Real Time Scale Measurement of Inorganic Phosphate Release by Fluorophore Labeled Phosphate Binding Protein

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Fluorescence change of coumarin labeled phosphate binding protein (PBP-MDCC) was monitored to measure the amount of released inorganic phosphate (P_i) during nucleoside triphosphate (NTP) hydrolysis reaction. After purification of PBP-MDCC, fluorescence emission spectra showed that fluorescence responded linearly to P_i up to about 0.7 molar ratio of P_i to protein. The correlation of fluorescence signal and P_i standard was measured to obtain [P_i] - fluorescence intensity standard curve on the stopped-flow instrument. When T7 bacteriophage helicase, double-stranded DNA unwinding enzyme using dTTP hydrolysis as an energy source, reacted with dTTP, the change of fluorescence was able to be converted to the amount of released P_i by the P_i standard curve. P_i release results showed that single-stranded M13 DNA stimulated dTTP hydrolysis reaction several folds by T7 helicase. Instead of end point assay in NTP hydrolysis reaction, real time P_i-release assay by PBP-MDCC was proven to be very easy and convenient to measure released P_i.

Key words – phosphate binding protein, inorganic phosphate, T7 bacteriophage helicase, stopped-flow instrument

Inorganic phosphate (P_i) is ubiquitous and very common constituent of living organisms. In fact, many enzymes use NTP as an energy source and obtain the driving force by NTP hydrolysis[6,7]. Actin, myosin, and motor proteins are good examples for the generation of forces by NTP hydrolysis[13,14]. Therefore, the ability to measure the amount of released P_i and monitor the changes of P_i during the NTP hydrolysis reaction contributes to understand an involved biological process. In fact, a variety of spectrophotometric assays have been developed to measure the released P_i quantitatively. They include the development of color after phosphomolybdate complex formation and enzymatic reaction by phosphorylation of fluorescent substrate[2,5]. Many kinetic studies of P_i release were done by these assays. In addition, another type of P_i release assay was also developed to follow rapid P_i release in real time. The assay method is based on *E. coli* phosphate binding protein (PBP), the product of the *phoS* gene, which is a member of proteins that are expressed and transported to the periplasm under the condition of P_i starvation[3]. PBP is a monomeric protein with a molecular mass of 35 kDa and the crystal structure for the P_i bound form revealed two domains with a P_i-binding cleft between them[12]. The single cysteine mutant (A197C) form of PBP made it possible to label selectively with a fluorophore, N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC). The resultant MDCC labeled PBP (PBP-MDCC) undergoes a large conformation change on P_i binding, so fluorescence signal is very sensitive to binding and amount of released P_i. PBP-MDCC is reported as rapid (1.36×10^8 $M^{-1}s^{-1}$) and tight (K_d of ~0.1 μM) binding to P_i[3], so that it is very sensitive in the nanomolar range of P_i.

Here, PBP-MDCC was used to measure the amount of released P_i from dTTP hydrolysis by T7 bacteriophage helicase. T7 helicase is a motor protein that translocates along DNA during DNA replication and recombination and unwinds the complementary DNA strand[14]. T7 helicase assembles into hexameric ring in the presence of NTP, preferably dTTP, and the hexamer has a high affinity for single-stranded DNA that binds within the central channel of the ring. The energy source for T7 helicase is dTTP and, whose function is equivalent to that of ATP in other motor proteins. The chemical energy of dTTP hydrolysis is used to move the T7 helicase unidirectionally along the DNA. Therefore accurate measurement of dTTP hydrolysis product is the first step to investigate the dTTP hydrolysis mechanism of T7 helicase.

Using a real time fluorescence stopped-flow assay, re-

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leased P_i from dTTP hydrolysis by T7 helicase was measured in the absence and presence of DNA. The results showed that PBP-MDCC turned out to be a good probe to follow rapid P_i changes in real time experiments.

Materials and Methods

Reagents

Bacterial purine nucleoside phosphorylase (PNPase), 7-methylguanosine (MEG), dTTP, and the standard solution of P_i were purchased from Sigma Chemicals. N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC) was purchased from Molecular Probes.

Purification of MDCC-labeled phosphate binding protein

The single cysteine mutant (A197C) phosphate binding protein (PBP) was expressed and purified as described[3] with the following modifications. Overnight grown cells in LB medium containing 12.5 mg/l tetracycline were placed in four liters of minimal media containing 100 mM Hepes (pH 7.5), 20 mM KCl, 15 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, 10 μM $FeSO_4$, 1 μg/ml thiamine, 0.25% (v/v) glycerol, 2 mM KH_2PO_4, and grown for two hours. 200 mM rhamnose was added to the minimal media, and cells were grown overnight at 37°C. The cells were pelleted by centrifugation and resuspended in a Tris/HCl buffer (10 mM, pH 7.6) including 30 mM NaCl. PBP was released from the cells' periplasm by osmotic shock[4], and the periplasmic extract was applied to a Q-Sepharose column (100 ml) that was equilibrated with 10 mM Tris/HCl (pH 7.6). PBP was eluted as the major protein with a linear gradient of 0 - 200 mM NaCl in the same buffer. PBP concentration was determined by absorbance measurement at 280 nm using an extinction coefficient of λ_{280nm} = 60,880 $M^{-1}cm^{-1}$.

Labeling of the A197C PBP protein with MDCC and further purification of the labeled protein (PBP-MDCC) were performed as described[3] except 200 μM of MEG and 0.2 units/ml of PNPase were included in the labeling reaction. The labeled protein after purification showed a 280 nm/430 nm absorbance ratio of 1.6, suggesting that most of PBP was labeled with MDCC[3].

T7 helicase protein and buffer

The T7 helicase used in this study is gp4A', which is a M64L mutant of T7 helicase-primase protein that was over-expressed and purified as described previously[8,15]. The protein concentration was determined both by absorbance measurements at 280 nm in 8 M urea (the extinction coefficient is 76,100 M^{-1} cm^{-1}), and by the Bradford assay using bovine serum albumin as a standard. Both methods provided similar protein concentrations. Tris buffer was used throughout the experiments unless specified otherwise, which contained 50 mM Tris/Cl (pH 7.6), 40 mM NaCl, and 10% (v/v) glycerol.

Fluorescence titrations of P_i with PBP-MDCC

1 ml of PBP-MDCC solution (5 μM PBP-MDCC, 300 μM MEG, and 0.5 unit/ml PNPase) was titrated with standard P_i solution and fluorescence emission spectra were obtained. Fluorescence was corrected with respect to volume change. Fluorescence emission at 465 nm was plotted versus P_i concentration to create correlation curve.

Real time measurement of released P_i

Fluorescence change experiments were performed using a stopped-flow instrument. Inorganic phosphate release reactions were performed in Tris buffer at 18°C. Since PBP-MDCC is sensitive to nanomolar quantities of P_i ubiquitously contaminated in all solutions, a coupled enzyme reaction (P_i-mop: 0.5 units/ml PNPase and 300 μM MEG) was used to sequester P_i chemically. Assay conditions were adjusted to ensure that the P_i-mop did not compete with PBP-MDCC for phosphate (k_{cat}/K_m for the PNPase reaction with P_i is 3.2×10^6 $M^{-1}s^{-1}$)[1]. The fluorescence signal of PBP-MDCC was calibrated using P_i standard on the stopped-flow apparatus. The amplitude of fluorescence increase was measured by conducting a control experiment in the absence of P_i and subtracting the maximum fluorescence of the control from the one with a known concentration of P_i. The amplitude thus calculated was plotted versus P_i concentration to create the calibration curve. The calibration curve was created using the same photomultiplier tube (PMT) voltage as used in the subsequent experiment, just before a set of P_i release experiments were performed. The slope was used to convert the observed fluorescence amplitude into molar P_i.

A 40 μl solution containing T7 helicase (0.4 and 0.2 μM hexamer in the absence and presence of single stranded DNA, respectively), EDTA (5 mM), P_i-mop (0.5 units/ml PNPase with 300 μM MEG), and various concentrations of dTTP in the absence of DNA and presence of single-stranded (ss) M13 DNA (3 nM) was rapidly mixed with 40

μl of $MgCl_2$, P_i-mop and 10 μM PBP-MDCC in the stopped-flow instrument at 18°C. The fluorescence changes of PBP-MDCC were monitored using an excitation wavelength of 425 nm and monitoring the emission above 450 nm using a cut-off filter (Corion LL-450 F). For each experiment, at least four fluorescence traces were averaged. The fluorescence changes were converted to the concentration of released P_i using the standard curve. The concentration of released P_i per T7 helicase hexamer was plotted versus time of reaction. A control experiment with all the components except Mg^{2+} was performed and subtracted. The resulting curves were fit to Eq. 1.

$$y = A[1 - \exp(-kt)] + mt \quad \text{(Eq. 1)}$$

Where y is observed fluorescence or molar amount of P_i released at time t, A and k are amplitude and rate of the burst phase, respectively; and m is the rate of the linear steady-state phase.

Results and Discussion

Titration of PBP-MDCC with P_i

When P_i was added to PBP-MDCC and the sample was excited at 425 nm, the emission maximum shifted from 475 nm to 465 nm and the fluorescence intensity increased about 6 fold (Fig. 1A). The titration curve with P_i showed that fluorescence responded linearly to P_i up to about 0.7 molar ratio of P_i to protein. (Fig. 1B). These measurements are in agreement with those reported previously[3]. The crystal structure of PBP in the absence and presence of P_i shows that conformational change between two structures make a cleft in the absence of ligand[11,12]. PBP consists of two domains with P_i situated at the domain interface[10]. Mutagenesis of wild type PBP to A197C PBP and MDCC labeling of A197C PBP resulted in structures that were identical to wild type PBP[12,17]. Labeling of PBP with MDCC is known as not disrupting either its overall folding or P_i binding site[10]. As in wild type PBP, P_i is monobasic and makes 12 hydrogen bonds with PBP-MDCC[10]. An interesting feature of the PBP-MDCC structure is its similarity to the wild type PBP at the MDCC binding site, which shows no major readjustment of either main chain or side chain residues responsible for anchoring coumarine. This is consistent with the observation that the pattern of P_i binding to wild type of PBP and PBP-MDCC is very similar[4]. Whereas the protein cleft is open in the absence of P_i, P_i binding causes cleft closure with changes in fluorescence.

Plotting fluorescence amplitude versus concentration of P_i created the P_i standard curve (Fig. 2). The slope was

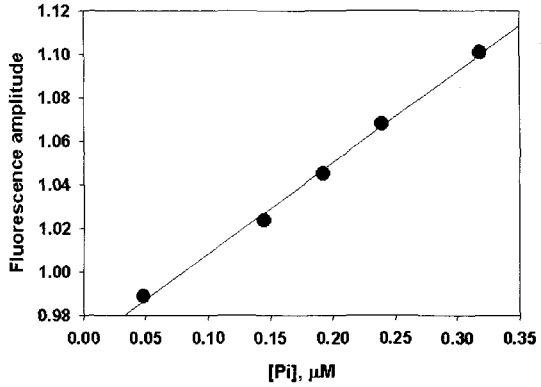

Fig. 2. P_i standard curve. The dependence on [P_i] of the fluorescence increase amplitude is shown. The amount of released P_i can be calculated from the slope of the line. The slope is 0.42 fluorescence/[P_i].

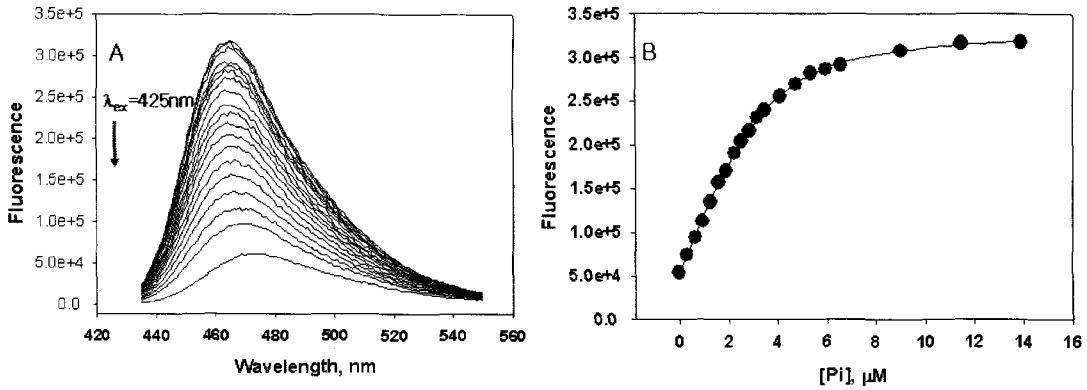

Fig. 1. Titration of PBP-MDCC with P_i. (A) Fluorescence emission spectra of PBP-MDCC in the presence of P_i. 5 μM PBP-MDCC in Tris buffer with 300 μM MEG and 1.0 unit/ml PNPase was titrated with standard P_i solution. Samples were excited at 425 nm. (B) Correlation between [P_i] and fluorescence intensity at 465 nm.

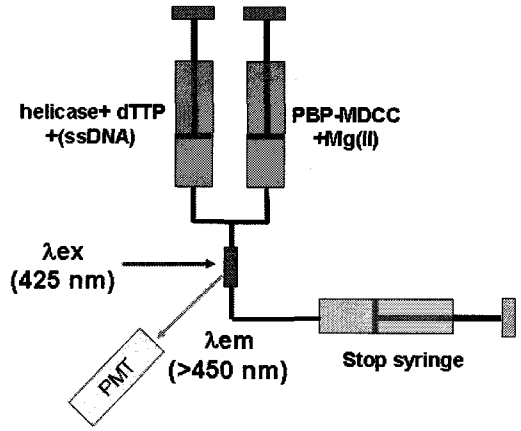

Fig. 3. Design of the stopped-flow P_i-release experiment. The two driving syringes are compressed to mix solutions, and then the mixture ages with time and the detector monitors the fluorescence change.

0.42 fluorescence/[P_i]. PMT voltage was same throughout the experiments.

Measurement of P_i release in a stopped-flow instrument

The stopped-flow instrument rapidly mixes reactants on a millisecond time scale and optical signal is obtained. Here, the optical signal is fluorescence and the goal in a stopped-flow experiment is that fluorescence signal correlates with P_i release in the reaction. The amount of released P_i can be measured in real time in a stopped-flow instrument by following the increase in fluorescence of MDCC[3]. In the presence of PBP-MDCC, dTTP-hydrolyzing protein, T7 helicase, was rapidly mixed in the stopped-flow instrument with $MgCl_2$ as shown in Fig. 3. T7 helicase assembles into hexamer in the presence of dTTP and binds ssDNA[8]. However Mg^{2+} is not required for hexamer formation and dTTP hydrolysis by preformed hexameric T7 helicase is negligible in the absence of Mg^{2+} (data not shown). T7 helicase was preincubated with various concentrations of dTTP (in the absence of Mg^{2+}) and mixed with $MgCl_2$ and PBP-MDCC in a stopped-flow instrument at 18°C. T7 helicase hexamer·dTTP complex was preformed and dTTP hydrolysis reaction was initiated by addition of $MgCl_2$. The magnitude of fluorescence increase was converted to P_i concentration, which was plotted as a function of time (Fig. 4A). A burst increase of P_i-release was observed at all concentration of dTTP. This indicate that the P_i-release rate is faster than the dTTP hydrolysis turnover rate, which is governed by the dTDP release rate or dTTP rebinding. When the slopes of the linear phase were plotted as a function of [dTTP], they increased in a hyperbolic manner (Fig. 4B). Therefore rate-limiting step shifts from dTTP binding to product release. The hyperbolic curve provided the V_{max} of 1.82±0.06 s^{-1} and K_m of 16.5±2.5 μM.

T7 helicase with dTTP in the presence of single-stranded M13 DNA was mixed with $MgCl_2$ and PBP-MDCC in a stopped-flow instrument at 18°C, and the P_i-release was measured as a function of time. Fig. 5A shows time tra-

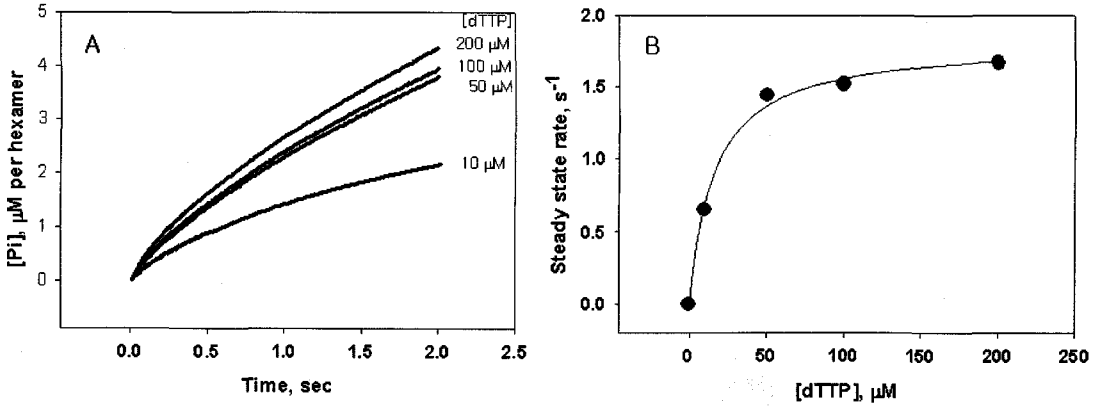

Fig. 4. P_i-release from dTTP hydrolysis by T7 helicase in the absence of DNA. (A) T7 helicase (0.4 μM hexamer), EDTA (5.0 mM), P_i-mop, and dTTP (5~400 μM) was mixed with $MgCl_2$ (9.1 - 9.4 mM) and PBP-MDCC (10 μM) in a stopped-flow instrument at 18°C. The final concentrations of helicase and free $MgCl_2$ were 0.2 μM hexamer and 2 mM, respectively. The fluorescence (em>450 nm) upon excitation at 425 nm was measured as a function of time. Molar amounts of P_i released per mole of hexameric helicase were shown. The P_i release curve fit best to the burst equation (Eq. 1). (B) The [dTTP] dependence of the steady state rate is shown. It shows a hyperbolic trend toward saturation with increasing [dTTP]. V_{max} and K_m are 1.82±0.06 s^{-1} and 16.5±2.5 μM, respectively.

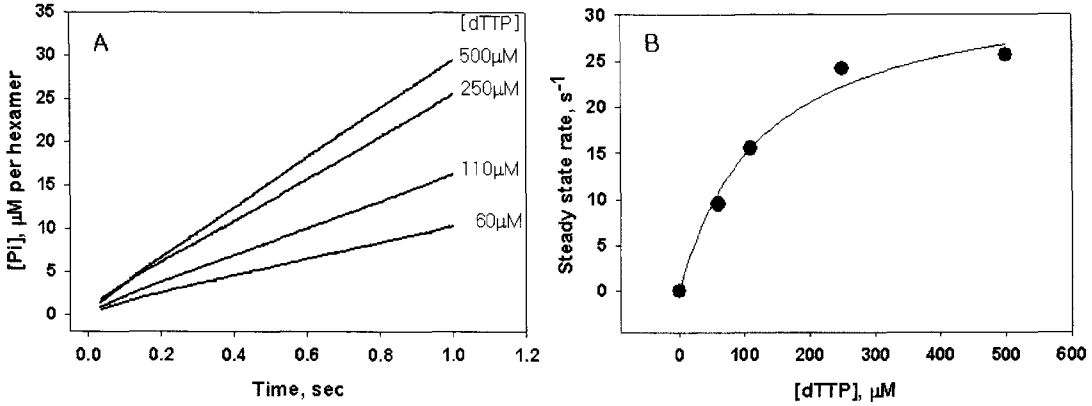

Fig. 5. P_i-release from dTTP hydrolysis by T7 helicase in the presence of single stranded M13 DNA. (A) T7 helicase (0.2 μM hexamer), EDTA (5.0 mM), P_i-mop, M13 ssDNA (3 nM), and dTTP (120~1000 μM) was mixed with $MgCl_2$ (45.2~48.2 mM) and PBP-MDCC (10 μM) in a stopped-flow instrument at 18°C. The final concentrations of helicase and free $MgCl_2$ were 0.1 μM hexamer and 20 mM, respectively. The fluorescence (em>450 nm) upon excitation at 425 nm was measured as a function of time. Molar amounts of P_i released per mole of hexameric helicase were shown. (B) The steady state rates from slope of linear phase in (A). It shows a hyperbolic trend toward saturation with increasing [dTTP]. V_{max} and K_m are 33.7±3.1 s^{-1} and 129.5±31.9 μM, respectively.

jectories of P_i release at different [dTTP]. When the slopes of the linear phase were plotted as a function of [dTTP], they increased in a hyperbolic manner (Fig. 5B). The hyperbolic curve provided the V_{max} of 33.7±3.1 s^{-1} and K_m of 129.5±31.9 μM.

Stopped-flow experiments showed that P_i release can be measured even millisecond scale of dTTP hydrolysis reaction. As shown in Fig. 4A and Fig. 5A, released P_i was about 2.3 μM at 1.0 s of 100 μM dTTP hydrolysis reaction in the absence of DNA and 16.3 μM at 1.0 s of 110 μM dTTP in the presence of M13 ssDNA, respectively. This indicate that ssDNA stimulate dTTP hydrolysis about 7-fold at 1.0 s reaction. Stimulation by ssDNA is clearly shown in Fig. 5B. Steady state rate reached 33 s^{-1} when [dTTP] increased up to 500 μM in the presence of M13 ssDNA. However steady state rate reached only 1.7 s^{-1} in the absence of DNA. The dTTP hydrolysis activity of T7 helicase is greatly stimulated by the presence of ssDNA, which is consistent with previous studies[15,16].

Here, the procedures and applications of PBP-MDCC as a P_i probe were described by dTTP hydrolysis reaction of T7 helicase. Instead of using radioactive material, fluorescence change made it possible to monitor real-time P_i release reaction by PBP-MDCC. Therefore it is expected to study the mechanism of nucleotide hydrolyzing enzymes in detail on a rapid time scale by real time measurement of P_i release.

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초록 : 형광단이 붙어 있는 인산결합 단백질에 의한 인산 배출의 실시간 측정

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Coumarine이 부착된 인산결합 단백질 (PBP-MDCC)의 형광변화가 뉴클레오사이드 삼인산 가수분해과정에서 배출된 무기 인산의 양을 측정하기 위해 관찰되었다. PBP-MDCC 정제후, 형광 방출 스펙트럼은 형광세기가 PBP-MDCC의 몰비율로 약 70%까지 직선형태로 증가하는 것을 보였다. 형광 신호와 인산 기준물질과의 상호관계 측정이 인산 농도-형광세기 표준곡선을 구하기 위하여 stopped-flow 기구에서 행하여졌다. dTTP 가수분해로부터 나오는 에너지를 이용하여 이중나선 DNA를 풀어주는 단백질인 T7 박테리오파지 나선효소를 dTTP와 반응시켰을때, 형광변화를 배출된 인산의 양으로 전환할수 있었다. 인산 배출 결과는 단일가닥 M13 DNA가 T7 나선효소에 의한 dTTP 가수분해반응을 여러배 증가시키는 것을 보인다. 뉴클레오타이드 삼인산 가수분해 반응에 있어서 종말점 분석 대신에, PBP-MDCC에 의한 연속적인 인산 배출 분석이 배출된 인산을 측정하는데 있어서 쉽고 편리한 방법임을 보였다.