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Bacteriophage T7 protein kinase: Site of inhibitory autophosphorylation, and use of dephosphorylated enzyme for efficient modification of protein *in vitro*

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ABSTRACT

Bacteriophage T7 encodes a serine/threonine-specific protein kinase that phosphorylates multiple cellular proteins during infection of *Escherichia coli*. Recombinant T7 protein kinase (T7PK), normally purified in phosphorylated form, exhibits a modest level of phosphotransferase activity. A procedure is described that provides dephosphorylated T7PK with an enhanced ability to phosphorylate protein substrates, including translation initiation factor IF1 and the nuclease domain of ribonuclease III. Mass spectrometric analysis identified Thr12 as the site of IF1 phosphorylation *in vitro*. T7PK undergoes Mg^{2+}-dependent autophosphorylation on Ser216 *in vitro*, which also is modified *in vivo*. The inability to isolate the presumptive autophosphorylation-resistant T7PK Ser216Ala mutant indicates a toxicity of the phosphotransferase activity and suggests a role for Ser216 modification in limiting T7PK activity during infection.

Introduction

Viral strategies of infection often involve expression of genes that are ordinarily nonessential, but confer a reproductive advantage under suboptimal growth conditions. Bacteriophage T7 is a lytic DNA phage that expresses a set of early genes that convert the infected *Escherichia coli* cell to an environment optimal for viral reproduction [1,2]. The T7 early gene 0.7 is dispensible under standard laboratory growth conditions, but is nearly essential for T7 growth at elevated temperatures, or in limited carbon/energy sources [3]. The 0.7 protein exhibits two distinct, biochemically separable functions: a serine/threonine-specific protein kinase (T7PK)[1] activity, and a host transcription shut-off (SO) activity [4,5]. The N-terminal domain possesses T7PK activity, while the SO activity is associated with the C-terminal region, and represses transcription by an unknown mechanism that is independent of T7PK activity [6]. T7PK uses ATP as phosphate donor and requires Mg^{2+} as a cofactor [7]. The T7PK domain of the 0.7 gene has been separately cloned and the polypeptide expressed in recombinant form [8]. The full-length 0.7 protein has been purified from T7-infected cells [9]. However, neither the full-length 0.7 protein nor the SO domain has been obtained in purified recombinant form, presumably reflecting the toxicity of the 0.7 protein, and the SO activity in particular.

A specific set of cellular proteins is phosphorylated during T7 infection in a T7PK-dependent manner [5,10]. The β′ subunit of the host RNA polymerase is modified at Thr1068 [11,12], which sensitizes transcription to otherwise weak terminators [12]. The RNA degradosome subunits RNase E and RNA helicase RhlB are T7PK targets, and their modification stabilizes transcripts synthesized by T7 RNA polymerase [13]. The dsRNA-specific processing enzyme RNase III is phosphorylated on serine during T7 infection [14,15], and the enhanced catalytic activity observed *in vitro* may optimize the maturation of the late transcripts that contain multiple RNase III processing sites, and that are synthesized at high levels [2]. Translation initiation factors IF1, IF2, and IF3, ribosomal proteins S1 and S6, and elongation factor G are *in vivo* targets [10,15], and their modification may enhance T7 late protein production by promoting the preferential translation of the mRNAs. While the T7PK-dependent modification of these proteins serves to maximize T7 gene expression, phosphorylation of proteins involved in other cellular pathways is likely [3,16].

Understanding how T7PK supports T7 growth requires knowledge of how phosphorylation alters the activity of the target proteins. In this regard, analyzing changes in activity of proteins phosphorylated *in vitro* can provide important information. However, recombinant T7PK directly purified from bacterial cells exhibits only modest levels of activity that may reflect an inhibitory *in vivo* phosphorylation [8]. We describe here a procedure for the preparation of dephosphorylated T7PK and its use in the efficient phosphorylation of substrate *in vitro*. We also show that T7PK

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[1] *Abbreviations used:* T7PK, T7 protein kinase; H-pT7PK, T7PK with the His-tag (H), and covalently attached phosphate (p); H-T7PK, T7PK with the His-tag, without covalently incorporated phosphate; IF1, translation initiation factor 1; NucD, nuclease domain.

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undergoes autophosphorylation, with the conserved serine 216 as the primary site of modification *in vitro* and *in vivo*, and the conserved serine 218 as an *in vivo* modification target.

Materials and methods

Water was deionized and distilled. Chemicals and reagents were molecular biology grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA) or ThermoFisher Scientific (Chicago, IL, USA). Standardized 1 M solutions of $MgCl_2$ and $MnCl_2$ were obtained from Sigma–Aldrich. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Perkin–Elmer (Boston, MA, USA). Lambda protein phosphatase was purchased from New England BioLabs (Beverly, MA, USA). Ni^{2+}-NTA affinity chromatography resin, biotinylated thrombin and streptavidin-agarose were purchased from Novagen (Madison, WI, USA). Protein assay kits and protein standards (low MW range) for SDS–PAGE were from Bio-Rad Laboratories (Hercules, CA, USA). ICON™ concentrators were obtained from Pierce (Rockford, IL, USA). Dialysis membranes (Spectra–Por CE 3500 and 10,000 MWCO) were purchased from ThermoFisher Scientific. Multisite-directed mutagenesis kits were from Agilent Technologies (Santa Clara, CA, USA). NuPAGE Precast Bis-Tris or Tricine gels (12% and 15%, respectively), agarose, and oligodeoxynucleotides were purchased from Invitrogen (Carlsbad, CA, USA). The oligodeoxynucleotides were obtained in deprotected form and were purified by denaturing gel electrophoresis, then stored at −20 °C in TE buffer (pH 8.0).

E. coli strains used included BL21(DE3) (Novagen), BL21(DE3)*recA,rnc105* [17] and DH10B (Invitrogen). Recombinant plasmids included pET-15b(T7PK), which encodes a truncated version of the T7 protein kinase, having a C-terminus defined by a point mutation (JS78) [6] that changes the Gln243 codon to a UAG codon [8]; pET-15b(NucD), encoding the N-terminal nuclease domain of RNase III [18]; and pET-15b(IF1), encoding the gene for *E. coli* translation initiation factor IF1 (a gift of P.R. Cunningham, Wayne State University, Detroit, MI, USA). All proteins expressed from the recombinant pET-15b plasmids carried an N-terminal hexahistidine [$(His)_6$] tag encoded by the vector.

Protein expression and purification

Protein purification followed a procedure described elsewhere [17], with some modification. A 5 ml overnight culture of LB broth containing ampicillin (100 μg/ml) (LB-Amp) was prepared using a freshly-transformed colony of BL21(DE3) or BL21(DE3)*recA,rnc105* cells containing pET-15b(T7PK). A portion of the overnight culture was used to inoculate 500 ml of LB-Amp, which was grown with vigorous aeration at 37 °C to an OD_{600} of ∼0.4. IPTG was added (1 mM final concentration) followed by vigorous aeration for 3 h at 37 °C. Aliquots were removed before and after IPTG addition and analyzed by 12% SDS–PAGE. Cells were collected by centrifugation (3500*g* for 20 min at 4 °C) and stored at −20 °C until further use. The following steps were carried out at ∼0–4 °C. Cells (∼1 g wet weight) were resuspended in 30 ml of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9) and subjected to repeated sonication bursts in an ice bath. The cell disrupter (Misonix, Inc.) was used at the "4–5" setting, with each sonication burst (1 min) followed by a 1 min pause with cooling, and the cycle repeated 20 times, or until lysis was judged complete. The sonicated material was centrifuged at 3500*g* for 20 min and the clarified lysate (∼30 ml) passed through a 0.2 μ filter (Corning). The solution was applied (∼1 ml/min) to a Ni^{2+}-NTA (Ni^{2+}-nitrilotriacetate) column (1 ml) prepared according to the supplier's instructions. The column was washed with 10 column volumes of binding buffer, followed by 10 column volumes of washing buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9). The protein was eluted with six column volumes of elution buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9). Most of the protein was obtained in the first three eluted volumes, which were combined and dialyzed (Spectrapor 10,000 MWCO) against 2 l of dialysis buffer 1 (100 mM NH_4Cl, 60 mM Tris–HCl, pH 7.9) for 12 h at 4 °C. The protein was further dialyzed against 2 l of dialysis buffer 2 (100 mM NH_4Cl, 60 mM Tris–HCl, pH 7.9, 2 mM EDTA–Na_2, 2 mM DTT) for 12 h at 4 °C. An equal volume of glycerol was added and the purified protein was stored at −20 °C.

Conversion of H-pT7PK to H-T7PK and T7PK

H-pT7PK (∼0.8–1 mg) was dialyzed at 5 °C against binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9), then incubated with 800 units of lambda protein phosphatase (2 h, 37 °C) in a total volume of ∼1 ml. An additional 800 units of phosphatase was added, followed by incubation for 2 h at 37 °C. The sample was loaded onto a His-bind column (1 ml), which was washed and protein recovered by elution with buffer containing 1 M imidazole as described above. H-T7PK was recovered in 10% yield; the source of the low yield is currently not known. To remove the affinity tag, H-T7PK was dialyzed against 40 mM imidazole, 500 mM NaCl, and 20 mM Tris–HCl (pH 8.4). The protein (∼800 μg) was treated with ∼10 units of biotinylated thrombin in a 1-ml reaction volume at room temperature for 16 h. When the reaction was judged complete by SDS–PAGE and Coomassie staining, streptavidin agarose was added (32 μl per unit of biotinylated thrombin), followed by incubation for 30 min at room temperature with gentle mixing. The reaction was loaded onto a spin column provided with the thrombin kit and centrifuged (500*g*, 5 min), and the filtrate dialyzed (Spectrapor, 3500 MWCO) against storage buffer. The cleaved affinity tag is eliminated in this step since its molecular mass is <2000 Da. Purified T7PK was stored at a concentration of ∼0.5 mg/ml in storage buffer containing 50% glycerol at −20 °C.

T7PK phosphorylation of protein in vitro

In a typical assay, ∼1 μg of substrate was incubated with ∼1 μg (33 pmol) of T7PK in reaction buffer (0.1 ml; 2 mM NH_4Cl, 30 mM Tris–HCl, 1 mM DTT, 0.1 mM EDTA) for 5 min at 30 °C. $MgCl_2$ was added (15 mM final concentration), followed by 1 mM [γ-^{32}P]ATP (12 Ci/mol). The reaction was incubated for 10 min (30 °C), followed by a second addition of T7PK (0.5 μg) and incubation continued for 5 min at 30 °C. The reaction was stopped by adding excess EDTA (20 mM final concentration). Aliquots were combined with sample loading buffer, heated 100 °C for 3 min, and analyzed by SDS–PAGE. Proteins were visualized by Coomassie Brilliant Blue R staining. The ^{32}P signal was detected by phosphorimaging (Typhoon 9400 System).

To determine the level of T7PK autophosphorylation, T7PK was incubated with 1 mM [γ-^{32}P]ATP (12 Ci/mol) as described above, and subjected to SDS–PAGE. The polypeptide was located by Coomassie staining. The gel band was excised and ^{32}P incorporation was measured by liquid scintillation counting of the gel slice using Scintiverse E (counting efficiency 99%). The incorporation of phosphate is reported as mol phosphate per mol protein, and assumes that 50% of the protein sample added to the gel lane was recovered in the excised gel band. This value is based on the observation (*vide infra*) that T7PK undergoes near-quantitative phosphorylation *in vitro* primarily on a single serine, providing a stoichiometry of ∼1 mol phosphate per mole T7PK. As the T7PK autophosphorylation reaction provides an internal control in the protein phosphorylation reaction, the level of phosphorylation of the target protein recovered in the gel slice also was able to be accurately determined