Studies on inorganic pyrophosphatase using imidodiphosphate as a substrate

Irina N. Smirnova, Alexander A. Baykov and Svetlana M. Avaeva

A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, USSR

Received 23 July 1986

Baker's yeast inorganic pyrophosphatase has been found to catalyze Mg^{2+}-dependent hydrolysis of imidodiphosphate yielding phosphate and amidophosphate. The reaction proceeds linearly in the presteady state. The catalytic constant is maximal at pH 9.0 and equals 0.5 min^{-1}. Kinetic titrations of the enzyme with imidodiphosphate and Mg^{2+} have provided direct evidence for the involvement of three Mg^{2+} per active site in the transition state of the pyrophosphatase reaction.

Inorganic pyrophosphatase *Imidodiphosphate* *Metal-ion cofactor* *Active-site titration*

1. INTRODUCTION

Inorganic pyrophosphatase (EC 3.6.1.1) is a ubiquitous enzyme splitting linear polyphosphates [1]. The specificity and intensity of the catalysis both depend on the metal-ion cofactor used. With Mg^{2+}, pyrophosphate and triphosphate were found to be the only substances hydrolyzed, the ratio of the two activities being 1:0.02 [2,3]. In the presence of Zn^{2+}, Mn^{2+} and Co^{2+}, monoesters of polyphosphoric acids (including ADP and ATP) are slowly split [4]. Essentially, a P–O bond is attacked in all these substrates.

The present results indicate that inorganic pyrophosphatase is also capable of hydrolyzing imidodiphosphate – a PP_i analog containing nitrogen in the bridge position. High affinity for pyrophosphatase in conjunction with a low catalytic constant make this new substrate a convenient tool for studies on enzyme mechanisms.

Abbreviation: PNP, imidodiphosphate

2. MATERIALS AND METHODS

Inorganic pyrophosphatase with a specific activity of 650 IU/mg was isolated from baker's yeast [5]. Stock solutions of the enzyme were made in 0.1 M Tris-HCl (pH 7.2) containing 1 mM $MgCl_2$. Enzyme concentration was estimated on the basis of $\epsilon_{280}^{0.1\%} = 93000\ M^{-1} \cdot cm^{-1}$ [1,6].

PNP was prepared as described [7,8]. Its solutions were standardized by measuring P_i on exhaustive hydrolysis with a mixture of sulfuric and perchloric acids.

PNP hydrolysis was assayed by measuring P_i in 0.1-ml aliquots of the reaction mixture using a highly sensitive Malachite Green procedure [9]. The reaction mixture contained 0.1 M Tris-HCl (pH 7.5 or 9.0), $MgCl_2$ and PNP in a total volume of 0.5–1 ml. If not stated otherwise, the reaction was carried out at 25°C for 5–10 min and terminated by adding an equal volume of 1 M perchloric acid. Denatured enzyme was sedimented for 2 min at 16000 × *g*, clear supernatant (0.18 ml) was transferred to a tube containing 0.045 ml

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50 © 1986 Federation of European Biochemical Societies

phosphate reagent, and the absorbance was measured in 2 min at 650 nm using a 1 cm cuvette. The phosphate reagent was prepared by adding successively 1.5 ml of 6% ammonium molybdate and 1.8 ml of 0.3% Malachite Green to 2.5 ml of 10 N H_2SO_4 while mixing vigorously.

In the thermal inactivation studies, the medium contained 0.05 M Tris-HCl (pH 8.25 at 50°C) and the enzyme. Aliquots were assayed for residual activity against PP_i and PNP at pH 9.0, 25°C in the presence of 5 mM $MgCl_2$.

3. RESULTS

Fig.1 illustrates the time course of P_i accumulation during PNP hydrolysis by pyrophosphatase. The reaction proceeded linearly, virtually until complete disappearance of the substrate, thus indicating high affinity of the enzyme to PNP. In fact, the K_m for PNP in the presence of 1 mM $MgCl_2$ was found to be 3 μM at pH 7.2 and less than 1 μM at pH 9.0. Lack of an initial burst of P_i in fig.1 was interpreted as showing that the breakdown of the enzyme-bound PNP is the rate-limiting step of the overall reaction.

The standard phosphate assay revealed 1.87 mol P_i per mol added PNP at the end of the enzymatic process. Treatment of the reaction mixture prior to P_i analysis with ethylene glycol, which is known to react with amidophosphate yielding an acid-stable phosphoric acid monoester [10], decreased this value to 1.05. These results indicate that the primary products of PNP hydrolysis by pyrophosphatase are P_i and amidophosphate and the latter product, which is unstable in the presence of acid molybdate [11], is converted non-enzymatically into P_i during the assay procedure.

The rate of PNP conversion was proportional to enzyme concentration. No catalysis was observed without Mg^{2+}. The hydrolysis rate was maximal at pH 9.0 (1–5 mM $MgCl_2$, 0.1 mM PNP) and decreased at pH 7.0 and 10.5 by 65 and 50%, respectively. The pH optimum of PP_i hydrolysis is 2 units below [1].

Several lines of evidence indicated that PNP hydrolysis was catalyzed by pyrophosphatase itself rather than by some contaminating enzyme. Thus, PNP- and PP_i-converting activities declined in parallel at 50°C and were equally protected by Mg^{2+} (fig.2). Fluoride (1 mM), a specific inhibitor of pyrophosphatase, caused an 80% decrease in the rate of PNP hydrolysis at pH 7.2.

The low catalytic constant of PNP hydrolysis by pyrophosphatase (0.5 min^{-1} at pH 9.0) provided the opportunity of measuring reaction rates at

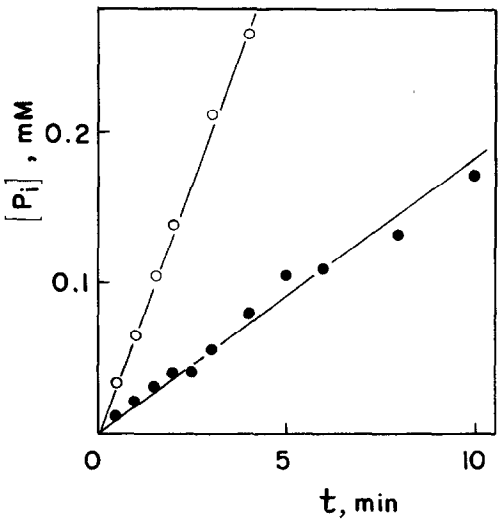

Fig.1. Time courses of P_i formation during PNP hydrolysis by pyrophosphatase at pH 9.0 (○) and 7.2 (●). Conditions: 40 μM enzyme, 1 mM $MgCl_2$, 0.5 mM PNP.

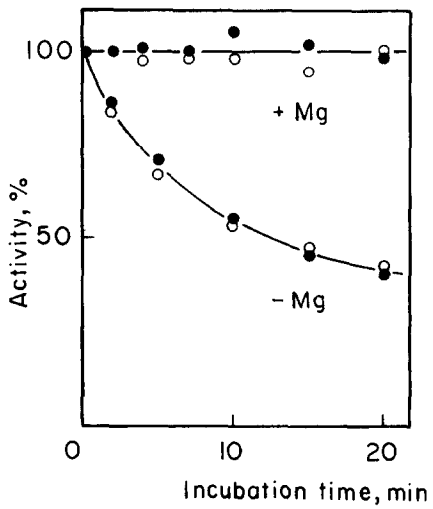

Fig.2. Thermal inactivation of pyrophosphatase in the absence and presence of 5 mM $MgCl_2$ at 50°C. Activity measured with PNP (○) and PP_i (●) as the substrates.

substrate to enzyme ratios below unity. Together with high affinity of pyrophosphatase to PNP, this allowed direct active-site titrations (fig.3). In these

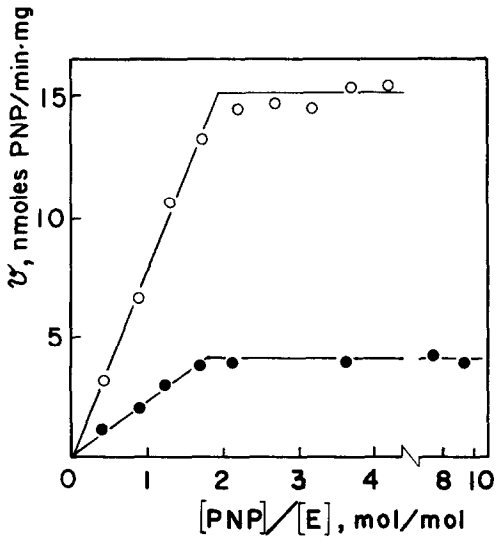

Fig.3. Rate of PNP hydrolysis as a function of its concentration at pH 9.0 (○) and 7.2 (●). Conditions: 20 μM enzyme, 1 mM (pH 7.2) or 5 mM (pH 9.0) $MgCl_2$.

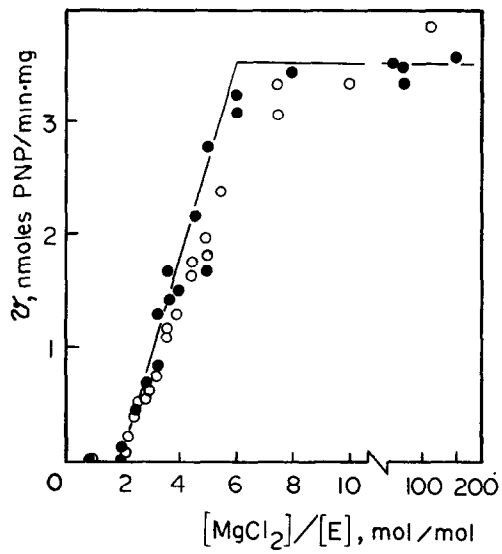

Fig.4. Rate of PNP hydrolysis as a function of $MgCl_2$ concentration. Conditions: pH 9.3, 15°C, 40 (○) or 100 (●) μM PNP, 20 (○) or 50 (●) μM enzyme.

experiments, the initial rate of PNP hydrolysis was measured at PNP to enzyme ratios below and above unity. The results suggest the presence of two independent active sites per enzyme dimer.

The same approach was used to titrate essential Mg^{2+}-binding sites on the enzyme (fig.4). These experiments were performed with equimolar amounts of the enzyme and PNP under conditions favoring tight binding of PNP and Mg^{2+} to the enzyme (15°C, pH 9.3). Analogous titrations were run at other proteins to PNP ratios. Noticeable hydrolysis only occurred if $MgCl_2$ was in excess over PNP, thus indicating that the metal ion must add both to the enzyme and substrate prior to catalysis, the latter complex being more stable. Equivalence points at two protein concentrations corresponded to about 4 mol $MgCl_2$ per mol enzyme, in addition to 2 mol/mol substrate-bound metal.

4. DISCUSSION

The results reported above show that PNP is a tightly bound, slowly converted substrate of pyrophosphatase. Equal affinities of the enzyme for PNP and natural substrate PP_i are in accord with the close similarity of their structures. Larsen et al. [12] found that the P–P distance and the P-X-P angle (X = O,N) differ in these compounds by as little as 0.06 Å and 1.5°, respectively. In contrast, the low value of the catalytic constant could have hardly been predicted since non-catalyzed hydrolysis of PNP proceeds faster compared to PP_i hydrolysis [13,14]. Low activity of pyrophosphatase towards PNP suggests that there is some kind of interaction between the enzyme and pyrophosphate bridge oxygen. It is tempting to speculate that the enzyme donates a proton to the bridge oxygen. Studies of the non-enzymatic hydrolysis of PP_i have clearly indicated that such protonation increases PP_i reactivity by a factor of 3000 [14]. This effect may thus be one of the major factors contributing to the high catalytic activity of pyrophosphatase. In this context, a low rate of the enzyme-catalyzed PNP conversion may be explained by its inability to be protonated at the nitrogen atom because of steric hindrance caused by the imido hydrogen atom.

The combination of the high affinity of pyrophosphatase towards PNP with a low catalytic

constant makes this substrate a very convenient tool for enzyme mechanism studies, in particular, for active-site and metal-binding site titrations. More than a decade ago, when the rate of PP_i hydrolysis in an acidic medium was found to be proportional to the third power of Mg^{2+} concentration [15,16], an idea was put forward that catalysis by pyrophosphatase involves three metal ions per subunit. Similar observations have been made recently in studies of the Mg^{2+}- and Mn^{2+}-supported hydrolysis of chromium(III) complexes of pyrophosphate [17]. This evidence is, however, indirect, and alternative explanations can be proposed, for instance, cooperative binding of the metal ions to two enzyme binding sites, one for each of the two subunits. The PNP data provide strong direct support for the participation in catalysis of three Mg^{2+} per pyrophosphatase subunit. One of these ions appears to be bound by the substrate and two others by the enzyme itself.

REFERENCES

[1] Kunitz, M. (1952) J. Gen. Physiol. 3, 423–449.
[2] Höhne, W.E. and Heitmann, P. (1974) Acta Biol. Med. Germ. 33, 1–14.
[3] Shafransky, Yu.A., Baykov, A.A., Andrukovich, P.F. and Avaeva, S.M. (1977) Biokhimiya 42, 1244–1251.
[4] Schlesinger, M.J. and Coon, M.J. (1960) Biochim. Biophys. Acta 41, 30–36.
[5] Braga, E.A., Baykov, A.A. and Avaeva, S.M. (1973) Biokhimiya 38, 344–350.
[6] Cohen, S.A., Sterner, R., Keim, P.S. and Heinrikson, R.L. (1978) J. Biol. Chem. 253, 714–725.
[7] Nielsen, M.L., Ferguson, R.R. and Coacley, W.S. (1961) J. Am. Chem. Soc. 83, 99–104.
[8] Kirsanov, A.V. and Zhmurova, N.N. (1958) J. Org. Khim. 28, 2478–2484.
[9] Itaya, K. and Ui, M. (1966) Clin. Chim. Acta 14, 361–366.
[10] Jencks, W.P. and Gilchrist, M. (1965) J. Am. Chem. Soc. 87, 3199–3209.
[11] Chanley, J.D. and Feageson, E. (1963) J. Am. Chem. Soc. 85, 1181–1190.
[12] Larsen, M., Willett, R. and Yount, R.G. (1969) Science 166, 1510–1511.
[13] Quimby, O.T., Narath, A. and Lowman, F.H. (1960) J. Am. Chem. Soc. 82, 1099–1106.
[14] Osterheld, R.K. (1958) J. Phys. Chem. 62, 1133–1142.
[15] Baykov, A.A. and Avaeva, S.M. (1973) Eur. J. Biochem. 32, 136–142.
[16] Braga, E. and Avaeva, S. (1972) Biochem. Biophys. Res. Commun. 49, 528–535.
[17] Knight, W.B., Dunaway-Mariano, D., Ransom, S.C. and Villafranca, J.J. (1984) J. Biol. Chem. 259, 2886–2895.