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A continuous spectrophotometric enzyme-coupled assay for deoxynucleoside triphosphate triphosphohydrolases

Deepa Singh [a], Roel M. Schaaper [a], Alejandro Hochkoeppler [b, c, *]

[a] Genome Integrity and Structural Biology Laboratory, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA
[b] Department of Pharmacy and Biotechnology, University of Bologna, 40136 Bologna, Italy
[c] CSGI, University of Firenze, 50019 Sesto Fiorentino, FI, Italy

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ABSTRACT

We describe a continuous, spectrophotometric, enzyme-coupled assay useful to monitor reactions catalyzed by nucleoside triphosphohydrolases. In particular, using *Escherichia coli* deoxynucleoside triphosphohydrolase (Dgt), which hydrolyzes dGTP to deoxyguanosine and tripolyphosphate (PPP_i) as the enzyme to be tested, we devised a procedure relying on purine nucleoside phosphorylase (PNPase) and xanthine oxidase (XOD) as the auxiliary enzymes. The deoxyguanosine released by Dgt can indeed be conveniently subjected to phosphorolysis by PNPase, yielding deoxyribose-1-phosphate and guanine, which in turn can be oxidized to 8-oxoguanine by XOD. By this means, it was possible to continuously detect Dgt activity at 297 nm, at which wavelength the difference between the molar extinction coefficients of 8-oxoguanine (8000 M^{-1} cm^{-1}) and guanine (1090 M^{-1} cm^{-1}) is maximal. The initial velocities of Dgt-catalyzed reactions were then determined in parallel with the enzyme-coupled assay and with a discontinuous high-performance liquid chromatography (HPLC) method able to selectively detect deoxyguanosine. Under appropriate conditions of excess auxiliary enzymes, the activities determined with our continuous enzyme-coupled assay were quantitatively comparable to those observed with the HPLC method. Moreover, the enzyme-coupled assay proved to be more sensitive than the chromatographic procedure, permitting reliable detection of Dgt activity at low dGTP substrate concentrations.

The turnover of dNTPs in vivo is an important factor affecting both the rate of DNA replication and its fidelity. The synthesis of dNTPs starts with the reduction of ribonucleoside diphosphates to the corresponding dNDPs by ribonucleoside diphosphate reductase, an allosteric enzyme whose regulation ensures a proper balance of the four dNDPs [1,2]. Subsequently, the nonspecific action of nucleoside diphosphate kinase (NdpK) yields dNTPs at the expense of ATP and dNDPs [3–5]. The presence of a properly balanced pool of dNTPs not only is important to replication velocity but also is essential to guarantee high-replication fidelity. Interestingly, although NdpK is not an essential enzyme in *Escherichia coli*, mutator phenotypes were linked to defects of this enzyme [6,7], indicating that imbalances in the dNTP pool are potentially mutagenic. The maintenance of a balanced dNTP pool is also affected by the dephosphorylation of dNTPs by the action of various nucleotidases, including the dNTP triphosphohydrolases [8]. These enzymes catalyze the release of tripolyphosphate and deoxynucleosides at the expense of the corresponding dNTPs [9–14]. In *E. coli*, deoxynucleoside triphosphohydrolase (Dgt) was early recognized as a dNTP triphosphohydrolase [9], and it was also demonstrated that Dgt strongly prefers dGTP as substrate. Remarkably, inactivation of the *dgt* gene triggers a mutator phenotype, suggesting that an increase in the dGTP pool reduces replication fidelity [15].

Currently, the activity of dNTP triphosphohydrolases is determined primarily using discontinuous assays. Usually, aliquots are withdrawn from reaction mixtures as a function of time, the reaction products are separated by chromatographic techniques, and they are finally revealed by ultraviolet (UV) spectroscopy or radioactivity counting [13,16,17]. A discontinuous high-throughput procedure has also been reported [18]. This method relies on the

Abbreviations used: NdpK, nucleoside diphosphate kinase; Dgt, deoxynucleoside triphosphohydrolase; UV, ultraviolet; FPBP, fluorescent phosphate-binding protein; PNPase, purine nucleoside phosphorylase; XOD, xanthine oxidase; AP, alkaline phosphatase; PPP_i, tripolyphosphate; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

* Corresponding author. Department of Pharmacy and Biotechnology, University of Bologna, Viale Risorgimento 4, 40136 Bologna, Italy.
E-mail address: a.hochkoeppler@unibo.it (A. Hochkoeppler).

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use of *E. coli* inorganic pyrophosphatase to release orthophosphate from tripolyphosphate and on the subsequent colorimetric quantitation of orthophosphate.

Recently, an elegant continuous coupled assay of dNTP triphosphohydrolases was proposed [19]. This method relies on the coupled release of orthophosphate from tripolyphosphate catalyzed by *Saccharomyces cerevisiae* Ppx1 exopolyphosphatase [19]. The released orthophosphate is then fluorimetrically determined using a modified phosphate-binding protein, *N*-(2-[1-maleimidyl] ethyl)-7-(diethylamino) coumarin-3-carboxamide phosphate-binding protein, the fluorescence of which is greatly increased when bound to the target [20,21]. On comparison with a reference discontinuous method, this procedure proved to be robust and accurate [19]. However, although the fluorescent phosphate-binding protein (FPBP) is commercially available, one must overexpress and purify the yeast Ppx1 exopolyphosphatase. In addition, the use of FPBP to detect orthophosphate faces at least two challenges. First, due to the low K_D of the FPBP–phosphate complex (30–80 nM, depending on pH), homogeneous FPBP needs to be further purified to remove the phosphate tightly bound to the protein [20]. Second, the fluorescence increase of the FPBP triggered by the binding of orthophosphate is negatively affected by the ionic strength of the assay solution [22].

Therefore, we thought it of interest to design a continuous coupled assay of dNTP triphosphohydrolases relying on enzymes commercially available and ready to use. In addition, we searched for a continuous method(s) able to detect both reaction products, that is, the tripolyphosphate and the deoxynucleoside.

Here we report on the continuous detection of *E. coli* Dgt activity. In particular, the detection of tripolyphosphate or deoxyguanosine was attempted, and the best conditions for performing a continuous coupled assay are described.

Materials and methods

Materials

Buffers, analytical enzymes, and chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). Analytical enzymes were used without any further purification. Sodium tripolyphosphate ($Na_5P_3O_{10}$) was obtained from Sigma–Aldrich and Ferrania (Cairo Montenotte, SV, Italy). Spectroscopic experiments and Dgt activity measurements were performed using PerkinElmer λ19 and Beckman Coulter DU 640 spectrophotometers, respectively.

Enzyme units

Here, 1 unit of purine nucleoside phosphorylase (PNPase, EC 2.4.2.1), 1 unit of xanthine oxidase (XOD, EC 1.17.3.2), and 1 unit of alkaline phosphatase (AP, EC 3.1.3.1) are defined as the amount of enzyme able to convert 1 μmol of substrate per minute at pH 7.4 (25 °C), pH 7.5 (25 °C), and pH 10.4 (37 °C), respectively. The substrates considered are inosine, hypoxanthine, and *p*-nitrophenyl phosphate for PNPase, XOD, and AP, respectively.

Assay for PPP_i hydrolysis by AP

The hydrolysis of tripolyphosphate (PPP_i) by AP was assayed using a previously described enzyme-coupled assay for inorganic phosphate (P_i) [23]. The reactions contained 100 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 0.25 mM inosine, 50 mU/ml PNPase, and 500 mU/ml XOD. PPP_i and AP concentrations were 100 μM and 50 mU/ml, respectively. The hydrolysis was monitored by quantitating the generated phosphate (P_i) via absorbance changes at 293 nm [23].

Difference spectra of guanine and its XOD-generated oxidation product

To determine the spectroscopic properties of oxidized guanine produced by XOD, the UV spectrum of 0.3 mM guanine in 100 mM Tris–HCl (pH 8.0) was recorded between 220 and 320 nm against a reference containing buffer only. XOD (5 mU/ml final concentration) was then added to both cuvettes, and spectra were recorded 5, 10, 20, and 40 min after enzyme addition.

Assay of XOD with guanine

The oxidation of guanine by XOD (125 mU/ml) was assayed in 100 mM Tris–HCl (pH 8.0). Initial velocities were determined as a function of guanine concentration, and reactions were monitored at 297 nm.

Assay of PNPase with deoxyguanosine and XOD

The phosphorolysis of deoxyguanosine catalyzed by PNPase (50 mU/ml) was assayed in 100 mM Tris–HCl (pH 8.0), 5 mM KH_2PO_4, and 500 mU/ml XOD. Initial velocities were determined as a function of deoxyguanosine concentration. The detection wavelength was 297 nm.

Expression and purification of E. coli Dgt

Dgt was overexpressed using *E. coli* BL21(DE3) transformed with the pET30–*dgt* vector [10]. Transformants were grown at 37 °C until mid-log phase. To trigger overexpression, the cultures were shifted to 18 °C, and 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) was added. After overnight incubation, cells were harvested and resuspended in 25 mM Tris–HCl (pH 8.0), 1 M NaCl, 10% (w/v) sucrose, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM β-mercaptoethanol, lysozyme, and EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). The cell suspension was sonicated at 4 °C, polyethyleneimine was added (0.3%, v/v), and the protein extract was centrifuged at 16,000 rpm for 30 min. Dgt was precipitated by adding ammonium sulfate at 50% (w/v) and centrifuging the sample. The protein pellet was resuspended in 25 mM Tris–HCl (pH 8.0), 2 mM EDTA, 15% (v/v) glycerol, and 1 mM β-mercaptoethanol. The dissolved pellet was then dialyzed against 25 mM Tris–HCl (pH 8.0) and 300 mM NaCl. The protein solution was mixed in batch with Ni–NTA (nitrilotriacetic acid) agarose resin (Qiagen, Venlo, Holland) previously equilibrated with 25 mM phosphate buffer (pH 8.0) and 300 mM NaCl (buffer A). After a 45-min incubation, the resin was washed with buffer A supplemented with 2 M NaCl and 5 mM imidazole. Dgt was then eluted from the resin using buffer A containing 200 mM imidazole. The hexahistidine tag was cleaved with enterokinase, and the tagless protein was dialyzed against 25 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, and 75 mM sodium citrate. The dialyzed sample was concentrated using ultrafiltration cells equipped with a 10-kDa molecular weight cutoff (MWCO) membrane.

Assay of Dgt activity using enzyme-coupled assay

The reaction was performed in 1.5-ml quartz cuvettes (1-cm path length) placed in a Beckman Coulter DU 640 spectrophotometer at ambient temperature. Reaction mixtures contained 100 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM P_i, 50 mU/ml PNPase, and 500 mU/ml XOD. dGTP substrate was included at a series of concentrations between 2 and 120 μM. Dgt enzyme was preactivated at 37 °C for 20 min, and the reactions were started by adding Dgt enzyme at 4 nM final (hexamer) concentration.