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A time-resolved FRET assay to measure activity of the deamidase of the prokaryotic ubiquitin-like protein

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Abstract

The modification of proteins in *Mycobacterium tuberculosis* (*Mtb*) by the prokaryotic ubiquitin-like protein (Pup) targets them for degradation by mycobacterial proteasomes. Although functionally similar to eukaryotic deubiquitylating enzymes, the deamidase of Pup (Dop) has no known mammalian homologs. Since Dop is necessary for persistent infection by *Mtb*, its selective inhibition holds potential for Tuberculosis therapy. In order to facilitate high-throughput screens for Dop inhibitors, we developed a time-resolved Förster resonance energy transfer (TR-FRET)-based assay for Dop function. The TR-FRET assay was successfully applied to determine the Michaelis constant for ATP-binding and to test the co-factor tolerance of Dop.

Keywords

Tuberculosis; Pupylation; Prokaryotic ubiquitin like protein; Ligase; Deamidase of Pup; TR-FRET

The human pathogen *Mycobacterium tuberculosis* (*Mtb*) infects about two billion people worldwide and poses a considerable threat to public health. The treatment of Tuberculosis requires an average of six months and failures in patient adherence to such extended drug regimens have led to the rise of multidrug resistant and extensively drug-resistant strains of *Mtb* [1]. Hence there is an urgent need to study biochemical pathways in *Mtb* that are not targeted by current therapeutics, and to devise new assays for identifying inhibitors of these pathways.

The prokaryotic ubiquitin-like protein, Pup, was discovered in *Mtb* as a post-translational modification of proteins that targets them for degradation by 20S proteasomes [2]. Pup is a 64-amino acid long polypeptide that is ribosomally expressed with a C-terminal glutamine that is first deamidated by the deamidase of Pup, called Dop, to generate a C-terminal glutamate residue. The γ -carboxylate of the nascent glutamate is then condensed with specific lysine ϵ -amines in target proteins by the Pup-ligase, proteasome accessory factor A or PafA [3]. The protein pupylation pathway is conserved across the actinomycete species of

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bacteria and mass spectrometry-based proteomic studies have confirmed ~200 proteins to be site-specifically pupylated in *Mtb*, *Mycobacterium smegmatis*, *Rhodococcus erythropolis* and *Corynebacterium glutamicum* [4, 5]. The importance of protein pupylation is highlighted by the fact that both PafA and Dop are required for efficient infection by *Mtb*, and inactivating mutagenesis of either enzyme is accompanied by a minimum 10-fold reduction in viable colony forming units of *Mtb* recovered from the lungs of infected mice [6, 7].

Given the key roles of PafA and Dop in facilitating *Mtb* survival within the host, we have embarked upon a program to identify fluorescent probes of PafA and Dop activity that facilitate the detection of these enzymes in complex mixtures. Our initial efforts led to the identification of the probe, N α -5-FAM-L-Lys (Probe **1**, Figure 1A) [8]. This simple fluorescent probe facilitated mechanistic studies of PafA in cell lysates and led to the identification of the minimal Pup sequence required for effective protein pupylation. Having established a robust assay for PafA activity, we turned our attention to developing a sensitive and high-throughput assay for Dop activity. Unlike PafA, which has the singular role of appending Pup to protein substrates, Dop is a bifunctional enzyme. It is responsible for both processing the C-terminal glutamine of Pup to glutamate as well as for *depupylating* proteins, in order to rescue Pup from proteasomal degradation and to permit its reuse for pupylation. Ovaa and co-workers previously reported the design of C-terminal aminomethylcoumarin (AMC) conjugates of Pup, obtained by the total chemical synthesis of H₂N-Pup(1-63)-Glu(AMC)-CO₂H, as probes for measuring Dop activity [9]. However, the synthesis of such conjugates required specialized chemistry and resulted in a modest yield. Moreover, the bulky hydrophobic compound AMC differs vastly from the lysine side-chain to which Pup is conjugated in protein substrates, which may influence both Dop kinetics and thus future applications toward inhibitor discovery.

Based on the fact that the single enzyme Dop depupylates a wide range of proteins *in vivo*, we wondered if a fluorescent conjugate of Pup with probe **1** could serve as a substrate for Dop (Figure 1A). The PupE-**1** conjugate was prepared by incubating 10 μ M of PupE (the C-terminal glutamate form of Pup) with 500 μ M of **1** in the presence of 1 μ M PafA and 5 mM ATP at pH 7.4 for 16 h at 37 °C [8]. The conjugate was purified by reverse-phase HPLC (RP-HPLC) and characterized by electrospray ionization mass-spectrometry (Supplementary Figure 1). The purified PupE-**1** conjugate was subsequently incubated with 1 μ M of purified Dop-His₆ (Supplementary Figure 2) in Buffer P which consisted of 50 mM Tris-HCl, 300 mM NaCl, 20 mM MgCl₂, 5 mM ATP, 1 mM DTT, and 10% (v/v) glycerol at pH 7.4. This led to the rapid depupylation of PupE-**1** that was easily followed by the disappearance of a fluorescent band in 15% SDS-PAGE gels (Figure 1B). Coomassie staining further confirmed the depupylation reaction by the presence of a free PupE band (Figure 1B). The PupE-**1** probe has several advantages over purely synthetic strategies in that an easily scalable enzymatic reaction is used to generate the probe, and the modular fluorophore may easily be varied to accommodate different screening strategies. With this exciting result in hand we turned our attention to developing a sensitive and robust assay that permits high-throughput screening of Dop activity.

Time-resolved Förster resonance energy transfer (TR-FRET)-based assays have been developed to follow a number of enzyme classes including kinases [10], peptidases [11], Ub ligases and deubiquitinases [12, 13]. One key aspect of the TR-FRET technique is the use of donor fluorophores with large Stokes' shifts and long fluorescence emission half-lives in the range of microseconds to milliseconds. This confers the ability to time-gate fluorescence measurements such that short-lived background fluorescence from compounds found in biological fluids and from small-molecules used in inhibitor screens is not observed. This results in high signal-to-background ratios and excellent assay sensitivity [14]. Hence a TR-FRET assay for Dop activity would be highly desirable. Commonly used donor fluorophores for TR-FRET are commercially available as lanthanide-cryptates that are pre-conjugated with highly-specific antibodies, or may be conjugated to any desired protein by standard protein-conjugation chemistries [15].

Our approach to developing the TR-FRET assay employed a commercially available mouse monoclonal anti-His₆-tag antibody conjugated with a Tb³⁺-cryptate fluorophore (Cisbio, Bedford, MA) that serves as the FRET-donor (Figure 1C). Upon antibody binding to the His₆-PupE-1, the Tb³⁺-cryptate donor and fluorescein acceptor are brought in close proximity such that irradiation of the donor with monochromatic light of wavelength 360 nm leads to the excitation of the acceptor and subsequent fluorescence emission at 528 nm. In a typical assay, 0.5 nM of antibody was incubated with 0.5 μM of His₆-PupE-1 in a final volume of 70 μL FRET buffer containing 5 mM Tris-HCl, 17.5 mM NaCl, and 0.005% (v/v) NP-40 for 15 minutes. The samples were measured in triplicate by transferring 20 μL aliquots to black 384-well small-volume assay plates (VWR, Radnor, PA) and the fluorescence at 485 nm from the FRET donor and 528 nm from the FRET acceptor were measured after irradiation at 360 nm on a BioTek Synergy 4 plate-scanner (BioTek, Winooski, VT). The TR-FRET signal remained stable over the course of several hours (Supplementary Figure 3), which indicates the robustness of our antibody-based approach. Several attempts to generate a His₆-tag-free Dop by Thrombin-mediated cleavage of the C-terminal His₆-tag were unsuccessful due to incomplete cleavage by Thrombin. The presence of a His₆-tag in Dop is undesirable as it competes with His₆-PupE-1 for antibody binding, giving rise to misleading reduction in TR-FRET signal that is independent of Dop-mediated depupylation. Therefore, an alternate approach was adopted wherein Dop was cloned with a C-terminal Strep-tag and purified by means of Strep-Tactin resin (Fisher Scientific, Waltham, MA) affinity chromatography (Supplementary Figure 4). A typical depupylation assay consisted of 1 μM His₆-PupE-1, 20 μM ATP, and 100 nM Dop-Strep in 35 μL of Buffer P, incubated for 15 minutes at 37°C. Depupylation assays were quenched by the addition of 35 μL 2× FRET buffer containing 1 nM antibody prior to TR-FRET measurement. We found that dilution in FRET buffer effectively inactivated Dop such that no significant depupylation was observed for the duration of fluorescence measurements.

The high utility of this straightforward assay was demonstrated by applying it to measure the Michaelis constant (K_M) for ATP-binding by Dop. Toward this goal, depupylation assays were undertaken with 1 μM His₆-PupE-1, 100 nM Dop, and ATP concentrations of 2 μM, 3.5 μM, 5 μM, 7 μM or 10 μM. For assays with 2-7 μM ATP, TR-FRET was measured at 30 s, 60 s, and 90 s (Figure 2A). In the case of assays with 10 μM ATP earlier time-points at 15

s, 30 s and 45 s were measured due to the faster kinetics of depupylation (Figure 2A). Non-linear fitting of the kinetics data using the Michaelis-Menten equation yielded values for $K_M = 7.8 \pm 3.5 \mu\text{M}$ and $k_{\text{cat}} = 8.6 \pm 2.1 \text{ min}^{-1}$ (Figure 2B). This indicates the slow turnover of pupylated substrates by Dop, and is consistent with the observation of a steady-state level of pupylated proteins inside cells [16]. We next turned our attention toward testing the ability of Dop to catalyze depupylation in the presence of various nucleotide co-factors. Dop requires ATP for function, but the co-factor is not hydrolyzed during depupylation [3]. Surprisingly, the non-hydrolyzable ATP-analog, ATP- γ -S, cannot effectively substitute for ATP in depupylation reactions [3]. An ability to detect subtle differences in Dop activity with different nucleotide co-factors would strengthen the suitability of a TR-FRET assay for identifying lead molecules of different potencies in high-throughput screens. With this in mind, depupylation assays were undertaken in 35 μL volumes with 1 μM His₆-PupE-1, 100 nM of Dop-Strep, and 20 μM of each co-factor, ATP, ATP- γ -S, ADP or AMP in Buffer P for 15 min at 37 °C. Assays were quenched by dilution with 35 μL FRET buffer containing 1 nM antibody and TR-FRET was measured in triplicate. Results from these experiments indicated that both ATP- γ -S and ADP enable Dop activity, albeit far less efficiently than ATP, with ATP- γ -S showing similar activity as ADP (Figure 2C). Interestingly, AMP alone was unable to activate Dop and did not lead to an appreciable decrease in the TR-FRET signal over 15 min. This suggests that while the γ -phosphate of ATP is important for Dop function and may be involved in positioning the nucleophile or substrate for optimum catalysis, the β -phosphate is strictly indispensable for Dop activity. This observation underscores the need for our assay, since X-ray crystal structures of the ATP-bound and apoenzyme forms of Dop do not reveal the basis for this requirement (Supplementary Figure 5).

In conclusion, we have designed and applied a highly sensitive and versatile TR-FRET assay to follow the activity of the deamidase of Pup enzyme. Our assay is readily amenable to a high-throughput format and has excellent signal-to-noise ratio that will facilitate screening for small-molecule inhibitors of Dop, an enzyme that is essential for the infectivity of *Mycobacterium tuberculosis*. Furthermore, mechanistic studies enabled by the TR-FRET assay revealed that Dop binds ATP with low micromolar affinity and that the β -phosphate of ATP is absolutely required for Dop function. Future studies in our lab will employ the depupylation assay in structure-function studies of the Dop-Pup interaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Development of a TR-FRET assay for measuring Dop activity

(A) Scheme depicting generation of the fluorescent PupE-1 substrate with the Pup ligase, PafA, and subsequent depupylation of PupE-1 by the deamidase Dop. (B) 15% SDS-PAGE gel showing the generation of PupE-1 (lane 1) and depupylation of PupE-1 by Dop (lane 2). (C) Scheme depicting the time-resolved Förster resonance energy transfer (TR-FRET) assay to follow Dop activity using a His₆-tag-specific antibody conjugated with fluorescent Tb³⁺-cryptate. The ratio of emission from the FRET acceptor at 528 nm to the FRET donor at 485 nm is inversely proportional to Dop activity.

Figure 2. Co-factor binding by Dop measured in the TR-FRET platform

(A) Initial time-course of 1 μM PupE-1 depupylation by 100 nM Strep-Dop at the indicated concentrations of ATP. (B) Non-linear regression analysis of reaction rates to determine the Michaelis constant (K_M) for ATP-binding by Dop. (C) Comparison of various nucleotide co-factors in a typical depupylation reaction. The y-axis shows the ratio of fluorescein emission to Tb^{3+} -cryptate emission, which is a measure of TR-FRET ($n = 3$, errors are s.d.). All assays were conducted with 20 μM of each co-factor for 15 min at 37 $^{\circ}\text{C}$.