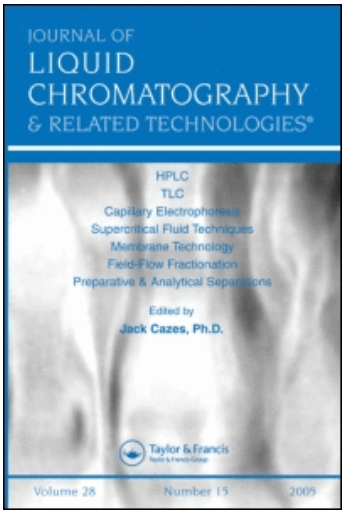

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G. Berger[a]; G. Girault[a]; S. Pezennec[a]; J. L. Zimmermann[a]
[a] Section de Bioenergetique, DBCM, CEA Saclay, Gif sur Yvette Cedex, France

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THE USE OF HPLC FOR THE STUDY OF CHLOROPLAST ATPase ENZYMATIC ACTIVITY AND ATP BINDING

G. Berger, G. Girault, S. Pezennec, J. L. Zimmermann

Section de Bioenergetique
DBCM, CEA Saclay
F-91191 Gif sur Yvette Cedex, France

ABSTRACT

The advantages of the use of HPLC for the purification of the activated chloroplast F_1 ATPase, for the measurement of enzymatic activity (by ADP-ATP separation on anion exchange column) and for ATP binding measurement (by the Hummel and Dreyer chromatographic method on gel filtration columns) are pointed out, by comparison with conventional methods such as ATP regenerating method or centrifugation on Sephadex columns.

The enzymatic data obtained at various ATP and Mg^{2+} concentrations and with different Mg^{2+} chelators and the ATP binding data led to the conclusion that metal free ATP is the true substrate of the enzyme and that Mg^{2+} is the activator. MgATP also binds to the enzymatic sites but with a weaker affinity (K_D = 180 μM) than ATP (K_D = 14 μM) and is not hydrolysed. Moreover, no cooperativity between the sites is needed to account for the results.

A schema is proposed in which the protonated form of the lysine of the enzymatic site interacts with the negatively charged γ phosphate of ATP. Complexation with Mg^{2+} would prevent this interaction.

These results and their interpretation are discussed and compared with those of the literature.

INTRODUCTION

Importance of Methods of Analysis

F_1 ATPase activity and ATP binding have been studied for more than 30 years and, in spite of the establishment of the structure[1] and of the binding change and rotational mechanisms,[2-5] several points remain obscure.

ATPase Activity

Various methods have been used to determine the enzymatic activity of ATPase: those in which the phosphate released from ATP was measured without separation from other reaction products,[6] or after solvent extraction of the complexed Pi,[7] precipitation of this complex,[8] or after adsorption of the radioactive unmodified ATP on charcoal.[9] The release of protons due to hydrolysis of ATP has also been measured by means of pH indicator[10] or by using a pH Stat.[11] However, the majority of the studies have been carried out with an ATP regenerating system involving phosphoenolpyruvate and pyruvate kinase, in order to maintain a constant substrate concentration.

The reaction was monitored either by the release of phosphate[12] or by the decrease of the absorbance of NADH in the presence of lactic dehydrogenase.[13] Nevertheless, the use of high concentrations of phosphoenolpyruvate modifies the free Mg^{2+} concentration and, hence, the enzymatic rate.[14] In fact, this widely used method cannot be applied for studies of the effect of substrate or Mg^{2+} ion concentrations.

We have developed an HPLC method based on ADP-ATP separation and measurement of ADP[15] which is simple, rapid, and without artifacts (see Experimental).

Nucleotide Binding

Nucleotide binding has been widely often measured using rapid separation by size exclusion chromatography on small Sephadex columns.[16-19] This method is simple and rapid; however, it has the major defect of modifying the equilibrium of binding since the free ligand concentration is not kept constant during the separation step. In the hypothetical case of a macromolecule ligand complex which obeys first order kinetics during dissociation and associates at the rate of 10^6 M^{-1} s^{-1}, the loss of binding during separation reaches ten per cent in 0.1 s for a dissociation constant $K_D = 10^{-6}$ M, or in 1s for $K_D = 10^{-7}$ M.[20] The separation techniques must then be extremely rapid in the case of loosely bound complexes. This is the reason why centrifugation is used to filter mixtures on small Sephadex columns. However, rapidity involves a decrease of the efficiency of the separation and several operations are then needed, during which dissociation may occur.

The methods which do not require separation are not subject to this constraint. They include physical methods, such as fluorescence emission of the tetracycline-CF_1 complex,[21] or of ethenoadenine bound to CF_1,[22] which are quenched by ADP binding, ultraviolet absorption spectroscopy of nucleotides, based on the modification of the spectrum due to the hydrophobic conditions of the binding pocket,[19,23,24] or circular dichroism of ADP, which is changed when fixed on CF_1.[25] The uv-visible spectral changes induced by binding of analogs of nucleotides (TNP ADP, TNP ATP) have also been studied[26,27] as well as the modification of fluorescence of the tryptophane probes incorporated by site-directed mutagenesis near the binding site,[28-30] or the steady state fluorescence intensity of maleimide attached to the cysteine at position 63 on β CF_1.[31] In all these cases, additional hypotheses are generally needed to relate the signal amplitude with the fraction of bound ligand and this requirement limits the validity of these methods.

Another method which is theoretically suitable in every situation, particularly for low affinity systems, is equilibrium dialysis. However, its sensitivity is poor since nucleotide binding is measured by the difference of concentrations between two compartments. Moreover, the results can be disturbed by the ligand adsorption on the dialysis membrane.

We have used, in the present study, the chromatographic method of Hummel and Dreyer.[32] The advantage of this method is that the complex does not dissociate during the course of the chromatography, even if the affinity constant is low, since the complex is always in equilibrium with the free ligand.

The initial method of Hummel and Dreyer has been extended to HPLC by Sebille et al.[33] for the study of the binding of different drugs on albumin and, in a preceeding work, we have applied it to the determination of the binding sites of CF_1 and of some of its subunits[34] (see Experimental).

HPLC Purification of Active ATPase

Heat activated CF_1 ATPase, as prepared by the Lien and Racker protocol,[35] and used by numerous authors, contains a large excess of ATP and dithiothreitol. Under these conditions, the fractions of ADP and ATP bound to the enzyme are not well defined and the effect of Mg^{2+} incubation can be largely perturbed.[36]

In this work, we used an activated CF_1 ATPase, obtained by ε subunit depletion and HPLC purification, which contains only one to two ADP per molecule (see Experimental).[37]

EXPERIMENTAL

CF_1 was extracted by EDTA, sucrose, and chloroform treatment from spinach chloroplasts as in ref. 38, then passed on a DEAE cellulose column, equilibrated with 50 mM Tris HCl, 2 mM EDTA, pH 7.8 and eluted with the same medium supplemented with NaCl 0.4 M, precipitated with $(NH_4)_2 SO_4$ at 50 % saturation, and eventually stored at 4°C.

HPLC was performed on a Waters apparatus, consisting in two pumps M 510, a solvent programmer M 660, an injector U_6K, and a spectrophotometer M 490. Crude extracts, prepared as above, were chromatographed on Protein Pak DEAE 5 PW columns 21.5 x 150 mm equilibrated with 20 mM Tris buffer pH 8.5. A linear ammonium sulfate gradient 0 to 1 M was applied during 30 min at 4 ml/min. Fractions corresponding to purified coupling factor were pooled, precipitated with $(NH_4)_2 SO_4$ at 50 % saturation and dialyzed against 20 mM Tris buffer pH 8.5. The solution was submitted to 3 cycles of freeze-thawing, centrifuged at 40 000 g during 15 min, and the supernatant was injected onto the Protein Pak DEAE column. Extensive washing with 20 mM Tris buffer (1 to 2 hours, 4 ml/min) led to partial fractionation of CF_1: β subunit, CF_1 - δ, CF_1 - ε complexes were eluted by ammonium sulfate gradient (0 to 0.8 M during 40 min).

Protein elution profile was monitored at 280 mm, protein concentrations were determined with Bio Rad protein assay using bovine serum albumin as a standard, or by uv absorption, assuming for CF_1 an $E_{1cm}^{1‰}$ of 0.48 at 280 nm. Fluorescence spectra (excitation at 280 nm, emission between 280 and 350 nm) were performed on spectrofluorimeter JYSD Jobin et Yvon. SDS polyacrylamide gel electrophoreses were performed as in ref. 39.

ATPase activity was measured at 37°C in 0.075 M Tris sulfate buffer pH 8.5, containing variable concentrations of ATP and Mg^{2+} ion (routinely, 1 mM and 0.2 mM, respectively). Aliquots of the reaction mixture were directly injected on a TSK DEAE 2 SW column (4 x 300 mm). The reaction was immediately stopped after injection by the lowering of pH 4.3. The nucleotides were separated by isocratic elution with PO_4H_2K 0.1 M, NaCl 0.25 M, at the rate of 1.2 mL/min, in about 3 minutes and with a resolution better than 2 (Fig. 1a). The concentration of the released ADP was measured by the height of the absorption peak at 260 nm, relative to a calibration curve established under the same conditions. ATP and ADP concentrations of standards were calculated assuming an E_{1cm}^{M} = 15,400 and were corrected for the presence of AMP and ATP in ADP and from ADP in ATP, measured by HPLC.

The amounts of released ADP increased linearly with time of action of the enzyme in the reaction mixture, up to a degree of hydrolysis of about 10 %. The initial enzymatic rate, for a period of at least several minutes, is thus well defined (Fig. 1b).

Beyond, the rate decreased because of the consumption of ATP and of the inhibition due to released ADP. No lag time, nor burst of activity were observed under these conditions, when CF_1 - ε was previously dialyzed against Tris buffer 0.075 M, even in the first minute (aliquots were taken every 10 s, frozen in liquid nitrogen, before analysis). The initial concentration of ADP (extrapolated to zero time) corresponded exactly to that brought by ATP as impurity. The amounts of ADP carried along with CF_1 - ε were negligible. Endogenous ADP or ATP bound to CF_1 - ε or CF_1 were measured by the same chromatographic method, after acid denaturation of the protein.

Nucleotide binding was measured according to the method of Hummel and Dreyer (32) : a known quantity of macromolecule (here, CF_1) is injected on a gel filtration column (here, TSK 2000 SW, 7.5 mm x 300 mm), equilibrated with a fixed concentration A of ligand (here, ATP or ADP, between 0.5 and 15 10^{-5} M, in Tris buffer 0.075 M, pH 8.5, containing different concentrations of Mg^{2+} ion). In the simpler case where there is a reversible binding of the ligand

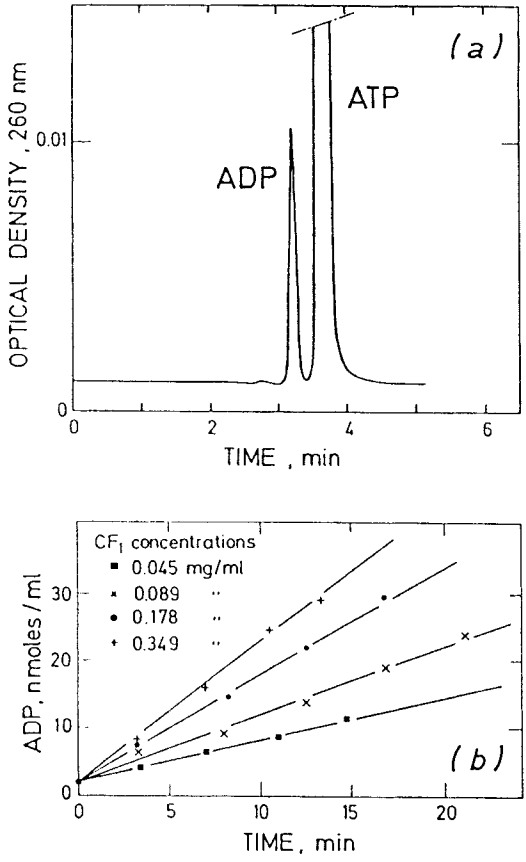

Figure 1. a) HPLC separation of ADP from ATP. Conditions: column TSK DEAE 2 SW, 4 x 300 mm; eluent $PO_4 H_2K$ 0.1 M, NaCl 0.25 M, 1.2 mL/min. b) Released ADP from ATP hydrolysis by CF_1, versus time. Conditions: ATP_t concentration 0.22 mM, Mg_t^{2+} 1mM; Tris buffer 0.075 M, pH 8.5, temperature 37°C.

on n independent identical sites, with a dissociation constant K_D, the number of molecules of bound ligand per molecule of protein is $nA/(K_D + A)$, the fraction of sites which is filled is $r = A/(K_D + A)$. This fraction is independent on the concentration of the protein and depends only on the concentration of the free ligand of the eluent and on the dissociation constant. The ligand which is bound to the macromolecule migrates with it and is withdrawn from the solvent. The local decrease of the ligand concentration migrates with the

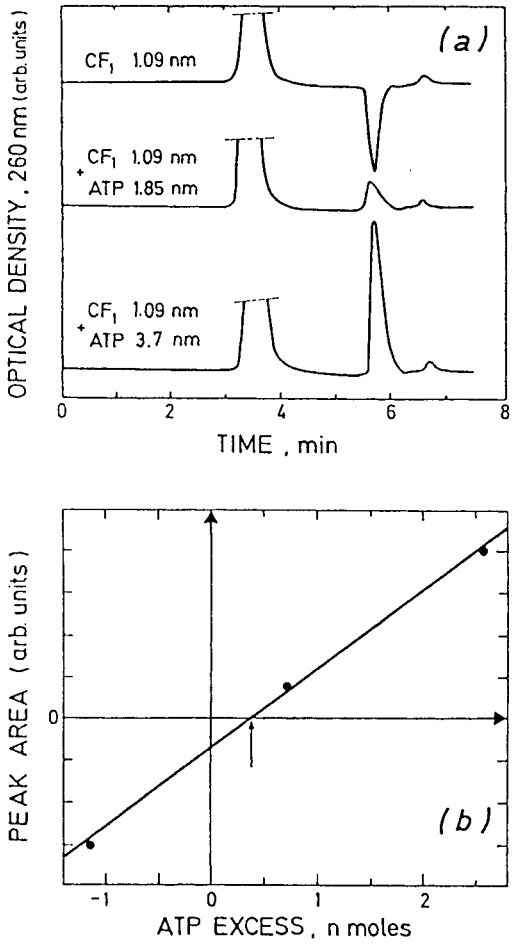

Figure 2. a) ATP-CF_1 binding measurement by the chromatographic method of Hummel and Dreyer. Conditions: column TSK 2000 SW, 7.5 x 300 mm. Eluent: Tris buffer 0.075 M, pH 8.5, ATP_t 0.228 mM, Mg_t^{2+} 0.4mM. The major peak (3.5 min) corresponds to the protein-ATP complex, the second one (5.8 min), to free ATP. b) ATP peak area versus ATP excess. The arrow indicates the quantity of ATP bound by the injected CF1 (same conditions as in a)).

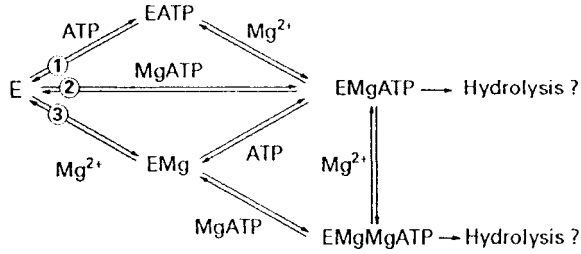

Figure 3. Possible mechanisms for ATP hydrolysis.

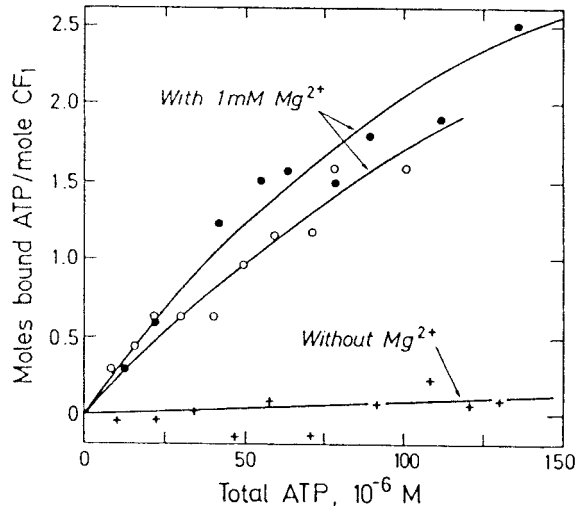

Figure 4. Number of moles of bound ATP per mole of CF_1, versus total ATP concentration, in presence of magnesium 1 mM, and without magnesium.

same rate as the ligand. It can be detected at the column outlet as a negative peak of the optical density at 260 nm and the bound quantity can be measured by the surface of the peak. Hummel and Dreyer[32] have proposed an internal calibration: when the same quantity of macromolecule is injected successively with increasing quantities of ligand, the size of the negative peak decreases progressively and becomes positive (Fig. 2a). Its area varies linearly with the excess of injected ligand over the quantity contained in the same volume of

eluent. The intersection with the X axis occurs when the bound ligand quantity compensates exactly the one that has been added in excess to the solvent (Fig. 2b).

Mg^{2+} binding on nucleotides and on CF_1 have also been determined by a spectrophotometric method which uses the modification of the visible spectrum of the dye Antipyrylazo III, when loosely bound to Mg^{2+}.[40,41] The difference ΔA (523 nm - 602 nm) is characteristic of this complex and is quite linear with Mg^{2+} concentration, up to 100 μM. In the presence of a Mg^{2+}, ligand with a dissociation constant sufficiently lower than that of Antipyrylazo III (~ 1 mM in 0.075 M Tris buffer pH 8.5), the binding of the dye does not perturb the equilibrium between Mg^{2+} and the chelating agent, and the concentration of free Mg^{2+} is satisfactorily measured by the ΔA (523 nm - 602 nm). The dissociation constants were calculated by the Scatchard method.[42]

RESULTS

Between ATP, Mg^{2+}, Mg ATP and E, reversible binding site of ATPase, the simplest equilibria which can be considered are described in Fig. 3.

The HPLC study of the influence of Mg^{2+} ion and ATP concentrations on the enzymatic rate and on the binding of substrate allows to choose between these different possibilities.

There is no appreciable binding of ATP in the absence of Mg^{2+} ion

Figure 4 illustrates clearly this point : the number of ATP moles per mole of CF_1 is less than 0.2 in the absence of Mg^{2+}, up to a concentration of ATP of 0.15 mM, when it reaches or exceeds 2, when Mg^{2+} is present.

$E + ATP \rightarrow EATP$. Pathway n° 1 is then impossible.

The binding of metal substrate complex on free enzyme is unlikely

The dissociation constant of the Mg ATP complex, measured by the Antipyrylazo III method has been found to be $0.36\ 10^{-4}$ M[43] in our conditions (Tris 0.075 M pH 8.5), which is close to the value given in the literature

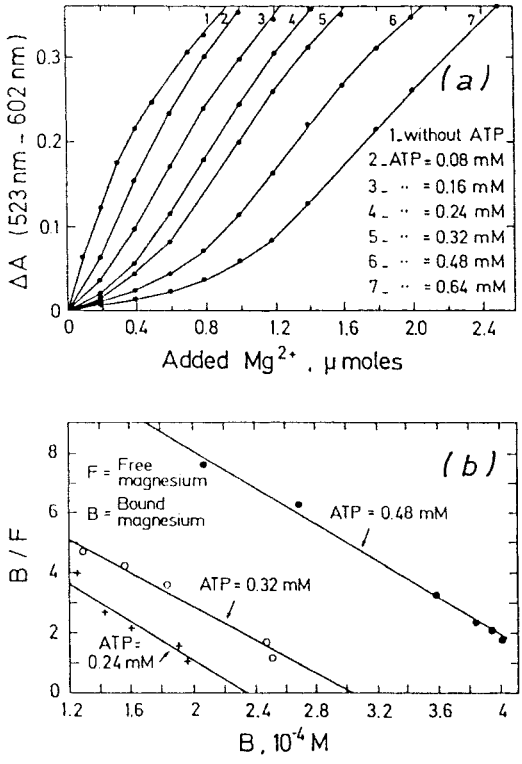

Figure 5. a) ATP-Mg binding measurements by the Antipyrylazo III method. Δ A (523 nm-602 nm) is characteristic of the magnesium not bound to ATP. Conditions: Tris buffer 0.075 M pH 8.5, dye 40 μM, for different ATP_t concentrations. b) Scatchard plot corresponding to a).

around 0.2 10^{-4} M,),[44] (Figure 5a and b). On the other hand, the dissociation constant between Mg^{2+} and CF_1, measured by the same method is lower than 0.8 10^{-5} M (43), involving 1.6 to 2 sites. Thus, the free enzyme cannot bind directly Mg ATP, since some Mg^{2+} from Mg ATP would immediately leave the complex to saturate the enzyme.

E + Mg ATP → E Mg ATP. Pathway n°2 is also impossible.

The remaining possibilities are then:

$$E + Mg \rightleftarrows EMg$$
$$EMg + ATP \rightleftarrows EMg\,ATP \rightarrow \text{hydrolysis ?}$$
$$EMg + Mg\,ATP \rightleftarrows EMg\,Mg\,ATP \rightarrow \text{hydrolysis ?}$$

Which is the true substrate? ATP, Mg ATP, or both? To our opinion, **only metal free ATP is hydrolysable** and the arguments are the following ones:

a) In the case of a mechanism involving the equilibria:

$$E + Mg \rightleftarrows EMg$$
$$EMg + ATP \rightleftarrows EMg\,ATP \rightarrow \text{hydrolysis}$$

the enzymatic rate is given by the expression:[45]

$$v = \frac{V_m}{1 + \left(1 + \frac{K_a}{a}\right)\frac{K_m}{s}}$$

where K_m is the Michaelis constant expressed with ATP concentrations, V_m the maximum rate, s and a, the free ATP and Mg^{2+} concentrations and K_a, the dissociation constant of the $E + Mg \rightleftarrows EMg$ equilibrium. On the other hand, if Mg ATP were the true substrate ($E + Mg\,ATP \rightleftarrows EMgATP \rightarrow$ hydrolysis), then the relationship giving the enzymatic rate would be:[45]

$$v = \frac{V_m}{1 + \left(1 + \frac{K_a}{a}\right)\frac{K_D^{MgATP}}{K_a}\cdot\frac{K'_m}{s}}$$

where K'_m is the Michaelis constant expressed with MgATP concentrations and K_D^{MgATP} is the dissociation constant of the MgATP complex. The two expressions are equivalent and the mechanisms cannot be distinguished by enzymatic data alone.

However, the comparison of the dissociation constant of the EMgATP complex $\left(K_D^{EMgATP}\right)$ with the Michaelis constant allows to choose between the two mechanisms.[45] In the first hypothesis:

$K_D^{EMgATP} = 2.5\mu M$, $K_m = 200\mu M$, and in the second hypothesis:

$K_D^{EMgATP} = 180\mu M$

$K'_m = 14\mu M$

Only the first couple is possible, since the Michaelis constant must be larger than the dissociation constant of the enzyme substrate complex.

b) Na^+ and K^+ are inhibitors of the ATPase activity.[45] This inhibition is competitive with Mg^{2+} concentration and it decreases as Mg^{2+} increases. As Na^+ and K^+ do not bind noticeably to ATP (dissociation constant of the complex with ATP on the order of 0.1 M),[46] the competition must occur at the level of the binding on the enzyme. Consequently, the role of activator must logically be attributed to Mg^{2+}, since there is inhibition by replacement of Mg^{2+} by monovalent ions. At constant total ATP concentration, the enzymatic rate first increases with total Mg^{2+} concentration, passes through a maximum for Mg^{2+}/ATP ratio well under the equivalence (~ 0.2) and then decreases,[45] (Fig. 6). If Mg^{2+} has an activating effect, the decrease of the rate must be attributed to the depletion of ATP, the true substrate, at the expense of the formation of Mg ATP.

c) The third argument is given by the enzymatic study using Mg^{2+} chelators. In this study, we always used a slight excess of Mg^{2+} over ATP (Mg^{2+}/ATP = 1.25). The residual activity of the ATPase was then around 25 % of the maximum. Then we added, progressively, Mg^{2+} chelators with different dissociation constants.

With low binding chelators like 8-hydroxyquinoline ($K_D^{Mg-Hydro.} = 1.5\ 10^{-3}$ M, measured by the spectrophotometric method), there was no modification of the enzymatic rate (Fig. 7): indeed, this chelator is unable to dissociate Mg ATP, nor Mg CF_1.

With tropolone (2 hydroxy - 2, 4, 6 cycloheptatrienone) ($K_D^{Mg-Trop} = 0.78\ 10^{-4}$ M) or pyrophosphate (first $K_D^{Mg-Pyr.} = 0.25\ 10^{-4}$ M), the activity increased and reached a plateau at 50 - 65 % of the maximal activity. These ligands have a dissociation constant with Mg^{2+} comparable to that of ATP ($K_D^{MgATP} = 0.36\ 10^{-4}$ M) and they compete with it for Mg^{2+} binding. Mg ATP is then partially

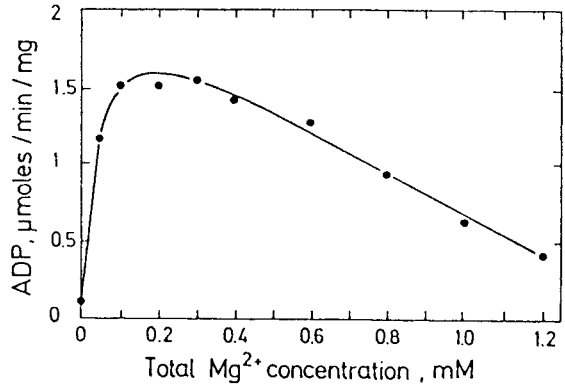

Figure 6. Enzymatic activity of CF_1 - ε versus total Mg^{2+} concentration. Conditions: Tris buffer 0.075 M, pH 8.5, ATP_t 1 mM, CF_1 - ε 6.8 ng/mL.

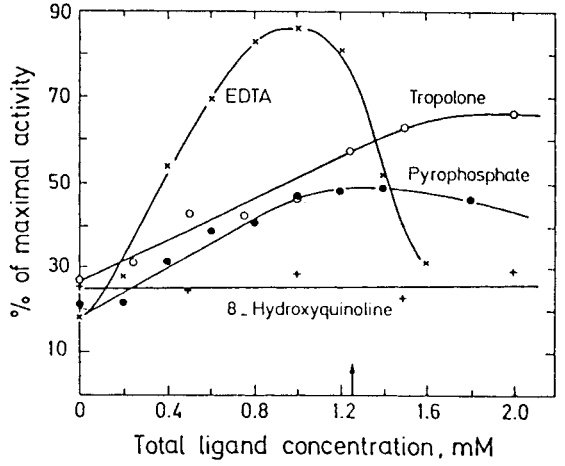

Figure 7. Enzymatic activity of CF_1 - ε in the presence of an excess of Mg_t^{2+} $\left(Mg_t^{2+} / ATP_t = 1.25\right)$, with different ligands of Mg_t^{2+}. Same conditions as in Figure 6. The arrow indicates the equivalence between the quantities of Mg_t^{2+} and chelating agents.

dissociated and more free ATP is available. This result gives evidence that ATP is the true substrate. It cannot be put forward that the increase of activity is due to the dissociation of Mg^{2+} from inhibitory sites on CF_1, since the dissociation constant of Mg^{2+} from CF_1 is well below that of the chelator ($0.8\ 10^{-5}$ M). If the MgATP complex was the substrate, the activity would decrease when the chelator dissociates MgATP without modifying the Mg CF_1 complex.

In the case of EDTA, a tight binding Mg^{2+} chelator ($K_D^{Mg-EDTA} < 0.2\ 10^{-6}$ M), the dissociation constant is smaller than those of Mg ATP and Mg CF_1. The activity of ATPase first increased, reached a maximum and then decreased by dissociation of Mg CF_1, when the concentration of EDTA exceeds that of Mg^{2+}.

Though only ATP is hydrolysable, ATP and Mg ATP competitively bind on CF_1

If metal free ATP is the only ligand of ATPase, the fraction r of the sites (supposed to be identical) which is filled is given by the relationship (see appendix 1) :

$$\frac{1}{r} = 1 + \left(1 + \frac{K_a}{a}\right)\frac{K_D^{EMgATP}}{s}$$

where a and s are the free Mg^{2+} and ATP concentrations, K_a and K_D^{EMgATP}, the dissociation constants of the Mg CF_1 and Mg CF_1 ATP complexes. As a is on the order of 10^{-4} M in the binding studies, well larger than K_a ($0.8 10^{-5}$ M or less, see above), this relationship becomes :

$$\frac{1}{r} \cong 1 + \frac{K_D^{EMgATP}}{s}$$

The different curves of r versus s, obtained with various Mg_t^{2+} concentrations would superimpose, which is not observed in reality.

If now MgATP is supposed to bind competitively with ATP, while maintaining that only metal free ATP is hydrolyzable, for the reasons mentioned above, r is given by the equation (see appendix 1):

$$\frac{1}{r}=1+\left(1+\frac{K_a}{a}\right)\frac{K_D^{EMgATP}}{s}\left(\frac{1}{1+\frac{a.K_D^{EMgATP}}{K_{D.}^{'EMgMgATP}K_D^{MgATP}}}\right)$$

where $K_D^{'EMgMgATP}$ is the dissociation constant of the Mg CF_1 Mg ATP complex. As above, the term $\frac{K_a}{a}$ is negligible and the relationship simplifies :

$$\frac{1}{r}=1+\frac{K_D^{EMgATP}}{s}\left(\frac{1}{1+\frac{aK_D^{EMgATP}}{K_{D.}^{'EMgMgATP}K_D^{MgATP}}}\right)$$

or $$1-r=\frac{r}{s}\left(\frac{1}{\frac{1}{K_D^{EMgATP}}+\frac{a}{K_{D.}^{'EMgMgATP}K_D^{MgATP}}}\right)$$

This last equation is similar to that used in the Scatchard plots, with a slope $-\left(\frac{1}{K_D^{EMgATP}}+\frac{a}{K_{D.}^{'EMgMgATP}K_D^{MgATP}}\right)$ instead of $-\frac{1}{K_D^{EMgATP}}$, in the case where only ATP could bind. In addition, since a is approximately constant in the conditions of the binding measurements $\left(Mg_t^{2+}\rangle\rangle ATP_t\right)$, the representation of r/s versus r must be linear, which is effectively shown on Figure 8 (with Mg_t^{2+} = 0.4 mM).

Furthermore, a plot of the slopes of these straight lines as a function of the mean concentration of free Mg^{2+} (approximately Mg_t^{2+}) allows to determine K_D^{EMgATP}=14μM and $K_D^{'EMgMgATP}$=180μM (Fig. 9). From these values, the fractions of ATP and Mg ATP bound on CF_1 can be calculated in different

Table 1

Repartition of Metal Free ATP and Mg ATP Bound on CF_1, for Different Total ATP and Total Mg^{2+} Concentrations*

Mg_t^{2-}	0.1 mM			0.2 mM			0.5 mM			1 mM		
ATP_t	0.1mM	0.2 mM	1 mM	0.1 mM	0.2 mM	1 mM	0.1 mM	0.2 mM	1 mM	0.1 mM	0.2 mM	1 mM
ATP_{free}	3.58 10^{-5}M	1.15 10^{-4}M	9.02 10^{-4}M	1.48 10^{-5}M	5.4 10^{-5}M	8.05 10^{-4}M	4.70 10^{-6}M	1.2 10^{-5}M	5.19 10^{-4}M	2.16 10^{-6}M	4.84 10^{-6}M	1.32 10^{-4}M
MgATP	6.42 10^{-5}M	0.85 10^{-4}M	0.98 10^{-4}M	8.52 10^{-5}M	1.46 10^{-4}M	1.95 10^{-4}M	9.53 10^{-5}M	1.88 10^{-4}M	4.81 10^{-4}M	9.78 10^{-5}M	1.95 10^{-4}M	8.68 10^{-4}M
$\frac{ATP_t}{Mg_t}$	1	2	10	0.5	1	5	0.2	0.4	2	0.1	0.2	1
$\frac{MgATP}{K_D}$	0.35	0.46	0.53	0.46	0.79	1.06	0.52	1.02	2.61	0.53	1.06	4.72
$\frac{ATP}{K_D}$	2.56	8.21	64.4	1.06	3.86	57.5	0.335	0.86	37	0.154	0.346	9.43
$\frac{boundATP}{boundMgATP}$ onATPase	7.31	17.8	121.5	2.3	4.89	54.2	0.644	0.84	14.2	0.28	0.33	2.0

* Calculated from $K_D^{EMgATP} = 14\ \mu M$ and $K_D'^{EMgATP} = 184\ \mu M$.

conditions. Table 1 shows that ATP is preferentially bound to CF_1, compared with MgATP, when the total ATP concentration is comparable or larger than that of Mg^{2+}.

When r (or nr, number of moles of ligand bound per mole of enzyme) is plotted versus $s\left(1+\frac{aK_D^{EMgATP}}{K_{D.}^{'EMgMgATP}K_D^{MgATP}}\right)$, the experimental points obtained with various Mg_t^{2+} concentrations, which should theoretically be gathered on the same curve, are in fact distributed within a sector, with a mean dispersion of 30% (Fig. 10). This rather high dispersion is perhaps due to differences in the intrinsic nucleotide content (ADP) of the CF_1 batches. Nevertheless, the fitting is much better than when only one entity (metal free ATP or Mg ATP) is supposed to bind.

DISCUSSION & CONCLUSION

All the results presented here with CF_1 are in favor of the role of metal free ATP as the true substrate of ATPase and consequently of the role of free Mg^{2+} as activator. The same conclusion has been reached with TF_1 from PS_3 (47). The ascending part of the curves of the enzymatic rate versus Mg_t^{2+} concentration is attributed to activation of the enzyme by Mg^{2+} and the descending part, to the depletion of the true substrate (metal free ATP), by complexation with Mg^{2+}. There is no reason that the maximum of the rate curve occurs at a stoechiometric value: in our conditions it occurs for $Mg_t^{2+}/ATP_t \cong 0.2$ (Fig. 6 and ref. 45). On the curves of the influence of total substrate concentration at fixed Mg_t^{2+} (Fig. 2 of ref. 45), the acceleration observed when ATP_t becomes larger than Mg_t^{2+} is conveniently explained by the hypothesis of metal free ATP as the true substrate : as K_D^{MgATP} is very low, the quasi totality of the nucleotide is complexed in the MgATP form when $ATP_t < Mg_t^{2+}$ and metal free ATP is only present in noticeable amounts when $ATP_t > Mg_t^{2+}$.

It must be pointed out that the opposite point of view has been generally developed. For Ulrich,[48] Mg^{2+} inhibition is a mixture of competitive and non competitive inhibition, Mg ATP is the substrate and free ATP acts as a

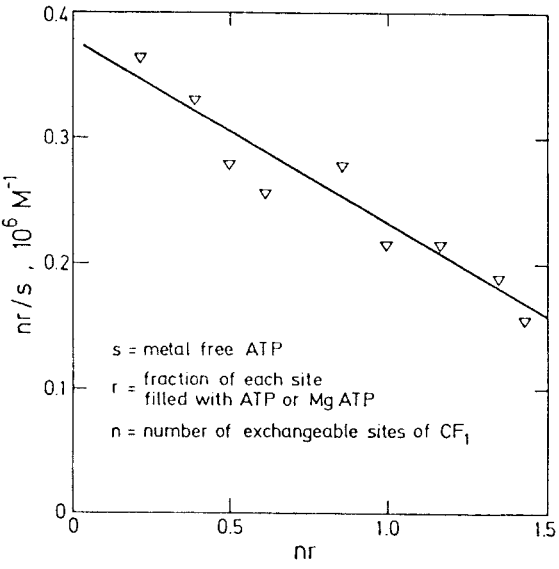

Figure 8. Scatchard plot of CF_1 - ATP binding measurements by the Hummel and Dreyer method: Conditions : Tris buffer 0.075 M, pH 8.5, Mg_t^{2+} 0.4 mM .

competitive inhibitor. The data of Adolfsen and Moudrianakis[49] are in favor of a non competitive binding of free Mg^{2+}, although a combination of non competitive and competitive kinetics is not ruled out. Free ATP would be a non competitive inhibitor of the MgATP hydrolysis. Anthon and Jagendorf[50] proposed that free Mg^{2+} inhibits the activity non competitively, the inhibition by high free ATP concentration being due either to substrate inhibition or to competition with MgATP. Uncompetitive inhibition by Mg^{2+} was observed by Malyan,[51] depending on micromolar concentrations of ADP. The majority of the authors now think that the presence of ADP is necessary for Mg^{2+} inhibition to develop, through the formation of MgADP bound to a catalytic site: Malyan,[51,52] Minkov,[53] Vasilyeva,[54] Drobinskaya,[55] Milgrom,[56] Bulygin,[57-59] Guerrero,[36] Murataliev.[60,61] Though ADP would be necessary for Mg^{2+} inhibition, the two compounds could bind independently to CF_1.[36] The induction and relief of the inhibition of the activity would be caused by slow binding and slow release of Mg^{2+},[36] or by the absence or presence of ATP on the regulatory sites.[62] For Hisabori,[63] the inhibitory effect of Mg^{2+} was attributed to a marked increase of k_{on} for the inhibitory ADP binding at the high affinity sites, induced by the previous binding of Mg^{2+}. Ahlers[64] and Schobert[65]

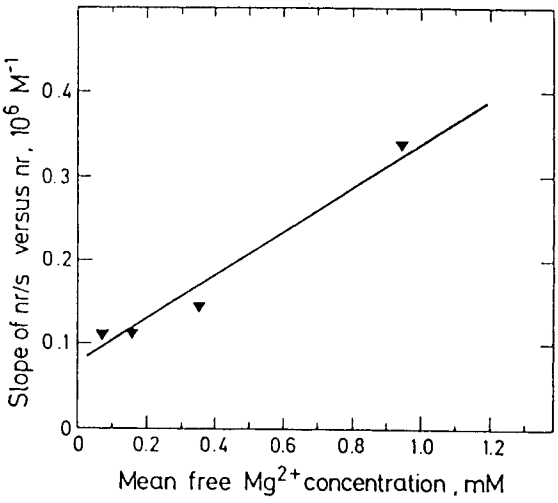

Figure 9. Slopes of the Scatchard plots of the CF_1 - ATP binding measurements, versus free magnesium concentration. The slope of the straight line is the reciprocal of $K_D^{Mg\ ATP} \cdot K_D^{'EMg\ Mg\ ATP}$ and the intersection with the Y axis is the reciprocal of $K_D^{EMg\ ATP}$ (see text).

proposed that two independent binding sites were responsible for the inhibitory and activatory effects of Mg^{2+}. To our knowledge, Adolfsen and Moudrianakis were the only authors to attribute to free Mg^{2+} the role of activator and to free ATP the role of substrate,[66] but they have later withdrawn their conclusions.[49]

The discrepancy with the commonly accepted model for which Mg ATP is the substrate may have been due to differences in the methods and conditions of measurement of the enzymatic activity, such as differences in the nature of the ATPases used and in the conditions used for their activation. We have pointed out, in the Introduction, the drawbacks of some of the methods of measurement of the enzymatic activity (ATP regenerating method) or of nucleotide binding (centrifugation on Sephadex columns).

The use of HPLC purified ε depleted CF_1, the HPLC determination of the released ADP and the simplicity of the reaction medium certainly reduce the risks of artifacts in the enzymatic rate measurement.

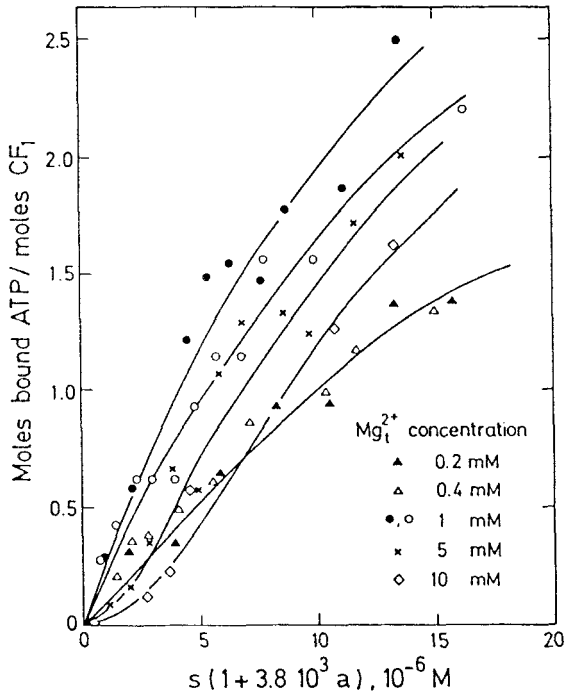

Figure 10. Number of nucleotides bound per mole of CF_1, versus the product $s.\left(1+\frac{a\,K_D^{EMg\,ATP}}{K_D^{Mg\,ATP}\;K_D^{'EMg\,Mg\,ATP}}\right)$, for different Mg_t^{2+} concentrations.

The enzymatic data obtained with CF_1 by our method have been conveniently interpreted within a model involving one category of enzymatic sites[45] with a single K_m, whereas two kinds of sites are necessary to account for the results obtained with TF_1.[47]

However, no cooperativity between the sites is needed to explain the acceleration of the rate when ATP concentration is increased at fixed Mg_t^{2+} concentration. This is in contradiction with the commonly admitted mechanisms,[67,68] (for a review, see Boyer, ref. 69), but a recent study by Reynarfarje and Pedersen[70] shows that all the catalytic sites of the F_1 moiety are kinetically equivalent in hydrolyzing ATP.

The binding data presented here have been obtained by the Hummel and Dreyer chromatographic method. This method allows to measure the binding of ligands on macromolecules, even when their mutual affinity is low, which is not the case when the centrifugation on Sephadex columns is used. With CF_1, there is apparently only one class of ATP binding sites (straight lines on Scatchard plots), characterized by its K_D^{EMgATP} and its $K_D^{'EMgMgATP}$ constants. CF_1, prepared according to Experimental, contains 1.5 to 2 moles of ADP per mole, which are irreversibly bound to it in the conditions of the chromatography and do not interfere with the measurement since they are not released when the CF_1 is injected on a column equilibrated without nucleotides. The total number of sites (intrinsic nucleotides + exchangeable ATP sites, extrapolated by the Scatchard plots) is around 6 (5.6 and 6.3 for an Mg_t^{2+} concentration of 1 mM). The same result has been obtained with ADP.[34] However, this value is not always reached, especially for low ATP binding, when extrapolation is not reliable.

In the two models for the mechanism, the classical one, $\left(E+MgATP \underset{\leftarrow}{\rightarrow} EMgATP \rightarrow hydrolysisproducts\right)$, and that one we propose $\left(E+Mg \underset{\leftarrow}{\rightarrow} EMg \text{ and } EMg+ATP \underset{\leftarrow}{\rightarrow} EMgATP \rightarrow hydrolysisproducts\right)$, EMg ATP is the complex which gives rise to hydrolysis products. But, its structure is not necessarily the same in the two hypotheses and the distinction between them does not reduce to a difference in the order of binding of the constituents but, rather, in the position of the magnesium ions: in the classical model, Mg^{2+} is linked to ATP and must be located at the enzymatic site; in the second model, Mg^{2+} corresponding to the activation of the enzyme is not necessarily located in the enzymatic site. Moreover other sites of binding are also to be considered: those corresponding to the enzymatic inhibition and those located in the non enzymatic sites, for the classical model; in the second model, Mg ATP competing with the true substrate ATP is located in the enzymatic sites. These multiple Mg^{2+} binding sites differ by their roles in the two mechanisms.

Physical methods such as X ray diffraction of protein crystal,[1] EPR, ESEEM and HYSCORE spectroscopy of Mg^{2+} analogs (Mn^{2+} and VO^{2+})[71-74] give information on the Mg^{2+} ligands (residue assignments of the bovine mitochondrial F_1 ATPase are designated to simplify the comparison between different ATPases).

With Mg^{2+}: ^{176}T, 2 oxygens from the γ phosphate and one oxygen from the β phosphate of ATP in the α subunit, ^{163}T, one oxygen from the γ phosphate and one oxygen from the β phosphate of ATP in the β subunit.[1]

With VO^{2+}: ^{176}T, ^{269}D or ^{270}D, ^{175}K, $2H_2O$ in α subunits without ATP. ^{163}T, ^{256}D or ^{188}E, $2H_2O$ in β subunit with ATP, but in the latent form, $^{163}_{\beta}T$, $^{344}_{\alpha}S$ and two phosphates, $^{162}_{\beta}K$ and $^{175}_{\alpha}K$ with ATP in the activated form.[71] $^{162}_{\beta}K$ and $^{328}_{\beta}H$ and two phosphates (β and γ) from ATP.[72] With Mn^{2+}: $^{162}_{\beta}K$ and phosphate from ATP.[73]

It must also be noted that the Mn-P distance measured by the EXAFS technique is significantly larger in the Mn ATP enzyme complex (4.75 Å), than in the Mn ATP complex in solution (3.36 Å),[74] which seems to exclude the binding of Mn ATP, just as it is.

On the other hand, the role of the lysine present in the enzymatic site of the β subunit has been singled out by several authors: the fixation of pyridoxal 5' phosphate (which is known to modify the lysine residues by formation of Schiff's bases with the ε amino group of lysine) on CF_1[75,76] or on chloroplast thylakoids[77] has been shown to inhibit the enzymatic activity. By the combined action of formaldelyde and cyanoborohydride (which is known to methylate the amino group of lysine and the terminal amino groups) we have reached the same conclusion (data not shown). From their study of the E. coli mutants β K 155 Q and β K 155 E, Senior et al.[78] have concluded that $^{155}_{\beta}K$ (analog of $^{162}_{\beta}K$ of MF_1, or $^{164}_{\beta}K$ of CF_1) contributes to the high affinity of ATP, through hydrogen bonding to the γ phosphate.

These data could be interpreted by the interaction of the negatively charged groups of the nucleotides with the positively charged groups of the lysine of the enzymatic site ($K_D^{EMgATP}=14\mu M$). When the nucleotide is metal complexed, the interaction would decrease, due to screen effect ($K_D^{EMgMgATP}=180\mu M$) (Fig. 11).

However, the interaction with lysine is certainly not completely suppressed, since Mn^{2+} and VO^{2+} have been shown to be coordinated to lysine (in the deprotonated form), by EPR and ESEEM.[72,73]

In order to activate the enzyme, to allow nucleotide binding and enzymatic activity, Mg^{2+} must be fixed on the enzyme, at a site which has not yet been determined, and which can be different than the enzymatic site. This metal binding site could involve a glutamic or an aspartic acid residue: $^{188}_{\beta}E$, $^{256}_{\beta}D$, $^{269}_{\alpha}D$, $^{270}_{\alpha}D$.[71]

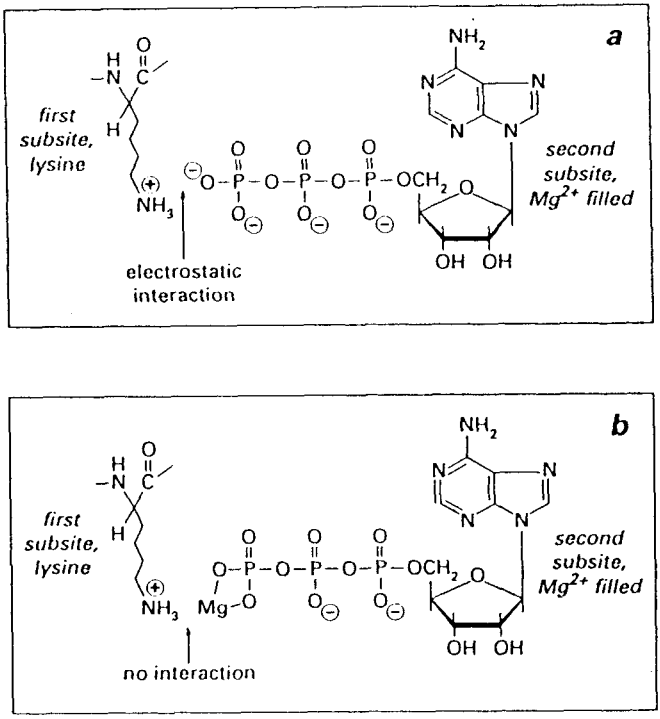

Figure 11. Proposition of mechanism for ATP hydrolysis: a) The phosphate group is metal free and interacts with the positively charged lysine: hydrolysis would be possible. b) The phosphate group is Mg^{2+} complexed and does not interact with lysine: there would not be hydrolysis.

APPENDIX 1

In the model we propose, metal free ATP binds to Mg^{2+} activated CF_1 and the ternary complex gives rise to hydrolysis products.

The equilibria are the following ones :

$$E + Mg \rightleftharpoons EMg \qquad K'_a = \frac{(e)(a)}{(ea)}$$

$$EMg + ATP \rightleftharpoons EMg\ ATP \qquad K_D^{EMgATP} = \frac{(ea)(s)}{(eas)}$$

$$Mg + ATP \rightleftharpoons Mg\ ATP \qquad K_D^{MgATP} = \frac{(a)(s)}{(as)}$$

where e, a, s are the concentrations of the enzymatic sites, magnesium ion and metal free ATP and ea, eas, as, the concentrations of the different complexes.

The total enzyme site concentration, e_t, is given by the formula:

$$e_t = e + ea + eas$$

$$= ea\left(1 + \frac{K_a}{a}\right) + \frac{(ea)(s)}{K_D^{EMgATP}}$$

$$= ea\left(1 + \frac{K_a}{a} + \frac{s}{K_D^{EMgATP}}\right)$$

$$= \frac{eas.K_D^{EMgATP}}{s}\left(1 + \frac{K_a}{a} + \frac{s}{K_D^{EMgATP}}\right)$$

$$= eas\left(1 + \left(1 + \frac{K_a}{a}\right)\frac{K_D^{EMgATP}}{s}\right)$$

The fraction of the sites which are filled, r, is given by:

$$r = \frac{eas}{e_t} = \frac{1}{1 + \left(1 + \frac{K_a}{a}\right)\frac{K_D^{EMgATP}}{s}}$$

$$r + \frac{r}{s}\left(1 + \frac{K_a}{a}\right)K_D^{EMgATP} = 1$$

$$1 - r = \frac{r}{s}\left(1 + \frac{K_a}{a}\right)K_D^{EMgATP}$$

Scatchard plots of $\frac{r}{s}$ versus r must give straight lines of slope:

$$-\frac{1}{\left(1 + \frac{K_a}{a}\right)K_D^{EMgATP}} \cong -\frac{1}{K_D^{EMgATP}}$$

If, now, competitive binding of Mg ATP, together with free ATP, is possible, while maintaining our major statement that only metal free ATP can be hydrolyzed, it becomes:

$$EMg + MgATP \rightleftharpoons EMg\ MgATP \rightarrow \text{no hydrolysis}$$

$$K_D^{EMgATP} = \frac{(eas)(as)}{eaas}$$

$$r = \frac{eas + eaas}{e_t} = \frac{\frac{(ea)(s)}{K_D^{EMgATP}} + \frac{(ea)(as}{K_D^{'EMgMgATP}}}{e_t}$$

$$r = \frac{ea}{e_t}\left(\frac{s}{K_D^{EMgATP}} + \frac{(a)(s)}{K_D^{'EMgMgATP}K_D^{MgATP}}\right)$$

$$e_t = e + ea + eas + eaas$$

$$e_t = ea\left(1 + \frac{K_a}{a} + \frac{s}{K_D^{EMgATP}} + \frac{as}{K_D^{'EMgMgATP}}\right)$$

$$r = \frac{1}{1+\left(1+\frac{K_a}{a}\right)\left(\frac{1}{\frac{s}{K_D^{EMgATP}}+\frac{(a).(s)}{K_D^{'EMgATP}K_D^{MgATP}}}\right)}$$

$$r = \frac{1}{1+\left(1+\frac{K_a}{a}\right)\frac{K_D^{EMgATP}}{s}\left(\frac{1}{1+a\frac{K_D^{EMgATP}}{K_D^{'EMgMgATP}K_D^{MgATP}}}\right)}$$

As before, neglecting the term $\frac{K_a}{a}$

$$r = \frac{1}{1+\frac{K_D^{EMgATP}}{s}\left(\frac{1}{1+a\frac{K_D^{EMgATP}}{K_D^{'EMgMgATP}K_D^{MgATP}}}\right)}$$

The Scatchard plot of $\frac{r}{s}$ versus r gives straight lines of slope:

$$-\frac{1}{K_D^{EMgATP}}\left(1+a\frac{K_D^{EMgATP}}{K_{D.}^{'EMgMgATP}K_D^{MgATP}}\right)$$
$$=-\left(\frac{1}{K_D^{EMgATP}}+\frac{a}{K_{D.}^{'EMgMgATP}K_D^{MgATP}}\right)$$

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