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Real-time Measurement of Pyrophosphate Release Kinetics

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Abstract

In order to measure the kinetics of pyrophosphate release, we have coupled pyrophosphatase to the fluorescently labeled phosphate binding protein developed by Webb. We show that the assay is capable of measuring rates of pyrophosphate release up to 100 s⁻¹.

There are over 1000 enzymes, including at least one from each of the six major enzyme commission categories, that generate pyrophosphate (PP_i) as a product of catalysis (1). Prominent among these are DNA and RNA polymerases in addition to enzymes that utilize ATP and release PP_i as one of the products of the reaction. In our ongoing efforts to characterize the kinetics and fidelity of DNA replication, it was necessary to have a sensitive technique for observing pyrophosphate dissociation from the active site of the polymerase following a single nucleotide incorporation reaction. A method was designed for reactions performed under pre-steady-state conditions using a fluorescence stopped-flow apparatus. The coupled assay described here is an adaptation of the methods developed by Webb for sensing the release of phosphate (P_i) using a site-specifically 7-diethylamino-3-(((2-maleimidyl)ethyl)amino)carbonyl)coumarin (MDCC) labeled phosphate binding protein (PBP) (2). The labeled protein exhibits an 8-fold increase in fluorescence upon the binding of phosphate that is both rapid ($> 3 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$) and tight ($K_d \sim 100 \text{ nM}$) (3;4) and has been used to measure the rate of phosphate release from a number of enzymes (5–10). To increase sensitivity, the assay employs a “P_i mop”, consisting of 7-methylguanosine (7-MEG) and purine nucleoside phosphorylase (PNPase) to remove free contaminating P_i from solution by its conversion to ribose 1-phosphate. Under most circumstances the P_i mop does not interfere with the real-time measurement because the rate of P_i consumption is slow in comparison to the rate at which it binds to the PBP, but it does limit the utility of the method for measuring rates less than 0.01 s⁻¹.

Here we report a detailed procedure for adapting this sensitive P_i assay for use in measuring PP_i release kinetics. The method for the preparation of the MDCC-labeled PBP used during this study was described previously (11). Shown in Fig. 1 are data from a series of experiments performed in a KinTek SF-2004 (Austin, TX) stopped-flow apparatus documenting the utility of the method. Our first experiment (Fig. 1A) was aimed at comparing the time dependence of the fluorescence change associated with directly mixing the PBP with P_i or PP_i. In these experiments 1.5 μM PBP was pre-incubated with 100 μM

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7-MEG and 0.02 units/mL PNPase for 15 minutes at room temperature. This solution was then rapidly mixed at 20°C with 10 μM P_i or PP_i . The sample was excited using a wavelength of 425 nm and observation of the emission was achieved with a 450 nm long pass filter. All concentrations listed refer to the final values after mixing (1:1 dilution) has occurred. The buffer system used for all experiments consisted of 50 mM Tris-base pH 7.5 at room temperature, 100 mM NaCl and 5 mM MgCl_2 .

The data show that PP_i does not induce a significant change in the PBP fluorescence (Fig. 1A), compared to the large signal seen after mixing with excess phosphate. The time dependence of the signal after mixing with P_i was biphasic and was therefore fitted using a double exponential equation to obtain the observed amplitudes (A_1 and A_2) and rates (k_1 and k_2) of the two phases: $\text{Fluorescence} = A_1 \cdot e^{-k_1 \cdot t} + A_2 \cdot e^{-k_2 \cdot t} + C$. Greater than 90% of the binding reaction occurred at a rate of $327 \pm 2 \text{ s}^{-1}$, while the remaining change occurred at $62 \pm 2 \text{ s}^{-1}$. The slow phase may represent a small fraction (less than 10%) of less active PBP, but because most experiments are conducted with PBP in excess over P_i , the slower component does not interfere. In contrast, when PP_i was mixed with the PBP there was only a small change in fluorescence of about 2% relative to that seen with P_i that could be attributable to small percentage (0.3%) of contaminating P_i in the PP_i .

To measure PP_i quantitatively, PP_i must be hydrolyzed to P_i in order to obtain a measurable signal. An experiment was performed in which various concentrations of yeast inorganic pyrophosphatase (PPase) (Sigma-Aldrich St. Louis, MO) were included in the solution containing the PBP. The concentration of the PPase was determined by using the Coomassie Plus assay available from Pierce Biotechnology (Rockford, IL) and assuming a molecular weight of 32,000 Da. In addition, the “ P_i mop” components were included in both syringes to eliminate trace P_i contamination and allowed to incubate for 15 minutes at room temperature prior to mixing in the stopped-flow. Two representative traces are shown in Fig. 1B., for the lowest and highest concentrations of PPase inspected (75 nM and 2.9 μM , respectively). The data were biphasic and therefore fitted using a double exponential equation to obtain the observed rates. The concentration dependence of the two phases of the reaction are plotted as a function of PPase concentration in Fig. 1C. The fast phase increased linearly with the concentration of PPase while the slower phase approached a constant value.

The kinetics of the coupled PP_i sensor reaction can be understood by considering the simplified pathway shown in Scheme I.

The faster phase of the reaction, under the conditions of the experiments (Figs. 1B and 1C), is defined by the rate of the PPase reaction, while the slower phase is defined by the rate of the P_i binding to the PBP. In general, experiments will be designed so that the concentrations of PPase and the PBP are in excess over the concentration of PP_i ; therefore, the rate of each step is governed by the product of the concentration of the PPase or the PBP and the corresponding second order rate constant (Scheme I). That is, to a first approximation the rate at which PP_i is converted to P_i is defined by the product, $k_2[\text{PPase}]$, and the rate at which P_i binds to the PBP is defined by the product, $k_3[\text{PBP}]$. The slope from the concentration dependence of the fast phase (Fig 1C) defines $k_2 = 70 \mu\text{M}^{-1}\text{s}^{-1}$, the apparent second order rate constant governing the conversion of PP_i to P_i by PPase and equal to k_{cat}/K_m .

The binding of P_i to the PBP is a two-step process with the formation of a rapid equilibrium collision complex ($K_d = 4.9 \mu\text{M}$) followed by isomerization to the highly fluorescent state at a rate of 317 s^{-1} (4).

This two-step reaction defines an apparent second order rate constant of $65 \mu\text{M}^{-1}\text{s}^{-1}$ for the binding of P_i to the PBP. Thus, at a concentration of $1 \mu\text{M}$ PBP, one expects a rate of slightly less than 65 s^{-1} , which is consistent with the observed rate of $61 \pm 5 \text{ s}^{-1}$ (Fig 1C). These data demonstrate that PPase and the PBP can be coupled to monitor PP_i at a rate sufficiently fast to be useful for a real-time assay.

The overall magnitude of the fluorescence change observed when the PBP is in excess of P_i should also allow for an accurate determination of the concentration of PP_i liberated during a reaction. An experiment was performed in which only the concentration of PP_i was varied to evaluate whether the amplitude of the signal was a linear function of concentration of PP_i . For this experiment, various substoichiometric concentrations of PP_i were mixed with $1.5 \mu\text{M}$ PBP in the presence of 190 nM of PPase. The total change in fluorescence (ΔF_{obs}) varied with the concentration of PP_i as shown in Fig. 1D. The data were slightly non-linear and were fitted using following hyperbolic function to generate the standard curve:

$\Delta F_{obs} = \frac{\Delta F_{max} \cdot [PP_i]}{K_{1/2} + [PP_i]}$. Fitting the data using this equation afforded a more accurate quantitative description of the trend so that the amount of PP_i released during the course of an enzymatic reaction can be reliably determined. However, the reaction was sufficiently linear at concentrations below 400 nM , that one can interpret the fluorescence time course as a linear function of PP_i concentration.

Experiments shown here and computer modeling show that at concentration of $1 \mu\text{M}$ PPase and $1 \mu\text{M}$ PBP, the assay can accurately measure rates of PP_i release up to 25 s^{-1} . Under these circumstances, one expects the time dependence to follow three exponentials with rates of 25 , 70 and 65 s^{-1} according to Scheme I. The two faster reactions blend together to form the “lag” phase with a rate of approximately 35 s^{-1} , computed by the approximation: $k_{net} = 1/(1/k_2 [PPase] + 1/k_3 [PBP])$. Due to the high signal:noise ratio provided by the fluorescently labeled PBP, the rate of PP_i release at 25 s^{-1} can be readily resolved from the lag phase at 35 s^{-1} . We have successfully measured the rate of release of PP_i from the active site of the human mitochondrial DNA polymerase γ following a single nucleotide incorporation reaction that occurred at a rate of approximately 20 s^{-1} (12).

Scheme I serves as a practical guide for understanding the kinetics of the couple assay, but it overlooks the rate-limiting hydrolysis of PP_i and release of P_i by PPase and the isomerization of the PBP after P_i binding. The maximum rate of PP_i release that can be measured with the PPase/PBP system is limited by the maximum rate of turnover of PPase ($k_{cat} = 270 \text{ s}^{-1}$) (13) and by the rate of isomerization of the PBP to the enhanced fluorescence state after P_i binding (317 s^{-1}) (4). We performed more comprehensive modeling by computer simulation including the rates of release of P_i from PPase and the two-step P_i binding to the PBP. Computer simulation was performed using KinTek Global Kinetic Explorer software (KinTek Corp, Austin, Tx). This analysis predicted that an assay with $10 \mu\text{M}$ PPase and $10 \mu\text{M}$ PBP would be able to resolve rates of PP_i release of at least 100 s^{-1} .

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Summary

Coupling pyrophosphatase to the fluorescently labeled phosphate binding protein allows rates of pyrophosphate release to be measured up to at least 100 s^{-1} . The assay provides a high signal:noise ratio capable of resolving the kinetics of PP_i release from the reactions of the coupled assay and with PP_i release amplitudes of 50 nM or less.

Fig. 1. Kinetics of the PP_i sensor using the PBP

(A) The time dependence of the fluorescence change is shown after mixing with either P_i or PP_i illustrating that PP_i does not cause a significant change in fluorescence of the MDCC-labeled-PBP. The fluorescence units given on the Y-axis are arbitrary. The data denoted with "+ P_i" was obtained by rapidly mixing a final concentration of 1.5 μM PBP with 10 μM P_i. The data denoted with "+ PP_i" was obtained similarly except that 10 μM PP_i was used in place of P_i. (B) A solution containing 1 μM PBP and 75 nM or 2.9 μM PPase were mixed with 400 nM PP_i in the presence of the P_i mop components. (C) The fast and slow phase rates from "(B)" were plotted as a function of PPase concentration. The fast phase data were best fitted using a linear function with a slope of $70 \pm 4 \mu\text{M}^{-1}\text{s}^{-1}$ and an intercept of $60 \pm 7 \text{ s}^{-1}$ and the slow phase data were best fitted using a hyperbolic function with a $K_{1/2}$ of $0.4 \pm 0.1 \mu\text{M}$ and a maximum rate of $61 \pm 5 \text{ s}^{-1}$. (D) A solution containing 1.5 μM PBP in the presence of 190 nM PPase was mixed with various concentrations of PP_i. The total change in fluorescence is plotted as a function of PP_i concentration. The data were fitted by non-linear regression using a hyperbolic function to obtain the solid line with a $K_{1/2}$ of $2.9 \pm 0.4 \mu\text{M}$.

Scheme I.