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Thermodynamic bookkeeping when nucleotides bind. Applications of the theory of linked functions

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The thermodynamic theory of linked functions was used to determine the numbers of modifier ions involved when nucleotides dissociate. Nucleotide dissociation constants, obtained spectrophotometrically using Dowex-1 resin as a model system, were plotted on log/log paper with respect to the modifier concentrations. The slopes of the lines represent the net number of modifier molecules/ions involved in the dissociation. Varying numbers of nucleotides are bound to the resin because the resin capacity is determined by the total number of charges bound. The nucleotides bind to the resin at comparable diffusion-limited rates, irrespective of how tightly they bind. When ATP binds at pH 6.8, 4 chlorides, 4 formates, 2 succinates or 1.4 citrates are displaced, indicating that the fully charged (ATP^{4-}) nucleotide binds. By comparing ATP, ADP and AMP it was possible to evaluate the contributions of the adenosine moiety and each phosphate to the binding. Between pH 2 and 3, where ATP has two negative charges, ATP binds largely as the trianion, displacing 2.7 chlorides and 0.7 protons. In the presence of 4 mM magnesium, 0.58 magnesiums facilitate the dissociation by chelating 58% of the liberated ATP. Calcium behaved similarly to magnesium but aluminum, at pH 6.8, promoted the binding of ATP as an $(Al \cdot ATP)^{3-}$ complex with the concomitant liberation of three chloride ions. These experimental thermodynamic stoichiometries were found to be independent of the concentrations of the other modifiers present. Thermodynamic linkage stoichiometries can be evaluated from log K vs. log (modifier) plots when a direct determination of modifier binding is impossible. The linkage stoichiometries permit one to write charge-balanced equations and to analyze the thermodynamic contributions of modifiers, such as protons, even when the chemical mechanism of the nucleotide dissociation is unknown. All of the proteins studied were found to bind more than 1 mol ATP per mol.

Introduction

This paper describes an experimental method for analyzing the thermodynamics of nucleotide binding to Dowex-1 resin. Although the results might be expected to vary, the method itself should be applicable to other resins, to protein binding and to the binding of nucleotides to enzymatic active sites, since it does not assume any specific chemical or physical mechanism.

The dissociation of ATP bound to Dowex-1 resin can be formulated

$$R \cdot ATP \rightleftharpoons R^{4+} + ATP^{4-} \quad (1)$$

and an apparent dissociation constant

$$K_{obs} = \frac{[R^{4+}][ATP^{4-}]}{[R \cdot ATP]} \quad (2)$$

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can readily be obtained experimentally. However, Eqn. 1 is chemically unsatisfactory for three reasons. First, neither the ATP form (R · ATP) nor the chloride form (R · Cl) of the resin dissociates, in the Arrhenius sense, for there is not the expected increase in electrical conductivity when they are added to a solution and removing the resin does not leave the ATP or chloride behind in solution. Second, Eqn. 1 does not account for the large effects of salt on the observed equilibrium constants (K_{obs}) as defined by Eqn. 2. Assuming that the sodium ions do not bind significantly to either the resin or the ATP, the effects of sodium chloride can be accounted for if the ATP dissociation is formulated as a ligand substitution [1,2] involving the utilization of a moles of chloride per ATP dissociated.

$$a \cdot Na^+ + a \cdot Cl^- + R \cdot ATP \rightleftharpoons R \cdot (Cl)_a + ATP^{-a} + a \cdot Na^+ \quad (3)$$

with a dissociation constant

$$K' = \frac{[R \cdot Cl_a][ATP^{-a}]}{[R \cdot ATP][Cl]^a} = \frac{K_{obs}}{[Cl]^a} \quad (4)$$

from which it follows that

$$d(\log K_{obs}) = a \cdot d(\log [Cl]) \quad (5)$$

The third reason why Eqn. 1 is unsatisfactory is that it does not account for the effects of either protons or chelating ions, such as Na, Mg or Ca, which interact differentially with the reactants and products of Eqn. 1 and hence are also formed or utilized in the reaction. For example, Mg increases the observed dissociation constant because it binds to the free ATP much tighter than it binds to the resin-bound ATP [3]. The effects of these other reactants can, however, be accounted for by other thermodynamic linkage equations such as

$$d(\log K_{obs}) = b \cdot d(\log [H^+]) \quad (6)$$

or

$$d(\log K_{obs}) = c \cdot d(\log [Mg^{2+}]) \quad (7)$$

in which b and c are the net numbers of protons and magnesium ions used per mol ATP dissociated. Eqns. 5–7 are only special cases of a more general thermodynamic linkage equation (Eqns. 4–7)

$$d(\log K_{obs}) = \frac{\delta \log K_{obs}}{\delta \log [X_i]} d(\log [X_i]) = n_i \cdot d(\log [X_i]) \quad (8)$$

where n_i are the number of moles of the component X_i which are consumed when the nucleotide dissociates, at a concentration of $X = [X_i]$.

Since the thermodynamic stoichiometry (n_i) is based on a partial differential, it can vary with the concentrations of both X_i and the other components present in the reaction mixture. Plots of log K_{obs} with respect to log $[X_i]$ can thus be non-linear. However, in order to write a charge balance for the ions left in solution when the resin is removed the summation $\Sigma n_i v_i$ must always be equal to zero, where v_i is the valence of each charged reactant or product, n_i moles of which are consumed in the dissociation reaction.

Note that the values of thermodynamic stoichiometries are not necessarily integral values. However, for the simplest cases, such as the ligand substitution reaction of Eqn. 3, integral values are often observed. Furthermore, since n_i for ligand substitution reactions is almost independent of the concentration of $[X_i]$, a plot of log K_{obs} with respect to log [Cl] is essentially linear.

Linkage equations have long been used to analyze the effects of protons on binding enzyme inhibitors [8,9] and on binding oxygen to hemoglobin [4]. They have also been used effectively to study the electrostatic and non-electrostatic components of the interactions between proteins and nucleic acids [6,10].

The ions, which are liberated or utilized in the reaction make large thermodynamic contributions (n_i RT ln X_i) to the overall dissociation energies. In order to understand these dissociations, and evaluate these contributions, it thus is necessary to determine not only which reactants (X) are involved but also their thermodynamic stoichiometry parameters (n_i). If the thermodynamic stoichiometries (n_i) of all reactants are known one can also write a charge-balanced equation for the nucleotide dissociation reaction.

Materials and Methods

Nucleotides were obtained from either Sigma or Boehringer-Mannheim. BioRad AG1-X2-chloride was washed twice with distilled water by decantation, filtered and air dried at room temperature for 48 h. The capacity of the resin was determined by passing a 5 mM nucleotide solution through a small (50 mg) column in a Pasteur pipette until no more nucleotide was absorbed. The appearance of the nucleotides was monitored at 286 nm to avoid dilutions for spectrophotometry.

The apparent dissociation constants were determined by measuring the amount in solution before (A_0) and after (A) the addition of sufficient resin to absorb between 10 and 90% of the nucleotide. Experiments were normally carried out with less than 10% of the total available resin sites occupied by the nucleotide so that, to a first approximation, the number of free sites was constant and possible electrostatic interactions between the bound nucleotides were minimized. Salt concentrations greater than 20 times that of the sites occupied but less than 0.4 M were used. Thus corrections were unnecessary for the amount of salt displaced from the resin or for differential hydration, respectively.

To study the binding of ATP to various proteins, solutions of about 0.06 mM were titrated with up to 0.5 mM ATP and the unbound ATP then measured in ultrafiltrates obtained with Amicon® Centricon-10©, microconcentrators. A plot of the filtrate ATP concentration (absorbance) with respect to the original ATP concentration yields an upward curving line, which has a limiting slope at high ATP concentrations, equal to that in the control without protein. This limiting tangent cuts the abscissa at a value equivalent to the molarity of ATP binding sites on the protein. Since the molarity of the protein is known, from the weight used, one calculates the average number of ATP molecules bound per protein molecule. Salt-free solutions of the proteins were adjusted to pH 4.1 with HCl; the same pH as the solution of $Na_2 \cdot$ ATP used.

Theory

If the total nucleotide is $[N_0]$ and the total resin sites available is $[R_0]$, then

$$[R_0] = [NR] + [RX_n]$$

and

$$[N_0] = [NR] + [N] = [N]\left(1 + \frac{[RX_n]}{K_{obs}}\right)$$

where [N] and [NR] are the concentrations of free and bound nucleotide, $[RX_n]$ the concentration of remaining resin binding sites, and $K_{obs} = [N]\,[RX_n]/[NR]$.

The ratio of absorbances before (A_0) and after (A) addition of resin is given by

$$\frac{A_0}{A} = \frac{[N_0]}{[N]} = 1 + \frac{[RX_n]}{K_{obs}}$$

from which it follows that

$$\frac{K_{obs}}{R_0} = \frac{[N]}{[N_0]-[N]} \frac{R_0 - [NR]}{R_0} = \frac{A}{A_0 - A} - \frac{A}{R_0'} \tag{9}$$

where R_0' is the resin capacity in absorbance units and A/R_0' is a small correction, which allows for the decrease in the number of free sites available as the nucleotide binds. It follows that

$$\log K_{obs} = \log R_0 + \log\left(\frac{A}{A_0 - A} - \frac{A}{R_0'}\right) \tag{10}$$

Plotting the right-hand side of this equation, either with or without the constant, log R_0, vs. log [X] yields a line whose slope n can be evaluated for any value of [X].

Record et al. [6] analyzed the effect of salt on binding a homologous series of charged DNP · lysine $(lysines)_n$ to nucleic acid [10]. They found that when log K_{obs} was plotted versus log [NaCl] a family of straight lines resulted, which intersected at a point corresponding to the free energy of binding the N-terminal ϵ DNP moiety ($\Delta G = -RT \ln K'$). Similarly, if one compares the resin binding of ATP, ADP and AMP, as a function of salt one would expect to get a family of straight lines, intersecting at a point corresponding to the binding of the adenosine moiety. Record et al.

also pointed out [6] that if such lines are extrapolated to cut the ordinate, at unit chloride activity, one can evaluate the non-electrostatic components of DNA binding each of the N lysines if the intersection points differ. Since the non-electrostatic contributions of phosphate binding to Dowex-1 resin would be expected to be negligible, the point of intersection for AMP, ADP and ATP should lie on the ordinate.

For simple ligand substitution reactions (Eqn. 3) the log/log plots yield straight lines; however, the degree of linearity depends on the mechanism. For example, with modifiers (X), which compete by complexing the free ligand ($K_x = [L][X]/[LX]$), the observed dissociation constant increases with modifier from a value K_0 according to the equation

$$K_{obs} = K_0\left(1 + \frac{[X]}{K_x}\right) \tag{11}$$

The log K_{obs} vs. log [X] plot has a slope

$$\text{slope} = \frac{[X]}{[X] + K_x} \tag{12}$$

which varies from zero at low concentrations of X to unity at high concentrations, where each ligand must necessarily lose one X before it can bind. However, the curves are still reasonably linear over less than an order of magnitude of modifier concentrations. Thus the slope, at a known concentration of the modifier, [X], can be easily measured and Eqn. 12 used to obtain the theoretical dissociation constant (K_x). This behavior is probably the major reason why non-integral values are obtained for the thermodynamic linkage stoichiometries. To minimize such competing reactions, and hence obtain straight lines when the anion concentration is varied, experiments should use only a single anion other than the nucleotide.

Results

Capacities for different nucleotides

Fig. 1 shows titrations of 50 mg of AG1-X2-chloride resin with 5 mM solutions of adenosine (A), Na · AMP, K_2ADP and Na_3ATP. When a small correction was made for the void volume of the column using adenosine (A), the capacities

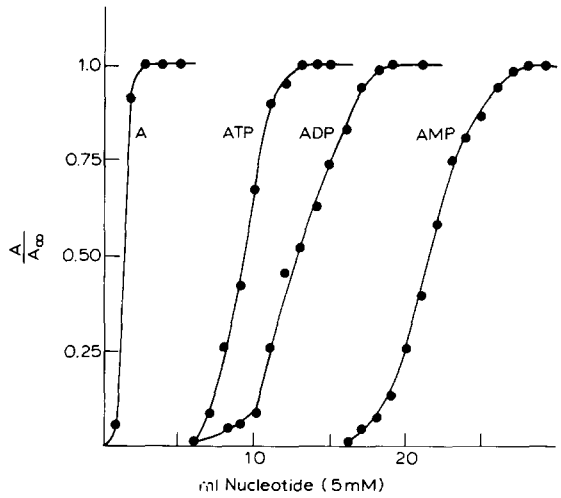

Fig. 1. Capacity of 50 mg AG1-X2 (200–400 mesh) Cl for nucleotides. Solutions (5 mM) were passed through a small column and the effluent monitored at 286 nm. The column was regenerated with 2 ml of 1 M HCl and washed with 4 ml water.

varied approximately in the ratios 2 AMP : 1.33 ADP : 1 ATP, indicating that, under these conditions, the resin binds the fully charged nucleotides as expected. In each case the resin bound approx. 3.5 mequiv. total charges per g, which is the resin capacity specified by the manufacturer.

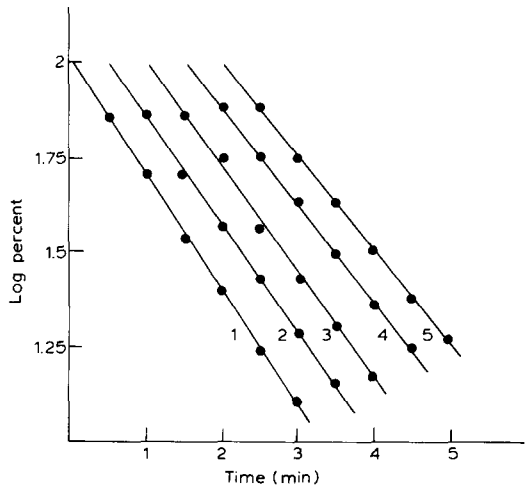

Fig. 2. Rates of nucleotide absorption by 50 mg of AG1-X2 (200–400 mesh) resin in 50 ml of stirred suspension. The initial concentration of nucleotides was 0.1 mM. The first-order plots have been offset 0.5 min for clarity. The experiments from left to right were for 1, ATP^{3-}; 2, $Mg{\cdot}ATP^{1-}$; 3, ADP^{2-}; 4, AMP^{1-} and 5, ATP^{2-}.

Reaction rates with the resin

When 50 mg of AG1-X2-chloride (200–400 mesh) resin was added to 50 ml of 0.1 mM nucleotide it was found that the nucleotide was only absorbed at a slow rate. Fig. 2 shows that under our experimental conditions the half lives for the different nucleotides do not vary significantly with the net charge of the nucleotide. In fact other compounds such as *p*-nitrophenylphosphate, benzoic acid and FMN also bind at a comparable rate. This, perhaps surprising, result is due to the fact that the rate-determining step is diffusion through the film surrounding the resin particle rather than the chemical exchange itself. Thus the rate of absorption is very dependent on the radius of the resin particle and the rate of stirring, but not on how tightly the nucleotide binds. To ensure equilibria a 15-min equilibration period was allowed, and we also used a finer resin (less than 400 mesh) which equilibrated faster ($t_{1/2} = 0.5$ min).

Anion effects

Fig. 3 shows the effects of formate, chloride, succinate and citrate on the binding of ATP at pH 6.8. At this pH the fully ionized ATP^{4-} is apparently bound, since either 4 chlorides, 4 formates, 2 succinates or 1.33 citrates are displaced. The lines for formate and chloride are significantly different. Chloride appears to affect the binding of the adenosine moiety in a manner different to the other anions, since the other lines all intersect on the ordinate (i.e., at 1 M anion).

Effect of number of phosphates

Fig. 4 shows the effect of sodium chloride on the binding of $Na_3 \cdot ATP$, $K_2 \cdot ADP$ and $Na \cdot AMP$ to AG 1-X2 chloride resin. As expected the affinities and slopes of the lines decrease ATP > ADP > AMP. Furthermore, the lines, when extrapolated to unit chloride activity, intersect. Thus, as anticipated from the work of Record et al. [6] the phosphates only make electrostatic contributions to the binding. Since the intersection is also at log $K_{obs} = -0.24$ the adenosine moiety appears to contribute little (1364 J) to the binding. The total chloride contribution to the dissociation can be calculated from the K_{obs} values ($\Delta G_{Cl} = 2.3\ RT$ (log $K_{obs} + 0.24$)). When this number is plotted with respect to the total number of chlorides in-

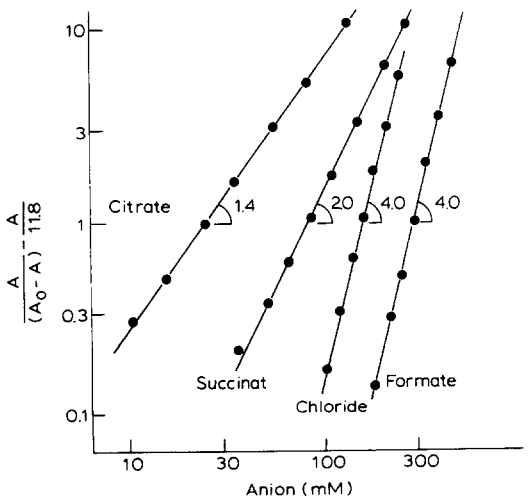

Fig. 3. Effects of different anions on the displacement of ATP from 50 mg AG1-X2 (400 mesh), Imidazole buffer (60 mM) (pH 6.8) at 25 °C. The lines for citrate, succinate and formate intersected at unit activity (1 M anion) and log $(A/(A_0 - A) - A/11.8) = 2.2$, whereas the chloride line intersected at 3.11. The slopes show that ATP^{4-} was bound. Citrate solutions contained 2 mol% citric acid to maintain the pH. $R_0 = 0.77$ mM.

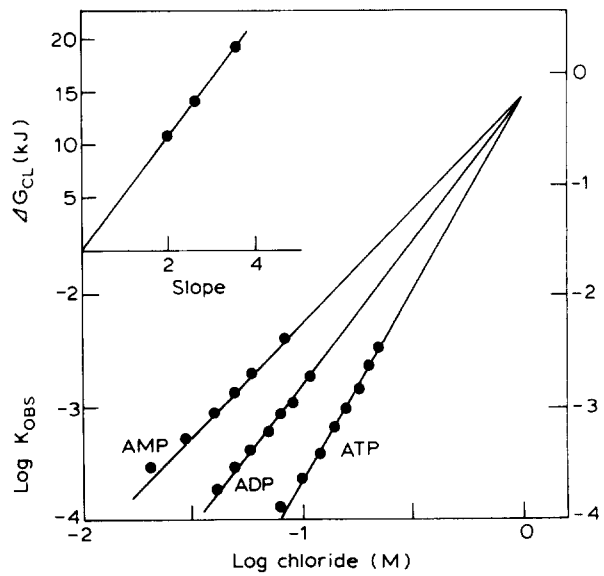

Fig. 4. Effects of sodium chloride on the displacement of $Na_3 \cdot ATP$, $K_2 \cdot ADP$, $K \cdot AMP$ from 50 AG1-X2 (200–400 mesh) chloride resin in 50 ml. The total amount of nucleotide was 0.1 mM in each case. Values for R_0 were obtained from Fig. 1.

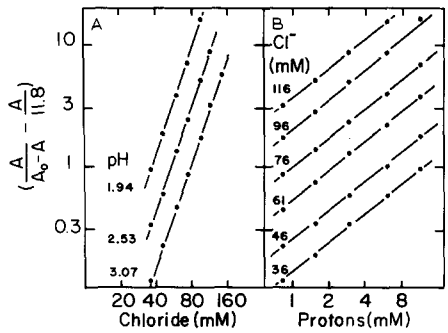

Fig. 5. Linkage plots for 0.1 mM ATP on 50 mg AG1-X2 (400 mesh) as functions of both chloride and pH. Glycine buffers (20 mM). $R_0 = 0.77$ mM. (A) Linkage with respect to chloride at constant pH values as indicated. (B) Linkage with respect to protons at constant chloride concentrations indicated.

volved, as indicated by the slopes, one gets the contribution for each chloride. The inset of Fig. 4 shows that, at 0.1 M, each chloride contributed 5.4 kJ to the energy of nucleotide dissociation.

Effect of pH on the binding of ATP between pH 2 and pH 3

Fig. 5 shows ATP binding at different chloride and pH values in the region where free ATP normally has two negative charges. When the data are plotted with respect to chloride, at constant pH values, a series of parallel lines of slope $3 = (n_{Na} + n_{Cl})$ results, indicating that ATP^{3-} is the species bound. Conversely, when the data were plotted with respect to pH at constant chloride

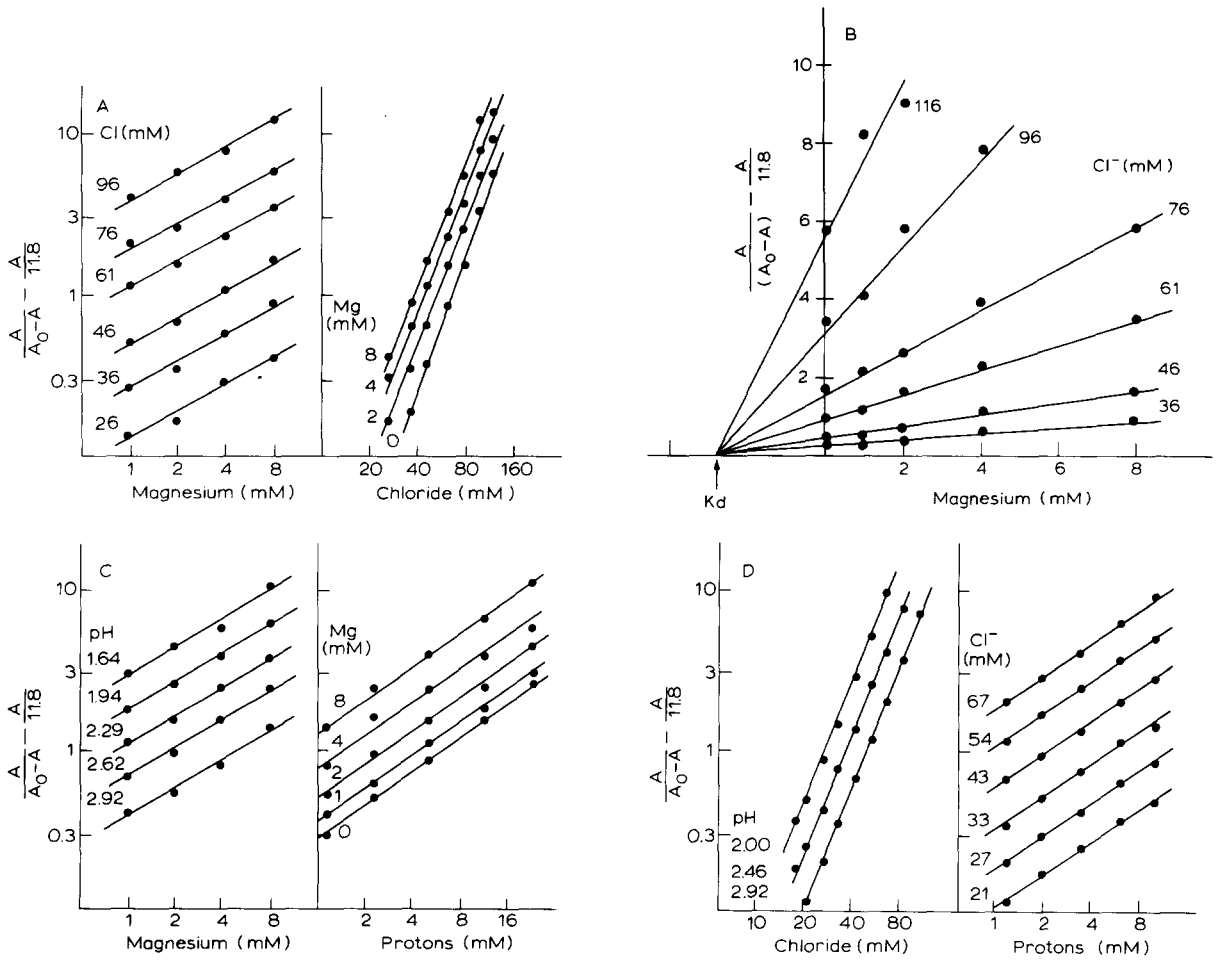

Fig. 6. (A) Plot similar to Fig. 5 but with magnesium varied with constant chloride concentrations or vice versa. 20 mM glycine buffer (pH 2.5). (B) Determination of the MgATP dissociation constants by use of Eqn. 11. (C) Plot similar to Fig. 5 but with magnesium varied at constant pH or vice versa, 52 mM chloride, 20 mM glycine buffers. (D) Plot similar to Fig. 5 but in the presence of 4 mM Mg, 10 mM HCl. The pH values were adjusted with glycine.

concentrations, another family of parallel lines results of slope 0.79 protons per ATP. The results can be summarized in the form of an empirical equation

$$\log K_{obs} = \log K + 3.0 \log [NaCl] + 0.79 \log [H^+] \quad (13)$$

Since ATP at pH 2.5 has two negative charges, one can write the following chemical equation both to account for the empirical equation and to balance the charges

$$0.2\ Na^+ + 2.8\ Cl^- + 0.79\ H^+ + R \cdot ATP$$

$$\rightleftharpoons 0.8\ ATP^{2-} + R \cdot Cl_{2.8} + 0.2\ Na \cdot ATP^{1-} \quad (14)$$

This equation is consistent with the known complexation of Na by ATP ($K_d = 0.2$ M). Note that the resin complexes are written with no formal charges since they have been removed from the solution.

Effect of magnesium on the binding of ATP

Fig. 6A shows a comparable experiment with chloride and magnesium ions at pH 2.5. From the magnesium slopes of 0.58 Mg/ATP at 4 mM Mg one can calculate (Eqn. 12) a dissociation constant $K_x = 2.9$ mM for Mg · ATP, and this was confirmed by plotting the same data according to Eqn. 11 (Fig. 6B) or according to Eqn. 6 of Ref. 3 (not shown). What is of particular interest is that when a comparable experiment was performed, either with magnesium and pH at a constant 52 mM chloride (Fig. 6C) or varying pH and chloride at constant Mg (4 mM) (Fig. 6D) the slopes for Mg, H^+ and chloride were essentially the same, so that one can write the following empirical equation

$$\log K_{obs} = \log K + 2.7 \log [Cl] + 0.58 \log [Mg] + 0.7 \log [H^+] \quad (15)$$

With 52 mM NaCl, 4 mM Mg and 4 mM protons (pH 2.4) one can calculate that the relative contributions ($n_i\ RT \log [X_i]$) of chloride, Mg and protons to the dissociation were 19.7 kJ (53%), 7.9 kJ (21%) and 9.5 kJ (26%), respectively.

To explain the results one can again write a balanced equation

$$R \cdot ATP + 2.7\ Cl^- + 0.7\ H^+ + 0.58\ Mg^{2+}$$

$$\rightleftharpoons R \cdot Cl_{2.7} + 0.58\ Mg \cdot ATP + 0.42\ ATP^{2-}$$

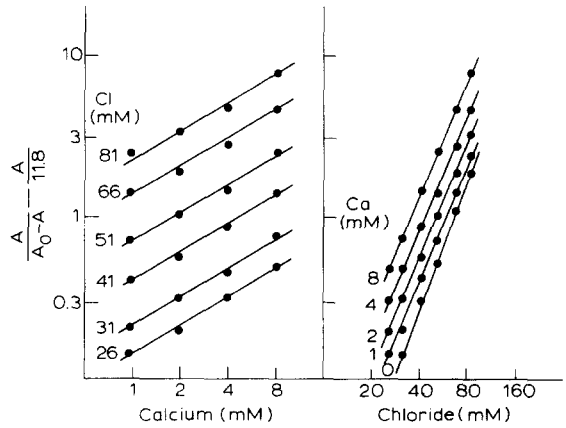

Fig. 7. Plot similar to Fig. 6A but using calcium. 20 mM glycine buffer (pH 2.5).

Effect of calcium

Fig. 7 shows a comparable experiment in which Ca and Cl were varied at pH 2.5. Calcium behaved very similarly to Mg with $K_x = 2.0$ mM.

Effect of aluminum

At low pH values (pH 2–3) aluminum ions displace ATP from the resin like Mg and Ca; presumably a less highly charged chelate is formed which does not bind. To our surprise Al behaved quite differently to Mg and Ca at pH 6.8 for it increased the affinity of ATP for the resin (Fig. 8). This was apparently due to binding a negatively charged $Al(OH)_3ATP^{3-}$ chelate for one Al was liberated for each ATP dissociated but this re-

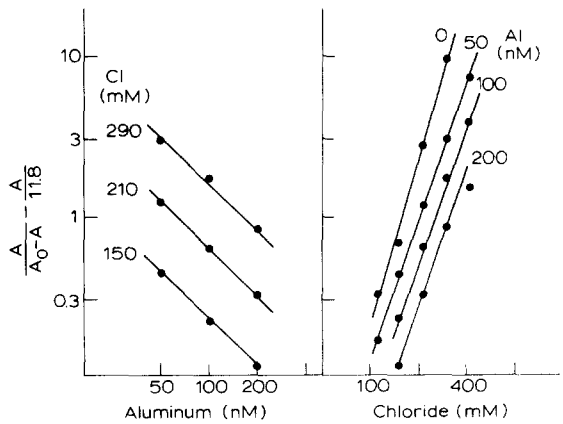

Fig. 8. Effect of aluminum on increasing the affinity of AG1-X2 for ATP at pH 6.8. 20 mM imidazole buffers.

quired three chloride ions. It is of interest that at pH 6.8 Al exists as a neutral $Al(OH)_3$ complex.

Discussion

The pH studies use proton activities and theoretically it would be preferable to use ion activities rather than their stoichiometric concentrations. However, Record et al. pointed out [6] that the activity coefficients do not have a major effect on the observed results because of the low concentrations and the fact that they tend to cancel. The validity of this conclusion was shown by the fact that the experimental dissociation constants for Mg · ATP and Ca · ATP (Figs. 6B and 7) did not change appreciably with ionic strength.

One big advantage of using thermodynamic linkages to measure reaction stoichiometries is that they can be evaluated under conditions where a direct determination is not possible. For example in these experiments only about 0.3 mM chloride would have been liberated for each ATP bound. This could not be measured directly, since the chloride concentration is about 100 mM. Another big advantage is that one does not need to know the actual chemistry involved in order to evaluate the thermodynamic contributions of the various reactants, such as pH, to the ATP dissociations. Chemically, it seems most reasonable to expect that the cations are reacting with the ATP and the anions with the resin, but other speculative mechanisms are not excluded by the thermodynamic data, as long as one can write a charge balanced chemical equation for the free reactants and products.

One could, conceivably, estimate the effect of ligand charge on the rates of nucleotide reactions with the resin (Fig. 2) from electrostatic theory. However, it has long been known [11] that, when the ligand concentration is low, the rates are controlled by diffusion through the immobile solvent surrounding the resin rather than by the electrostatic attraction. Many enzymes are known where, judging by the ratio V_m/K_m, diffusion is also limiting [12,13]. Diffusion effects are also known to be important when comparing soluble and immobile enzymes [14].

Non-integral linkage stoichiometries arise from several causes. First the number of bound counter-ions per ionic site varies from less than 0.2 in ATP to 0.88 as in DNA [6] to almost 1 in ion-exchange resins. Second, the linkage stoichiometry represents the difference between the number bound to the free ligand and the number interacting with the bound ligand. If the free ligand is only partially titrated with protons or cations such as Mg/Ca, there will be fractional differences. In the case of salts another factor is that one must take into account the binding of both the anion and the cation, the linkage stoichiometry being the sum of the two numbers (Eqn. 14).

A detailed analysis of enzymatic reactions involving ATP and ADP requires that the linkage stoichiometries of binding both nucleotides be evaluated. For example, the synthesis of enzyme-bound ATP from enzyme-bound (ADP + P_i) implies that ATP binds much tighter than do the products of hydrolysis [15]. In order to elucidate the contributions of these interactions it is necessary to identify the other reactants involved and their individual linkage stoichiometries. To ensure that there is no unidentified reactant remaining one can take advantage of the important fact that the positive and negative charges must balance,

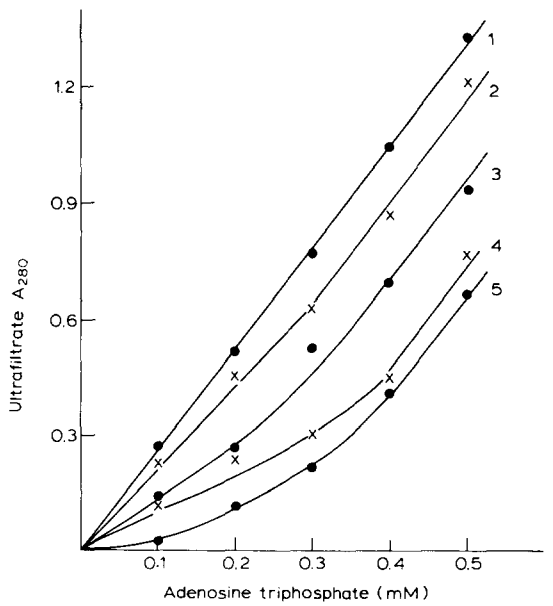

Fig. 9. Protein binding of ATP. Solutions were adjusted to pH 4.1. Additions: 1, none; 2, pepsin (2.5 mg/ml); 3, chymotrypsin (2.0 mg/ml); 4, trypsin (1.5 mg/ml); and 5, bovine serum albumin (4.5 mg/ml).

TABLE I

PROTEIN BINDING OF ATP AT PH 4.1

Protein	p*I*	Molecular mass (kDa)	ATP/mol
Lysozyme	10.0	14.4	3.6
Ribonuclease	8.8	14.7	4.4
Trypsin	10.5	24.0	3.9
Chymotrypsin	9.0	25.0	2.1
Pepsin	2.9	35.0	1.1
Bovine serum albumin	5.0	66.0	4.4

even when non-integral stoichiometries are involved.

This thermodynamic analysis also applies to proteins and to enzymes, since it assumes no mechanism. With enzymes there is a problem, however. Even when they have a net negative charge, proteins bind anions [16,17] because there are local electropositive regions [18]. Binding is further enhanced by a 'chelate effect' when there are multiple electrostatic interactions as with ATP. As pointed out by Rapoport et al. [19,20] most proteins will thus bind at least one molecule of ATP per molecule or subunit; a prediction that is readily confirmed by ultrafiltration experiments (Fig. 9, Table I). This poses a severe technical restraint on attempts to determine the linkage stoichiometries for nucleotides at the active site of an enzyme by simple titration type experiments, for other binding sites may have intrinsically different linkage stoichiometries. This problem can be overcome if the linkage stoichiometries are determined by kinetic methods [15]. By analogy, one cannot determine the role of protons in an enzymatic reaction from the acid/base titration curve but one can do so kinetically from plots of log V_m/pH and log (V_m/K_m)/pH [8,15,21].

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References

1 Jenkins, W.T., (1982) Adv. Enzymol. 53, 307–344.
2 Jenkins, W.T. (1984) in Chemical and Biological Aspects of Vitamin B-6 Catalysis, Part B (Evangelopoulos, A.E., ed.), pp. 89–96, A.R. Liss, New York.
3 Jenkins, W.T. (1985) Anal. Biochem. 145, 362–366.
4 Wyman, J., Jr. (1964) Adv. Prot. Chem. 19, 223–286.
5 Jensen, D.E. and Von Hippel, P.H. (1976) J. Biol. Chem. 251, 7198–7214.
6 Record, M.T., Jr., Lohman, T.M. and De Haseth, P. (1976) J. Mol. Biol. 107, 145–158.
7 Tanford, C. (1969) J. Mol. Biol. 39, 539–544.
8 Dixon, M. (1953) Biochem. J. 55, 161–170.
9 Massey, V. (1953) Biochem. J. 55 172–177.
10 Latt, S.A. and Sober, H.A. (1967) Biochemistry 6, 3293–3306.
11 Boyd, G.E., Adamson, A.W. and Myers, L.S., Jr. (1947) J. Am. Chem. Soc. 69, 2836–2848.
12 Cleland, W.W. (1975) Acc. Chem. Res. 8, 145–151.
13 Knowles, J.R. and Albery, W.J. (1977) Acc. Chem. Res. 10, 105–111.
14 Engasser, J.M. and Horvath, C. (1973) J. Theor. Biol. 42, 137–155.
15 Jenkins, W.T., Marshall, M. and Lewin, A.S. (1984) Arch. Biochem. Biophys. 232, 496–504.
16 Liljas, A. and Rossmann, M.G. (1974) Annu. Rev. Biochem. 43, 475–507.
17 Bradford, M. (1976) Anal. Biochem. 72, 248–254.
18 Mathew, J.B., Gurd, F.R.N., Garcia-Moreno, E.B., Flanagan, M.A., March, K.L. and Shire, S.J. (1985) CRC Crit. Rev. Biochem. 18, 91–197.
19 Rapoport, S., Gerber, G., Ruckpaul, K., Janig, G.-R., Frunder, H. and Jung, F. (1972) in Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status (Roeth, M., Astrup, P., eds.), pp. 147–156, Munksgaard, Copenhagen.
20 Jänig, G.-R., Gerber, G., Ruckpaul, K., Rapoport, S. and Jung, F. (1970) Eur. J. Biochem. 17, 441–444.
21 Alberty, R.A. and Massey, V. (1954) Biochem. Biophys. Acta 13, 347–353.