Video Article

Measuring *In Vitro* ATPase Activity for Enzymatic Characterization

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URL: https://www.jove.com/video/54305
DOI: doi:10.3791/54305

Keywords: Biochemistry, Issue 114, ATP, ATPase, phosphate, activity, enzyme, protein

Date Published: 8/23/2016

Citation: Rule, C.S., Patrick, M., Sandkvist, M. Measuring *In Vitro* ATPase Activity for Enzymatic Characterization. *J. Vis. Exp.* (114), e54305, doi:10.3791/54305 (2016).

Abstract

Adenosine triphosphate-hydrolyzing enzymes, or ATPases, play a critical role in a diverse array of cellular functions. These dynamic proteins can generate energy for mechanical work, such as protein trafficking and degradation, solute transport, and cellular movements. The protocol described here is a basic assay for measuring the *in vitro* activity of purified ATPases for functional characterization. Proteins hydrolyze ATP in a reaction that results in inorganic phosphate release, and the amount of phosphate liberated is then quantitated using a colorimetric assay. This highly adaptable protocol can be adjusted to measure ATPase activity in kinetic or endpoint assays. A representative protocol is provided here based on the activity and requirements of EpsE, the AAA+ ATPase involved in Type II Secretion in the bacterium *Vibrio cholerae*. The amount of purified protein needed to measure activity, length of the assay and the timing and number of sampling intervals, buffer and salt composition, temperature, co-factors, stimulants (if any), *etc.* may vary from those described here, and thus some optimization may be necessary. This protocol provides a basic framework for characterizing ATPases and can be performed quickly and easily adjusted as necessary.

Video Link

The video component of this article can be found at https://www.jove.com/video/54305/

Introduction

ATPases are integral enzymes in many processes across all kingdoms of life. ATPases act as molecular motors that use the energy of ATP hydrolysis to power such diverse reactions as protein trafficking, unfolding, and assembly; replication and transcription; cellular metabolism; muscle movement; cell motility; and ion pumping[1-3]. Some ATPases are transmembrane proteins involved in transporting solutes across membranes, others are cytoplasmic and may be associated with a biological membrane such as the plasma membrane or those of organelles.

AAA+ ATPases (ATPases associated with various cellular activities) make up a large group of ATPases that share some sequence and structural conservation. These proteins contain conserved nucleotide binding motifs such as Walker-A and -B boxes and form oligomers (generally hexamers) in their active state[1]. Large conformational changes in these proteins upon nucleotide binding have been characterized among diverse members of the AAA+ family. EpsE is a AAA+ ATPase and member of the bacterial Type II/IV secretion subfamily of NTPases[4-6]. EpsE powers Type II Secretion (T2S) in *Vibrio cholerae*, the causative agent of cholera. The T2S system is responsible for the secretion of a wide variety of proteins, such as the virulence factor cholera toxin that causes profuse watery diarrhea when *V. cholerae* colonizes the human small intestine[7].

Techniques for quantitating *in vitro* ATPase activity are varied, but commonly measure phosphate release using colorimetric, fluorescent, or radioactive substrates[8-11]. We describe a basic method for determining *in vitro* ATPase activity of purified proteins using a colorimetric assay based on a commercially available malachite green-containing substrate that measures liberated inorganic phosphate (Pi). At low pH, malachite green molybdate forms a complex with Pi and the level of complex formation can be measured at 650 nm. This simple and sensitive assay may be used to functionally characterize new ATPases and to evaluate the roles of potential activators or inhibitors, to determine the importance of domains and/or specific residues, or to assess the effect of particular treatments on enzymatic activity.

Protocol

1. Perform ATP Hydrolysis Reaction with Purified Protein

1. Prepare Stocks of All the Necessary Reagents for Incubation with Purified Protein.
    1. Prepare 5x HEPES/NaCl/glycerol (HNG) buffer containing 100 mM HEPES pH 8.5, 65 mM NaCl, and 5% glycerol (or other assay buffer as appropriate).
    2. Prepare 100 mM $MgCl_2$ (or other metal, if ATPase is metal-dependent) in water.
    3. Prepare fresh 100 mM ATP in 200 mM Tris Base (do not adjust pH further) using high purity ATP. Aliquot and store the ATP stock at -20 °C for no longer than a few weeks, as ATP will break down over time, and refrain from freezing and thawing the ATP stock.

4. Premix $MgCl_2$ and ATP at a 1:1 ratio just before setting up the ATP hydrolysis reaction.

2. Prepare and label 1.5 ml tubes for collecting samples at regular intervals throughout the reaction. Prepare tubes to collect samples at time 0 and at time 15, 30, 45, and 60 min.
   NOTE: As an alternative, collect samples only at time 0 and the endpoint.
   1. Add 245 µl 1x HNG buffer to each tube in order to dilute samples collected from the ATP hydrolysis reactions 1:50.

3. Prepare a bath of dry ice and ethanol for quickly freezing samples to stop the reaction. In a rubber ice bucket or other safe (non-plastic) container, add several pieces of dry ice and carefully pour enough 70-100% ethanol to cover the dry ice.
4. Dilute purified protein in 1x HNG buffer, as appropriate (typically 5-10 µM), and keep on ice.
5. Set up the ATP hydrolysis reactions.
   1. In separate 0.5 ml tubes for each sample, add the following reagents (in order): H_2O (up to a final volume of 30 µl), 6 µl 5x HNG or other buffer, 3 µl 100 mM $MgCl_2$-ATP mixture, and 0.25-5 µM protein.
   2. Include a buffer-only negative control condition in which no protein is added.

6. Remove 5 µl from the reaction at time 0, dilute 1:50 in the prepared 1.5 ml tube containing 245 µl HNG buffer, and immediately freeze the sample in the dry ice/ethanol bath.
7. Incubate reactions at 37 °C to allow ATP hydrolysis to occur for 1 hr. At each interval (15, 30, 45, and 60 min), remove 5 µl aliquots from the reaction and add to labeled sample tubes containing HNG buffer as in step 1.6.
8. At the end of the ATP hydrolysis reaction, move diluted samples to a -80 °C freezer for storage. To ensure all samples are completely frozen, wait at least 10 min before proceeding.

2. Incubate Samples Containing Free Pi with Detection Reagent

1. Thaw diluted samples containing ATP hydrolysis reaction aliquots at each time point (obtained from steps 1.6 and 1.7) at room temperature.
2. Set up a 96-well plate containing samples and phosphate standards.
   1. In a 0.5 ml tube, dilute the phosphate standard (provided with Pi detection reagent) from 800 µM to 40 µM by adding 5.5 µl of the 800 µM standard to 104.5 µl HNG buffer. Mix well, and add 100 µl of this 40 µM Pi standard to well A1 of a 96-well plate.
   2. Add 50 µl HNG buffer to wells B1-H1 for 1:1 serial dilutions of the Pi standard.
   3. Remove 50 µl of 40 µM Pi from well A1 and add to 50 µl assay buffer in well B1, mix, and remove 50 µl from well B1 and add to well C1, continuing dilutions through well G1. Discard 50 µl from well G1 after mixing to ensure each well has the same volume. Well H1 should contain only buffer to create a Pi standard from 40 to 0 µM.
      NOTE: If an additional factor (such as an inhibitor) has been added to the samples, create a standard curve containing that factor to control for changes in phosphate release or absorbance under those conditions.
   4. Add 50 µl of each sample in duplicate to the plate. Add samples from the same time point in columns vertically (sample 1 time 0 = A2, A3; sample 2 time 0 = B2, B3) and different time points horizontally. This allows for up to 8 samples and 5 time points per plate.

3. Use a sterile pipet to remove enough malachite green/molybdate Pi detection reagent to add 100 µl to each of the wells containing samples and standards (reagent needed (ml) = 0.1 x number of samples and standards) and add to a dish for easy pipetting using a multichannel pipet. Do not pour the detection reagent directly into the dish, as Pi contamination is likely to occur.
4. Using a multichannel pipet, add 100 µl of the Pi detection reagent to each well and mix by carefully pipetting up and down a consistent number of times without introducing bubbles, preferably in order from the last time points to the first time points.
5. Incubate the plate for 25 min at room temperature, or according to the manufacturer's directions.

3. Quantitate Results Using a Microplate Reader

1. Read the absorbance of the samples at 650 nm using an absorbance microplate reader.
2. Make a Pi standard curve. Using a graphing software, graph the absorbance values for the Pi standard samples versus concentration in order to find an equation used to solve for the amount of phosphate in each sample.
3. Calculate the ATPase activity for each sample by calibrating with the phosphate standard. Phosphate released = (OD_{650} - Y intercept)/slope.
   1. Average the total Pi from duplicates of each sample. Subtract the buffer-only control's absorbance reading from this number. Multiply this by the dilution factor (50 in our example).
   2. Determine the nmol Pi released per µmol protein. Graphing these values for each time point in a kinetic assay should yield linear fits of at least R = 0.99 (**Figure 1**); If not, the assay may be adjusted with more or less protein or a longer or shorter incubation time.

4. Represent the results as nmol Pi/µmol protein/min (**Figures 2**, **3**), or as nmol Pi/µg protein/min if desired.

Representative Results

The *in vitro* activity of the T2S ATPase EpsE can be stimulated by copurification of EpsE with the cytoplasmic domain of EpsL (EpsE-cytoEpsL) and addition of the acidic phospholipid cardiolipin[12]. It is also possible to determine the role of particular EpsE residues in ATP hydrolysis by comparing activity of wild type (WT) to variant forms of the protein using this assay. Here, the effect of substituting two lysine residues in the EpsE zinc-binding domain is measured by comparing ATPase activity of purified WT EpsE-cytoEpsL to the EpsE K417AK419A-cytoEpsL variant. In **Figure 1**, phosphate release proceeds linearly over the course of a 1 hr kinetic assay, a requirement for accurate quantification of kinetic ATPase activity. **Figure 2** shows data from the same purified protein samples as **Figure 1** that were assayed three times in duplicate and quantitated in terms of the mean rate of phosphate release. These data show that K417 and K419 appear to be important for EpsE ATPase activity. However, comparison of unstimulated (no cardiolipin) ATPase activity rates (**Figure 3**) shows that K417 and K419 do not contribute to EpsE's basal activity but rather to the ability of the protein to be stimulated by cardiolipin. The positively charged lysines in the EpsE zinc-binding domain may directly interact with the negatively charged phospholipids, thus contributing to the phospholipid-mediated stimulation of EpsE ATPase activity.

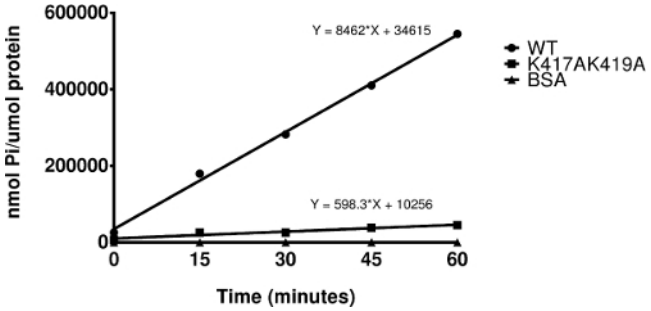

Figure 1: Phosphate is Released Linearly in a Kinetic ATPase Assay. One hour kinetic cardiolipin-stimulated ATPase assay comparing phosphate release of 0.5 µM WT EpsE-cytoEpsL to EpsE K417AK419A-cytoEpsL with bovine serum albumin (BSA) assayed as a negative control. Data [amount of released phosphate (y-axis) versus time (x-axis)] were plotted and subjected to linear regression analysis. The slope represents the rate of ATP hydrolysis. A representative graph with linear regression equations for the three proteins is shown. Please click here to view a larger version of this figure.

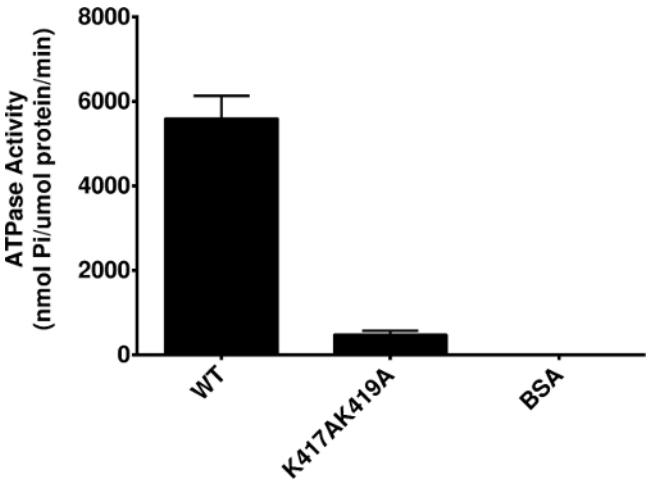

Figure 2: Double Lysine Mutations in EpsE Zinc-Binding Domain Reduce Stimulated ATPase Activity. Results of the 1 hr kinetic cardiolipin-stimulated ATPase assay with the same proteins and conditions as in **Figure 1**. Assays were performed three separate times in technical duplicate and the rate of ATP hydrolysis was calculated as nmol phosphate generated per minute per µM protein using linear regression equations. The mean results with standard error are displayed. Please click here to view a larger version of this figure.

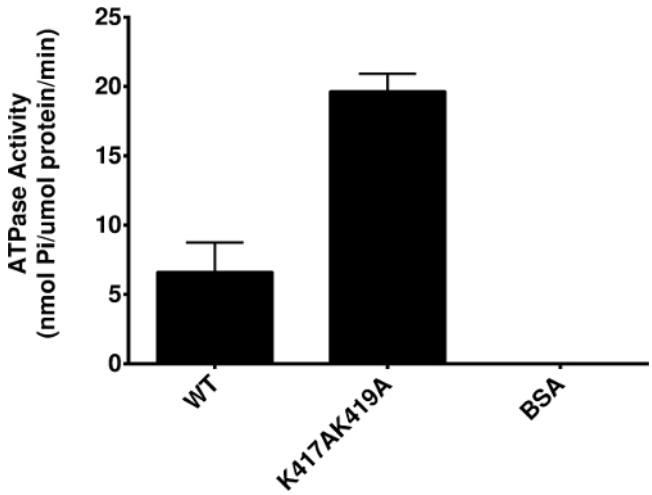

Figure 3: Double Lysine Mutations in EpsE Zinc-Binding Domain Do Not Interfere with Unstimulated ATPase Activity. Overnight (16 hr) endpoint unstimulated (no cardiolipin) ATPase assay comparing the activity of 5 µM WT EpsE-cytoEpsL to EpsE K417AK419A-cytoEpsL with BSA as a negative control. Assays were performed three separate times in technical duplicate and mean results with standard error is shown. The basal level of unstimulated EpsE ATPase activity assayed over a 16 hr period is ~1,000-fold lower than 1 hr kinetic cardiolipin-stimulated activity. Please click here to view a larger version of this figure.

Discussion

This is a general protocol for measuring *in vitro* ATPase activity of purified proteins for biochemical characterization. This method is easily optimized; for example, adjusting the amount of protein, buffer and salt compositions, temperature, and varying the assay length and intervals (including increasing the total number of intervals) can improve activity quantitation. Commercially available malachite green-based reagents are highly sensitive, and can detect small amounts of free phosphate (~50 pmol in 100 µl). Because of this assay's sensitivity, it is crucial to use disposable plastic ware, ultrapure water, buffers, and reagents devoid of contaminating phosphate. After purifying proteins, size exclusion or ion exchange chromatography is recommended to improve protein purity and remove contaminants.

For proteins displaying weak *in vitro* ATPase activity, stimulants may be added to the reaction to enhance enzymatic activity. Many factors that stimulate ATPase activity have been characterized. For example, cardiolipin and other membrane lipids, client proteins of chaperones such as Hsp90, and other proteins involved in supporting particular conformations or environments in which ATPases function[13-15]. Our laboratory first characterized EpsE by purifying monomers with weak ATPase activity compared to homologous ATPases[6]. We later discovered that when EpsE was copurified with the cytoplasmic domain of EpsL, a transmembrane protein and binding partner of EpsE in the T2S system, addition of acidic phospholipids such as cardiolipin to the reaction mixture greatly increased the ATPase activity of EpsE[12]. This likely mimics the conditions EpsE experiences at the cytoplasmic membrane that may promote oligomerization.

Many techniques have been used to quantify *in vitro* ATPase activity of purified proteins. Radioactive γ-^{32}P has been frequently and successfully implemented to quantify ATP hydrolysis[14,16], however, safety is a concern and requires approval for the laboratory use of radioactivity. While highly sensitive, the short half-life of γ-^{32}P is also a disadvantage. Other commercial phosphate detection methods are available, such as those that rely on the formation of a fluorescent product. Some of these methods are also very sensitive, but frequently require the addition of other enzymes to the reaction, resulting in reagents that are less stable over time. Additionally, kits are available that detect ADP released during ATP hydrolysis using a stable luminescence-based reagent, but these may be less ideal for ATPases with low levels of activity[17].

The assay described here consists of only one phosphate release measurement step, is highly sensitive, and can typically be performed within a few hr. It is also possible to prepare a malachite green-containing substrate to avoid purchasing a kit from a commercial vendor[6,9]. One consideration before undertaking this assay is that the step between adding the phosphate detection reagent and taking absorbance readings is relatively time-sensitive and must be between 20-30 min. This basic protocol can be used to determine the role of stimulants (as described), antagonists, subunits, domains, and specific residues in ATPase activity[15,16,18,19]. This assay can also be extended to measure the activity of phosphatases or other enzymes that release phosphate during catalysis. Additionally, this method can be applied to high-throughput screening for ATPase inhibitors[20].

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors would like to acknowledge funding from a National Institutes of Health grant RO1AI049294 (to M. S.).

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