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A green fluorescent protein-based assay for high-throughput ligand-binding studies of a mycobacterial biotin protein ligase

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ABSTRACT

Biotin protein ligase (BirA) has been identified as an emerging drug target in *Mycobacterium tuberculosis* due to its essential metabolic role. Indeed, it is the only enzyme capable of covalently attaching biotin onto the biotin carboxyl carrier protein subunit of the acetyl-CoA carboxylase. Despite recent interest in this protein, there is still a gap in cost-effective high-throughput screening assays for rapid identification of mycobacterial BirA-targeting inhibitors. We present for the first time the cloning, expression, purification of mycobacterial GFP-tagged BirA and its application for the development of a high-throughput assay building on the principle of differential scanning fluorimetry of GFP-tagged proteins. The data obtained in this study reveal how biotin and ATP significantly increase the thermal stability (ΔT_m = +16.5 °C) of *M. tuberculosis* BirA and lead to formation of a high affinity holoenzyme complex (K_{obs} = 7.7 nM). The new findings and mycobacterial BirA high-throughput assay presented in this work could provide an efficient platform for future anti-tubercular drug discovery campaigns.

1. Introduction

The fatty acid biosynthetic pathway is an effective drug target, exemplified with the first line anti-tubercular treatment isoniazid that specifically inhibits the enoyl reductase (Lu and Tonge, 2008). However, the continual emergence and growth of highly drug-resistant mycobacterial strains and the recent appearance of totally and extremely drug-resistant strains is fuelling the need for new drugs and the validation of new targets (Dheda et al., 2014). The first committed step in fatty-acid synthesis is performed by the acetyl-CoA carboxylase (ACC) which is an attractive drug target (Freiberg et al., 2004). ACC catalyses the conversion of acetyl-CoA to malonyl-CoA (Freiberg et al., 2004) and relies on a biotin molecule being attached to the biotin carboxyl carrier protein (BCCP). BCCP is one of the three subunits of ACC (Samols et al., 1988). The biotinylation of BCCP is catalysed by biotin protein ligase (BirA) in prokaryotes (Wilson et al., 1992). BirA is ubiquitous and conserved throughout the vast majority of living organisms (Wilson et al., 1992; Chapman-Smith et al., 2001), and is essential for cell survival.

A number of studies have recently focussed on the application of BirA as a drug target (Payne et al., 2006; Raman et al., 2008) for development of a new class of anti-tubercular agents (Duckworth et al., 2011; Shi et al., 2013). Analogues of biotinyl-5′-AMP have recently been shown to be potent inhibitors of BirA but further developments are needed to improve their specificity over human biotin protein ligase (Duckworth et al., 2011; da Costa et al., 2012b,a; Soares da Costa et al., 2012; Tieu et al., 2013; Paparella et al., 2014; Tieu et al., 2014; Bockman et al., 2015; da Costa et al., 2015; Tieu et al., 2015). A promising biotin analogue drug candidate was identified by Soares da Costa et al. (2012) that inhibits *Staphylococcus aureus* BirA with a K_i = 0.09 μM and has > 1100 fold selectivity over the human protein. No toxicity was observed in HepG2 cells with this compound, further validating bacterial BirA as a promising drug target. More recently, Tieu et al. (2015) showed that biotinol-5′-AMP had activity against *Mycobacterium tuberculosis* (Mt) and that here again the compound was devoid of cytotoxicity to human HepG2 cells. Crystal structures of BirA are available for the apo- and holo-enzyme of Mt providing essential tools for structure-based drug design (Gupta et al., 2010; Ma et al., 2014). A somewhat unique feature of Mt BirA is that it biotinylates three different protein subunits (encoded by *accA1-3*) containing a BCCP domain, which are shared for the biosynthesis of the structurally diverse array of lipids of Mt (Cole et al., 1998; Gago et al., 2006). Mt BirA only shares 25% sequence identity with *Escherichia coli* (Ec) BirA (Bond et al., 2017). Furthermore, the affinity of BirA varies significantly for its natural ligands depending on the species, highlighting possible differences in activity of BirA inhibitors between species (Wilson et al., 1992; Bower et al., 1995; Polyak et al., 1999; Clarke et al., 2003; Bagautdinov et al., 2005; Pendini et al., 2008; Purushothaman et al.,

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2008; Gupta et al., 2010; Mayende et al., 2012; Soares da Costa et al., 2012; Feng et al., 2015). As a result, there is an urgent need for fast and effective primary binding assays specifically designed for screening large libraries of compounds targeting Mt BirA.

Differential scanning fluorimetry (DSF) and Thermofluor assays (Pantoliano et al., 2001), have been widely adopted in the field of protein chemistry and drug discovery due to their high throughput. A more recent DSF-based technology called DSF-GTP was specifically developed for thermal stability profiling of GFP-tagged proteins in the presence of other contaminating proteins in high throughput (HT) (Moreau et al., 2010, 2012; Moreau and Schaeffer, 2013). The technology was recently applied for the functional characterisation of *Burkholderia pseudomallei* (Bp) BirA (Bond et al., 2017) as well as for the screening of Ec BirA:ligand interactions in HT for which it demonstrated an excellent Z' factor (Askin et al., 2016).

Here, we describe for the first time the production of a soluble Mt GFP-tagged BirA that is fully functional and its application for the development of a DSF-GTP assay for HT screening of Mt BirA ligands. Our study reveals how the binding of biotin and ATP increases the thermal stability of Mt BirA and leads to formation of a much tighter holoenzyme complex than previously reported (Purushothaman et al., 2008). The data and Mt BirA DSF-GTP assay offer a new toolkit for mechanistic studies and future screening of anti-tubercular compounds.

2. Materials and methods

2.1. Cloning, expression, and purification procedures

The Mt BirA sequence (UniProtKB entry code: P96884) was codon optimized (Bioneer) for optimal expression in *E. coli*, and cloned into pIM013 (pET-uvGFP) (Moreau et al., 2010) to create pAC286 (pET-N-6HIS-MtbBirA-GFP-C). The Mt BirA-GFP was expressed in *E. coli* BL21(DE3)RIPL using Overnight Express TB Medium (Novagen) containing 100 μg/mL ampicillin. 100 mL of medium in a 1 L conical flask was inoculated with 1 mL of an overnight starter culture and incubated at 37 °C with shaking at 200 rpm until the optical density reached 0.5. The culture was switched to 16 °C and further incubated for ~3 days at 200 rpm. Bacterial lysis and protein purification procedures were performed as previously described for Ec BirA-GFP (Askin et al., 2016). Mt BirA-GFP concentration was determined by Bradford Assay and its purity assessed by SDS-PAGE (Fig. 1).

2.2. DSF-GTP assay

DSF-GTP reactions (50 μL) were performed in iCycler iQ 96 well PCR plates (Bio-Rad) sealed with Microseal B Adhesive sealer (Bio-Rad). Reactions were left at room temperature for 10 min before starting the melt curve analysis in a iQ5 iCycler (Bio-Rad). Temperature range was set from 25 to 90 °C, increasing in 0.5 °C increments every 30 s and a stabilization phase of 30 s between increments as previously described (Bond et al., 2017). T_m peaks were identified in the Bio-Rad iQ5 Standard edition program or using an in-house peak identification program (Moreau and Schaeffer, 2013). T_m peak data were analysed with GraphPad Prism. Control reactions were performed in parallel to test reactions in identical buffer conditions. The net change in thermal stability (ΔT_m) of Mt BirA-GFP was determined by subtracting the T_m of a control reaction (*i.e.* Mt BirA-GFP alone) from the T_m of a test reaction. Reactions were performed in duplicates or triplicates.

2.3. Protein refolding studies

Mt BirA-GFP was subjected to two consecutive DSF-GTP melt curve analysis as for Bp BirA-GFP (Bond et al., 2017) but using a temperature range of 45–60 °C. Reactions consisted of 1 μM Mt BirA-GFP in BirA buffer (Bond et al., 2017).

2.3.1. Biotin and ribonucleotide dependence

The effect of biotin, ribonucleotides and combinations thereof on the T_m of Mt BirA-GFP was analysed as for Bp BirA-GFP according to Bond et al. (2017). Briefly, all reactions consisted of 3 μM Mt BirA-GFP in 15 mM Tris (pH 8), 10 mM NaCl, and 0.5% (v/v) glycerol in the presence of ribonucleotides, biotin or combinations thereof in concentrations ranging from 977 nM to 1 mM and were run independently at least in duplicate. Apparent binding constants (K_{obs}) were determined as for Ec and Bp BirA-GFP in conditions of [ligand] > [protein] (Askin et al., 2016; Bond et al., 2017).

3. Results and discussion

3.1. Development of DSF-GTP assay for Mt birA-GFP thermal stability analysis

DSF-GTP detects the unfolding of a protein tethered to a GFP in the form of a measurable transition midpoint (T_m) peak. The essential requirement for developing of a new Mt BirA-GFP DSF-GTP assay was to ascertain that Mt BirA is producing a T_m peak that is distinct to the T_m peak resulting from unfolding of the tethered GFP which serves as an internal control (Moreau et al., 2012; Moreau et al., 2012, 2013; Moreau and Schaeffer, 2013). Recently, we demonstrated the utility of our DSF-GTP principle for Ec BirA ligand-binding studies in HT (Askin et al., 2016) as well as for the functional characterization of Bp BirA-GFP (Bond et al., 2017). These studies demonstrated that Ec and Bp BirA-GFP were soluble and fully functional. We applied the same strategy and a similar workflow for the development of the Mt BirA DSF-GTP assay. The Mt BirA-GFP variant was obtained in high yields and soluble form (Fig. 1) and tested using the same DSF-GTP protocol as

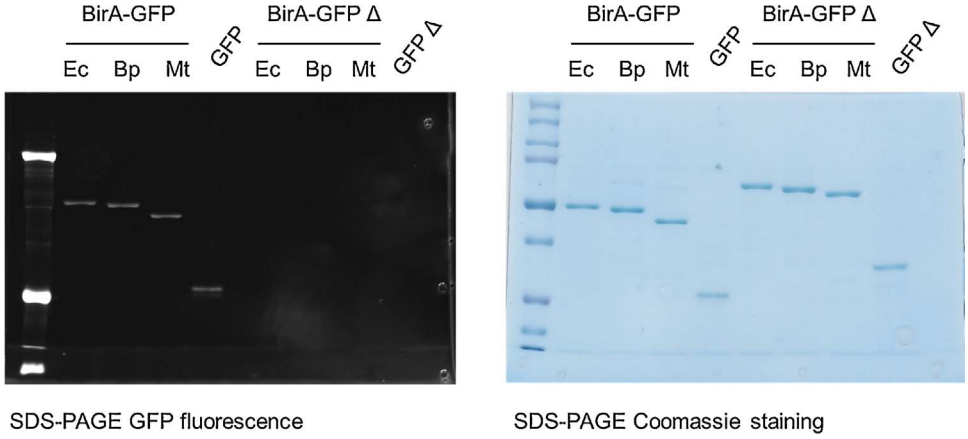

Fig. 1. SDS-PAGE comparing the electrophoretic mobility of heat-treated (Δ) and untreated samples of *M. tuberculosis* (Mt) BirA-GFP with *E.coli* (Ec) and *B. pseudomallei* (Bp) BirA-GFP, and GFP (proteolysis control). Identical amounts of proteins were loaded (1 μg). As expected, untreated proteins produce a discreet fluorescent band upon exposure of the gel to UV light (Left panel). Right panel shows the same gel after Coomassie blue staining. All proteins migrate at their expected size with Ec BirA-GFP being the largest and Mt BirA-GFP the smallest. No protein degradation is visible, *i.e.* no presence of additional fluorescent bands such as GFP is present in the BirA-GFP lanes (left panel). Purity is essentially the same across all proteins.