Determination of ATPase Activity Using BioTek's ELx808 Microplate Reader

The level of enzymatic ATP hydrolysis activity in biological samples is an important measurement in many different fields of research. Here we describe the use of a BioTek Instruments' ELx808 microplate reader to determine calcium dependent ATPase activity in cardiac myofibril samples.

Introduction

Many metabolic processes that require energy do so by coupling that process with the enzymatic hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) with the liberation of free orthophosphate. This cellular process is carried out by enzymes generally referred to as ATPases. The release of some of the energy contained in ATP can be described simplistically by the following reaction.

$$ATP + H_2O \rightarrow ADP + Pi + Energy$$

Muscle contraction, such as occurs in the heart, is a classic example of the conversion of the chemical energy contained in molecules, such as ATP, to mechanical movement. Cardiac muscle investigators have long observed reduced levels of calcium (Ca^{+2}) activated magnesium dependent ATPase activity in the failing heart. Investigation of the etiology of this reduction of ATPase activity requires that enzymatic ATP hydrolysis be measured accurately and reliably. Here we describe the use of a BioTek Instruments ELx808 microplate reader to carry out ATPase assays on cardiac myofibril samples.

Enzymatic ATP hydrolysis has been measured by several different means, including the determination of ADP by coupling enzymes (1), measurement of ^{32}P release from [γ-^{32}P] ATP hydrolysis (2), or by colorimetric reactions (3-5). 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 (5,6). The determination of ATPase activity in this report is based on the measurement of free phosphate from ATP.

Materials and Methods

Cardiac myofibrillar suspensions were purified from bovine ventricular tissue by treatment with Triton X-100 as described by Solaro et al. (7). The purified myofibrils were suspended in ATPase buffer (60 mM KCl, 20 mM MOPS, pH 7.0, 2 mM MgCl$_2$, 7 mM NaN$_3$, 28 μM cyclopiazonic acid, 0.7 mg/ml leupeptin, 0.7 mg/ml pepstatin A, 48.72 µg/L PMSF) to a protein concentration of 0.75 to 1.75 mg/ml. Total protein determinations were made by the method of Lowry (8) modified for determination in microplates (9).

The ATPase assay was essentially performed as described by White (3) with the modification of Dobrowolski et. al. (10). In order to generate a standard curve, inorganic phosphorous standards (0, 2.5, 5, 10, 25, 50, 100, 150 nmole Pi) in a volume of 70 μl were dispensed in triplicate into a 96-

well microplate. To each of the wells in which experimental myofibrillar samples were to be received 30 µl of ATPase buffer and 10 µl of the appropriate Ca^{+2}-EGTA mixture to achieve the desired free calcium ion concentration were added. Each calcium concentration was represented in replicates of 4. After the addition of ATPase buffer and Ca^{+2}-EGTA solutions, 20 µl of myofibrillar sample was added and the samples were mixed by placing the microplate in the ELx808 reader and shaking for 30 seconds using an intensity setting of 4. After mixing, ATP hydrolysis was initiated by the addition of 10 µl of a 14 mM Na$_2$ATP solution resulting in a final reaction volume of 70 µl and an ATP concentration of 2 mM. ATP hydrolysis was terminated after 10 minutes by the addition of 30 µl of a stop solution consisting of 13% SDS and 120 mM EDTA. Previous experiments had determined that the rate of phosphorous production was linear with respect to time for at least 15 minutes (data not shown). Control wells (stop solution added prior to the addition of ATP) were run in order to correct for any non-enzymatic hydrolysis of ATP or any orthophosphate contamination of the reagents. Following the addition of stop solution, the reaction was mixed again by placing the microplate back into the ELx808 with shaking for 30 seconds at an intensity setting of 4.

Color development was initiated with the addition of 200 µl of freshly prepared color reagent warmed to 30°C. Color reagent consists of 0.5% (w/v) ferrous sulfate, 0.5% (w/v) ammonium molybdate, 0.5M sulfuric acid. Upon the addition of color reagent, the reaction was shaken for 30 seconds at a setting of 4, using the ELx808 to affect mixing. The reaction was then incubated in the EL$_X$808 for 20 minutes at 30°C. Following incubation, samples were shaken again for 30 seconds, followed immediately by the determination of absorbance at 650 nm.

Results

The absorbance of the ATPase assay standard curve was determined for ortho-phosphate concentrations ranging from 0.0 to 150 nmoles per well. Over this range the absorbance increased in a hyperbolic fashion. Using KC3 data reduction software (BioTek Instruments) to perform regression analysis, a polynomial equation describing the standard curve can be generated (Figure 1).
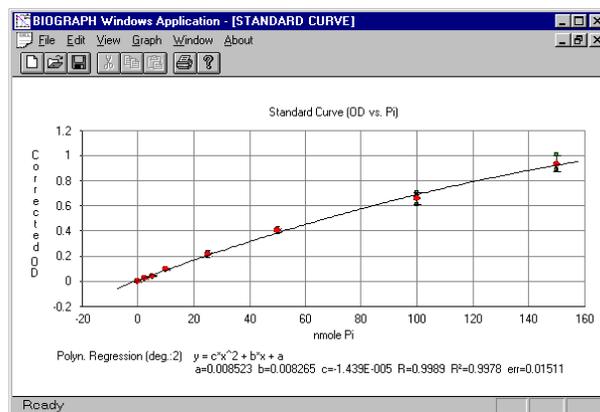

Figure 1. Linearity of the assay. Concentration curve from 0 to 150 nmoles/well of Pi with polynomial regression analysis. Image depicts the screen output from KC3 of a typical standard curve of an ATPase assay performed on myofibrils. Note that the equation describing the regression curve is provided along with statistics concerning the curve.

Although the curve begins to plateau at a orthophosphate concentration of 100 nmoles per well, determinations can be made with a high level of confidence ($r^2 = 0.999$). Determinations in the lower portion of the curve offer the greatest degree of accuracy with a polynomial fit due to the greater change in signal verses change in Pi concentration. As demonstrated in Figure 2, if only low ATPase activity (i.e., concentrations of orthophosphate below 50 nmole/well) then a calibration

curve determined using linear regression analysis rather than a polynomial analysis can be used with confidence (r^2 = 0.998). Routine dilution of each sample would be expected to provide determinations at an appropriate concentration.
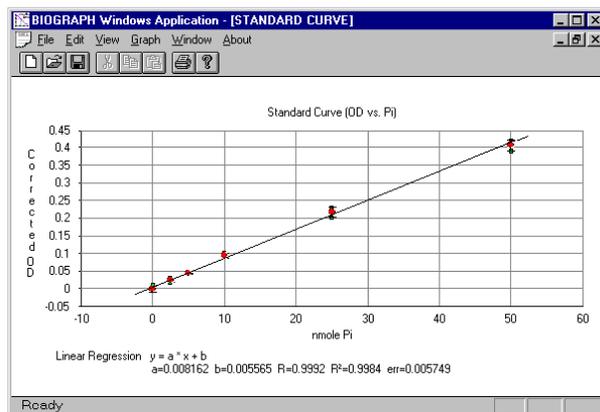
Figure 2. Calibration curve. Concentration curve from 0 to 50 nmoles of Pi with linear regression analysis. Using the data depicted in Figure 1 a linear regression analysis was performed using the 0-50 nmole/well standards. Image depicts the screen output from KC3 of a typical standard curve of an ATPase assay performed on myofibrils. Note that the equation describing the regression curve is provided along with statistics concerning the curve.
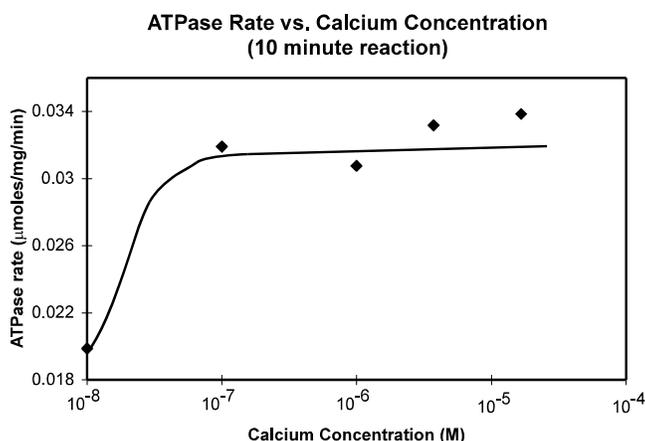
Figure 3. Calcium dependence of ATPase Activity. Myofibril samples assayed under various free calcium concentrations and the ATPase activity rates determined. ATPase rate data was normalized for time and protein content using Microsoft Excel.

The dependence of calcium for activation of enzymatic ATPase activity in myofibrils is demonstrated in Figure 3. Increasing the free calcium ion concentrations from 10^{-8} M to 10^{-7} M results in an almost two fold increase in the rate of enzymatic ATPase activity. Further increases in free calcium above 10^{-7} M did not result in any further appreciable increase in ATPase rate. By subtracting the absorbance of a sample blank containing all of the reaction components, but with stop solution added at time zero, samples were corrected for non-enzymatic ATP hydrolysis.

Discussion

Temperature control of the reaction mixtures is critical, as ATP hydrolysis is temperature sensitive. Consistent and uniform temperature control across the entire plate are necessary for reliable assay performance. The BioTek ELx808 microplate reader, which is equipped with an incubation chamber as well as a shaker, is an excellent choice for this micro-ATPase assay.

The use of KC3 software to control the reader allows the user a great deal of flexibility in regards to data reduction capabilities. The software allows 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.

Like most assays that are read in microplates, the ability to read all of the samples simultaneously greatly reduces the manual labor required to obtain the data. Likewise it ensures that all of the experimental samples are treated similarly in regards to incubation time and temperatures. The microplate format also lends itself to "off the shelf" automation for laboratories with high volume requirements. The smaller reaction volumes in microplates will lead to lower per assay costs by reducing the amount of expensive and/or precious reagents necessary to perform the assay.

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