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Microcalorimetric Study of Magnesium-Adenosine Triphosphate Ternary Complex

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Abstract

Using an original microcalorimetric method, the existence of the Mg_2ATP ternary chelate has been studied. The thermodynamic parameters of this complex are $\Delta H = 7.2 \pm 0.5$ kJ mole^{-1} and $K = 49 \pm 9$ M^{-1}. These values are compared with those previously obtained for binary chelate Mg ATP^{2-}. A possible regulation role of Mg_2ATP is discussed.

Key Words: Magnesium adenosine triphosphate ternary complex; microcalorimetric study.

Introduction

The $MgATP^{2-}$ binary complex has been extensively studied because of its importance in a great many biological reactions. Certain authors have, however, underlined the fact that excess Mg^{2+} can inhibit certain enzymatic reactions in which this complex participates. This effect has been measured for adenylate cyclase (Blair, 1970), adenyl cyclase (Drummond *et al.*, 1971), phosphofructokinase (Garner and Rosett, 1973), and different hexokinases (Bachelard, 1971; Noat *et al.*, 1970; Purich and Fromm, 1972). These results have been interpreted, in certain cases, as competitive inhibition by free Mg^{2+} when the Mg/ATP ratio is much greater than 1. On the other hand, Noat *et al.* (1970) have suggested that Mg^{2+} can form, with ATP, an inactive ternary chelate Mg_2ATP which, in order to agree with their results, should have an association constant of 40 M^{-1}. Several authors have studied this possibility (Burton, 1959; Frey *et al.*, 1972; Liebecq and Jacquemotte-Louis, 1958),

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although no direct evidence for this complex existence has yet been published. Burton *et al.* (1959) have stated that its equilibrium constant K_2 should not be greater than 70 M^{-1} for an ionic strength 0.11 at pH 8. The K_1 constant of the binary $MgATP^{2-}$ complex varies, according to the authors, from 10^4 to 10^5 M^{-1} in similar experimental conditions (Phillips, 1966).

Quantitative results for K_2 have been determined by Mohan and Rechnitz (1974) using Mg-sensitive electrodes. They obtained a $K_1 = 1.15 \times 10^6$ $M^{-1} \pm 1.56 \times 10^5$ and $K_2 = 409 \pm 39$ M^{-1} with a very low ionic strength ($\simeq 5 \cdot 10^{-3}$). These experimental conditions also imply the use of the extended Debye–Hückel equation to adjust Rechnitz's K_2 value to biological conditions. More recently Bishop *et al.* (1981), using NMR results, obtained $K_2 = 33 \pm 3$ M^{-1} ($\theta = 25°C$).

The microcalorimetric method allows the determination of all the thermodynamic parameters of a complex with only one set of binding sites. This technique has already been used by one of the authors of this paper to study various complexes, in particular metal/nucleotide complexes (Belaich and Sari, 1969; Ragot *et al.*, 1977b; Sari and Belaich, 1973). However, in order to use this method to study the ternary chelate Mg_2ATP, it was necessary to overcome certain difficulties caused by the simultaneous existence of two species, $MgATP^{2-}$ and Mg_2ATP, and by the low value of K_2 compared to that of K_1. A microcalorimetric technique was therefore developed and used in conjunction with an iterative calculation method recently devised by one of the authors for the determination of low constants (Coassolo *et al.*, 1980).

Experimental

Reagents

Na_2H_2ATP was purchased from Boehringer or Sigma. This product contains a small amount (about 5–10%) of AMP, ADP, and P_i. These impurities must be eliminated to avoid competitive Mg^{2+} binding and, therefore, for all experiments, freshly purified ATP was used. Sodium has an affinity constant of 20 M^{-1} for ATP. This ion was therefore exchanged for tetramethylammonium (TMA), which has a negligible affinity for ATP (O'Sullivan and Perrin, 1964; Smith and Alberty, 1956). The purification was carried out on a Sephadex DEAE A25 column (30 mm × 10 mm), using 15 ml of a solution containing 50 mg ATP sodium salt at pH 3. The column was eluted with a 0–0.15 M TMA chloride linear gradient. The fraction containing the ATP was buffered at pH 8.00 with 10^{-2} M triethanolamine (TEA) and the ionic strength was adjusted to 0.2 with TMA chloride. TMA and TEA

were Merck puris. products. TEA was used as it does not complex Mg. The final ATP concentration was determined by UV absorption using an extinction coefficient of 15 $cm^2\ \mu mol^{-1}$ at 260 nm and pH 7.

$MgCl_2 \cdot 6H_2O$ was purchased from Prolabo. As this salt is extremely hygroscopic, concentrations were determined by atomic absorption (Perkin Elmer) with a $Mg(OOCCH_3)_2$ standard 1000 ppm (Alfa products). $MgCl_2$ was dissolved in 10^{-2} M TEA and the pH value was adjusted at 8.00 with TMAOH.

Apparatus

An LKB (type 107001) flow microcalorimeter, thermostatically regulated at a temperature of 37°C, was used.

The thermal flux dQ/dt was continuously recorded as a solution of constant ATP concentration was mixed with a solution of increasing Mg concentration. The Mg^{2+} linear gradient was obtained by classical means. The ionic strength was adjusted to 0.2 in the two flasks using different amounts of TMA. If we take into account the ATP concentration and pK values, we see that the amount of protons evolved during the complex formation reaction is so weak that the pH value of the buffered medium remains constant through the experiment.

LKB pumps, thermostatically regulated at 37°C, were set at $v_1 = 4.3 \times 10^{-6}$ liter s^{-1} for the ATP solution, and $v_2 = 7.5 \times 10^{-6}$ liter s^{-1} for the Mg^{2+} gradient. The thermal flux $dQ_i/dt = kD_i$, where D_i is the measured deviation recorder and k is determined by electrical and chemical calibration ($k = 0.3\ \mu W\ mm^{-1}$).

The heat of reaction is calculated from

$$Q_i(\text{J liter}^{-1}) = \frac{kD_i\ (\text{W})}{v_1 + v_2\ (\text{liter s}^{-1})}$$

Under these conditions thermal flux of the order of magnitude of 70 μW can be measured with greater than $\pm 1\%$ accuracy. Corrections were performed, taking into account the heats of dilution of the reagents. To correct thermal inertia of the apparatus, we can use Tian's equation. However, for $0 < R < 5$ the slope of the curve is very important; therefore Tian's correction is difficult to make accurately. So we performed experimental corrections and for this purpose the heats of reaction for $R = 0.5$, 1, 1.5, 2, 3, and 5 were determinated separately. For $R > 5$ the slope of the curve becomes so weak that the correction is negligible:

$$R = \frac{[\text{Mg}]}{[\text{ATP}]}$$

Methods

a. Enthalpic Titration Method. This method, devised by one of the authors (Belaich and Sari, 1969; Ragot *et al.*, 1977b), is as follows. The association constant and the enthalpy variation can be calculated for all the reactions involving the formation of binary complexes fom two heat measurements. The free energy ΔG and entropy ΔS variations can be deduced by means of classical thermodynamic relationships. However, as we have already shown (Ragot *et al.*, 1977a), enthalpic titration does not allow an accurate measurement of low association constants. This method was therefore used for the K_1 (MgATP) determination only. To determine K_2, a modified iterative calculation method, recently developed by one of the authors (Coassolo *et al.*, 1980), was used.

b. Iterative Enthalpic Titration Method. This technique (Coassolo *et al.*, 1980) was devised to calculate the weak association constants encountered in the formation of bimolecular complexes. Let a be the constant concentration of the first reagent; c_i, the variable concentration of the second reagent; x_i, the concentration of the bimolecular complex corresponding to c_i; Q_i, the heat measured for each c_i value (J liter^{-1}). As the temperature, pH, and ionic strength are constant for each concentration, the apparent association constant for a given ionic strength is

$$K = \frac{x_i}{(a - x_i)(c_i - x_i)} \tag{1}$$

The heat evolved for each c_i value is

$$Q_i = x_i \Delta H \tag{2}$$

Combining relationships (1) and (2), we obtain the mathematical expression of the enthalpic titration curve $Q_i = f(c_i)$:

$$Q_i = \left\{ \frac{1}{K} + a + c_i - \left[\left(\frac{1}{K} + a + c_i \right)^2 - 4a\, c_i \right]^{1/2} \right\} \frac{\Delta H}{2} \tag{3}$$

The hyperbola described by relationship (3) is not equilateral; so, a direct iteration of $Q_i = f(c_i)$ using the least-squares technique to determine both K and ΔH values is very difficult (Hardee *et al.*, 1978; Landau *et al.*, 1977; Otagiri *et al.*, 1978).

The interest in the iterative enthalpic titration method resides in the fact that the K value is optimized, the ΔH being deduced from the best K value.

The following procedure is used: first a K value is chosen arbitrarily; then, using relationship (1), an x_i value can be calculated for each c_i value. It is then possible to form a linear regression $Q_i = f(x_i)$ from the totality of the experimental points of the enthalpic titration curve. The following relation-

ship is obtained:

$$Q_i = Ax_i + B \tag{4}$$

The Q_i value for $x_i = 0$ is called the "residue" B. The correlation coefficient r and the B value for such a straight line are generally very different from 1 and 0 respectively.

By means of a computer, the K values are iterated until the correlation towards coefficient and the residue value tend towards 1 and 0 respectively. The K value corresponding to the best correlation coefficient and the lowest residue is selected.

The residue value being generally lower than 10^{-12} J liter^{-1}, relationships (2) and (4) can be identified, and the slope of the straight line allows calculation of the ΔH value.

The confidence intervals for ΔH and B values are calculated from the variances of the linear regression coefficients. The extremes of the K values are deduced from confidence intervals for the B value.

Results

Figure 1A shows an example of the type of experimental curve obtained with a linear Mg^{2+} gradient, from 0 to a final concentration giving R =

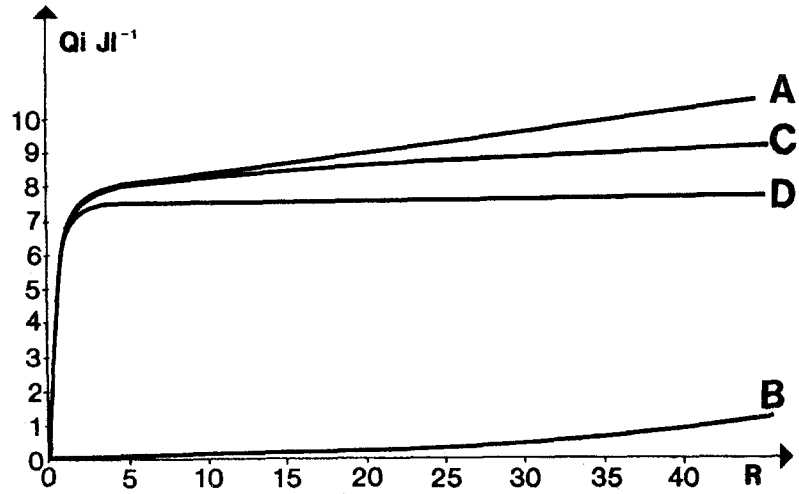

Fig. 1. Variation of the heat of reaction as a function of R = [Mg]/[ATP]. $\theta°$ = 37°C, pH = 8.00, μ = 0.2. (A) Experimental curve (continuous recording); (B) curve corresponding to dilution of the Mg^{2+} gradient; (C) corrected heat of dilution curve; (D) curve corresponding to formation of the $MgATP^{2-}$ binary complex.

Fig. 2. Variation of the heat of reaction corresponding to formation of the ternary chelate Mg_2ATP.

[Mg]/[ATP] = 40. In this case the constant ATP concentration in the cell was 0.49×10^{-3} M. This curve must be constantly corrected to allow for the heats of dilution of ATP and Mg. Under our experimental conditions, however, the ATP heat of dilution is negligible (10^{-3}J liter^{-1}). The Mg heat of dilution is shown in Fig. 1B, and, using this, we obtain the corrected curve in Fig. 1C. As the ATP concentration is 0.49×10^{-3} M, it is easy to verify that, for a K_1 constant of 5×10^4 M^{-1} the percentage of $MgATP^{2-}$ complex present will be greater than 99.5% when $R > 5$. However, from Fig. 1C, when $R > 5$, the exchanged heat continues to increase, which would not be the case if only the $MgATP^{2-}$ complex was present. Also, as the ratio of the constants K_1/K_2 is in the 10^3 range, the Mg_2ATP concentration can be considered negligible when $R < 1$. This fact shows that the heat recorded in this zone corresponds to the $MgATP^{2-}$ complex only.

Using the heats obtained for $R = 0.5$ and $R = 1$, the enthalpic titration method outlined above allows one to calculate the apparent measured values:

$$\Delta H_{ap} = +15.8 \text{ kJ mole}^{-1}, \qquad K_{ap} = 5.3 \times 10^4 \text{ M}^{-1}$$

These results must be corrected as about 8% of the ATP H^{3-} form persists at pH 8. In fact, we have demonstrated that the amount of H^+ is proportional to the concentration of $MgATP^{2-}$ complex (Ragot *et al.*, 1977a). So it is possible to correct the apparent measured values taking into account the ionization ΔH values of ATP H^{3-} and TEA and the pK value of ATP H^{3-}.

We obtain

$$\Delta H_1 = 18.3 \pm 0.1 \text{ kJ mole}^{-1}, \qquad K_1 = 5.4 \pm 0.4 \times 10^4 \text{ M}^{-1}$$

These results are slightly different from those previously obtained (Sari and Belaich, 1973). This could be due to a difference in temperature (37°C instead of 30°C) and the use of $MgCl_2$ in place of $MgSO_4$.

Using Eq. (3) and K_{1ap} and ΔH_{1ap}, we can trace the theoretical enthalpic titration curve corresponding to the formation of a single complex $MgATP^{2-}$ (Fig. 1D). Figure 2, which represents the exchanged heat variation during the fixation of the second Mg atom, is obtained from the difference between the curves in Figs. 1C and 1D. The iterative calculation method (Coassolo *et al.*, 1980) was then applied to the results derived from this curve. For this purpose, pairs of Q_i and $c_i = [Mg^{2+}]$ values were chosen corresponding to $R - 1 = 4, 8, 12, 16, \ldots, 40$. Under these conditions the influence of the $MgATP^{2-}$ binary complex can be ignored as it is completely formed when $R \geqslant 5$. This complex is therefore equivalent to the first reagent defined in the iterative method. We obtain

$$\Delta H = 7.2 \pm 0.5 \text{ kJ mole}^{-1}, \qquad K_2 = 49 \pm 9 \text{ M}^{-1}$$

These results are taken from the linear regression coefficients of the straight line $Q_i = f(x_i)$ which has the best correlation coefficients and residue values (Fig. 3).

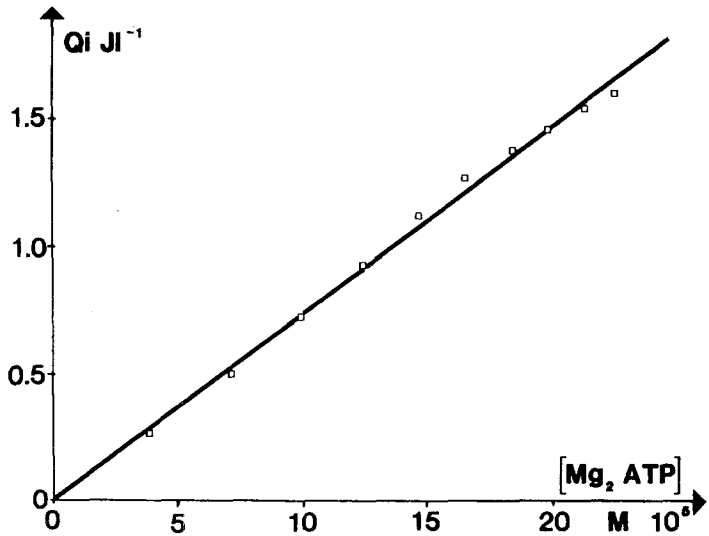

Fig. 3. Experimental heat measurement versus complex concentration calculated from the best microcalorimetric K value. $K = 49 \text{ M}^{-1}$, $B = 3 \cdot 10^{-13}$ J liter^{-1}, $r = 99.7$.

Table I. Thermodynamic Parameters of Mg/ATP Complexes[a]

Complex	ΔH (kJ mole^{-1})	K (M^{-1})	ΔG (kJ mole^{-1})	ΔS (J K^{-1} mole^{-1})
$MgATP^{2-}$	18.3 ± 0.1	5.4 ± 0.4 × 10^4	28.0 ± 0.2	+149 ± 1
Mg_2ATP	7.2 ± 0.5	49 ± 9	$10.0^{+0.5}_{-0.4}$	$+55.5^{+3.0}_{-3.3}$

[a] $\mu = 0.2$; $\theta = 37°C$.

Discussion

Our results give the values of the thermodynamic parameters of the Mg_2ATP complex under conditions approximating the human biological medium. These results are listed in Table I together with those obtained for the $MgATP^{2-}$ binary complex.

The measured K_2 constant is close to the theoretical value indicated by the authors for the same ionic strength. The fact that a knowledge of these parameters is essential for all thermodynamic studies of the enzymatic reactions in which ATP and Mg are involved should be underlined. Thus Goldberg (1976), in his study of hexokinase-catalyzed reactions, stressed the importance of obtaining calorimetric measurements of this parameter.

The results recorded for Mg_2ATP are remarkably close to those determined for MgAMP under similar conditions (Sari and Belaich, 1973): ΔH = 7.4 kJ mole^{-1}, $K = 64$ M^{-1}. The fixation of the second Mg atom by ATP seems therefore only to affect a single orthophosphate group in the phosphoric chain whereas it is widely accepted that several groups are involved in the fixation of the first Mg^{2+}. The AMP phosphorus atom has an analogous structure to that of the terminal phosphorus of ATP. This seems to indicate therefore that the second Mg atom binds to the Pγ of ATP. This nucleotide is classically considered as having a folded structure (Evans and Sarma, 1974; Melchior, 1954; Sundaralingam, 1967; Yathindra and Sundaralingam, 1973) in which the terminal phosphorus is brought close to the adenine ring. Under these conditions the fixation of the second Mg atom at the end of the chain will increase the interactions already existing between the ring and the first Mg atom (Glassman *et al.*, 1973; Granat and Fiat, 1977). This additional constraint, imposed on the molecule, could explain why the ternary chelate is no longer a substrate for enzymes which specifically recognize the $MgATP^{2-}$ complex.

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