Vol. 117, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1983 Pages 210-216

MEASUREMENT OF THE DISSOCIATION CONSTANT OF MgATP AT PHYSIOLOGICAL NUCLEOTIDE LEVELS BY A COMBINATION OF ^{31}P NMR AND OPTICAL ABSORBANCE SPECTROSCOPY*

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Received October 20, 1983

To resolve a controversy in the literature concerning the affinity of Mg^{++} for ATP to be used in our noninvasive ^{31}P NMR procedure for the determination of free Mg^{++} in living cells, we have reinvestigated the apparent dissociation constant of MgATP under physiologic ionic conditions and over the cellular range of ATP concentrations by a combination of NMR and optical absorbance techniques. The new combination method utilizes ^{31}P NMR chemical shifts to determine the degree of Mg^{++} chelation of ATP in a solution containing free ATP and MgATP, and uses a properly calibrated indicator dye, antipyrylazo III, for optical measurement of free Mg^{++} in the same solution. The data yield an average value of 50±10 μM for the apparent dissociation constant of MgATP which indicates low levels of free Mg^{++} (<1 mM) in several different types of tissues, including perfused heart muscle, contrary to a recent report in the literature.

Knowledge of the free magnesium concentration of living cells and tissues is of fundamental biochemical interest. We previously introduced a noninvasive ^{31}P NMR method for measuring the concentration of free Mg^{++} in intact cells and tissues based on the Mg^{++}-dependent separation between the αP and βP resonances in the ^{31}P NMR spectrum of intracellular ATP (1). Applications of this method to a number of tissues have been described (1-7). In all of our studies (1, 4, 5) we obtained values for the concentration of free Mg^{++} in the range 0.4 ±0.2 mM and we concluded that Mg^{++} would limit the rates of cellular reactions in which the substrate is the Mg^{++} complex of ADP or another compound that binds Mg^{++} weakly. Although our NMR technique would appear to be acceptable in concept, the validity of our conclusions has been challenged by Wu

*This work was supported by National Institutes of Health Grants AM-32030 and GM-19875, and by NCI Core Grants CA-13330 and CA-06927.

0006-291X/83 $1.50

et al (7) who asserted that we have used an inappropriate value for the dissociation constant for MgATP. Wu et al (7) redetermined the dissociation constant using hydroxyquinoline sulfonate and then used our method to study the free Mg^{++} in heart muscle. They reported that the free Mg^{++} in normal heart is 2.5 mM which is too high to regulate most Mg^{++}-dependent reactions. In addition they expressed concern that we did not account for the complexation of the total Mg^{++} in each tissue.

Since an accurate and appropriate MgATP dissociation constant is crucial for free Mg^{++} calculations based on our NMR technique, we have reinvestigated its exact value under physiological ionic conditions and over the cellular range of ATP concentrations using a combination of ^{31}P NMR and optical absorbance techniques. The ability to measure the apparent dissociation constant of MgATP at physiological levels of the nucleotide may be important since it is possible that higher order complexes form at cellular levels of ATP. We reinterpret literature data concerning free Mg^{++} in perfused and ischemic heart muscle in the light of our new results and point out that misleading conclusions would result from attempting to account for the total Mg^{++} in a cell by considering its binding only to a few known cellular components.

MATERIALS AND METHODS

The dyes 8-hydroxyquinoline-5-sulfonic acid and bis(4-antipyrylazo)-4,5-dihydroxy-2,7-naphthalene disulfonic acid (antipyrylazo III) were purchased from Aldrich (Milwaukee, WI) and from K & K Rare Chemicals Co. (Plainview, NY), respectively. All measurements were made at 25°C and at pH 7.2 in 0.05 M N-tris[hydroxymethyl]methyl-2-aminoethane sulfonate (TES) buffer in the presence of 0.15 M K^{+}. Optical measurements were carried out using a Gilford 250 spectrophotometer. Phosphorus-31 NMR spectra were recorded at 81 MHz with a Varian XL-200 FT NMR instrument with proton noise decoupling. Each spectrum was obtained as a time-average of 1000 to 5000 transients of free induction signal following a 90° pulse.

RESULTS

A method of determining the dissociation constant of MgATP at millimolar levels of ATP has been devised which involves measuring the ^{31}P chemical shifts of ATP resonances to determine the proportion of ATP complexed to Mg^{++} in the presence of various nonsaturating Mg^{++} levels and then measuring free Mg^{++} in the same solution optically using a properly calibrated indicator dye, antipyrylazo III. Since the binding of this dye to Mg^{++} is weak and upon

Mg^{++}-complexation the change in its optical absorbance at certain wavelengths is large (24 cm^{-1} M^{-1} at 600 nm), it is possible to measure small concentrations of free Mg^{++} (<100 μM) with reasonable precision. The data from a titration of antipyrylazo III with Mg^{++} can be fitted to a curve with a dissociation constant of 2.5 mM for the Mg-dye complex. At low concentrations of the dye (20-50 μM) and Mg^{++} (50-200 μM), therefore, the amount of Mg^{++} complexed to the dye is negligibly small (0.4 to 4 μM) and does not cause any significant perturbation in the free Mg^{++} level. Because of the high dissociation constant, i.e. weak binding, the dye absorbance change at low levels of the dye and Mg^{++} is proportional to the concentration of free Mg^{++} permitting the dye to serve as a good indicator for free Mg^{++} in vitro.

^{31}P NMR spectra were recorded for solutions containing no magnesium, at several partially saturating magnesium levels such that 51 to 93% of the ATP was complexed, and at a Mg^{++} level that was saturating for ATP. The extent of Mg^{++}-complexation of ATP was quantitated by measuring $\delta_{\alpha\beta}$, the chemical shift difference between the αP and βP resonances (Table I and Fig. 1). ATP levels were in the range of 2-6 mM. The free Mg^{++} values were based on measurements with the indicator dye. The dissociation constant was then obtained from the following equations:

$$\phi = \frac{[ATP]_f}{[ATP]_T} = \frac{\delta_{\alpha\beta} - \delta_{\alpha\beta}^{MgATP}}{\delta_{\alpha\beta}^{ATP} - \delta_{\alpha\beta}^{MgATP}} \qquad [1]$$

$$K_D^{MgATP} = [Mg]_f \left\{ \frac{\phi}{1 - \phi} \right\} \qquad [2]$$

where ϕ is the fraction of total ATP that is not chelated to Mg^{++}. $[ATP]_f$ means the sum of all ATP species not chelated to Mg^{++}; i.e., $[KATP^{3-}]$, $[HATP^{3-}]$, as well as $[ATP^{4-}]$. $\delta_{\alpha\beta}$ is the measured separation between the αP and βP resonances; $\delta_{\alpha\beta}^{MgATP}$ and $\delta_{\alpha\beta}^{ATP}$ are the values of $\delta_{\alpha\beta}$ in the presence of a saturating level of Mg^{++} and no Mg^{++}, respectively. An average value of 50 ± 10 μM was obtained in this way at physiological ionic strength ($[K^+]$ = 0.15 M) and pH 7.2 (Table I). This value is similar to those obtained by EPR (8) and ^{31}P NMR (9) methods but is significantly different from the values of Wu

TABLE I

Determination of MgATP dissociation constant by the combination method using NMR to determine the extent of ATP chelation and the dye antipyrylazo III as an indicator for measuring free Mg in the same solution.

$\delta_{\alpha\beta}$ Hz	ϕ	Δ(o.d.)	$[Mg]_f$ μM	K_D^{MgATP} μM
873	1.00	0.000	0	
735	0.27	0.072	147	55
715	0.17	0.141	288	58
710	0.14	0.160	327	54
697	0.07	0.251	512	41
683	0.00		>10,000	
875	1.00	0.000	0	
776	0.49	0.025	51	49
719	0.19	0.106	216	50
702	0.10	0.182	371	41
683	0.00		>10,000	

Average K_D^{MgATP} = 50±10 μM

All measurements were made at pH 7.2 in 50 mM TES buffer containing 20% by volume D_2O and 0.15 M K^+ (T=25 ± 1°C). ^{31}P NMR measurements were made at 81 MHz and $\delta_{\alpha\beta}$ values are expressed in Hz. For the measurement of free Mg^{++}, the indicator dye antipyrylazo III was added to a final concentration of 50 μM. Total ATP concentration in the solution was in the range 2.7 to 6.3 mM. In a control under the same conditions but lacking ATP it was determined that the relationship of the absorbance change, Δ(o.d.), at 595 nm vs. added free Mg^{++} concentration at free Mg^{++} values up to 500 μM was approximately linear with a slope of 0.049/100 μM of Mg^{++}. ATP had no measureable absorbance at 595 nm.

et al (7) obtained using the dye hydroxyquinoline sulfonate. Our attempts to measure the dissociation constant of MgATP with hydroxyquinoline sulfonate following the procedure of Burton (10) led to variable results which depended strongly on the concentrations of the dye, Mg^{++}, and ATP used. Similar problems in the use of such dyes were noticed previously by Adolfsen & Moudrianakis (11), and were ascribed in part to an aggregation of the dye.

A unique advantage of the combination method is that the methodology is identical to that used for estimation of free Mg^{++} in intact cells. Actually it is the same approach run in reverse to obtain the dissociation constant instead of the free Mg^{++} value. Since the free Mg^{++} calculation in the intact cell is based on the same method as that used for the measurement of MgATP dissociation constant, all systematic errors should cancel out. Possible breakdown of ATP is also not a problem since free Mg^{++} in a solution containing several components must satisfy all existing equilibria.

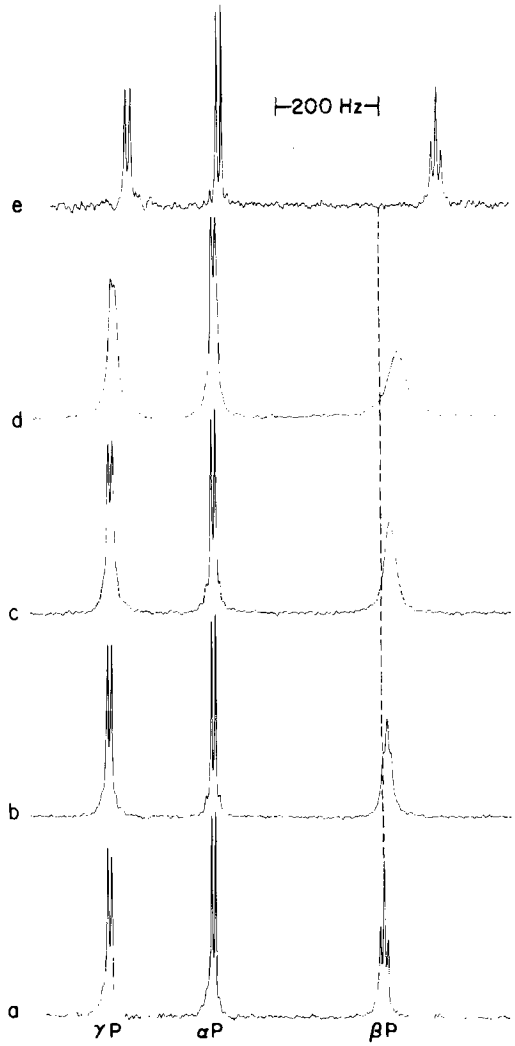

Figure 1. NMR spectra showing the ^{31}P resonances of an equilibrium mixture containing ATP and MgATP at 81 MHz. Components present in all cases were: KCl (0.13 M), K^+-TES buffer pH 7.2 (50 mM), 20% by volume D_2O, 3.5 mM ATP, and Mg in the range 0 (trace e) to 14 mM (trace a). Free Mg^{2} values as estimated using the dye were: b, 512 μM; c, 327 μM; d, 147 μM. To obtain these spectra, 5000 transients of free induction signal with a flip angle of 90° and a pulse recycle time of 2 sec. were used. Artificial line broadening of 3 to 5 Hz was used to improve the signal to noise ratio of the resulting spectra.

DISCUSSION

The estimation of intracellular free Mg^{++} levels in several types of living cells from the phosphorus NMR spectrum of intracellular ATP was discussed thoroughly by us previously (1, 4, 5, 12). The method requires measurement of the chemical shift difference between the $^{\alpha}P$ and $^{\beta}P$ resonances, and only simple calculations not dependent on the determination of total cellular Mg^{++}

content. As long as only a single set of resonances is observed, consistent with fast exchange-averaging of the resonances of ATP and MgATP, the frequencies of phosphorus resonances reflect the state of Mg^{++} complexation of ATP.

The values of the dissociation constant of MgATP determined in this paper are significantly lower than those in the paper by Wu et al (7) determined using the hydroxyquinoline-5-sulfonate dye. Wu et al compared their value with a few selected literature values, but neglected to consider the published values of MgATP dissociation constant from our laboratory based on the EPR method (8). Adolfsen and Moudrianakis (11) have previously measured the MgATP dissociation constants using a divalent cation electrode. They compared these results with those obtained from an indirect spectrophotometric method using hydroxyquinoline as an indicator of free Mg^{++} concentration. While the electrode measurements conformed to theoretical expectations, the MgATP dissociation constants obtained from the spectrophotometric method were markedly different. The latter continuously increased as the pH was raised, with 30-fold variation over the pH range 7.5 to 9.0. Possible sources of trouble may be the formation of a $Mg(dye)_2$ aggregate which precipitates as well as the formation of a Mg-dye-ATP ternary complex. A precipitate was indeed detected visually in some of the solutions in this study. Even small amounts of precipitate which may not be detectable visually may introduce substantial error into the data. It should also be mentioned that Na^+ and K^+ ions bind to the metal-ATP complex almost as well as to the metal-free ATP (Gupta, R. K. & Gupta, P., unpublished results). Thus the assumption of purely competitive binding of Mg^{++} and K^+ or Na^+ ions which is generally made in the literature may not be correct.

In support of our low value for free Mg^{++} in skeletal muscle, recent measurements of free magnesium concentration in muscle cytoplasm using skinned muscle fibers indicate that of the total of 6.2 mmol Mg^{++} per Kg whole muscle, only 60% is in the diffusable form (13). Thus the remaining 40% of the Mg^{++} in the living muscle must be bound to various subcellular structures. These results clearly demonstrate that attempting to account for total Mg^{++} in terms of its interactions with only a few known components would be misleading. Maughan (13)

concluded from his data that free Mg^{++} concentration in frog muscle is less than or equal to 0.4 mM which agrees fairly well with our published value of 0.6±0.2 mM in this tissue and argues strongly against the higher value of 3 mM suggested by Wu et al (7).

From the NMR chemical shift data on perfused guinea pig heart muscle in the paper by Wu et al (7) and the value obtained in this work for the dissociation constant of MgATP, we estimate, using equations [1] and [2], the free Mg^{++} value to be 0.8 mM which is 4-fold lower than values calculated in that paper. A low free Mg^{++} value in heart muscle is also indicated by computer modeling of energetic and metabolic processes and fluxes (14-16). The free Mg^{++} level of erythrocytes was also found by Flatman & Lew (17) to be low (~ 0.4 mM) using an ionophore A23187, in agreement with NMR results (1). The low intracellular concentrations of free Mg^{++} indicated by our studies support a regulatory role for free Mg^{++} in various Mg^{++}-requiring metabolic processes.

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