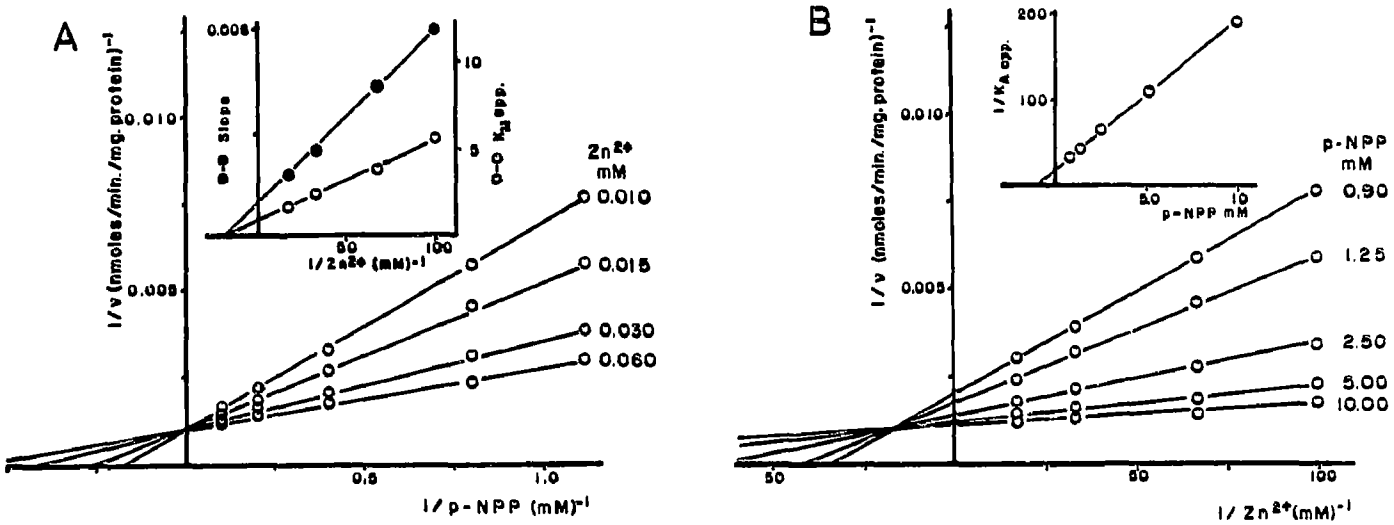

Fig. 1. Double reciprocal plots from saturation curves of *P. aeruginosa* acid phosphatase by the sodium salt of *p*-NPP (A) or Zn^{2+} (B) in the presence of variable concentrations of Zn^{2+} or *p*-NPP, respectively. Insets show replots of data taken from the same figures. Other experimental conditions are described in section 2 and Table II. Data correspond to a representative experiment.

apparently do not have any adverse effect on either of them.

The effect produced by the Al^{3+} ion on the phosphatase activity indicated that it is a good inhibitor of the enzyme activity. At present, we do not known its importance on the physiological behavior of *P. aeruginosa* under different nutritional conditions. It is possible that in the presence of high concentrations of Al^{3+}, the coordinated action of phospholipase C and phosphorylcholine phosphatase may be interrupted. If it occurs, then the question arise whether the bacteria open a new metabolic way in the presence of this ion in order to survive.

Since *P. aeruginosa* is capable to colonize at various sites in different animal hosts, the establishment of its infection is dependent on various factors where the role of the metal ions may not be ignored.

Table II

Kinetic constants of *P. aeruginosa* acid phosphatase obtained with different metal ions

	Mg^{2+}	Zn^{2+}	Cu^{2+}
K_m (*p*-NPP, mM)	1.4	1.0	3.5
K_A (mM)	1.25	0.05	0.03
V_{max}	863	952	1769
V_{max}/K_m	616	952	505
K'_{app} (Al^{3+}, mM)	0.26	0.24	0.24
n_{app} (Al^{3+}, mM)	4.2	4.3	4.0

K_m, K_A and V_{max} were obtained from primary and secondary plots from saturation curves performed with the sodium salt of *p*-NPP in the presence of variable concentrations of metallic ions. (as in Fig. 1). The enzyme activity was measured in the presence of acetate buffer, pH 5.0 (70 mM), EDTA (1 mM), *p*-NPP (0.3–10 mM) and free cations (0.05–2.0 mM for Mg^{2+}; 0.005–0.06 mM for Zn^{2+} and Cu^{2+}). V_{max} is expressed as nmol *p*-nitrophenol/min·mg protein. K'_{app} is the constant of the Hill equation and defined as the Al^{3+} concentration that produced 50% inhibition of the reaction rate. n_{app} (Hill coefficient) values are calculated from the maximal slope (obtained with Al^{3+} concentration in the range of 0.2–0.35 mM) by plotting log $v_i/v_o - v_i$ vs. log $[Al^{3+}]$. v_o and v_i are the enzyme activities measured with saturating substrate (10 mM *p*-NPP) and free cation concentrations (3.0 mM Mg^{2+}, 0.2 mM Zn^{2+} and 0.1 mM Cu^{2+}) in the absence (v_o) or in the presence (v_i) of Al^{3+} ion (0.05–0.35 mM). 1 mM EDTA was present in the assay mixture to obtain the indicated free cation concentrations. The constants represent an average from at least three independent experiments performed with partially purified enzyme obtained from cells of different cultures.

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