



Phosphate and ATPase Determination Using the μ Quant Scanning Microplate Spectrophotometer

There are a large number of assays in cellular and molecular biology that either produce or consume inorganic phosphate. Therefore, the ability to quantitatively measure these phosphorous compounds can allow the assessment of the associated enzymes. Here we describe the use of the mQuant Scanning microplate spectrophotometer to quantitate ATPase activity by measuring inorganic phosphate in solution.

Introduction

Many metabolic processes that require energy do so by coupling that process with the enzymatic hydrolysis of high-energy phosphate bonds. For example, the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) with the liberation of free orthophosphate does so with a liberation of energy. Enzymes generally referred to as an ATPase or GTPase, depending on the nucleoside triphosphate utilized carry out these cellular processes. The release of some of the energy contained in ATP can be described simplistically by the following reaction.



Muscle contraction is a classic example of the conversion of the chemical energy contained in molecules, such as ATP, to mechanical movement (1). ATP hydrolysis also serves as the energy source for active cellular transport, glycogenesis, and nerve excitation (1). GTP hydrolysis has been shown to be intimately associated with intracellular signaling, such as ras protein activation, and cyclic AMP formation (2). Tubulin polymerization also required GTP hydrolysis (2).

Enzymatic ATP hydrolysis has been measured by several different means, including the determination of ADP by coupling enzymes (3), measurement of ^{32}P release from [g- ^{32}P] ATP hydrolysis (4), or by colorimetric reactions (5-7). Most of the colorimetric reactions measure the release of free orthophosphate (Pi) and are based on the formation of a phosphomolybdate complex in an acid medium followed by a reduction or complexation with basic dyes that yield colored complexes (7,8).

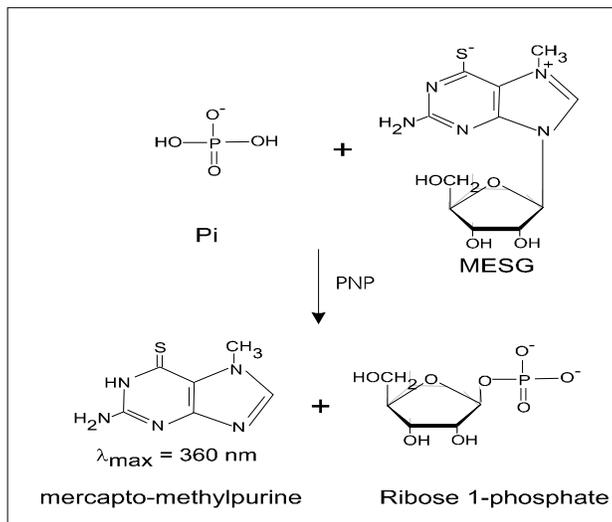


Figure 1. Enzymatic Conversion of MESG to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by PNP. Inorganic phosphate consumption is proportional to the formation of 2-amino-6-mercapto-7-methylpurine, which has a peak absorbance wavelength of 360 nm.

The determination of ATPase activity in this report is based on the measurement of free phosphate released from ATP. The assay reported here is based on the method described by Webb modified to be performed in microplates (9). Briefly, the enzyme, purine-nucleoside: orthophosphate ribosyltransferase, EC 2.4.2.1 (PNP) uses inorganic phosphate (Pi) to convert the substrate 2-amino-6-mercapto-7-methylpurine riboside (MESG) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine (Figure 1). The enzymatic removal of the ribose moiety from MESG results in a shift in the wavelength of maximum absorbance (I_{max}) from 330 nm to 360 nm (9,10). Because conversion of MESG requires inorganic phosphate (Pi), the increase in absorbance at 360 nm can be used to measure Pi concentration. When PNP enzyme and MESG substrate are in excess relative to phosphate, the increase in absorbance at 360nm is quantitative for inorganic phosphate. Assuming there is no preexisting phosphate, any increase in the absorbance at 360 nm must be the result of Pi liberation from ATP hydrolysis.

Materials and Methods

An EnzChek[®] Phosphate Assay Kit, catalogue number E-6646, was purchased from Molecular Probes (Eugene, OR). Sodium phosphate, potassium phosphate, ATPase enzyme, catalogue number A-7510, and Adenosine 5'-triphosphate, catalogue number A-7699, were obtained from Sigma Chemical Company (St. Louis, MO). Clear UV transparent 96-well microplates, catalogue number 3635, were from Corning Costar (Cambridge, MA).

Phosphate assays were performed according to the kit instructions except that the volumes were reduced to accommodate microplates. Briefly, a series of dilutions of phosphate, either sodium or potassium salts, were made using deionized water as the diluent. Dilutions were made such that the assay amount was contained in a 50 μ l volume and dispensed into microplate wells in replicates of four. Several stock solutions were prepared and stored separately according to the kit instructions. A 5X MESG stock solution was prepared and aliquoted and stored frozen at -20°C. Aliquots were thawed as needed immediately prior to use. Lyophilized PNP enzyme was reconstituted to a concentration of 100 U/ml and stored at 5°C. A 20X-reaction buffer was also supplied with the kit. A master substrate mix was prepared using these stock solutions immediately prior to use such that the mix contained everything necessary for a final volume of 200 μ l reaction mix, with the exception of the phosphate dilution, in a 150 μ l volume. Final assay mixture in a 200 μ l volume contained: 20 mM Tris-HCl, pH 7.5, 1mM MgCl₂, 0.2 mM MESG, 0.2 U PNP and various phosphate dilutions. Assays were initiated by the addition of 150 μ l of the substrate reaction mixture to the previously dispensed 50 μ l phosphate dilution.

Reactions were incubated at room temperature for 30 minutes after which their endpoint absorbance at 360 nm was determined.

The measurement of ATPase was performed by combining the following reagents in a 200 μ l volume: 400 μ M ATP, 1 mM KCl, 1 mM NaCl, 0.2 units of PNP, various dilutions of ATPase enzyme, and 1X reaction buffer. Assays were initiated by the addition of assay mixture to the ATPase enzyme dilution and incubated for 30 minutes at room temperature. Following incubation, the absorbance at 360 nm was determined using a mQuant scanning microplate spectrophotometer (Bio-Tek Instruments).

Kinetic assay experiments were performed as described for endpoint determinations. Assays were initiated by the addition of reaction substrate and immediately placed in a mQuant Scanning Microplate spectrophotometer and kinetic readings begun. Data capture and reader control were carried out using KC4 software (Bio-Tek Instruments). Absorbance measurements were made as described in the figure legends at 360 nm.

Results

The absorbance was determined for sodium phosphate concentrations ranging from 0 to 500 nmoles per well. Over this range the absorbance increased in a hyperbolic fashion, with phosphate concentrations above 100 nmoles per well resulting in very little increase in absorbance signal (Figure 2A). However, using KC4 data reduction software (Bio-Tek Instruments), a 4-parameter logistic best-fit equation describing the standard curve can be generated. When lower concentrations are examined (0 to 90 nmoles), the relationship between absorbance at 360 nm and phosphate concentration is linear (Figure 2B). Under these conditions, a least-means-squared linear regression can be utilized to describe the data. The coefficient of determination ($r^2=0.998$) value indicates that concentration determinations can be made with a high degree of confidence at these concentrations. In terms of sensitivity, as little as 2 nmoles of phosphate can be reliably detected using this assay.

A spectral scan of the reaction substrate and reaction products demonstrates the basis of the assay (Figure 3). The conversion of (MESG), with the consumption of inorganic phosphate, to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine results in a change in the absorbance peak from 330 nm to 360 nm. Because there is very little background absorbance at 360 nm from un-reacted substrate, the formation of reaction products can be followed by an increase at this wavelength.

The speed of the assay is demonstrated when the assay is followed kinetically. While the assay is routinely allowed to incubate for 30 minutes at room temperature, under the conditions used, steady-state levels of absorbance are observed in less than 2 minutes (Figure 4). This suggests that while a 30-minute incubation can be used, considerably shorter times may actually be necessary. In addition, the stability of the absorbance values for at least 2 hours (data not shown) suggests that longer incubations may be used with no loss of sensitivity or linearity if desired.

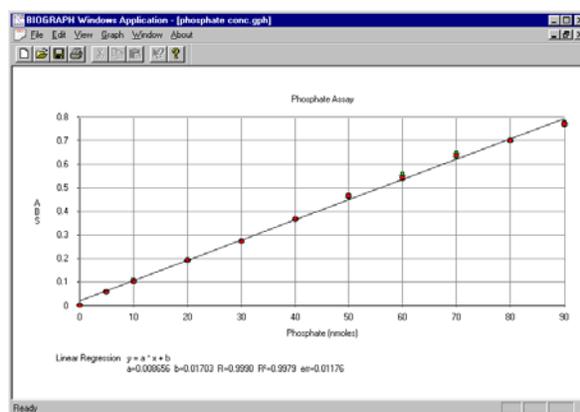
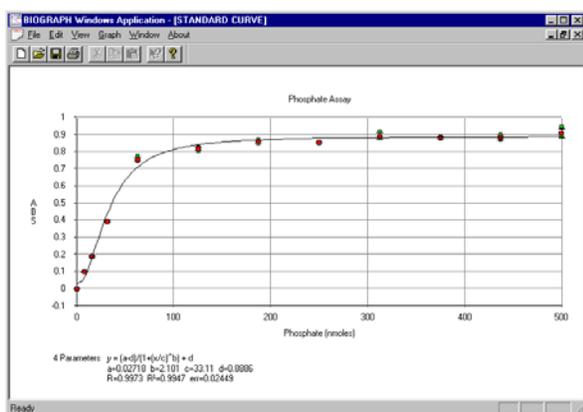


Figure 2. Phosphate Concentration curves. (A) The absorbance of sodium phosphate concentrations ranging from 0 to 500 nmoles per well was measured and a 4-parameter logistic best fit used to describe the data. (B) Sodium phosphate concentrations ranging from 0 to 90 nmoles per well were assayed and linear regression analysis performed.

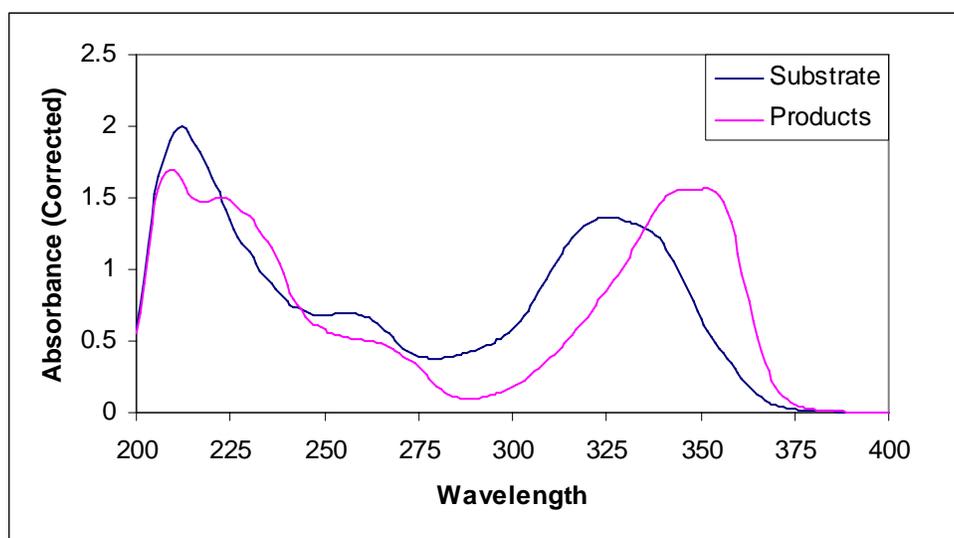


Figure 3. Spectral Scan of phosphate assay reaction substrates and products. Reaction mixture containing all of the necessary components with either 0 nmoles (substrate) or 500 nmoles (products) of inorganic phosphate. Both reactions contained a final volume of 200 ml and were allowed to proceed for 30 minutes at room temperature. The absorbance of the reactions was scanned from 200 nm to 400 nm in 1 nm increments using a mQuant scanning microplate spectrophotometer. Subsequent absorbance measurements were exported to Microsoft Excel and the background absorbance of the microplate subtracted.

The significance of different counter ions was examined. When equal molar amounts of either sodium phosphate or potassium phosphate were assayed, very little difference in absorbance is observed (Figure 5). Both salts produce linear results for phosphate levels below 100 nmoles per well. Values above 100 nmoles result in a plateau similar to that observed in Figure 2A (data not shown). These data indicate that neither counter ion interferes with PNP enzymatic activity or they do so with equal efficacy.

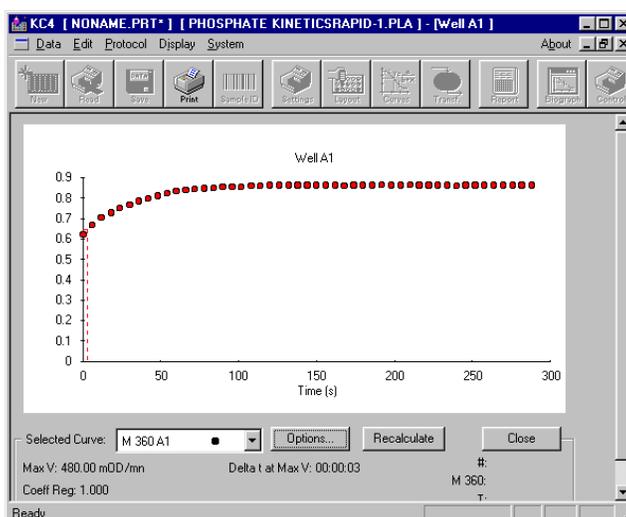


Figure 4. Kinetics of phosphate assay. The absorbance at 360 nm of a phosphate assay containing 200 nmoles of phosphate was measured every 6 seconds for 300 seconds.

The utility of the phosphate assay to measure ATPase activity was examined. As demonstrated in Figure 6, there is a linear relationship between ATPase enzyme concentration and absorbance. ATPase enzyme hydrolyzes ATP to ADP and liberates inorganic phosphate, which in turn serves as a substrate for the phosphate assay previously described. Increasing amounts of enzyme results in the formation of more phosphate over a defined period of time, which leads to the conversion of more MESG and a subsequent increase in absorbance at 360 nm.

Kinetic analysis of the ATPase reaction demonstrates that ATPase enzyme hydrolyzes ATP to ADP and Pi at a constant rate over time (Figure 7). Comparison of a reaction that contains 0.05 U/ml of ATPase with a reaction that does not contain enzyme shows that the increase in signal is not the result of non-enzymatic hydrolytic breakdown of ATP. The reaction lacking any enzyme demonstrated no increase in absorbance from the initiation of the reaction; whereas the reaction containing ATPase enzyme demonstrated a very rapid initial increase in absorbance followed by a more gradual consistent increase over the 30-minute kinetic reading.

The initial increase above background (from 0.35 to 0.6 ODs) occurred in the 10-15 seconds between the time the reaction was initiated and the first absorbance determination was made.

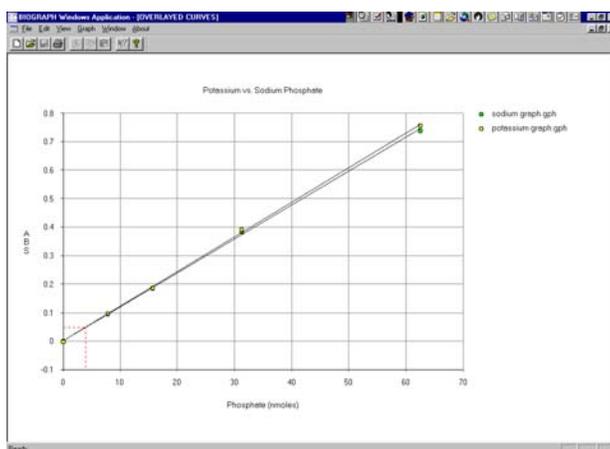


Figure 5. Comparison of sodium and potassium phosphates. Equal molar amounts of sodium phosphate and potassium phosphate were assayed in parallel and their absorbances compared.

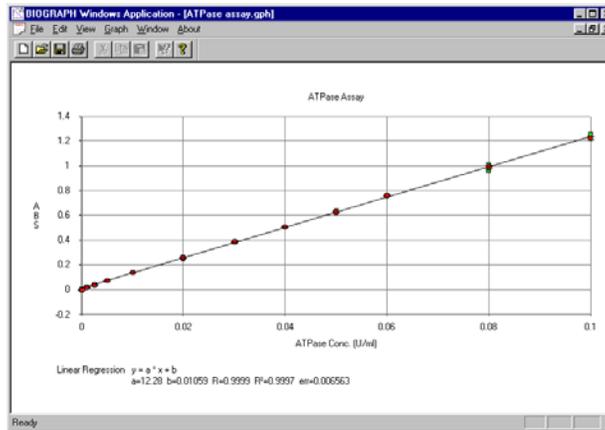


Figure 6. ATPase Enzyme Concentration Curve. ATP hydrolysis to ADP and inorganic phosphate by various concentrations of the enzyme ATPase were assayed. Endpoint absorbance measurements after a 30-minute incubation were determined in replicates of 8 for each enzyme. Absorbance values were corrected by subtraction of a reagent control, plotted against enzyme concentration and a linear regression performed.

Both the initial rapid increase and the slower reaction rate were observed to be concentration dependent. The initial absorbance determination demonstrates a linear relationship with enzyme concentration (Figure 8.) Reagent corrected absorbance measurements ranged from 0 to 0.525 for 0 and 0.1 U/ml enzyme concentrations respectively with a high correlation coefficient ($r^2=0.998$) for the linear regression equation. Likewise, when the rate (mean V) of the reactions over a 30-minute kinetic reaction is plotted against enzyme concentration, a linear relationship is also observed (Figure 9). However, with a 30-minute kinetic determination, enzyme concentrations above 0.06 U/ml were observed not to increase in rate, suggesting that ATP substrate was not in sufficient excess relative to the enzyme.

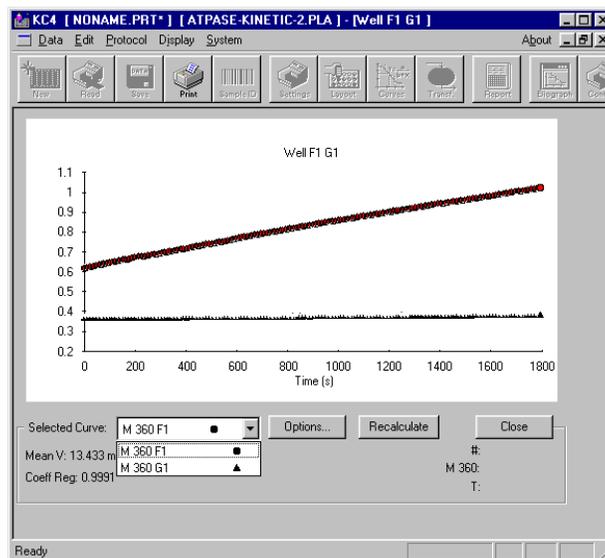


Figure 7. Kinetic Absorbance Measurements of ATPase Assay. The absorbance at 360 nm was determined every 6 seconds for 30 minutes. A comparison of the signal generated by a sample having 0.05 U/ml ATPase enzyme (upper plot) and that obtained from a sample lacking ATPase enzyme (lower plot).

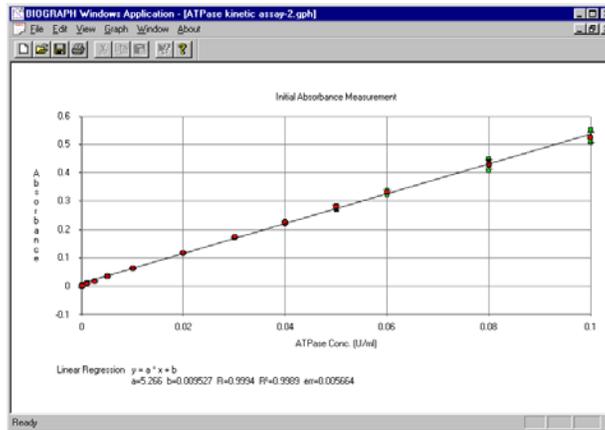


Figure 8. Initial Absorbance Measurement of ATPase Assay. The absorbance of an ATPase assay was measured within 15 seconds of the initiation of the reaction. Absorbance measurements were corrected by subtracting the absorbance of a reagent control and plotted against enzyme concentration.

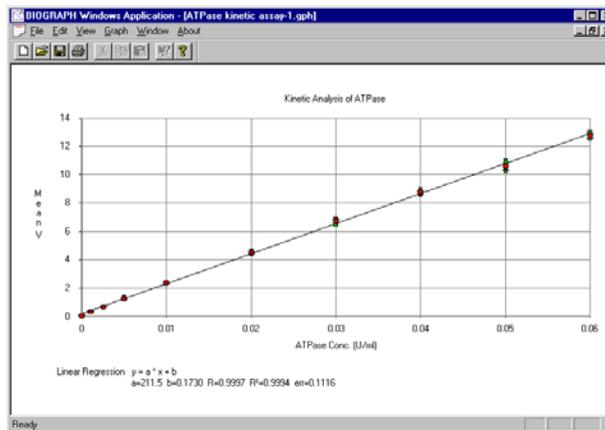


Figure 9. Kinetic Analysis of ATPase Assay. The average mean velocities of various ATPase enzyme concentrations were plotted. Kinetic absorbance measurements were made every minute for 30 minutes and the mean velocity calculated using KC4 data reduction software. Kinetic Data at each concentration represent the average of eight determinations.

Discussion

We have demonstrated the utility of this assay to determine phosphate concentration in solution. The determination of inorganic phosphate can in turn be used to measure enzymatic ATP hydrolysis. While we have only demonstrated the utility of this assay with ATPase enzyme, there are multitudes of different ATP- and GTP-dependent enzymes that can be measured using this system.

Unlike most assays that quantitate inorganic phosphate, this assay is performed under physiologic conditions. This allows for real-time kinetic analysis of ATP-dependent enzymatic reactions. Traditional colorimetric reactions depend on the formation of a phosphomolybdate complex in an acid medium followed by a reduction or complexation with basic dyes that yield colored complexes. As such, only static end-point determinations can be performed.

In regards to kinetic analysis, this assay has the added advantage of consuming one of the products of ATPase activity, namely inorganic phosphate (Pi). This effectively prevents any form of bidirectional steady-state condition from existing and essentially causes the reaction to go in one direction. This also has the added advantage of preventing any feedback inhibition that Pi might have on the enzyme system being measured.

Several factors can influence background absorbance of the assay. Due to the sensitivity of this assay, phosphate contamination needs to be avoided. Buffers containing phosphate (e.g., PBS)

cannot be used for obvious reasons. Tris or other non-phosphate physiologic buffers are more acceptable. Contamination of reagents with small amounts of Pi can be accounted for by subtraction of a reagent blank, if the standard curve and samples all contain equivalent amounts. The presence of phosphate from cell or tissue extracts is much more difficult to address, as differing amounts of contamination can be expected from different samples. As demonstrated in Figure 3, the MESH substrate mixture itself is not totally devoid of absorbance at 360 nm. In addition, many microplates have a significant amount of background absorbance at 360 nm. However, the use of UV transparent microplates and the subtraction of a reagent blank can virtually eliminate these problems.

The use of KC4 software to control the reader allows the user a great deal of flexibility in regards to data reduction capabilities. The software enables the user to define any configuration of plate map necessary. With several different curve fit algorithms to choose from, regression analysis of the standards and the subsequent concentration determinations of samples can be accomplished with a high degree of confidence. Likewise, the software is capable of performing statistical analysis on sample groups, as well as any mathematical calculation required by the user. In the case of the experiments demonstrated, the standard curve was corrected using the water (0 nmoles Pi) blank, while at the same time the non-enzymatic hydrolysis of ATP in samples was corrected for by subtracting the absorbance of control wells from the experimental samples.

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Rev. Date: 2/6/01