Spectrophotometric Assays to quantify the activity of T4SS ATPases

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i. Abstract

Biogenesis of T4SS apparatus and substrate transport require energy. Conjugative T4SS have three ATPases, which enable DNA processing and transport of the nucleoprotein complex to the recipient cell. In the case of conjugative R388 plasmid these ATPases are named TrwB, TrwK and TrwD. Here, three different spectrophotometric assays to measure the enzymatic properties of these ATPases are described. The choice of any of the assays will depend on the specific requirements of each enzyme.

ii. Key Words: ATPase activity, molecular motor, coupled assay, spectrophotometric assay

1. Introduction

Bacterial conjugation is the main mechanism for the dissemination of antibiotic resistance genes [**1-2**]. Bacteria use a sophisticated machinery for the propagation of these genes [**3**]. This machinery, named Type IV Secretion System (T4SS) is formed by at least 10 different proteins that assemble in one apparatus that spans across the inner and outer membrane and the space in between [**4**]. This secretion apparatus is involved in the transfer of the plasmid as a nucleo-protein complex to the recipient bacteria. Both, the biogenesis apparatus and the substrate transport are powered by three different hexameric ATPases [**5**]. The three ATPases, named TrwB, TrwD and TrwK in plasmid R388, are inserted or associated to the inner membrane of the bacteria and are involved in DNA translocation, protein unfolding and protein transport through the secretion channel, respectively.

These molecular engines belong to the family of RecA/AAA+ ATPases (ATPases Associated with diverse cellular Activities) and consist of a common ring-shape hexameric structure. Each subunit in the hexamer presents the characteristic Walker A and Walker B nucleotide binding motifs. Nucleotide binding takes place in the interface between two adjacent subunits, so that the engines are only active in the hexameric form. This ring-shape formation allows cooperativity in the ATP hydrolysis process. Binding and hydrolysis of ATP give rise to conformational changes that, in each engine, is coupled to a specific function.

This chapter describes the use of three different spectrophotometric assays to follow ATP hydrolysis rates by T4SS ATPases. As each ATPase presents specific requirements, protocols suitable for different conditions are described. A short introduction for each technique is provided.

1.1 Real time, coupled assay

This assay, which is adapted from [**6**], has the particularity of maintaining the ATP concentration constant during the reaction. Under these conditions, ATP is constantly regenerated from ADP and PEP by pyruvate kinase and, therefore, there is not inhibition by the product ADP.

The hydrolysis of an ATP molecule is coupled to the oxidation of a molecule of NADH, which absorbs at 340 nm (Figure 1). Therefore, the rate of ATP hydrolysis is concomitant with the decrease in optical absorbance of the reaction at 340 nm. We have extensively used this coupled assay to analyze the DNA-dependent ATPase activity of TrwB, the VirD4 homolog in R388 plasmid [**7-8**] and also for measuring the activity of TrwK, which is the VirB4 homologue in this system [**9-10**]. The assay allows the continuous monitoring of the ATPase activity.

1.2 Enzcheck™

Since pyruvate kinase requires magnesium for maximal activity in a coupled assay, this method is not suitable to measure the ATPase activity of enzymes that are inhibited by magnesium, such as the T4SS traffic ATPase TrwD [**11**]. The EnzCheck™ kit (Molecular Probes, Invitrogen, ThermoFisher Scientific) is an assay that allows for a fast quantification of inorganic P_i in solution. The method, based on a protocol initially described by Webb [**12**] , consists in monitoring the change in absorbance at 360 nm upon reaction of inorganic P_i with 2-amino-6-mercapto-7-methylpurine riboside (MESG), in a reaction catalyzed by a purine nucleoside phosphorylase (PNP). The products of the reaction are 2-amino-6-mercapto-7-methylpurine and ribose 1- phosphate (Figure 2). This assay is very sensitive (within the range of 2 μM to 150 μM P_i) at a pH between 6.5 and 8.5.

1.3. Malachite green/molybdate ATPase assay

This assay, initially described by Taussky & Shorr [**13**], is based on the complex formed between malachite green, ammonium molybdate, and inorganic free phosphate (Pi) under acidic conditions. The formation of the malachite green phospho-molybdate complex is directly related to the free Pi concentration. Therefore, activity can be measured as the amount of inorganic phosphate liberated. Although this assay is not a good option to study the kinetics of the protein, it is very useful to check rapidly different parameters in order to find the optimal conditions for ATP hydrolysis. It is also a convenient assay when the enzyme works better with nucleoside triphosphates different from ATP, such as GTP, CTP or TTP.

There are many versions of the Malachite Green/molybdate reagent in the literature [**14-16**]. They all contain molybdate and malachite green but differ in other components. Some of them are commercially available as ready-to-use Pi-detecting reagents. However, the assay can be easily performed by preparing some stock solutions as follows:

2. Materials

2.1 Materials and stock solutions for a coupled assay

An UV-visible spectrophotometer thermo-jacketed or equipped with a thermoelectrically temperature controlled cell holder is required in order to maintain a constant temperature of 37ºC. Ideally, the spectrophotometer should be connected to a computer with a kinetics program pack or software to facilitate data processing. Here, the conditions used for 1cm path-length quartz cuvettes holding a volume of 150 µl (Hellma) are described.

Stock solutions

Pyruvate kinase and Lactate Dehydrogenase

PK/LDH enzymes from rabbit muscle can be purchased as a soluble mixture in a 5 ml buffered aqueous glycerol solution (solution in 50% glycerol containing 10 mM HEPES pH 7.0, 100 mM KCl and 0.1 mM EDTA, Sigma, Ref P-0294).

ATP

A stock solution of 500 mM buffered ATP can be made by dissolving 280 mg of the ATP disodium salt (Roche) in 500 µl of buffer 100 mM Tris pH 7. The solution is then neutralized to pH 7.0 by adding NaOH and the final volume is adjusted to 1ml (see note I for more clarity). The ATP stock solution is stored frozen in small aliquots of 50 µl.

Phosphoenolpyruvate

A stock solution (5 mM) of phosphoenolpyruvate, PEP (Roche), is made by dissolving 10 mg in 10 ml of Milli-Q water. Aliquots (1 ml) are stored frozen at -20 ºC.

NADH

NADH is purchased as a disodium salt (grade I, Roche). A 10 mM solution is made by dissolving 7.1 mg in 1 ml of Milli-Q water. In contrast to the other compounds, the NADH solution is made up freshly before use. It can be kept for just a few hours in ice, since NADH is prompt to oxidation.

2 X Reaction Buffer

The 2X reaction buffer consists of 100 mM Pipes pH 6.3, 100 mM NaCl, 10 mM $MgCl_2$ and 10 % glycerol (note II). Once prepared, it can be stored for several weeks at 4 ºC. These components can be modified depending on the optimal pH value of each particular enzyme, magnesium requirements, etc.

TrwB

Protein can be stored at -80 ºC in small aliquots. 10 – 20 % of glycerol should be added to the sample first to avoid freezing.

DNA

In order to analyze a ssDNA-dependent ATPase activity, oligonucleotides of different lengths can be purchased from Sigma-Aldrich. For dsDNA substrates, complementary oligonucleotides are incubated at 95 ºC for 3 min and cooled down at room temperature for 2h. When secondary G-quadruplex DNA structures are required, we follow the protocol described in Matilla et al. (2010).

2.2 Materials and stock solutions for an Enzcheck assay

An UV-visible, temperature controlled, spectrophotometer is required to measure the absorbance of the samples at 360 nm. Although plastic cuvettes could been used, reactions are better measured in 1cm path length quartz cuvettes.

Stock solutions

MESG

Dissolve MESG in dH_2O to a final concentration of 1 mM, prepare small aliquots (50-200 µl) and store at -20 ºC. Special care must be taken to avoid precipitates (extensive mixing, no heating). After thawing the solution is stable for at least 4 h in ice.

PNP

Prepare a purine nucleoside phosphorylase (PNP) stock solution at a final concentration of 100 U / ml and keep it in ice. Dilute this stock solution 100 times in dH_2O to get a 500 µM

working solution. This PNP can be used as a control to check that everything in the reaction is working perfectly. In the specific assay described here, the phosphorylase will be the conjugative traffic ATPase TrwD.

TrwD

TrwD is purified as described in (Ripoll-Rozada et al., 2012). Samples are usually prepared in a buffer consisting of 20 mM Hepes / NaOH, pH 7.6, 0.2 mM NaCl, 1 mM PMSF and 5 % glycerol (w/v), and stored at -80 ºC at a 50 μM protein concentration.

ATP

Prepare a 500 mM stock solution as previously described.

Reaction buffer

Although the commercial kit comes with a reaction buffer solution, the pH conditions and ionic strength can be modified depending on the specific requirements of each ATPase. In the case of TrwD, the final ATP hydrolysis buffer consists of 50 mM Tris/HCl, pH 8.5, 75 mM potassium acetate and 10 % glycerol, with special care of avoiding Mg^{2+} to prevent inhibition of the enzyme (see note III).

2.3. Materials and Stock Solutions for a Malachite green/molybdate ATPase assay

As for the previous assays, an UV-visible spectrophotometer is required to measure the absorbance of the samples at 740 nm. However, in this case, a temperature controlled cell holder is not required since data acquisition is performed once the reaction has been stopped and the enzyme is denaturalized. Instead, a multi-cell holder results more efficient for accurate measurements. Reactions are measured in plastic disposable 1-cm pathlength cuvettes holding a volume of 1.5 ml.

Stock Solutions

Ammonium Molybdate solution

A stock solution of ammonium molybdate is made by dissolving 16 g in 100 ml of a 5 N solution of sulfuric acid (note IV).The solution must be stored protected from exposure to light.

Ferrous sulfate- ammonium molybdate solution

This solution is prepared directly before the ATPase assay is performed by dissolving 1 g of ferrous sulfate in 18 ml of Milli-Q water and adding 2 ml of the ammonium molybdate stock solution

SDS solution

A 10 ml stock solution of 10 % sodium dodecyl sulfate (SDS) in water (wt/vol) is prepared and stored for several weeks at room temperature

Potassium dihydrogen phosphate solution

A 10 mM stock solution of potassium dihydrogen phosphate in water is prepared to be used as a standard to calculate phosphate concentrations in the ATPase assay.

Reaction buffer

In the case of TrwB, the reaction buffer consists of 100 mM Pipes pH 6.3, 50 mM NaCl, 2 mM $MgCl_2$ and 5 % glycerol but, as mentioned in the previous assays, buffer composition can be modified according to the specific requirements of each enzyme.

3. Methods

3. 1 Coupled assay

I. Prepare 2 ml of reaction mixture in ice by adding: 1 ml of 2 X reaction buffer, 706 µl of Milli-Q water, 200 µl of the 5 mM PEP stock solution, 50 µl of the 10 mM NADH solution, 20 µl of the 500 mM ATP stock solution and, finally, 24 µl of the PK/LDH mixture. Since ATPase reactions are carried out in 150 µl quartz cuvettes, this volume is enough for 12 reactions plus a control.

II. Place 150 µl of the reaction buffer in the quartz cuvette, at the spectrophotometer cell holder previously tempered at 37 ºC. Make a blank with this sample and start collecting data at 340 nm. Absorbance should be cero during the time of the reaction

III. Stop data collection and add 1-2 µl of the protein of interest. Make a blank again and start collecting data immediately. A linear decrease in absorbance should be observed and the slope of the curve will be correlated to ATP hydrolysis rates(note V). Note that protein is stored at high concentration, so 1-2 µl do not variate the final concentration significantly. If the protein is diluted or more substrates need to be added to the reaction, the final volume should be adjusted to avoid variations in buffer composition (see note VI).

IV. The difference in absorbance (ΔA) per minute can be converted to moles of ATP hydrolyzed by min and mg of protein by following the Beer-Lambert formula:

A= ε c l, where "l" is the path length (1 cm) and *epsilon* is the wavelength-dependent molar extinction coefficient of NADH (6.22 x 10^3 M^{-1} cm^{-1}).

$$\text{mol ATP/min} = \frac{\Delta\text{Absorbance/min}}{6.22 \times 10^{3}\ \text{L} \times \text{mol}^{-1}} \times 150 \times 10^{-6}\ \text{L}$$

3.2. Enzcheck

A standard curve made with inorganic phosphate is needed each time

Inorganic Phosphate Standard Curve

I. Prepare a 50 mM KH_2PO_4 stock solution.

II. Add variable amounts of the phosphate stock solution to the reaction buffer (without ATP) to comprise a range from 2 μM to 150 μM. Always include a blank without phosphate in the stock solution.

III. Incubate for 30 minutes at 22 ºC.

IV. Measure the absorbance at 360 nm. Perform a background subtraction of a blank solution without phosphate.

ATP hydrolysis reaction

I. Prepare a reaction mixture dissolving 200 μl of 1 mM MESG in 1ml of reaction buffer (50 mM Tris/HCl, pH 8.5, 75 mM potassium acetate).

II. Add ATP and $MgCl_2$ (or other magnesium salt, see note VII). In the case of TrwD, maximum ATP hydrolysis rates are obtained in the presence of 1 mM ATP and 10 μM Mg^{2+}(note VIII).

III. Add TrwD protein to a final concentration of 1 μM.

IV. Keep the temperature of the reaction at 37 ºC (5-10 minutes in the case of TrwD).

V. Measure the absorbance at 360 nm.

3. 3. Malachite green/molybdate ATPase assay

I. Protein is pre-incubated with its specific substrate for 15 min at 37°C in 600 µl of the optimal reaction buffer. Several samples can be prepared to check different parameters, such as different pH values, different nucleoside triphosphate, etc.

II. Reactions are started by the addition of ATP (5 mM) or the respective NTP, incubated at 37°C for 10 min, and then stopped by the addition of 400 µl of 10% SDS (w/v).

III. The ferrous sulfate–ammonium molybdate solution (500 µl) is added, and the amount of inorganic phosphate is estimated by absorbance at740 nm (note IX).

IV. ATPase rates can be calculated by carrying out a series of absorbance measurements as a function of time in duplicate (note X).

V. The relationship between the absorbance and phosphate concentration is established by using the potassium dihydrogen phosphate stock solution as a standard. Prior to the assay, a standard curve with different Pi concentrations is calculated by adding different volumes of the stock solution up to 600 of buffer and then following the same protocol as for the ATPase assay, adding 400 µl of the 10% SDS solution and 500 µl of the ferrous sulfate–ammonium molybdate solution.

4. Notes

I. ATP is not soluble in water or buffer at acidic pH values. Only upon adjustment to neutral pH the solution becomes clear. In this particular case, the pH value is adjusted to 7 with 0.5 N NaOH.

II. Although NADH is unstable at pH values below 7.0, spontaneous oxidation at pH values of 6.2 during the time of the reaction (5 min) is not observed.

III. For ATP hydrolysis rates catalyzed by TrwK, the VirB4 homologue, the conditions were similar to the above described, with some modifications in the ATP assay. Reaction buffer consisted of 50 mM PIPES [piperazine-N,N´-bis(2-ethanesulfonic acid)]-NaOH, pH 6.45, 75 mM potassium acetate, 5 % glycerol (w/v) 10 mM magnesium acetate, 1 mM potassium chloride, 1 mM dithiothreitol, and 0.1 mM EDTA.

IV. Caution is required to prepare the 5 N sulfuric acid solution by mixing water and sulfuric acid in the right order (i.e. adding the water on the sulfuric acid).

V. For data processing, the slope of the linear initial part of the reaction should be used.

VI. Where possible, it is preferable to add the ATP to the reaction mixture before adding the enzyme, so if there is some ADP in the solution, this is converted back to ATP before the reaction starts.

VII. In the case of adding magnesium is preferable to add an acetate salt rather than a chloride one.

VIII. TrwD ATPase activity is inhibited by magnesium. Therefore, special care is needed to avoid the presence of this cation (maximum ATP rates by TrwD are obtained in the presence 10 μM Mg^{2+}). Other enzymes may require higher concentrations.

IX. The assay requires accurate timing between the measurements. Color changes will occur with time, so it is very important to measure the results immediately and, in particular, maintain always the same time intervals once the ATPase reaction has been stopped. It is also highly recommended to repeat the standards with each new experiment, since small variations in buffer composition can also be an important source of error in the experiment.

X. It is important to check the background of the ATP solution, since it might have significant Pi amounts already. A sample with ATP but not protein should be taken as a blank.

Figure legends

Figure 1. In a coupled assay the production of ADP is coupled to NADH oxidation via pyruvate kinase (PK) and lactate dehydrogenase (LDH) enzymes. Following each cycle of ATP hydrolysis, pyruvate kinase converts the phosphoenolpyruvate (PEP) to pyruvate and the ADP back to ATP. Pyruvate is subsequently converted to lactate by lactate dehydrogenase, in a reaction in which NADH is oxidized to NAD+. The assay measures the rate of NADH absorbance decrease at 340 nm, which is proportional to the rate of steady-state ATP hydrolysis.

Figure 2. Enzcheck^{tm} assay consists in a spectrophotometric assay in which ATP hydrolysis rates are measured as the variation of absorbance at 360 nm accompanying the increment of 2-amino-6-mercapto-7-methylpurine (MES) upon the reaction of inorganic Pi with MESG (2-amino-6-mercapto-7-methylpurine riboside). The reaction is catalyzed by a purine nucleoside phosphorylase (PNP), which uses the Pi released by the ATPase. The other product of the reaction is ribose 1-P.

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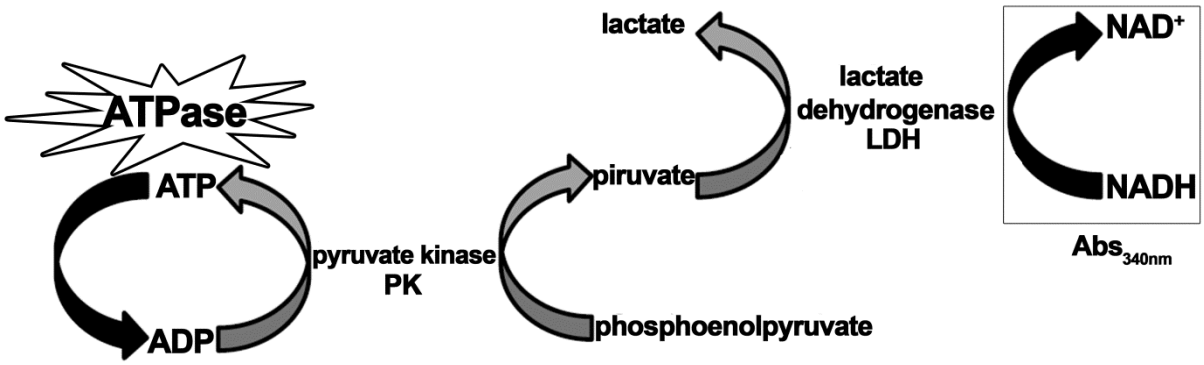
ATPase
ATP
ADP
pyruvate kinase
PK
phosphoenolpyruvate
piruvate
lactate
lactate
dehydrogenase
LDH
NAD+
NADH
Abs340nm

Figure 1

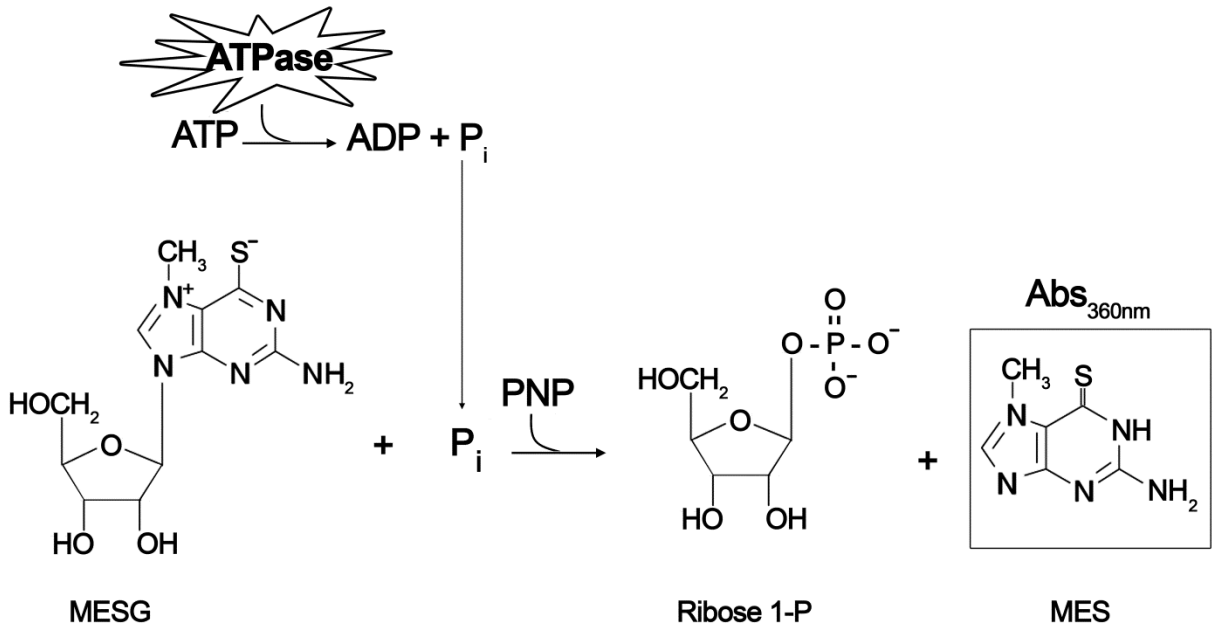

Figure 2