Ion-Electrode Study of the Calcium–Adenosine Triphosphate System

M. S. Mohan and G. A. Rechnitz*

Contribution from the Chemistry Department, State University of New York, Buffalo, New York 14214. *Received July 10, 1971*

Abstract: Association constants of the complexes $CaATP^{2-}$ and Ca_2ATP have been measured by direct potentiometry using a calcium ion selective membrane electrode. The second complex has not previously been reported.

Adenosine triphosphate (ATP), when present in biological systems as an energy-transfer agent, is generally associated with divalent ions[1] such as Ca^{2+} or Mg^{2+}. Divalent metal ions act as activators and, frequently, as inhibitors of enzymic reactions involved in the metabolism of ATP. Indeed there is evidence now that the divalent metal ion–ATP complexes are the actual substrates in ATPase reactions.[2] In addition, the formation of these complexes would be expected to control the free metal ion activity in the cell fluids. It is not surprising, therefore, that a number of studies have been made of the interaction of divalent cations with ATP both from the point of view of measuring the formation constants and, also, determining the site of metal ion binding at the ATP molecule.[3–6]

An examination of the literature shows considerable discrepancies in the values of the formation constants of Ca^{2+}–ATP complexes. In the case of the $CaATP^{2-}$ species the values range from $\log K = 3.29 \pm 0.08$ (obtained from pH titrations[7] at an ionic strength of 0.1 maintained with tetrapropylammonium bromide at 25°) to 4.51 (obtained at an ionic strength of 0.1 maintained with tetraethyl bromide and also from spectrophotometric determination of Ca^{2+} in 0.1 *M* *N*-ethylmorpholine buffer[8]). There are two reasons for these discrepancies. Firstly, most of the previously used methods have been indirect methods which introduce into the measurements the uncertainties of another system and its relation to the system of interest.[3] Secondly, all the measurements have been carried out in the presence of a variety of "inert" electrolytes some of which have more recently been demonstrated to bind to the ATP molecule with considerable affinity.[9] The literature also indicates an absence of values determined at zero ionic strength. The interaction of a multivalent anion like ATP^{4-} with a divalent cation would be expected to show considerable dependence on the ionic strength of the medium.

A direct determination of the thermodynamic formation constants of calcium–ATP complexes thus seems both pertinent and desirable. In recent years, ion-selective electrodes have emerged as versatile concentration and activity probes in several thermodynamic and kinetic studies.[9–12] The Orion calcium electrode[13] has by now been proved to be a reliable tool in several chemical and biochemical studies.[14] In the work reported here the thermodynamic formation constants of the $CaATP^{2-}$ species and a hitherto undetected Ca_2ATP^0 species have been measured with this electrode.

Experimental Section

Reagents and Apparatus. The sigma-grade disodium salt of adenosine 5-triphosphate ($Na_2H_2ATP \cdot 3H_2O$) from Sigma Chemicals (lots 100C–7620 and 110C–7410) was used without further purification. Calcium stock solutions were prepared by dissolving reagent grade $Ca(NO_3)_2 \cdot 4H_2O$ in water and were standardized by potentiometric titration against EDTA. Ca^{2+} activity measurements were made with an Orion 92–20 calcium electrode in conjunction with an Orion Model 90–02 double-junction reference electrode with 1 *M* potassium nitrate in the outer chamber. A Corning glass electrode (Catalog No. 476022) was used to monitor pH. Potential measurements were made with a Corning Model 104 digital electrometer. Distilled water deionized by passing through a mixed-bed resin column was used to prepare all solutions. The cell assembly and the experimental conditions were similar to those described in an earlier study.[9] Computations were made with the aid of a CDC6400 computer.

Results

The pH glass electrode was calibrated with equimolar phosphate buffer, pH 6.865, and 0.01 *M* borax, pH 9.180, prepared according to the method of Bates.[15] The calcium electrode was calibrated *in situ* by adding increments of 0.01 *M* calcium nitrate to 50 ml of water to which a known amount of 0.05 *M* NaOH had been added to bring the pH to 9.1 ± 0.1. The activity coefficients were obtained by the Davies equation.[9,16] The electrode slope and E^0 values were determined by fitting the data to a linear least-squares program using a Hewlett-Packard 9100-A calculator. Potential measurements were made by adding increments of a freshly prepared solution of $\sim 10^{-2}$ *M* Na_2H_2ATP and 0.05 *M* NaOH so that the resultant pH was 9.1 ± 0.1.

The total metal (T_M) to total ligand (T_{ATP}) ratios varied from about 10 at the beginning of the titration to 1 toward the end. Attempts were made to calculate the formation constant of $CaATP^{2-}$ assuming that

(1) J. M. Melancon, Jr., and H. F. Deluca, *Biochemistry*, **9**, 1658 (1970).
(2) H. W. Wolf and L. Adolph, *Eur. J. Biochem.*, **8** (1), 68 (1969).
(3) R. C. Phillips, *Chem. Rev.*, **66**, 501 (1966).
(4) F. L. Khalil and T. L. Brown, *J. Amer. Chem. Soc.*, **86**, 5113 (1964).
(5) M. J. Heller, A. L. Jones, and A. T. Tu, *Biochemistry*, **9**, 4981 (1970).
(6) M. E. Heyde and L. Rimai, *ibid.*, **10**, 1121 (1971).
(7) R. M. Smith and R. A. Alberty, *J. Amer. Chem. Soc.*, **78**, 180 (1956).
(8) W. J. O'Sullivan and D. D. Perrin, *Biochemistry*, **3**, 18 (1964).
(9) M. S. Mohan and G. A. Rechnitz, *J. Amer. Chem. Soc.*, **92**, 5839 (1970).
(10) G. A. Rechnitz, *Accounts Chem. Res.*, **3**, 69 (1970).
(11) K. Srinivasan and G. A. Rechnitz, *Anal. Chem.*, **40**, 1818 (1968).
(12) G. A. Rechnitz and Z. F. Lin, *ibid.*, **40**, 696 (1968).
(13) J. W. Ross, *Science*, **156**, 3780 (1967).
(14) E. W. Moore, *Nat. Bur. Stand. (U. S.), Spec. Publ.*, No. **314**, 215 (1969).
(15) R. G. Bates, "Determination of pH," Wiley, New York, N. Y., 1963.
(16) C. W. Davies, "Ion Association," Butterworths, London, 1962, p 41.

Table I. Formation Constants of $CaATP^{2-}$ Species Measured at 25°[a]

pH	$T_M \times 10^3$ M	$T_{ATP} \times 10^3$ M	$[Ca^{2+}] \times 10^5$ M	$[CaATP^{2-}] \times 10^3$ M	K_1, l. mol^{-1} $\times 10^{-6}$	Ionic Strength $\times 10^3$, M
9.047	0.881	0.898	3.09	0.850	2.358	4.823
9.078	0.928	0.963	2.78	0.900	2.229	5.187
8.963	1.000	1.022	3.25	0.967	2.462	5.473
8.945	1.043	1.083	3.03	1.013	2.270	5.826
9.035	1.110	1.137	3.300	1.077	2.680	6.077
9.143	1.150	1.193	3.03	1.119	2.571	6.415
9.103	1.213	1.245	3.44	1.179	2.764	6.645
9.085	1.250	1.298	3.28	1.217	2.553	6.957
9.035	1.310	1.345	3.68	1.273	2.706	7.171
8.964	1.344	1.395	3.45	1.310	2.596	7.460
9.179	0.665	0.732	1.43	0.651	2.063	4.102
9.131	0.734	0.806	1.49	0.719	2.161	4.496
9.132	0.800	0.874	1.62	0.783	2.210	4.853
9.142	0.863	0.937	1.78	0.845	2.234	5.184
9.165	0.923	1.011	1.65	0.906	2.413	5.594
9.126	0.982	1.067	1.97	0.963	2.233	5.822
9.015	1.039	1.128	2.36	1.015	1.899	6.225
9.018	1.145	1.251	2.27	1.122	2.080	6.892
9.056	1.243	1.365	2.23	1.221	2.220	7.515
9.021	1.315	1.464	2.26	1.292	2.077	8.107

[a] Mean $K_1 = 2.339 \times 10^6$ l. mol^{-1}; standard deviation, $\pm 2.43 \times 10^5$ (56 determinations).

this was the only species present, making use of the following equations (1–5), where S is the experimental

$$E_{cell} = E^0 + S \log \{Ca^{2+}\} \quad (1)$$

$$T_M = [CaATP^{2-}] + [Ca^{2+}] \quad (2)$$

$$T_{Na} = 2T_{ATP} + T_{base} \quad (3)$$

$$T_{ATP} = [ATP^{4-}] + [CaATP^{2-}] + [NaATP^{3-}] \quad (4)$$

$$K_{Na} = \frac{[NaATP^{3-}]}{[Na^+][ATP^{4-}]} \frac{f_3}{f_1 f_4} \quad (5)$$

electrode slope, T_{Na} the total sodium in the solution, T_{base} the concentration of the added NaOH in the solution, K_{Na} the thermodynamic formation constant of $NaATP^{3-}$, and f_z the activity coefficient of the z-valent ion. Ca^{2+} ion concentrations were calculated from the activities by the use of the Davies equation assuming an approximate ionic strength. K_{Na} was assumed to be equal to 229 l. mole^{-1} as obtained by us in a previous study.[9] After making suitable substitutions, a quadratic equation in $[NaATP^{3-}]$ was obtained in terms of known quantities and from this the value of $[ATP^{4-}]$ was derived. The first formation constant

$$K_1 = \frac{[CaATP^{2-}]}{[Ca^{2+}][ATP^{4-}] f_4} \quad (6)$$

was obtained by the successive approximation of the ionic strength: $I = 0.5\{16.0[ATP^{4-}] + 9.0[NaATP^{3-}] + 4.0[CaATP^{2-}] + 4.0[Ca^{2+}] + 2T_M + [OH^-]\}$. An examination of the data revealed that both $[ATP^{4-}]$ and K_1 values were negative in most cases except where the T_M/T_{ATP} ratio approximated 1.0. The $\bar{n}$ values, where $\bar{n} = (T_M - [Ca^{2+}])/T_{ATP}$, also indicated that, on the average, more than one metal ion was bound per ligand molecule. In order to obtain K_1 values under conditions where $CaATP^{2-}$ was the predominant species, the experiments were redesigned to make the T_M/T_{ATP} ratios less than or equal to unity at each titration point. The $[Ca^{2+}]$ levels at such ratios ranged between 1×10^{-5} and 4×10^{-5} M which necessitated calibration of the electrode at $[Ca^{2+}]$ lower than usual. Though the slopes of the calibration plots in this range were generally sub-Nernstian and depended on the conditioning of the electrode, accurate interpolations could be easily made in the activity range of interest. Potentiometric measurements were then made by additions of equal volumes of nearly equimolar solutions ~0.01 M Na_2H_2ATP and $Ca(NO_3)_2$ with sufficient 0.05 M NaOH added to bring the pH to 9.1 ± 0.2. The K_1 values obtained by this method are given in Table I.

Examination of the data at T_M/T_{ATP} ratios greater than one indicated additional complexes besides $CaATP^{2-}$. Under such experimental conditions, Ca_2ATP^0 would be the species most likely to be present; hence, the data were treated accordingly. From eq 1, 3, 5, and 6 and the mass balance equations (7 and 8) a quadratic

$$T_M = [CaATP^{2-}] + 2[Ca_2ATP^0] + [Ca^{2+}] \quad (7)$$

$$T_{ATP} = [ATP^{4-}] + [CaATP^{2-}] + [Ca_2ATP^0] + [NaATP^{3-}] \quad (8)$$

equation in $[ATP^{4-}]$ in terms of known quantities was obtained. By a method similar to the one used for calculating K_1, the thermodynamic constant of the Ca_2ATP^0 species was evaluated.

$$K_2 = \frac{[Ca_2ATP^0]}{[CaATP^{2-}][Ca^{2+}] f_2^2} \quad (9)$$

The values of K_2 thus obtained are given in Table II.

Discussion

Most of the previous determinations of the stability constants of the alkaline earth adenine nucleotide ion pairs were carried out in the presence of buffers and a variety of other supporting electrolytes. This fact reflects the difficulties involved in measuring these constants at low ionic strengths by the indirect methods employed. However, for a complete understanding of the energetics of ATP metabolism a knowledge of the thermodynamic association constants of all the species involved is essential. Our K_1 value of 2.34×10^6 l. mol^{-1} is considerably higher than the literature values obtained at higher ionic strengths. However, on the

Table II. Formation Constants of Ca_2ATP^0 Species Measured at 25°[a]

$T_M \times 10^3$ M	$T_{ATP} \times 10^4$ M	$[Ca^{2+}] \times 10^4$ M	$[CaATP^{2-}] \times 10^4$ M	$[Ca_2ATP^0] \times 10^5$ M	K_2, l. mol^{-1}	Ionic strength $\times 10^3$, M	pH
1.207	1.345	10.01	0.751	5.93	1321	3.695	9.232
1.630	2.311	13.06	1.374	9.53	955	5.009	9.135
1.177	3.935	6.72	2.808	11.21	1016	3.846	9.161
1.585	5.618	8.39	3.763	18.48	1083	5.172	9.047
1.023	3.710	5.73	2.898	8.05	815	3.559	8.933
0.668	2.895	3.35	2.445	4.42	889	3.289	9.246
1.163	5.184	5.12	3.831	13.42	1178	3.927	8.903
1.561	7.379	6.70	5.812	15.52	753	5.571	8.963
1.148	6.397	3.67	4.939	14.38	1380	4.082	8.950
1.538	9.084	4.86	7.595	14.59	759	5.893	9.033
0.659	4.284	1.96	3.883	3.75	828	3.554	9.076
1.133	7.578	2.62	6.365	11.75	1247	4.384	8.894
0.987	7.153	1.81	6.149	9.54	1484	4.035	9.205
0.654	4.962	1.21	4.488	4.24	1325	3.677	9.171
1.119	8.728	1.75	7.872	7.82	1023	4.751	8.893
0.980	7.698	1.40	6.844	7.78	1422	4.219	9.250
0.974	8.230	1.05	7.555	5.65	1262	4.432	9.344

[a] Mean $K_2 = 1.10 \times 10^3$ l. mol^{-1}; standard deviation, ±240 (49 determinations).

basis of the simple Debye–Hückel theory, a reaction involving large charge cancellation as in the case of $CaATP^{2-}$ formation is expected to show considerable ionic strength effects, the concentration quotients increasing with decreasing ionic strength. These effects are very well illustrated in the analogous case of the formation of $MgATP^{2-}$ species. Phillips, George, and Rutman,[17] employing an empirical modification of the Debye–Hückel equation, and making certain simplifying assumptions, extrapolated the data of Burton[18] obtained at an ionic strength of 0.11 M to zero ionic strength.

The formation constant (eq 10) increased from $3.8 \times$

$$K_M = \frac{[MgATP^{2-}]}{[ATP^{4-}][Mg^{2+}]} \qquad (10)$$

10^4 l. mol^{-1} at the higher ionic strength to 5.8×10^5 l. mol^{-1}. Their own data[19] obtained at ionic strengths of 0.065 M and higher yielded an even higher value of 6.8×10^5 l. mol^{-1} on extrapolation to zero ionic strength. These values are of the same order of magnitude as the K_1 values obtained in the present study, although values for the $CaATP^{2-}$ formation constants are generally lower than the corresponding values for the $MgATP^{2-}$ species. While Burton's[18] value was 38,000 l. mol^{-1}, O'Sullivan and Perrin[8] obtained a value of 100,000 l. mol^{-1} under nearly comparable conditions. Thus, it seems prudent to conclude that this reversal of order in the magnitude of the constants is not significant until a direct determination of the latter constant has been made. It is pertinent to mention the analogous instances of the $CaPO_4^-$ and $CaP_2O_7^{2-}$ ion pairs[20,21] where the thermodynamic stability constants have been found to be $3.46 \pm 0.2 \times 10^6$ l. mol^{-1} and 6.0×10^6 l. mol^{-1}, respectively.

It is not surprising that the neutral Ca_2ATP^0 species has not been observed previously since most of the earlier work has been done in solutions equimolar with respect to the total metal and total ligand. Under these conditions the concentration of the neutral species is expected to be small and of the same order as the experimental error. In addition, if the association of the $CaATP^{2-}$ species with a second Ca^{2+} ion does not result in the displacement of a proton, the formation of $CaATP^0$ would go undetected by methods which depend strictly on proton balance considerations. Indirect evidence for the presence of the analogous Mg_2ATP complex has been obtained in a study of the enzyme-catalyzed transphosphorylation reaction between adenosine triphosphate and creatine (eq 11),

$$ATP + Cr \underset{\text{transphosphorylase}}{\overset{\text{ATP-Cr}}{\rightleftharpoons}} ADP + Cr\text{-}P \qquad (11)$$

where Kuby, Noda, and Lardy[22] found that the reaction velocity reached a maximum value with an Mg:ATP ratio of 1:1, but with increasing Mg concentration asymptotically reached a value 75% of the maximum. The authors attributed this behavior to the presence of a less active species, *e.g.*, Mg_2ATP. A similar conclusion has been reached in a study of the hexokinase reaction.[23] The only semiquantitative estimate of K_2 for Mg_2ATP has been made by Burton[18] who gave a value of 70 M^{-1} as the upper limit at an ionic strength of 0.11 M. If one assumes that the ionic strength effects observed in the case of K_1 are present here as well, though probably to a lesser extent, our value of 1100 M^{-1} at zero ionic strength is not unreasonable. Though the potentiometric technique used does not permit a determination of the site of binding, the adenosine moiety is probably implicated in the binding of the second metal ion.

Acknowledgment. The support of the Environmental Protection Agency and the National Science Foundation is gratefully acknowledged. The second author is an Alfred P. Sloan Fellow, 1966–1972. We thank the Corning Co. for use of their Model 104 meter.

(17) P. George, R. C. Phillips, and R. J. Rutman, *Biochemistry*, 2, 508 (1963).
(18) K. Burton, *Biochem. J.*, 71, 388 (1959).
(19) R. C. Phillips, P. George, and R. J. Rutman, *J. Amer. Chem. Soc.*, 88, 2631 (1966).
(20) A. Chughtai, R. Marshall, and G. H. Nancollas, *J. Phys. Chem.*, 72, 208 (1968).
(21) J. A. Wolhoff and J. T. Overbeek, *Recl. Trav. Chim. Pays-Bas*, 81, 3201 (1959).
(22) S. A. Kuby, L. Noda, and H. A. Lardy, *J. Biol. Chem.*, 210, 65 (1954).
(23) E. A. Robbins and P. D. Boyer, *ibid.*, 224, 121 (1957).