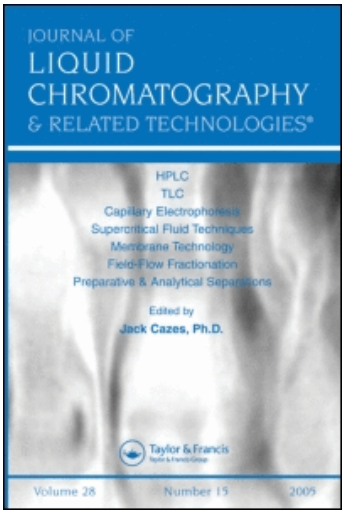

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USE OF HPLC FOR THE STUDY OF ADP BINDING TO CHLOROPLAST ATPase. I. INFLUENCE OF EXPERIMENTAL CONDITIONS AND PROPOSITION OF MECHANISM

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ABSTRACT

The binding of ADP to chloroplast ATPase (CF_1) has been studied by chromatographic methods (Hummel and Dreyer method and chromatographic determination of the enzymatic inhibition).

The influence of different factors has been studied: temperature, ionic strength, activation treatments, pyrophosphate fixation. From the comparison of the dependence of ADP binding (and in certain cases, of ATP binding), with that of the enzymatic activity, it is shown that the conclusions drawn for the dissociation constants cannot be extended to the Michaelis constant.

The role of the lysine present in the nucleotide binding sites, already emphasized by several authors, has been proposed to be the ligand, in the protonated form, of the negatively charged phosphate groups of ADP or ATP.

INTRODUCTION

Several different methods have been used to determine ADP binding to F_1 ATPase, both in the presence or absence of ATP, and sometimes in relation with

enzymatic activity (for a review, see accompanying paper). Large differences can be noted between the K_D values reported in the literature (Table 1).[2-12]

A part of these differences can be ascribed to the nature of the ATPases and to the methods employed.

Table 1

Dissociation Constants for ADP - CF_1 Complex, Expressed in μM Mg ADP

Binding On	Enzymatic Sites	Separated β Subunit	Regulatory Sites	Separated α Subunits
CF_1	0.23 (1) 15-25 (2)	0.7 (3)	1.6 (4) 0.021 (4) 0.17 (1)	
TF_1	3 sites 87 (5) with Mg^{2+} 1 site 100 (5) 1 site 100 (5) 1 site 100 (5) without Mg^{2+} 1 site 30(5) 1 site 30 (5)	11(5) with Mg^{2+} 25 (5) 8.5 (5) without Mg^{2+} 24 (5)	3 sites 0.061 (5) with Mg^{2+} 3 sites 10 (5) 1 site 25 (5)	18 (5) with Mg^{2+} 5.2 (5) 120 (5) without Mg^{2+} >100 (5)
MF_1	0.3 (6) 0.001 (7) 0.007 (7)		with Mg^{2+} {0.05 (8)	
EF_1	1 site 0.05 with P_1 (9) 1.4 site 29 1 site 0.14 without P_1 1 site 20 (10) 1 site 20		3 sites 24 (10) 105 (11) 2.6 sites 1300 (11) 7400 (11) 3 sites 88 (11)	
ATPase of Alcaligenes faecalis	**15 (12)**			

In this study, we have used the chromatographic method of Hummel and Dreyer for the binding measurements. It has the advantage that the complex which is formed by binding 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.[13] 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 β subunit and of some of its partial complexes (CF_1 - δ, CF_1 - ε).[2]

The enzymatic rates have been determined by chromatographic separation of ADP from ATP which is less subject to artifacts than classical methods.[14]

However, the major cause of discrepancy between the data 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, are exposed in this article.

EXPERIMENTAL

CF_1 was purified and CF_1 - ε was prepared by HPLC, as described.[15]

Nucleotide binding was measured according to the method of Hummel and Dreyer, adapted to ATPase:[2,16-19] 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 quantity of protein is injected successively with increasing quantities of ligand, the size of the negative peak decreases progressively and becomes positive. 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. This quantity does not depend on the size of the injected volume and is strictly proportional to the protein quantity (see accompanying paper, Fig. 1a and 1b).

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:[14] 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. The amount of released ADP increased lin-

early with the time of action of the enzyme in the reaction mixture, up to a degree of hydrolysis of about 10%. 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 (see accompanying paper, Fig. 2a and 2b).

RESULTS

Influence of the Ionic Strength

ADP binding decreases when the ionic strength of Tris buffer pH 8.5 is increased, between 50 mM and 125 mM (Fig. 1). This suggests that ionic bonds are certainly implied, for instance between the positively charged lysine[18] and

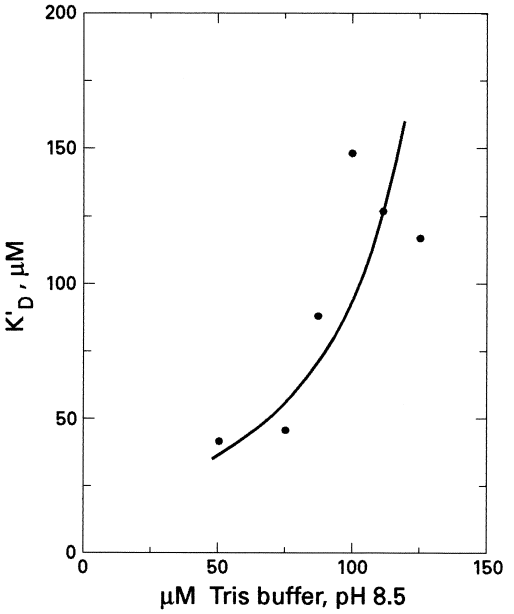

Figure 1. Influence of ionic strength on $K_D^{'EMgMgADP}$ dissociation factor. Conditions: Tris buffer pH 8.5, temperature 25°C, Mg_t^{2+}1mM.

the negatively charged terminal phosphate group of the metal free ADP, or perhaps through competition with Mg^{2+}, preventing the binding of this cation on ATPase (at the level of Glu or Asp groups for instance).[20]

The influence of the ionic strength on ATP binding has not been studied. However, there is only a moderate increase of the enzymatic rate between 25mM and 200 mM Tris buffer pH 8.5 (unpublished data).

Influence of pH

The binding of ADP is more efficient at pH 6.5 than at pH 8.5, but it is the reverse with ATP (data not shown). In the latter case, the γ phosphate is completely ionized at pH 8.5, which allows ionic interaction with positively charged lysine, while this is not the case at pH 6.5 (higher pK of phosphate groups of ATP = 6.5). However, the same reasoning cannot explain the results obtained with ADP.

Influence of the Temperature

Temperature has virtually no influence on the binding of ADP (mostly in the Mg ADP form) on CF_1, between 0°C and 37°C. There is a decrease between 0°C and 10°C and a minimum at around 20°C (Fig. 2). The influence of the temperature has not been studied in the case of ATP binding, but it is likely to be the same. This property is to be compared with the effect of the temperature on the enzymatic rate (20 fold increase between 30°C and 70°C)[21] due to the effect on the rate coefficient k.

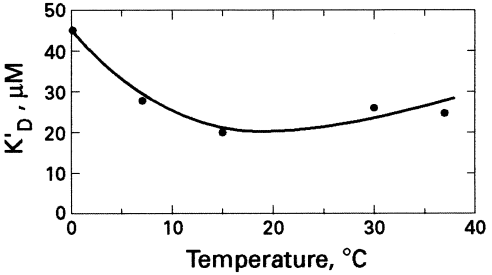

Figure 2. Influence of temperature on $K_D^{'EMgMgADP}$ dissociation factor. Conditions: Tris buffer 0.075 M, pH 8.5, Mg_t^{2+}1mM.

Influence of Pyrophosphate

Both the binding of pyrophosphate to ATPase[22] and its effect on the enzymatic activity have been extensively studied. Jault et al.[23] found that pyrophosphate stimulates the ATPase activity by binding to non catalytic sites, which in turn promotes the dissociation of inhibitory Mg ADP from a catalytic site, whereas, for Weber et al.,[24] the inhibitory effect is not due to the binding of pyrophosphate on catalytic sites, but to the complexation of Mg^{2+}. Moreover, magnesium pyrophosphate bound at non catalytic sites was found not to affect the ATP hydrolysis rate.

In the presence of an excess of Mg_t^{2+} over ATP_t, where most of the nucleotide is in the complexed form, we have attributed the activating effect of the pyrophosphate to the chelation of Mg^{2+} and dissociation of Mg ATP, with more metal free ATP being available.[25] This effect is to be compared with that of other chelating agents, such as tropolone, with similar K_D. However, competition of pyrophosphate with ADP (and to a lesser extent with ATP) on CF_1 binding sites also occurs, as can be seen from the results shown in Fig 3: there is a decrease of binding of ADP for a concentration of pyrophosphate (0.2 mM) which does not modify significantly free Mg^{2+}(Mg^{2+}_{total} = 1mM).

Remark

It must be noted that phosphate has comparatively low effect on binding (Fig. 3) and on enzymatic rate (data not shown), in the same conditions and concentration range.

Activated CF_1

The activation of CF_1 obtained by depletion of the ε subunit following chromatography or by dithiothreitol treatment, only slightly modifies the binding of ADP to CF_1:

$K_D^{'EMgMgADP}$ for CF_1: 30 μM (see Fig. 4)

From the study of the influence of Mg_t^{2+}, the value of 64 μM has been obtained)

for CF_1-ε: 60 μM

for DTT activated CF_1: 55 μM

Although the binding is not significantly affected, the enzymatic rate is enhanced about ten fold by activation.[14] These results show that the ε subunit

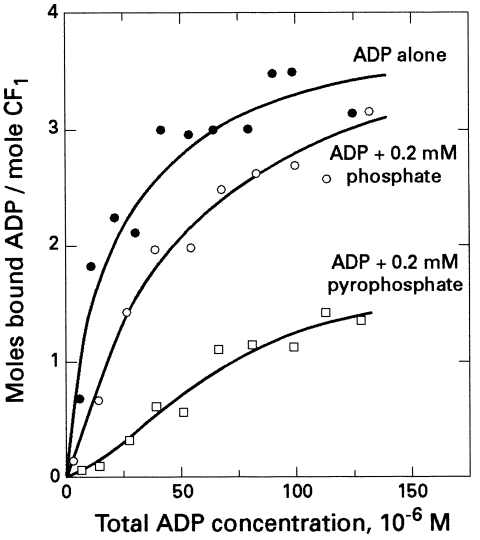

Figure 3. Influence of pyrophosphate (0.2 mM) or phosphate (0.2 mM) on the binding of ADPon CF_1. Conditions: Tris buffer 0.075 M, pH 8.5, temperature 25°C, Mg_t^{2+}1mM.

does not intervene in the binding of the substrate, but prevents the intermediary complex from dissociation into ADP and Pi. On the other hand, depletion of the δ subunit, obtained by washing CF_1 at low ionic strength on a TSK 5 PW DEAE column, followed by ammonium sulfate gradient elution[15] is without effect on ADP binding as well as on enzymatic rate.

DISCUSSION

The influence of different parameters on the binding of ADP to CF_1 has been studied: ionic strength, pH, temperature, activation treatments, presence of pyrophosphate. The corresponding effects of these treatments on the binding of ATP have not been studied in every case. When it has been done, the effect on the binding of each nucleotide was similar. However, these effects do not allow to predict those on the enzymatic activity. Indeed, temperature has a classical effect on the enzymatic activity, according to the Arrhenius law, with CF_1 as with TF_1,[21] whereas nucleotide binding is only slightly modified. The enzymatic rate moderately increases when the ionic strength increases, while the binding of ADP is dramatically decreased. Activation treatments enhance more than ten fold the enzymatic activity but the binding of ADP is only slightly

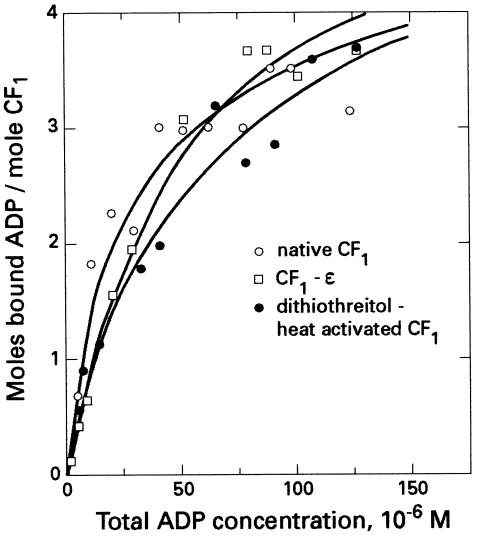

Figure 4. Influence of activation of CF_1 on the binding of ADP. Conditions: same conditions as in Fig 3, the activation was performed by ε subunit removal or by dithiothreitol and heat treatment.

decreased. Pyrophosphate binds on CF_1, competing with nucleotides but its effect on enzymatic activity is mainly due to Mg^{2+} depletion (when this ion is in excess over ATP_t). All these examples show that the conclusions drawn for the dissociation constant K_D cannot be applied to

$$K_m : K_m = \frac{k_{-1} + k_2}{k_1} \text{ and } K_D = \frac{k_{-1}}{k_1}, \text{ they differ by the term } \frac{k_2}{k_1}.$$

The role of the lysine residue, which is present in the nucleotide binding sites, has been emphasized by several authors: the binding of pyridoxal-5 phosphate (which is known to modify the lysine residues) has been shown to inhibit the enzymatic activity of isolated CF_1[26,27] and of chloroplast thylakoids.[28] The use of β K 155 Q and β K 155 E E. coli ATPase mutants has led Senior et al.[29] to the conclusion that the lysine contributed to the ATP binding, through hydrogen bonds with phosphate groups. In our case, we have observed an important inhibition of the enzymatic rate of CF_1-ε after methylation of the lysines with formaldehyde and cyanoborohydride and we have observed, by the chromatographic method of Hummel and Dreyer, the particular affinity for ADP of lysine and arginine, compared to other aminoacids (data not shown).

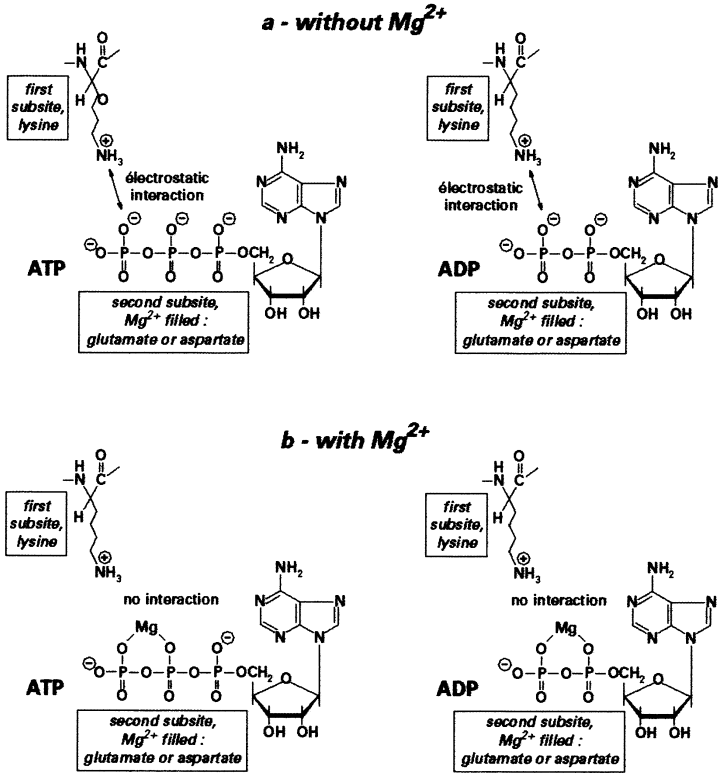

Figure 5. Proposition of mechanism of the nucleotide binding in absence or presence of Mg^{2+}. a) In absence of Mg^{2+}, the terminal phosphate group of the nucleotide is metal free and interacts with the positively charged lysine. There is tight binding (dissociation constant on the order of 10 μM) and in the case of ATP, the hydrolysis would be possible. b) In presence of Mg^{2+}, the terminal group is metal complexed and does not interact with the lysine. The dissociation constant is on the order of 100 μM and ATP would not be hydrolysed.

These latter data and those concerning the influence of ionic strength and Mg^{2+} concentration (accompanying paper) can be interpreted by the interaction of the negatively charged phosphate groups of the nucleotides, with the positively charged group of the lysine of the enzymatic site ($K_D^{EMg\ ATP}$ = 14 μM, $K_D^{EMg\ ADP}$ = 5 to 15 μM). When the bound nucleotide is complexed with Mg^{2+}, the interaction with the lysine would be smaller, due to screening effects ($K'_D{}^{EMg\ Mg\ ATP}$ = 184 μM, $K'_D{}^{EMg\ Mg\ ADP}$ = 64 μM) (Fig. 5). However, the interaction of nucleotides with lysine is not completely suppressed in the latter conditions,

since Mg^{2+} analogs such as Mn^{2+} and VO^{2+} have been suggested, by EPR spectroscopy,[30,31] to be coordinated to this lysine residue at least when it is deprotonated. Moreover, Mg ADP is better fitted to the binding pocket than Mg ATP, perhaps because of its smaller size.

On the other hand, one must admit that the nucleotide binding must depend on another subsite, which must be Mg^{2+} filled (or filled with Mn^{2+} or Ca^{2+}), since there is neither appreciable enzymatic activity nor nucleotide binding in their absence. This metal binding site could be a glutamic or an aspartic residue: β E 188, β D 256, α D 269, α D 270.[20]

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