# Determination of the apparent dissociation constant of Mg-ATP (at T=37°C, pH=7.2, I=0.15) using a combination of $^{31}$P NMR spectroscopy and the fluorescence indicator Mag-fura-2

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## Purpose

The purpose of this study was to determine $K_D^{MgATP}$, the apparent dissociation constant of Mg-ATP, at T=37°C, pH=7.2, and I=0.15 using $^{31}$P NMR and the magnesium-sensitive fluorescence indicator Mag-fura-2.

## Introduction

*In-vivo* research has concentrated on the use of $^{31}$P NMR for the non-invasive determination of intracellular free magnesium. The chemical shift difference between the $\alpha$ and $\beta$-ATP peaks in the phosphorus spectrum, $\delta_{\alpha\beta}^{obs}$, is used to calculate $\phi_{\alpha\beta}^{obs}$ where $\phi_{\alpha\beta}^{obs} = (\delta_{\alpha\beta}^{obs} - \delta_{\alpha\beta}^{MgATP})/(\delta_{\alpha\beta}^{ATP} - \delta_{\alpha\beta}^{MgATP})$ [1]. Multiplication of $[(1 - \phi_{\alpha\beta}^{obs})/ \phi_{\alpha\beta}^{obs}]$ by $K_D^{MgATP}$, the apparent dissociation constant of the magnesium-ATP complex [1-5], yields the final free magnesium level, $[Mg^{2+}]$, in the sample studied.

Alternatively, the $^{31}$P NMR $\phi_{\alpha\beta}^{obs}$ value combined with independent knowledge of the $[Mg^{2+}]$ level enables a direct calculation of $K_D^{MgATP}$. This study used Mag-fura-2 for measurement of $[Mg^{2+}]$. Similar studies have combined $^{31}$P NMR measurements with $[Mg^{2+}]$ data provided by electron paramagnetic resonance spectroscopy [2-3] and optical absorbance spectroscopy [4].

Rearrangement of the chemical equations governing the equilibrium of the magnesium-ATP complex [1] enables an alternative calculation of $\phi$. This calculation is independent of the $^{31}$P NMR $\phi_{\alpha\beta}^{obs}$. Regression of this alternative $\phi$ with the $\phi_{\alpha\beta}^{obs}$ would ideally yield a slope and $r^2$ of 1. The calculation does require, however, an accurate knowledge of the total magnesium, $[Mg]_{Total}$, and ATP, $[ATP]_{Total}$.

## Methods

Test solutions were made up using the following reagents: $MgCl_2$, 2M (Quality Biological, Inc., Gaithersburg, MD), $Na_2ATP$ (Sigma, St. Louis, MO), KCl, (Mallinckrodt, Paris, KY), MOPS, 1 M (Calbiochem, La Jolla, CA), NaOH, 1M ( J.T. Baker, Phillipsburg, NJ), HCl, 1 M ( J.T. Baker, Phillipsburg, NJ), Mag-fura-2, tetrapotassium salt, 1 mM, (Molecular Probes, Eugene, OR).

Each of the seven experiments consisted of analysis of five $[Mg^{2+}]$ levels with 3 mM ATP in solution at pH 7.2 and I = 0.15. Preliminary mag-fura-2 experiments estimated the solutions so as to obtain $[Mg^{2+}]$ levels of approximately 40 μM, 80 μM, 120 μM, 160 μM and 200 μM. Each $[Mg^{2+}]$ solution was then divided into aliquots for parallel analysis by Mag-fura-2 (as detailed in [6]) and $^{31}$P NMR. A magnesium-free ATP solution and one containing a saturating level of magnesium [7] were used to determine $\delta_{\alpha\beta}^{ATP}$ and $\delta_{\alpha\beta}^{Mg-ATP}$ for each experimental run.

Accurate determination of $[Mg^{2+}]$ with Mag-fura-2 requires a careful calibration of the fluorescence signal through determination of the dissociation constant of the fluorescence dye [6]. The dissociation constant of the dye was determined to be 2.26 mM for the experimental conditions of this study. A previous study combining $^{31}$P NMR and Mag-fura-2 did not calibrate the dye but used a literature value of 1.5 mM [7].

The amount of magnesium in each sample was estimated from volumetric dilutions of a 2M $MgCl_2$ standard solution. True magnesium levels were determined for similar test solutions using Flame Atomic Absorption (FAA) Spectroscopy ($r^2$ =0.984, d.f.=7, data not shown). FAA data from the test solutions was used to correct the magnesium content of the original volumetric data.

$^{31}$P NMR: Spectra were acquired at 121.5 MHz using a Bruker AC-300 wide bore spectrometer maintained at 37°C with heated $N_2$. Pulse sequence parameters: PW = 3.2 μsec (Flip angle=61°), 4096 acquisitions, 8192 data points, TR=0.85 sec, resolution of 0.005 ppm/pt, SW=5000 Hz, DW=101μsec. The spectra were not proton decoupled.

Each spectrum was post-processed five times from the original data through the final peak fit. WIN-NMR™ (Bruker Instruments, Inc., Billerica, MA) was used to line broaden (LB=20 Hz), Fourier transform, and phase the data. Peak Fit™ (Jandel Scientific Software, San Rafael, CA) was used to determine the $\alpha$ and $\beta$-ATP peak locations.

## Results

Figure 1 plots $[Mg^{2+}]$, as measured by fluorescence, against $(1 - \phi_{\alpha\beta}^{obs})/\phi_{\alpha\beta}^{obs}$. The slope of the line, $K_D^{MgATP}$, equals 27.7±0.4 μM ($r^2$=0.957, d.f.=34). Results of the regression of the calculated $\phi$ against the $\phi_{\alpha\beta}^{obs}$ (slope=0.955, $r^2$=0.967, d.f.=34, data not shown) confirms this determination of $K_D^{MgATP}$.
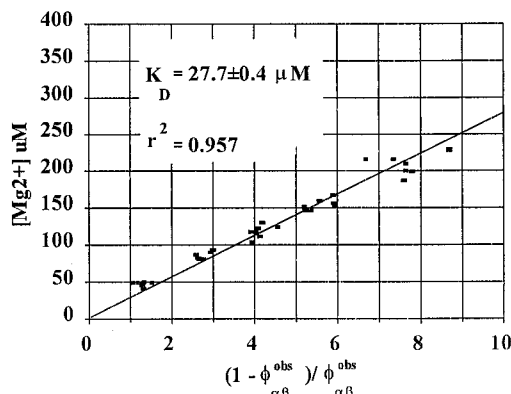


**Figure 1.** $[Mg^{2+}]$ plotted against $(1 - \phi_{\alpha\beta}^{obs})/\phi_{\alpha\beta}^{obs}$ to obtain $K_D^{MgATP}$.

## Discussion

This study used the two methodologies of $^{31}$P NMR and the fluorescence indicator, Mag-fura-2, to determine a $K_D^{MgATP}$ of 27.7 ± 0.4 μM (T=37°C, pH=7.2, and I=0.15). The $K_D^{MgATP}$ reported here is also uniquely confirmed by regression of the $\phi_{\alpha\beta}^{obs}$ to the $\phi$ calculated using $[Mg]_{Total}$ and $[ATP]_{Total}$. This value is almost half of the current value of 50 μM used in the literature [4]. However, that value was determined at 25°C, which most likely contributed to the discrepancy. Other studies support a $K_D$ on the order of 30 μM [2, 5].

## References

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