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ORIGINAL RESEARCH PAPER

The indigoidine synthetase BpsA provides a colorimetric ATP assay that can be adapted to quantify the substrate preferences of other NRPS enzymes

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Abstract
Objectives To develop a colorimetric assay for ATP based on the blue-pigment synthesising non-ribosomal peptide synthetase (NRPS) BpsA, and to demonstrate its utility in defining the substrate specificity of other NRPS enzymes.
Results BpsA is able to convert two molecules of L-glutamine into the readily-detected blue pigment indigoidine, consuming two molecules of ATP in the process. We showed that the stoichiometry of this reaction is robust and that it can be performed in a microplate format to accurately quantify ATP concentrations to low micromolar levels in a variety of media, using a spectrophotometric plate-reader. We also demonstrated that the assay can be adapted to evaluate the amino acid substrate preferences of NRPS adenylation domains, by adding pyrophosphatase enzyme to drive consumption of ATP in the presence of the preferred substrate.
Conclusions The robust nature and simplicity of the reaction protocol offers advantages over existing methods for ATP quantification and NRPS substrate analysis.

Keywords BpsA · ATP · Indigoidine · NRPS · Phosphopantetheinyl transferase · Glutamine · Microplate assay · Adenylation

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Introduction

ATP is a vital source of metabolic energy and fuels a vast number of reactions that are essential to life (Khakh and Burnstock 2009). The ability to accurately measure ATP levels in complex biological solutions is important to many diverse areas of medicine, industry and research. The most accurate methods to achieve this employ firefly luciferase (Ishida et al. 2008), which catalyses a two-step oxidation of luciferin and yields a light signal at 560 nm with an intensity proportional to the starting concentration of ATP in a test solution (De Wet et al. 1985). With a sufficiently sensitive luminometer, attomolar levels of ATP can be quantified in this manner, enabling detection of the ATP produced by a single bacterial cell (Okanojo et al. 2017). However, many laboratories do not have access to luminometers, and despite their greatly reduced sensitivity relative to luminescence-based methods, there is a strong demand for colorimetric ATP detection kits (Ishida et al. 2008). The most widely

available colorimetric method uses the ATP in a test sample to fuel a glycerol kinase catalysed transformation of glycerol to glycerol-3-phosphate, which is converted to a visible product in subsequent reactions (Hayashi and Lin 1967). This assay format requires multiple reaction steps and cumbersome before-and-after measurements if measuring ATP in a solution that could potentially also contain glycerol-3-phosphate.

To address these limitations, we have developed a rapid single-enzyme assay using the single-module non-ribosomal peptide synthetase (NRPS) BpsA as a colorimetric reporter. Once activated to a *holo*-form by a partner 4′-phosphopantetheinyl transferase (PPTase) enzyme (Fig. 1a), BpsA can convert two molecules of L-glutamine into an easily detected blue pigment, indigoidine, in an ATP-dependent fashion (Takahashi et al. 2007) (Fig. 1b). This capability has underpinned several applications of *bpsA* as an in vivo reporter gene (e.g. Müller et al. 2012; Owen et al. 2012; Knirschova et al. 2015) or a screening tool for discovery or re-engineering of NRPS enzymes (Owen et al. 2011, 2016). At a protein level, we have previously shown that BpsA can be purified in the active *holo*-form and used in an in vitro assay to accurately quantify unknown levels of L-glutamine (Brown et al. 2017). Here we demonstrate that BpsA can be used in a similar fashion to measure unknown ATP concentrations, following addition of excess L-glutamine. We further show that the system can be applied to elucidate the substrate specificity of purified NRPS adenylation (A-) domains, by using the consumption of ATP to report on adenylation activity (Fig. 1c). NRPSs are biotechnologically important enzymes that incorporate specific amino acids (as defined by their A-domains) into natural products. Biochemical characterisation of adenylation domain specificity is therefore an important step in understanding natural product biosynthesis, as bioinformatics prediction tools are not always accurate, especially with rarer substrates or fungal NRPSs (Lee et al. 2010).

Methods

Expression and purification of constructs

BpsA was expressed, purified and converted into the *holo* form as previously described (Brown et al. 2017). EntF-A was expressed and purified as a N-terminal six-histidine-tagged construct using the same purification strategy as described for PPTases by Owen et al. (2011) with the following amendments: cultures were

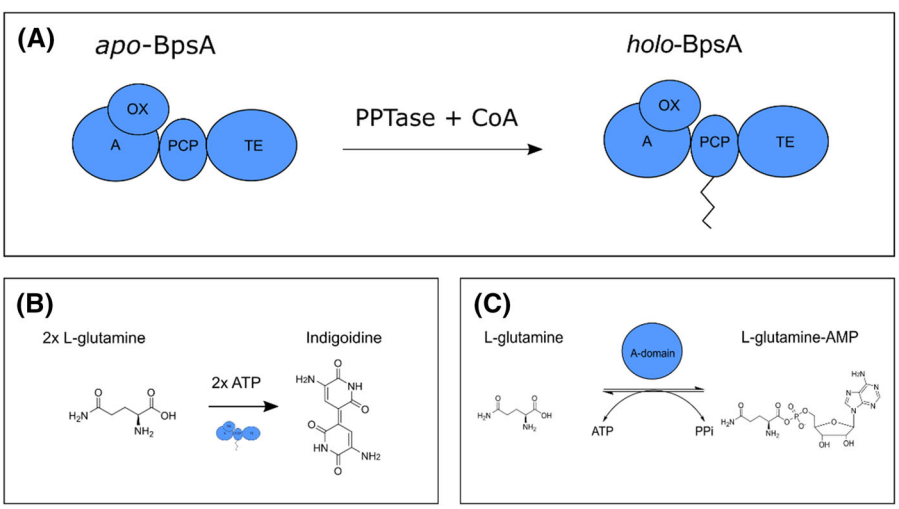

Fig. 1 Schematic diagram of BpsA: **a** To enable indigoidine synthesis, BpsA first needs to be converted to its active *holo* form by the attachment of 4′-phosphopantetheine arm to the PCP domain by a PPTase. **b** BpsA synthesises the blue pigment indigoidine from two molecules of L-glutamine in an ATP powered reaction that can readily be detected both in vivo and in vitro. **c** The A-domain is responsible for the initial recognition of the substrate and catalyses the formation of the aminoacyl-adenylate intermediate and its subsequent attachment to the free thiol of the phosphopantetheine arm. The adenylation reaction is an equilibrium reaction that consumes one molecule of ATP and releases one molecule of pyrophosphate

grown in media supplemented with 2.5 mM Betaine and 1 M sorbitol, and the pH of buffers was adjusted to pH 7.8.

Measurement of ATP using BpsA

To measure ATP, a reaction master mix was established containing 50 mM Tris–Cl pH 8.5, 10 mM $MgCl_2$, 5 mM L-glutamine and 3 μM holo-BpsA in ddH_2O. Next, 30 μL of reaction mix was added in triplicate to a 96 well plate containing 10 μL of ATP standards in ddH_2O to initiate the reaction. To facilitate indigoidine synthesis, the plate was incubated at 25 °C while shaking at 200 rpm for 60 min. Next, to solubilise the indigoidine, 200 μL of anhydrous DMSO was added to each well (giving a final concentration of 83% (v/v)) and mixed at 2000 rpm for 20 min. The A_{590} value of each well was recorded and the triplicate reactions were averaged and used to generate a standard curve. GraphPad Prism® (Graphpad Software Inc.) was then used to extrapolate ATP values from the standard curve.

Measurement of adenylation activity

A modified malachite green assay was used to measure adenylation according to the method of McQuade et al. (2009). Briefly, a reaction for each substrate was established in a 96 well plate in a final volume of 100 μL per well, comprising 50 mM Tris–Cl (pH 7.8), 10 mM $MgCl_2$, 1 mM ATP, 20 μg enzyme, 0.03 U of inorganic pyrophosphatase and 1.25 mM of test amino acid substrate. Plates were then incubated for 30 min at 30 °C, after which reactions were terminated by addition of 20 μL 4.2% (w/v) sodium molybdate in 4 M sulfuric acid in each well. Plates were then shaken for 5 s at 500 rpm, and 20 μL 0.135% (w/v) brilliant green dye in ddH_2O was added to each well. The plate was again shaken for 5 s at 500 rpm and the A_{650} value was measured immediately using a microplate reader.

To measure adenylation using BpsA, the purified EntF A-domain was used. Triplicate reactions were established for each substrate in a microfuge tube containing the following master mix (MM1); 50 mM Tris–Cl pH 7.8, 10 mM $MgCl_2$, 1 mM test amino acid substrate, 500 μM ATP, 0.03 U inorganic pyrophosphatase (IP) and 40 μg of the EntF A-domain in a final volume of 30 μL. The reactions were incubated at 30 °C, 200 rpm for 30 min to enable adenylation. A second master mix (MM2) containing 50 mM Tris–Cl pH 8.5, 10 mM $MgCl_2$, 4 mM L-Gln and 3 μM *holo*-BpsA was established and 20 μL was dispensed into each well to initiate indigoidine synthesis. The 96 well plate was then incubated at 25 °C with shaking at 200 rpm for 30 min. After this, 200 μL of DMSO was dispensed into each well to solubilise the indigoidine and the plate was shaken at 2000 rpm for 10 min. A_{590} values were then recorded for each well and compared to a standard curve to establish the level of ATP consumption per well. Welch's T-tests were performed using Graphpad Prism® (Graphpad Software Inc.).

Results and discussion

We first sought to confirm that BpsA could be used to establish a linear standard curve across a range of ATP concentrations, as linearity across a broad range would ensure complete consumption of the ATP had occurred in each case, and therefore that ATP concentrations in unknown samples could reliably be obtained by interpolation. To do this, 10 μL standards of ATP spanning a concentration range of 0–1000 μM were combined with 30 μL of reaction mix containing BpsA and excess L-glutamine. Following incubation for one hour at 25 °C, the synthesised indigoidine was resolublised by addition of DMSO, the absorbance measured at 590 nm, and a standard curve generated (Fig. 2a). The standard curve had an excellent r^2 value (0.9939), confirming linearity up to at least 1 mM ATP.

We next sought to estimate the minimal detection levels, considering the possibility that basal levels of ATP hydrolysis might occur without the corresponding synthesis of a molecule of indigoidine. For this, a range of concentrations from 0 to 100 μM were tested as before (Fig. 2b). Using 10 μL standards, the minimal detection range was conservatively estimated to be *ca*. 20 μM, the same as previously reported for glutamine (Brown et al. 2017). However, we consider that greater sensitivity would likely be achievable by increasing the ratio of test sample to reaction mix.

To assess the accuracy and performance of our ATP assay in more complex media, we established standard curves in lysogeny broth (LB; Fig. 2c) and Rosewell Park Memorial Institute (RPMI; Fig. 2d) medium, common bacterial and human cell culture growth

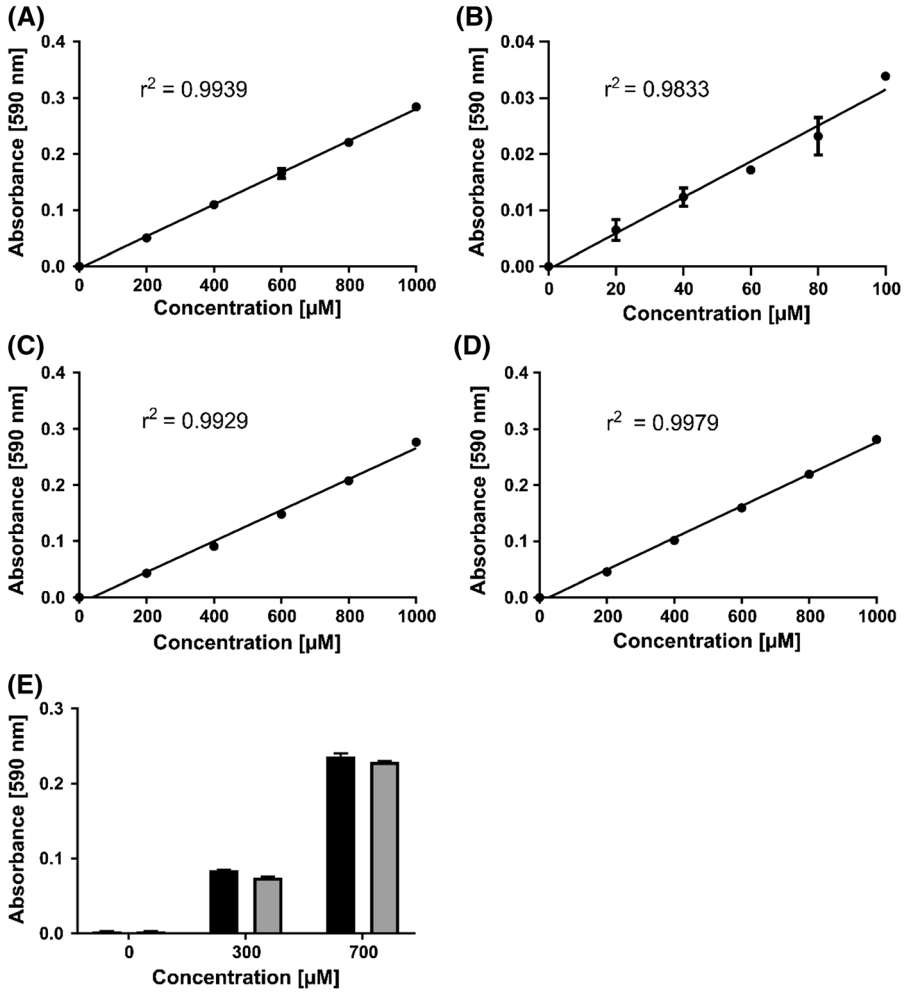

Fig. 2 ATP detection assay performance: **a** A robust standard curve can be generated between 0 μM and 1000 μM. Data are the mean of three independent replicates and were zeroed against the 0 μM ATP sample. Error bars indicate ± 1standard deviation. **b** Between 0 and 100 μM ATP the r^2 value was lower and the standard deviation of individual measurements was higher; nevertheless, a clear linear relationship was still observed. Data are the mean of three independent replicates and were zeroed against the 0 μM ATP sample. Error bars indicate ± 1standard deviation. **c, d** Linear standard curves generated in **c** LB and **d** RPMI media, each yielding excellent r^2 values that allowed the accurate interpolation of spiked ATP samples. For each of panels C and D, data are the mean of two independent replicates, comprising of three technical replicates and were zeroed against the 0 μM sample. Error bars indicate ± 1 standard deviation. **e** Comparison of indigoidine levels produced using the BpsA assay system in ddH_2O (black bars) or sheep serum (grey bars). Data are the mean of three independent replicates, zeroed against an unspiked ddH_2O control. Error bars indicate ± 1 standard deviation

media respectively. Standard curves with excellent r^2 values were established for both media types (0.9929 and 0.9979 respectively). Fresh samples of each media type were then spiked with a concentration of 300 μM ATP and interpolating the A_{590} values measured for the spiked media from these standard curves resulted in estimated concentrations of 299 μM for the LB sample and 297 μM for the RPMI sample. Finally, to test whether we could measure the levels of ATP in a complex biological fluid, we spiked replicate samples of either sheep serum or ddH_2O with 300 μM or 700 μM ATP and compared the relative levels of indigoidine production in each paired sample (Fig. 2e). In each case, the amount of indigoidine in the serum sample was within 10% of that measured in the corresponding ddH_2O sample, indicating that the performance of the BpsA ATP assay is not substantially impaired in this complex fluid.

A key goal for us in developing this assay was to provide a cost-effective and reliable means to characterise NRPS A-domains of unknown substrate specificity. The two most widely described methods for this are the ATP-PPi exchange assay (Lee and Lipmann 1975) and the malachite green assay (McQuade et al. 2009), which each have limitations; the former requires radioactive ^{32}P and specialised equipment to detect beta particle emission, while the product measured in the malachite green assay (a green molybdophosphoric acid complex) is not always stable.

We therefore sought to determine whether we could quantify adenylation activity by using the consumption of ATP as a surrogate. We used the well-characterised EntF A-domain (EntF-A) as a test system (Ehmann et al. 2000). EntF-A was expressed and purified as a standalone His_6-tagged protein. To confirm the excised adenylation domain was properly folded and retained activity, 40 μg of purified EntF-A was assayed for activity using a modified malachite green assay. A strong and specific signal for serine, its native substrate was detected (Fig. 3a), confirming it was functional.

We next tested whether BpsA could quantify EntF-A adenylation activity in terms of ATP consumption. First, replicates comprising 40 μg of EntF-A and 1 mM of amino acid test substrate (serine, glutamine or tryptophan) were established in individual wells of a 96-well plate and a final concentration of 300 μM ATP added to each well. At this concentration, ATP provides a strong signal that is detectable within the linear range of the assay but is still a rate-limiting step in the adenylation reaction. After 30 min a master-mix containing L-glutamine and BpsA (MM2, Methods) was dispensed into each well to initiate indigoidine synthesis, and the reactions were incubated for a further 30 min at 25 °C. The indigoidine was then resolublised by the addition of DMSO and the A_{590} value was recorded. We were surprised to discover no difference in indigoidine production could be observed for any of the three amino acid, even when the concentration of EntF-A was increased to 400 μg ($p > 0.05$, Welch's T-test; Fig. 3b).

We reasoned that EntF-A might not have consumed a significantly different quantity of ATP when incubated with serine *versus* the glutamine and tryptophan controls owing to the equilibrium nature of the adenylation reaction, and that it might therefore be necessary to drive the reaction towards a more complete consumption of ATP. In the malachite green assay, pyrophosphatase is added to convert the PP_i to P_i to enable the formation of the malachite green complex. We considered that pyrophosphatase might have similar utility in the BpsA assay to drive the reaction forwards. To test this, the experiment was repeated as above only with 0.03 units of pyrophosphatase and 40 μg EntF-A added to each well during the initial amino acid substrate incubation The ATP concentration in the reaction was also increased to 500 μM to increase the dynamic range. Following the final indigoidine resolubilisation step, we observed that the experimental samples containing EntF-A and serine had synthesised only 12% of the amount of indigoidine relative to the no amino acid control ($p = 0.0002$; Welch's T-test), indicating that almost all the ATP had been consumed. In contrast, the tryptophan and glutamine samples yielded levels of indigoidine that were not significantly different ($p > 0.05$, Welch's T-test) to the no amino acid control, indicating that the EntF-A domain had not catalysed adenylation of either of these amino acids (Fig. 3c), in agreement with the results of the malachite green assay (Fig. 3a).

Conclusions

We describe here the application of BpsA as a flexible and elegant colorimetric reporter to measure ATP concentrations in unknown solutions. We show that it can be readily adapted to accurately quantify ATP across a wide concentration range in complex solutions including bacterial and human cell media. It also provides a novel method to assess the adenylation activity of NRPS A-domains to rapidly define their substrate preferences. While this method does not readily permit continuous monitoring of adenylation activity over time, it does avoid key disadvantages associated with other NRPS substrate assays, i.e. the usage of radio-labelled PPi and the issues of product instability associated with the malachite green assay.

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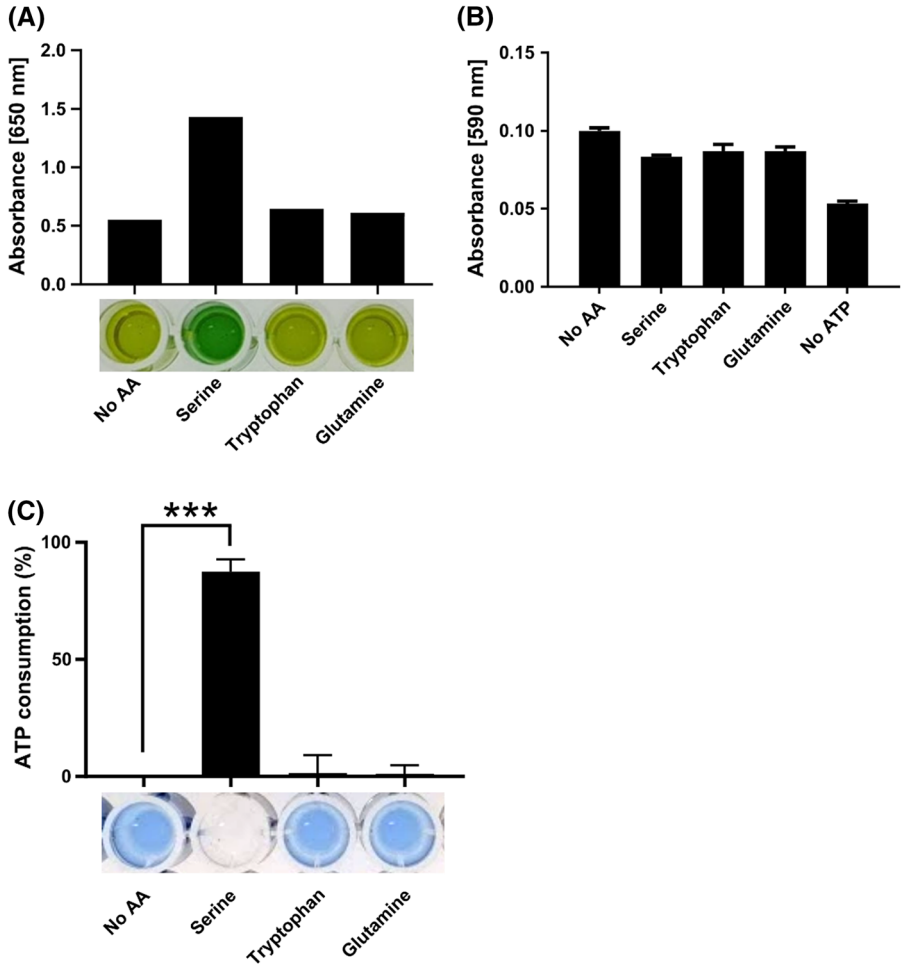

Fig. 3 Adaptation of the BpsA ATP assay to report on NRPS A domain adenylation activity in the presence of preferred or non-preferred amino acid substrates. **a** As an initial determination of EntF-A activity, a malachite green assay was used to report on substrate specificity. A strong signal was detected for the established substrate serine, while no signal was detected for the negative control (No AA), glutamine or tryptophan. **b** Initial tests of the BpsA assay indicated no detectable ATP consumption, using 400 µg EntF-A. Data are the mean of three independent replicates, and error bars indicate ± 1 standard deviation. **c** With the addition of pyrophosphatase the adenylation reaction is driven forward and ATP is consumed. The tryptophan and glutamine samples now produce the same amount of indigoidine as the no amino acid control (No AA), indicating they are not adenylated by EntF-A. In contrast, the serine sample produces no indigoidine showing that ATP has been consumed and confirming it is recognised as substrate by EntF-A (*** indicates $p < 0.001$ in a pairwise comparison with the No AA control; Welch's T-test). Data are the mean of three independent replicates, and error bars indicate ± 1 standard deviation. Inset images were acquired from individual 96 well plate assays and are representative of the plotted mean data

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