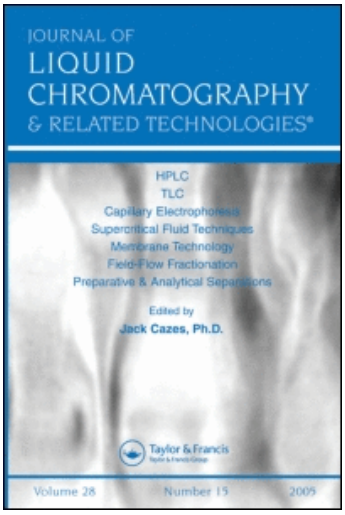

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G. Berger[a]; G. Girault[a]; J. L. Zimmermann[a]
[a] CEA Saclay, France

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USE OF HPLC FOR THE STUDY OF ADP BINDING TO CHLOROPLAST ATPase. II. ITS EFFECT ON ENZYMATIC ACTIVITY

G. Berger,* G. Girault, J. L. Zimmermann

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

ABSTRACT

The binding of ADP to chloroplast ATPase (CF_1) has been measured by the chromatographic method of Hummel and Dreyer which allows low affinity binding determinations. Besides about 1.5 endogenous ADP molecules that are irreversibly bound to CF_1 or slowly exchangeable in the experimental conditions, ADP binds to CF_1 in the presence of Mg^{2+} with an apparent unique dissociation constant of 64 μM, up to a total of 6 ± 0.5 moles/mole, when Mg^{2+} is in excess over ADP. Under these conditions, the major part of the bound nucleotide is in the metal complexed form. Metal free ADP also binds to CF_1, with a dissociation constant of 5 to 15 μM, measured by competitive inhibition of ATPase activity, at low Mg^{2+} concentration.

ADP and ATP appear to bind competitively on CF_1, the extent of binding of each nucleotide can be shown and measured by the chromatographic method of Hummel and Dreyer, using an anion exchange column which separates ADP from ATP. The fractions of each nucleotide have been calculated, using the dissociation constants determined in the conditions of the measurement.

INTRODUCTION

Importance of the Methods of Analysis

The binding of ADP to F_1 ATPase has been extensively described, both in the presence or absence of ATP, and sometimes in relation with the enzymatic activity. The structure of the enzyme, from bovine heart mitochondria, filled with ADP and AMP PNP[1] and that from rat liver mitochondria, filled with ADP and ATP[2], have been determined, and have brought an insight of the two kinds of binding sites.

Different methods have been used for studying nucleotide binding, the most widely used being the separation on Sephadex centrifuge columns:[3-6] the nucleotide-ATPase mixture is laid on a small Sephadex column and centrifuged rapidly. The complex is excluded from the gel and free nucleotides are retained. The major drawback of this method is that dissociation of the complex during the separation may be suspected. 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 of 10^{-6} M or in 1 s for 10^{-7} M.[7] The separation must then be extremely rapid in the case of loosely bound complexes and it is the reason why centrifugation is used. However, rapidity involves also 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 submitted to this constraint. The ultra-violet spectral changes, due to the hydrophobicity of the nucleotide binding pocket of the ATPase,[6,8,9] the circular dichroism of ADP,[10] have been used as a measure of the nucleotide binding. The fluorescence emission of the tetracycline-CF_1 complex[11] or of ethenoadenine bound to CF_1,,[12] the fluorescence or the uv-visible spectral changes of analogs of nucleotides (TNP-ADP, TNP-ATP), have also been followed,[13,14] as well as the modifications of the fluorescence spectrum of tryptophan probes placed near the binding sites by site directed mutagenesis,[15-17] or the steady state fluorescence intensity of maleimide attached to the cysteine at position 63 on β CF_1.[18] However, for all these cases, additional hypotheses are needed (such as the total number of sites) to relate the signal amplitude to the fraction of bound ligand.

Another method, which is theoretically suitable for low affinity binding systems, is equilibrium dialysis.[6,9,14] Nevertheless, its sensitivity is poor since the nucleotide binding is measured by the difference of the concentrations between two compartments, and the results can be disturbed by ligand adsorption on the dialysis membrane.

Lastly, the binding of ADP may also be studied by the inhibition of the rate of ATP hydrolysis. This has been ascribed either to the binding on regulatory sites[4,19-22] or on catalytic sites[5,23-30] or both, depending on the latent or activated state of ATPase.[31] On the other hand, interconversion of the different sites has been proposed in the binding change mechanism (for a review, see Boyer,[32]): in the three steps of the cycle, each site passes through tight and loose ATP or ADP + Pi binding configurations. In this respect, it must be emphasized that the inhibitory effect of ADP binding on regulatory sites was not observed with E. coli ATPase,[33] nor with rat liver mitochondrial ATPase, when less than 5 sites are filled with ADP.[34]

Adenosine diphosphate is always supposed to be bound in the Mg^{2+} complexed form. However, Kironde and Cross[4] have shown that the uncomplexed nucleotide preferentially binds at the unique non catalytic site.

In this study, we have used the chromatographic method of Hummel and Dreyer[35] to measure binding of ADP to chloroplast ATPase: a known quantity of protein is injected on a chromatographic gel filtration column equilibrated with a fixed concentration of ligand. The advantage of this method is that the complex which is formed does not dissociate during the separation, even if the affinity constant is low, since it is always in equilibrium with the free ligand. The initial method of Hummel and Dreyer has been extended to HPLC by B. Sebille et al.[36] for the study of the binding of different drugs on albumin, and in a preceding work, we have applied it to the study of the nucleotide binding sites of CF_1, of its isolated β subunit and of some of its subcomplexes (CF_1 - δ, CF_1 - ε).[37] The simultaneous binding of ADP and ATP was measured by the use of an anion exchange column which separates the two nucleotides. The validity of the method and the comparison with the data obtained with the gel filtration column have been discussed in detail previously.[38]

EXPERIMENTAL

CF_1 was purified and CF_1 - ε was prepared by HPLC, as described.[39]

Nucleotide binding was measured according to the method of Hummel and Dreyer,[35] adapted to ATPase.[37,38,40,41] A known quantity of ATPase is injected on a gel filtration column (TSK 2000 SW, 7.5 mm x 300 mm) or on an anion exchange column (TSK DEAE 2 SW, 4.6 mm x 250 mm) equilibrated with a fixed concentration of ADP, in Tris buffer 0.075 M, pH 8.5 containing variable concentrations of Mg^{2+}. The ligand, which is bound to the protein, migrates with it and is withdrawn from the solvent. The local decrease of the ligand concentration migrates with the same rate as the ligand and is detected at the column outlet as a negative peak of the optical density at 260 mm. When the same

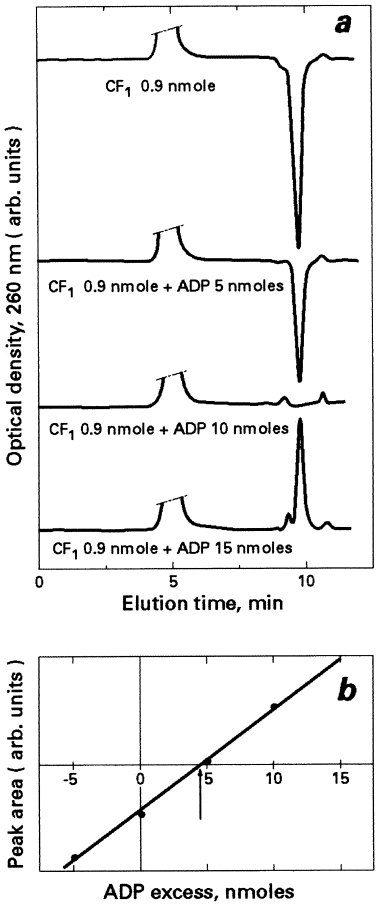

Figure 1. a) ADP-CF_1 binding measurement by the chromatographic method of Hummel and Dreyer. Conditions : column TSK 2000°SW (7.5 x 300 mm); eluent: Tris buffer 0.075 M, pH 8.5, ADP_t 0.099 mM, Mg_t^{2+} 1mM. b) ADP peak area versus ADP excess. The arrow indicates the quantity of ADP bound by the injected CF_1.

quantity of protein is injected successively with increasing quantities of ligand, the size of the negative peak decreases progressively and becomes positive (Fig. 1a). 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 measures the quantity of bound ligand (Fig. 1b). This quantity does not depend on the size of the injected volume and is strictly proportional to the protein quantity. In the case of a mixture of ADP and ATP, anion exchange column (TSK DEAE 2SW) is used, which allows the separation of the two nucleotides.[39]

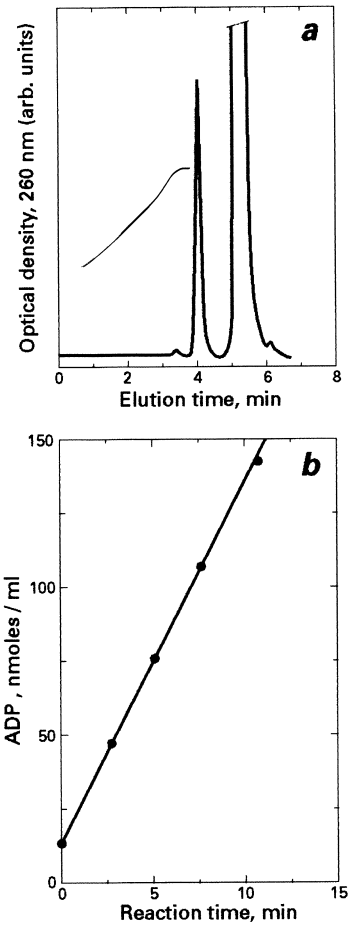

Figure 2. a) HPLC separation of ADP from ATP. Conditions: column TSK DEAE 2 SW (4.6 x 250 mm), eluent : PO_4H_2K 0.1 M, NaCl 0.25 M, 1.2 mL/min. b) Released ADP from ATP, versus time. Conditions: ATP 1.052 mM, Mg^{2+} 0.2 mM, Tris buffer 0.075 M pH 8.5, CF_1-ε 33,2 μg/mL.

ATPase activity was measured at 37°C in 0.075 M Tris buffer pH 8.5, containing variable concentrations of ATP and Mg^{2+}, by a chromatographic method:[42] the released ADP was separated from ATP by isocratic elution with PO_4H_2K 0.1 M, Na Cl 0.25 M, on a TSK DEAE 2 SW column, in about 3 minutes, with a resolution better than 2 (Fig 2a). The amount of released ADP increased linearly with the time of action of the enzyme in the reaction mixture,

up to a degree of hydrolysis of about 10% (Fig. 2b). The initial enzymatic rate is then well defined, for a period of at least several minutes. Beyond, the rate decreased, because of the consumption of ATP and of the inhibition due to the released ADP. No lag time, nor burst of activity were observed under these conditions, when CF_1 - ε was previously dialysed against Tris buffer 0.075 M, even in the first minute (aliquots were taken every 10 s and frozen in liquid nitrogen, before analysis). The initial concentration of ADP (extrapolated to zero time) corresponded to that which is brought by ATP as impurity.

RESULTS

ADP Binding, in Relation to Mg^{2+} Concentration

Mg^{2+} concentration has been shown by the chromatographic method of Hummel and Dreyer to be an important factor for ATP binding on CF_1[42,43] and TF_1.[40] With CF_1, it was shown that metal free ATP binds with high affinity ($K_D^{EMg\ ATP}$ = 14 μM), while the Mg ATP complex binds with low affinity ($K_D^{1EMgATP}$=180 μm). The ratio of ATP to Mg ATP bound to CF_1 depends, then, on the respective concentrations of total ATP and total Mg^{2+}.

In the case of ADP, the binding on CF_1 depends also on the Mg^{2+} concentration, but to a lesser extent: there is a slight binding of ADP in the absence of Mg^{2+}, then a marked increase of binding in the presence of Mg^{2+}, but no important change between 0.2 mM and 5mM Mg^{2+} (Fig 3). Assuming that,[43] the CF_1 binding sites are in the Mg^{2+} complexed form and can bind both metal free and the Mg ADP complex, we have:

$$1 - r = \frac{r}{s}\left(\frac{K_D^{EMgADP}}{1 + a\dfrac{K_D^{EMgADP}}{K_D^{'EMgMgADP}.K_D^{MgADP}}}\right) \qquad (1)$$

Where r is the fraction of sites which are filled

a is the free Mg^{2+} concentration

s is the free ADP concentration

K_D^{EMgADP} is the dissociation constant of the EMg - ADP complex.

$K_D^{'EMgMgADP}$ is the dissociation constant of the EMg - Mg ADP complex.

K_D^{MgADP} is the dissociation constant of the Mg ADP complex = $7.76\ 10^{-5}$ M.[44]

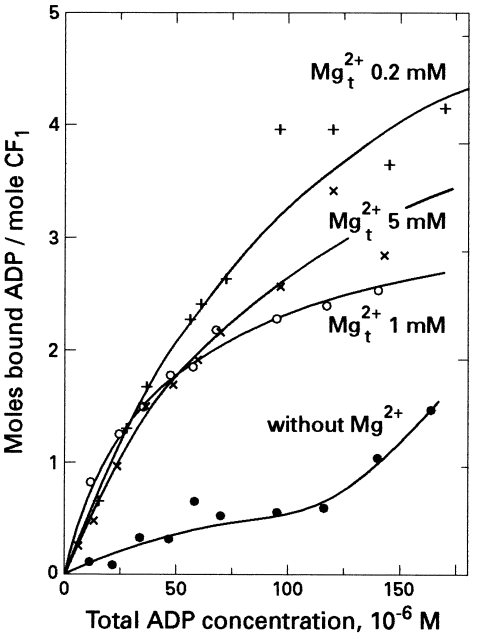

Figure 3. Number of moles of ADP bound per mole of CF_1, versus total ADP concentration, for different total Mg^{2+} concentrations. Conditions: Tris buffer 0.075 M, pH 8.5.

The Scatchard plots (bound ADP per mole of CF_1/s versus bound ADP per mole of CF_1, nr/s versus nr) yield straight lines, which means that, apparently, only one category of sites binds ADP in a reversible manner (Fig. 4). However, 1.5 ± 0.5 sites are already filled with ADP, in an irreversible way, but they do not intervene in the Hummel and Dreyer measurements: no ADP is released from CF_1, when the chromatography is performed without ADP in the eluent. From the plot of the slope of the Scatchard diagrams versus the mean concentration of Mg^{2+}, $K_D^{'EMgMgADP}$ can be calculated with sufficient accuracy (eq. 1).[43]

$$K_D^{'EMgMgADP} = 64\mu M$$

However, the dispersion of the results does not allow the determination of K_D^{EMgADP}. This will be calculated below, from the inhibition of the enzymatic rate by ADP.

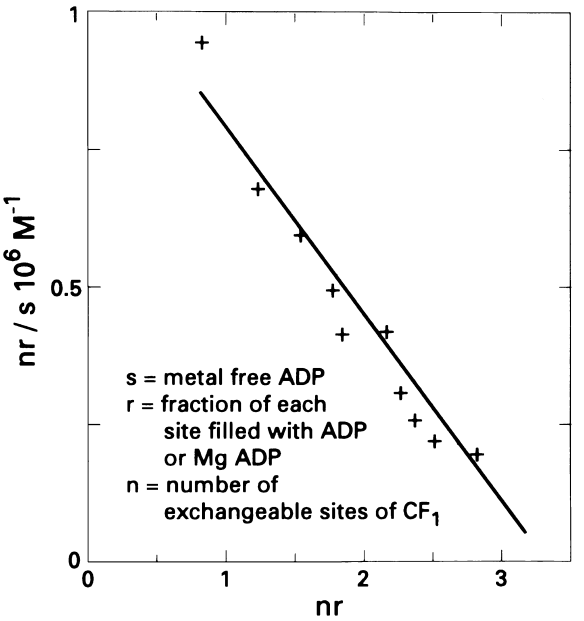

Figure 4. Scatchard plot of CF_1-ADP binding measurements by the Hummel and Dreyer method. Conditions: Tris buffer 0.075 M, pH 8.5, Mg_t^{2+}.

Competition ADP - ATP, Effect on the Enzymatic Rate

Assuming that only metal free ATP is the substrate of ATPase,[42,43] the relationship giving the enzymatic rate v is: (see Annex 1)

$$v = \frac{ke_t}{1 + \left(1 + \frac{K_a}{a} + \frac{s_2}{K_D^{EMgADP}} + \frac{as_2}{K_D^{'EMgMgADP}} + \frac{as_1}{K_D^{'EMgMgATP}}\right)\frac{K_D^{EMgATP}}{s_1}}$$

where index 1 refers to ATP and index 2 to ADP and with:

$$k_a = \frac{(e)(a)}{(ea)} = 1.4\,10^{-6}\ M$$ [42]

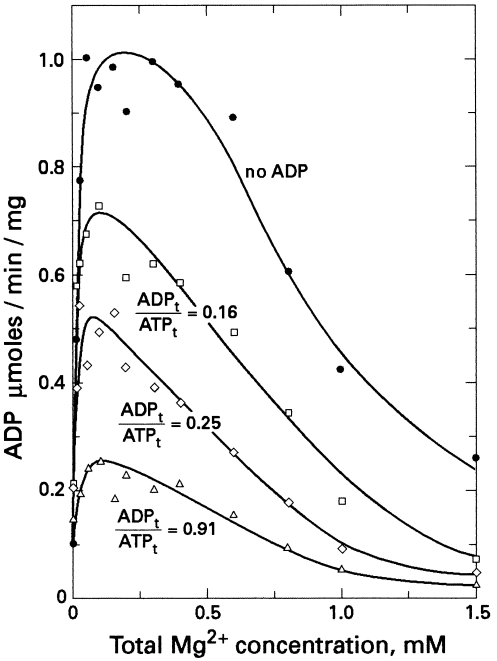

Figure 5. Enzymatic activity of CF_1-ε versus Mg_t^{2+} 1mM, for different ADP_t/ATP_t ratios. Conditions: ATP_t 0.96 mM, t° 37°C, Tris buffer 0.075 M pH°8.5, CF_1-ε: 27 µg/mL.

Fig. 5 shows the influence of the ADP_t/ATP_t ratio on the enzymatic rate measured as a function of Mg_t^{2+} concentration. 50% inhibition is obtained at ADP_t = 0.25 mM for Mg_t^{2+} 0.2 mM and at ATP_t = 0.12 mM (extrapolated value obtained from Fig. 5) for Mg_t^{2+} = 1 mM. In these conditions, free and metal complexed ADP concentrations can be calculated from the values of the dissociation constants

$(K_D^{MgATP} = 20\mu M, K_D^{MgADP} = 77.6\mu M)$.[42]

In a first approximation, one can assume that when ADP_t is added at low level concentration, free Mg^{2+} is not significantly modified, since $ADP_t << ATP_t$ and $K_D^{MgATP} < K_D^{MgADP}$.

Replacing K_D^{EmgATP}, $K_D^{'EMgMgATP}$, $K_D^{'EMgMgAD}$, s_1, s_2, as_1, and as_2 by their values, the last constant K_D^{EMgADP} can be calculated.

For Mg_t^{2+} 0.2 mM, $K_D^{EMg\ ADP}$ = 16.4 μM and for Mg_t^{2+} = 1 mM, $K_D^{EMg\,ADP}$ = 5.5 μM.

Remark

It must be emphasized that, with ATP_t = 1 mM and Mg_t^{2+} = 0.2 mM, the inhibition by ADP reaches 50% with ADP_t = 0.25 mM and that more than 95% of this nucleotide is then in the free metal form.

Competition ADP - ATP - Effect on the Binding

Simultaneous binding of ADP and ATP on CF_1 can be shown and measured by the Hummel and Dreyer method, using an anion exchange TSK DEAE column separating the two nucleotides. Two negative peaks are seen when CF_1 is injected in an eluent containing an equimolecular mixture of ADP and ATP (Fig. 6), which correspond to the binding of each nucleotide. These peaks decreased and become positive when known amounts of ADP and ATP are added to CF_1. The bound quantity is determined by the excess of added nucleotide corresponding to a null area peak. On the other hand, the bound fraction can be calculated from the values of the dissociation constants (see Annexe 2), and the fractions of sites filled with each nucleotide (alone or in equimolecular mixtures) are given in Table 1. Figure 7 shows, as predicted, that ADP binding decreases when ATP is present, but it is not the same with ATP binding, which is not significantly modified by the presence of ADP. However, the determination of ATP binding is less accurate than that of ADP and the comparison between two batches of CF_1 can be difficult if their content of residual nucleotides is different.

DISCUSSION

ADP binding on CF_1 has been measured by the chromatographic method of Hummel and Dreyer which enables low affinity binding determinations. It has been shown that, beside 1 to 1.5 endogenous ADP molecules, almost irreversibly bound in the conditions of the measurement, Mg ADP bound to CF_1 with an apparent unique dissociation constant of 64 μM, up to a total of 6 molecules per molecule of CF_1.[37] Metal free ADP bound also to CF_1 with a dissociation constant around 10 μM (measured by inhibition of ATP hydrolysis). However, in the conditions used for the Hummel and Dreyer method (excess of Mg_t^{2+} over ADP_t), the major part of the bound nucleotide is in the Mg^{2+} complexed form.

These data are to be compared with those of the literature: comparable values of K_D have been found with TF_1,[9] EF_1,[15,16] and CF_1,[37] but several orders lower values have also been given with CF_1,[21] and MF_1.[3,5] Part of the differences

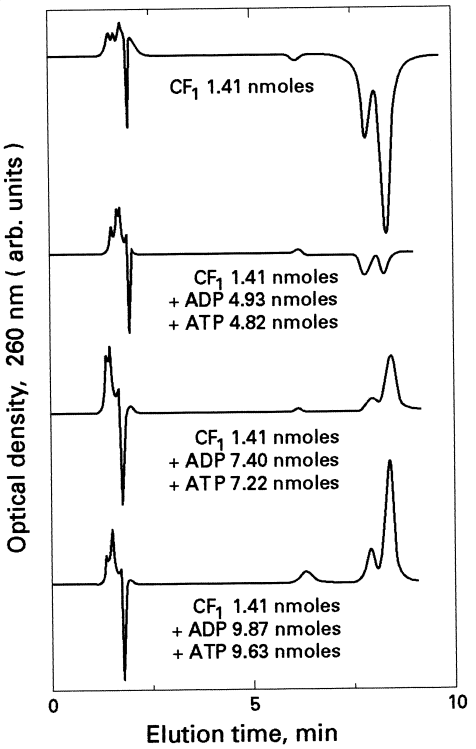

Figure 6. Hummel and Dreyer method, applied to a quasi equimolar mixture of ADP and ATP (respectively 0.969 10^{-4} M and 0.913 10^{-4} M). Conditions: Tris buffer 0.075 M, pH 8.5, Mg_t^{2+} 1 mM, flow rate 1.5 mL/min, column TSK DEAE 2 SW (4.6 x 250 mm).

can be ascribed to the nature of the ATPases and to the methods employed. However, the major cause of discrepancy is certainly due to differences in the conditions of measurement, such as pH, ionic strength, temperature, Mg^{2+} concentration, etc. The influence of these parameters on ADP binding, in relation with enzymatic activity, will be exposed in a following paper.

APPENDIX I

Enzymatic Rate

The binding of ATP on CF_1 , and practically also that of ADP, needs the presence of Mg^{2+}, so we can write:

Table 1

Fraction of Sites of CF_1 Filled with Each Nucleotide*

	ATP Alone	ADP Alone	In Equimolar Mixture ATP	ADP
Fraction of sites filled	0.41	0.71	0.19	06

* Alone or in equimolar mixture (ADP 51.5%, ATP 48.5%) calculated for a total concentration of nucleotide of 10^{-4} M, and a total concentration of Mg^{2+} of 10^{-3} M, from the dissociation constants determined in this work and in Reference 43.

$e_t = e + ea + eas_1 + eas_2 + ea\ as_1 + ea\ as_2$

with (e_t) = total enzymatic sites concentration

(e) = free enzymatic sites concentration

(ea) = metal complexed enzymatic sites concentration

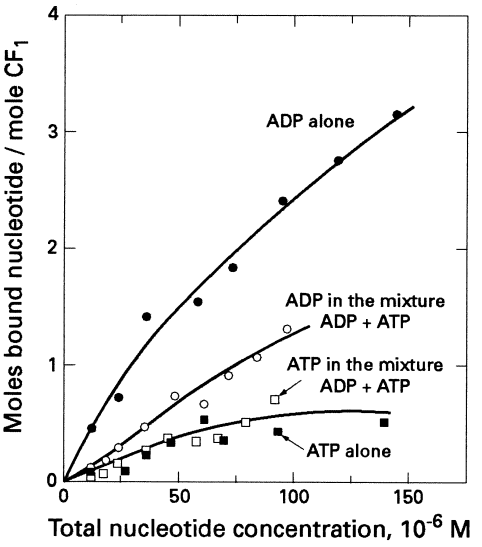

Figure 7. Number of moles of nucleotides bound on CF_1, versus total nucleotide concentrations. Same conditions as in Fig 6.

(eas) = ternary enzyme-metal-nucleotide complex concentration

(ea as) = enzyme metal-metal nucleotide complex concentration

in which index 1 refers to ATP and index 2 refers to ADP.

$$K_D^{EMg\ ATP} = \frac{(ea)(s_1)}{(eas_1)} \qquad K_D^{'EMg\ Mg\ ATP} = \frac{(ea)(as_1)}{(ea\ as_1)}$$

$$K_D^{EMg\ ADP} = \frac{(ea)(s_2)}{(eas_2)} \qquad K_D^{'EMg\ Mg\ ADP} = \frac{(ea)(as_2)}{(ea\ as_2)}$$

$$K_a = \frac{(e)(a)}{(ea)}$$

$$e_t = \left(1 + \frac{K_a}{a}\right)(ea) + \frac{(s_1)(ea)}{K_D^{EMg\ ATP}} + \frac{(s_2)(ea)}{K_D^{EMg\ ADP}} + \frac{(as_1)(ea)}{K_D^{'EMg\ Mg\ ATP}} + \frac{(as_2)(ea)}{K_D^{'EMg\ Mg\ ADP}}$$

Assuming that only eas_1 is able to dissociate into products, the enzymatic rate v is given by:

$$v = \frac{k_{et}}{1 + \left(1 + \frac{K_a}{a} + \frac{s_2}{K_D^{EMg\ ADP}} + \frac{as_1}{K^{'EMg\ Mg\ ATP}} + \frac{as_2}{K^{'EMg\ Mg\ ADP}}\right)\frac{K_D^{EMg\ ATP}}{s_1}}$$

APPENDIX II

Nucleotide Binding

$e_t = (e) + (ea) + (eas_1) + (eas_2) + (ea\ as_1) + (ea\ as_2)$

In the particular case where $s_{1t} = s_{2t}$ and when Mg_t^{2+} is in excess over s_{1t} and s_{2t}, it becomes:

$as_1 \cong s_{1t} = s_{2t} \cong as_2$

$$K_D^{Mg\ ATP} = \frac{(s_1)(a)}{(as_1)} \qquad K_D^{Mg\ ADP} = \frac{(s_2)(a)}{(as_2)}$$

$$\frac{s_1}{K_D^{Mg\,ATP}} \approx \frac{s_2}{K_D^{Mg\,ADP}}$$

$$e_t = ea\left(1+\frac{K_a}{a}\right)+ea\left(\frac{s_1}{K_D^{EMg\,ATP}}+\frac{s_2}{K_D^{EMg\,ADP}}\right)+ea\left(\frac{as_1}{K_D^{'EMg\,Mg\,ATP}}+\frac{as_2}{K_D^{'EMg\,Mg\,ADP}}\right)$$

$$e_t = ea\left(1+\frac{K_a}{a}\right)+\frac{s_1}{K_D^{Mg\,ATP}}\left(\frac{K_D^{Mg\,ATP}}{K_D^{EMg\,ATP}}+\frac{K_D^{Mg\,ADP}}{K_D^{EMg\,ADP}}\right)+as_1\left(\frac{1}{K_D^{'EMg\,Mg\,ATP}}+\frac{1}{K_D^{'EMg\,Mg\,ADP}}\right)$$

$$eas_1 = \frac{(ea)(s_1)}{K_D^{EMg\,ATP}}$$

$$ea\,as_1 = \frac{(ea)(as_1)}{K_D^{'EMg\,Mg\,ATP}}$$

$$eas_1 + ea\,as_1 = ea\left(\frac{s_1}{K_D^{EMg\,ATP}}+\frac{as_1}{K_D^{'EMg\,Mg\,ATP}}\right)$$

$$eas_1 + ea\,as_1 = \frac{e_t\left(\frac{K_D^{Mg\,ATP}}{K_D^{EMg\,ATP}}+\frac{a}{K_D^{'EMg\,Mg\,ATP}}\right)}{\left(1+\frac{K_a}{a}\right)\frac{K_D^{Mg\,ATP}}{s_1}+\frac{K_D^{Mg\,ATP}}{K_D^{EMg\,ATP}}+\frac{K_D^{Mg\,ADP}}{K_D^{EMg\,ADP}}+a\left(\frac{1}{K_D^{'EMg\,Mg\,ATP}}+\frac{1}{K_D^{'EMg\,Mg\,ADP}}\right)}$$

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