ENZYME-CATALYZED CONVERSION OF PYROPHOSPHATE TO ORTHOPHOSPHATE : KINETIC CHARACTERIZATION BY FLOW INJECTION ANALYSIS AND P-31 NMR

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Abstract Flow injection method for the selective detection of orthophosphate in the presence of pyrophosphate(diphosphate) was designed to characterize the inorganic pyrophosphatase (EC3.6.1.1) -catalyzed hydrolysis of pyrophosphate. Fast blue-coloration of molybdophosphate by a mixed reductant of Sn(II) and hydrazine sulfate was employed. The proposed method provided kinetic data that were enough to determine a catalytic rate constant and a Michaelis constant. P-31 NMR spectroscopy was also employed to confirm the conversion of pyrophosphate to orthophosphate.

INTRODUCTION

Much attention has been focussed on the bioavailability or physiological contribution of inorganic phosphorus compounds in biosystems and ecosystems^{1, 2)}. It is of special attention that pyrophosphate (diphosphate, P₂) and monofluorophosphate (MFP) are catalytically hydrolyzed by inorganic pyrophosphatase (EC3.6.1.1, P₂ase) and alkaline phosphatase (EC3.1.3.1, APase) to produce orthophosphate(P₁), respectively, at neutral pH³⁻⁸⁾. The bond cleavages of P-O-P and P-F in equations 1 and 2 are extremely accelerated by the enzymes by a factor of ten billion.

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High-performance liquid chromatography (HPLC) has been successfully employed to characterize the kinetic processes of these enzymatic reactions.^{3, 5-7)} More rapid and sensitive analytical methods such as flow injection analysis (FIA) are very often required to make multipoint measurements of fast kinetic processes especially when kinetic parameters in Michaelis-Menten equation^{1, 5, 7)} are to be determined. An improved FIA methodology with more than 10-fold enhancement in sensitivity was designed by using a mixed solution of tin(II) chloride and hydrazine sulfate as a reductant of molybdophosphoric acid. A photodiode array detector (200-800 nm)^{3, 4)} was employed to survey the spectral characteristic of the blue-colored species in the FIA flow cell. A catalytic rate constant K_3 and a Michaelis constant K_m of the enzymatic P_2 hydrolysis were evaluated by the proposed FIA method. ^{31}P NMR method was also employed to confirm the enzymatic conversion of P_2 to P_1 by measuring both the substrate and the product.

EXPERIMENTAL

Inorganic pyrophosphatase (EC3.6.1.1) from bakers yeast (Sigma, St Louis) was used for enzymatic hydrolysis of pyrophosphate.^{6, 7)}

Main components of an FIA system were indicated in Fig.1. The Mo(V) reagent was prepared by dissolving 5.3 g of ammonium molybdate, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, in about 900 ml of water, with subsequent addition of 17 ml of sulfuric acid (ca.18M). Total volume is then adjusted to 1000 ml with water. This reagent was stable at room temperature for at least one month. The Sn(II)-hydrazine sulfate solution as a reductant was prepared as follows: Two grams of hydrazine sulphate, $\text{N}_2\text{H}_6\text{SO}_4$, and 0.2 g of tin(II) chloride, $\text{Sn(II)Cl}_2 \cdot 2\text{H}_2\text{O}$, were dissolved in this order in 900 ml of water, with subsequent addition of 17 ml of sulfuric acid (ca.18M). The total volume was adjusted to 1000 ml with water. The order of dissolution

was important, because tin(II) chloride in sulfuric acid solution in the absence of hydrazine sulfate tended to produce turbidity. The reductant was stable for a few days at room temperature.

Fig.1. Flow injection system. S: sample injector. RC: reaction coil (5 m). D: photodiode-array detector. H₂O: pure water. Mo(VI): molybdenum(VI) reagent. Red: Sn(II)-hydrazine sulphate. All lines consist of PTFE tubings (0.5 mm i.d.; 1.5 or 2.0 mm o.d.).

³¹P NMR spectra were recorded at 30 °C by using 10 mm NMR tubes on a JEOL-GX-400 spectrometer operating at 162 MHz. Chemical shifts, δ values in ppm, were presented with respect to an external reference of 85% phosphoric acid.

RESULTS AND DISCUSSION

Design of FIA system

The three-channel FIA system in Fig.1 was designed so that the enzymatic reaction in eqn. 1 could be stopped instantaneously and the product P₁ could be detected selectively in the presence of the substrate P₂. A sample solution (S) was injected into the water stream (1ml/min) via a loop-valve injector (100 μ l). The molybdenum (VI) reagent from the second channel was acidic enough to stop the enzymatic reaction by acidifying the sample zone at the confluence point. Of the three phosphate species tested, P₁, P₂ and P₃ (triphosphate), only P₁ reacted rapidly with the Mo(VI) reagent to form molydophosphoric acid. Selective detection of P₁ could be achieved by measuring the absorbance of the yellow complex at wavelength around 400 nm.^{6, 7)} The third channel of a reductant was installed in Fig.1 to achieve 20-fold enhancement in sensitivity by

converting the yellow complex to a blue complex . The colored complex that was passing through the FIA flow cell was monitored by a photodiode array detector to present a three-dimensional FIA profile, time-wavelength-absorbance (Fig.2). The spectrum with a broad absorption maximum around 700 nm was different from a spectrum obtained by using a Mo(V)-Mo(VI) reagent.³⁾ Good linearity of calibration at 700 nm was observed for orthophosphate over the wide working range, 2×10^{-4} – 2×10^{-7} M P_1 .

Fig.2.
Three-dimensional
FIA profile.
(*1) 1×10^{-5} M P_1
(*2) 2×10^{-5} M P_1
(*3) 3×10^{-5} M P_1
Corresponding FIA
signals at 700 nm
are inserted.

^{31}P NMR spectroscopic monitoring of pyrophosphate hydrolysis

As can be seen in the following section only the progressive formation of P_1 from P_2 is monitored by the FIA technique, without direct evidence for the degradation of P_2 . Prior to the quantitative discussion with kinetic data from FIA experiments a simple ^{31}P NMR experiment was made to ascertain the simultaneous variation of both P_2 and P_1 . The result in Fig.3 with the P_2 -enzyme incubate indicates that the ^{31}P NMR signal intensity of orthophosphate at +2 ppm (singlet) increased with the decrease in signal intensity of pyrophosphate at -7 ppm (singlet). No NMR signals other than P_2 and P_1 were observed in the wide δ range from +20 to -25 where most monomeric, dimeric and trimeric phosphates might indicate their signals.^{9, 10)} These facts rationalize that the time course of P_2 hydrolysis can be followed by measuring the variation in P_1 concentration, which can be easily and rapidly achieved by FIA experiments.

Fig.3. Kinetic ^{31}P NMR profile for inorganic pyrophosphatase-catalyzed hydrolysis of pyrophosphate. Sample: $1.0 \times 10^{-2} \text{ M P}_2 + 1.0 \times 10^{-8} \text{ M P}_2\text{ase} + 1.0 \times 10^{-3} \text{ M Mg}^{2+} + 5 \times 10^{-3} \text{ M Tris-HCl (pH 7.2)}$. Incubation time of the $\text{P}_2\text{-P}_2\text{ase}$: a, 0 min, before the addition of P_2ase ; b, 10 min; c, 40 min; d, 60 min. Each run required the scan time of 8 min (160 transients). The spectrum in the absence of the enzyme(a) remained unchanged for at least 24 h.

Catalytic rate constant and Michaelis constant

P_2ase -catalyzed hydrolysis of P_2 in equation 1 is represented by the following scheme;

k_1 , k_2 and k_3 are rate constants. The second forward step with k_3 is assumed to be rate-determining process.

The enzymatic hydrolysis of P_2 to P_1 in Fig.4 was initiated by the addition of P_2ase to the buffered $\text{P}_2\text{-Mg}^{2+}$ mixture at 30°C . After 0.5 min incubation the first aliquot of the P_2 -enzyme incubate was injected to obtain the first FIA signal. The second signal and subsequent signals were obtained by the injections of the same P_2 -enzyme incubate at 1.5 min intervals. The P_1 signal grew with the

increase in incubation time and became constant towards the completion of hydrolytic reaction. Such kinetic profiles were analyzed according to the Michaelis-Menten equation^{1, 6, 7)} to evaluate a rate constant, $k_3 = 2.4 \times 10^4 \text{ min}^{-1}$, and a Michaelis constant, $K_m = 5 \times 10^{-6} \text{ M}$.

Fig.4. Kinetic FIA profile for the enzyme-catalyzed hydrolysis of pyrophosphate. Sample: $1 \times 10^{-4} \text{ M P}_2 + 2 \times 10^{-10} \text{ M P}_2\text{ase} + 1 \times 10^{-3} \text{ M Mg}^{2+} + 5 \times 10^{-3} \text{ M Tris-HCl (pH7.2)}$. Recording was made at 700 nm.

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REFERENCES

1. L. Stryer, Biochemistry, W. H. Freeman, New York, 1988.
2. A. Torriani-Gorini, E. Yagil, and S. Silver(Eds), Phosphate in Microorganisms, ASM Press, Washington, D.C., 1994.
3. N. Yoza, I. Akazaki, T. Nakazato, N. Ueda, H. Kodama, and A. Tateda, Anal. Biochem., 199, 279(1991).
4. N. Yoza, S. Nakashima, T. Nakazato, N. Ueda, H. Kodama, and A. Tateda, Anal. Chem., 64, 1499(1992).
5. N. Yoza, S. Nakashima, I. Akazaki, N. Ueda, T. Nakazato, N. Tokushige, and T. Yokota, Mem. Fac. Sci., Kyushu University, Ser. C, 18, 203(1992).
6. N. Yoza, H. Hirano, Y. Baba, and S. Ohashi, J. Chromatogr., 325, 385(1985).
7. H. Hirano, Y. Baba, N. Yoza and S. Ohashi, Anal. Chim. Acta, 179, 209(1986).
8. N. Yoza, H. Hirano, Y. Baba, and S. Ohashi, Phosphorus Sulfur, 30, 605(1987).
9. N. Yoza, S. Nakashima, N. Ueda, T. Miyajima, T. Nakamura, and P. Vast, J. Chromatogr., 664, 111(1994).
10. N. Yoza, N. Ueda, and S. Nakashima, Fresenius J. Anal. Chem., 348, 533(1994).