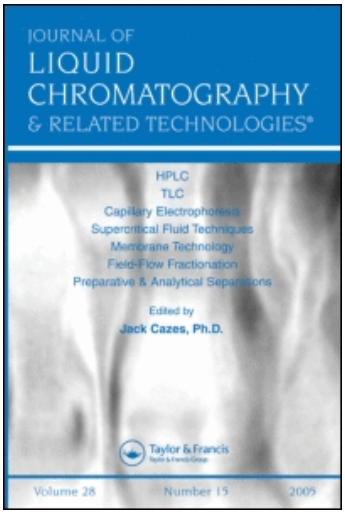


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# Study of the Simultaneous Binding of ADP and ATP on Coupling Factor CF-1 by a Modification of the Hummel and Dreyer Method

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# STUDY OF THE SIMULTANEOUS BINDING OF ADP AND ATP ON COUPLING FACTOR CF1 BY A MODIFICATION OF THE HUMMEL AND DREYER METHOD

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## ABSTRACT

A modification of the Hummel and Dreyer method[1], based on anion exchange separation is used here for the study of the simultaneous binding of ADP and ATP on spinach coupling factor $CF_1$.

This method gives the same results as gel filtration (dissociation constant and number of sites) when ADP alone is present.

The extent of binding of ADP and ATP is approximately the same when mixed in equimolecular ratio, but since endogenous ADP is irreversibly bound, this nucleotide is predominant on $CF_1$.

The binding of one nucleotide is partly prevented by prealable mixing of $CF_1$ with the other nucleotide. This phenomenon occurs likely at the level of high affinity sites, where binding would not be entirely reversible, contrarily to low affinity sites.

This method is of potential application for other ligands separable by anion exchange chromatography and for other types of chromatography (reversed phase).

## INTRODUCTION

The study of the binding of a ligand on a macromolecule is carried out by different methods which fall into two categories : those for which the bound ligand is

measured in presence of the free ligand and those which involve previous separation of the two forms.

In the latter case, separation can be achieved by different means (centrifugation, filtration, size exclusion chromatography, etc.) and it is assumed that the equilibrium state is not modified during the separation step. This condition is not necessarily fullfilled, especially when the dissociation constant is sufficiently high. 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 0.1s for a dissociation constant $K_D=10^{-6}M$, or in 1s for $K_D=10^{-7}M$ (2). Separation techniques must then be extremely rapid in the case of loosely bound complexes.



The methods which do not require separation are not submitted to this constraint. They include physical methods such as fluorescence emission of the tetracycline-$CF_1$ complex (3), which is quenched by ADP binding, or of ethenoadenine bound to $CF_1$ (4). Ultraviolet absorption is modified in the case of fixation of ADP on $TF_1$ (5). Also the circular dichroism of ADP is changed when fixed on $CF_1$ (6). However these methods are often difficult to relate the signal amplitude with the fraction of bound ligand. Another method which is theoretically suitable in every situation, particularly for low affinity systems, is equilibrium dialysis. Sensitivity is relatively poor, however, since the difference of ligand concentration is measured between two compartments. Moreover, the results can be disturbed by ligand absorption on the dialysis membrane.

In this work, we have used the chromatographic method of Hummel and Dreyer (1) and modified it in order to study the simultaneous binding of ADP and ATP on coupling factor $CF_1$. In the original method, a known quantity q of macromolecule is injected on a gel filtration column which is preequilibrated with a fixed concentration (A) of ligand.

In the case of reversible binding of the ligand with $n_i$ independent sites of class i, of respective binding affinity constants $K_i$, the quantity of bound ligand is :

$$q \sum_{i=1}^{i=m} \frac{n_i K_i [A]}{1 + K_i [A]}$$

where m is the number of site classes (7,8).

This quantity is withdrawn from the solvent and migrates with the macromolecule since the size of the complex is not significantly different from that of the macromolecule. There is then a local decrease of the ligand concentration of the solvent. This depression migrates with the same rate as the ligand. It can be detected at the column outlet as a negative peak in the ligand concentration.

The mean number $\bar{r}$ of moles of bound ligand/mole of macromolecule is independent of the concentration of macromolecule, which varies all along the chromatographic peak. It depends only on the concentration of free ligand in the eluent and on association constants.

The bound ligand quantity can be measured by the surface of the above-mentioned negative peak. It can also be evaluated from the peak of the complex when the participation of the free macromolecule to the detector response is known or is null. Hummel and Dreyer have proposed an internal calibration (1), based on the measurement of the ligand peak : the same quantity of macromolecule is injected together with increasing quantities of ligand. The size of the negative peak decreases progressively and eventually it becomes positive. Its surface 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 exactly compensates what has been added to the solvent.

The advantage of the method is that the complex does not dissociate during chromatography, even if it lasts a long time and if the affinity constant is low, since the complex is always in equilibrium with the free ligand. There are a few drawbacks to the method. Indeed the measurements are time consuming and the number of determinations is limited to 10-15/day. Moreover, the method requires a large

amount of ligand. Artifacts can also occur when the ionic strength or the pH of the injected mixture is different from that of the eluent.

The initial method of Hummel and Dreyer has been extended to HPLC by Sebille and al. (9–11) for the study of the binding of different drugs on human serum albumin. In a precedent work we have applied it to the fixation of ADP and ATP on chloroplast coupling factor $CF_1$ and on some of its subunits (12).

This protein is a part of a proton translocating multisubunit enzyme found in photosynthetic membranes and is able to hydrolyse and synthesize ATP. We have shown by this method that $CF_1$ possesses six binding sites : two high affinity sites for ADP or ATP ($K_D$=1–5 $10^{-6}$M) in addition to one site where endogenous not exchangeable ADP is bound, and three low affinity sites binding ADP or ATP with a dissociation constant of 15–20 $10^{-6}$M, responsible for the catalytic activity.

In the original method, the separation between the free ligand and the complex is based on the difference of size. When there is simultaneous fixation of two ligands of similar molecular weights, separation by gel filtration is difficult or impossible. The respective concentrations of each ligand can be determined if they have different optical characteristics or if one of them is radioactive. In the particular (but frequent) case of an ADP–ATP mixture, the situation is unfavourable : same optical spectra, similar sizes. In order to avoid important levels of radioactivity, we have adapted the Hummel and Dreyer method by using anion exchange separation of the protein and of the two nucleotides.



## MATERIALS AND METHODS

Coupling factor is extracted from spinach chloroplast by sucrose–chloroform treatment, then adsorbed on a batch of DEAE cellulose (Whatman DE 52) (13) and eluted by 0.4M NaCl. $CF_1$ is further purified by HPLC on a Protein Pak 5 PW Waters column, eluted with ammonium sulfate gradient in 20 mM Tris pH 8 (14). For storage, $CF_1$ is precipitated with 50% saturation ammonium sulfate. Before use, it is dialysed over night against adequate buffer.

The purity of the different protein fractions is checked by slab SDS-PAGE (8 M urea, 12% polyacrylamide) according to the method of Piccioni et al. (15) and by the fluorescence emission ratio at 308 nm and 340 nm, when excited at 280 nm. The purified fractions used for nucleotide binding experiments have a fluorescence ratio between 1.6 and 1.8.

Protein content is obtained from the U.V. absorption spectrum, assuming an $E^{1\%}_{1cm}$ of 4.8 at 280 nm for $CF_1$ and by the BioRad protein assay (16), using bovine serum albumin as a standard. Molecular weight of 400 000 (17) is used to calculate molar concentration of $CF_1$.

1 to 2 moles ADP/mole of $CF_1$ are firmly bound and resist to ammonium sulfate precipitation and dialysis. They are released by acidic treatment (0.5 N HCl) which precipitates the protein, separated, after neutralisation, by HPLC on an anionic TSK DEAE 2SW column, with 70mM $P0_4H_2K$ and 300 mM NaCl as eluent, and measured by absorption at 260 nm. ADP and ATP concentrations of standard or eluent solutions are calculated from the absorption at 260 nm, with $E^{M}_{1cm}$=15,400. They are corrected for traces presence of AMP and ATP in ADP or of ADP in ATP, determined by HPLC.

Nucleotide binding to $CF_1$ is measured according to Hummel and Dreyer, with a gel filtration TSK SW 2000 (30 cm x 0.75 cm) column, and in the modified method, with an anion exchange TSK DEAE 2SW (25 cm x 0.46 cm) column. Eluents are ADP or ATP solutions, from 0.5 to 10 $10^{-5}$M in 0.075M Tris sulfate pH 8.5, $1.10^{-3}$M $MgCl_2$. A constant volume (50 $\mu$l) containing 1 to 2 nmoles of $CF_1$ and variable quantities of ADP or ATP is injected on the column. Nucleotide concentration of the eluent is recorded by absorption spectrophotometry at 260 nm and the areas of the negative or positive peaks are determined by weighing.

HPLC were performed on a Waters Assoc. liquid chromatograph, consisting of a M510 pump, an U6K injector and a M481 detector system.

## RESULTS AND DISCUSSION

### Comparison of the gel filtration and the anion exchange methods

The binding of ADP alone is quite identical when measured by the gel filtration method or the anion exchange separation (fig. 1). The total number of sites (respectively 6.04 and 5.87) and the dissociation constant of low affinity sites (45 and 52 $\mu$M) are similar in both cases (fig. 2).

That means that the binding of $CF_1$ on the DEAE column does not perturb the nucleotide fixation on $CF_1$ and thus that the two kinds of sites present on $CF_1$ are independent.



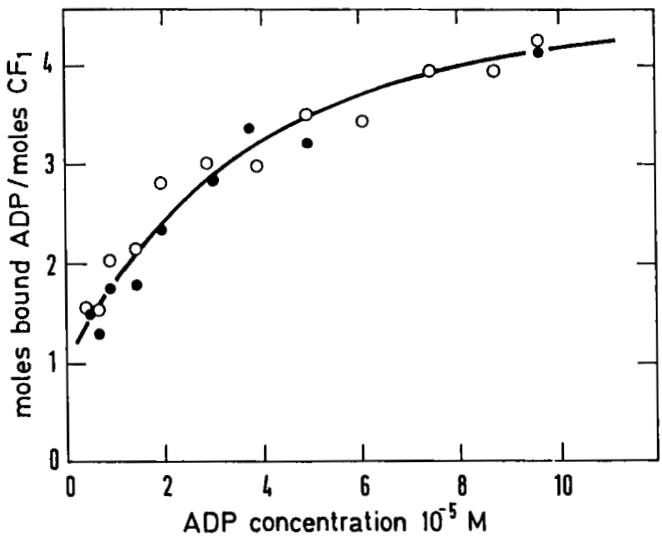


Figure 1 : ADP binding on $CF_1$ in function of free ADP concentration, measured by gel filtration (•) or by anion exchange separation (o). Eluent 0.075M Tris, pH 8.5, 1 mM $MgCl_2$. Temperature 26°C. The content of $CF_1$ in endogenous ADP has been included. Injected $CF_1$, respectively 2.5 (•) and 1.66 (o) nmoles in 50 $\mu$l.

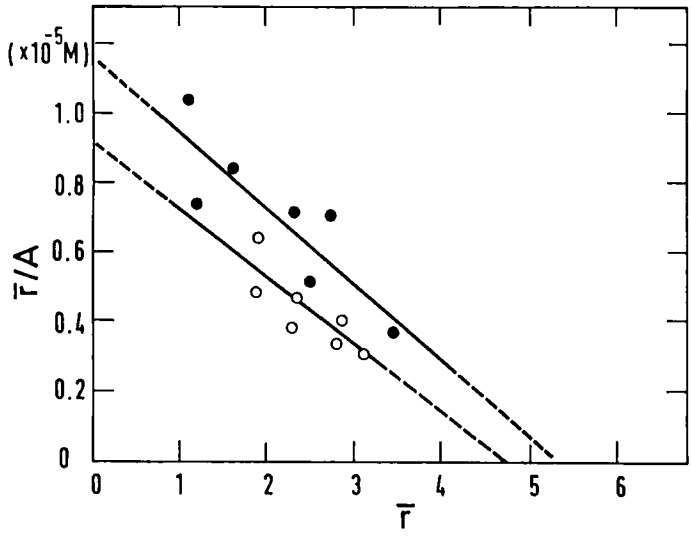


Figure 2 : Scatchard plot of ADP binding on $CF_1$. The measurements have been obtained by gel filtration (•) or by anion exchange chromatography (o). Eluent 0.075M Tris pH 8.5, 1 mM $MgCl_2$. Temperature 26°C. $\bar{r}$=number of moles of ADP bound per mole of $CF_1$, A=free ligand molar concentration.

The dissociation constants for low affinity sites, calculated from the slopes of the Scatchard plots, are respectively : 45 and 52 $\mu$Moles for the gel filtration method and the anion exchange separation. The total number of sites are 5.3 (intercept of the Scatchard plot with the x axis) + 0.74 (endogenous ADP) = 6.04 for the first method and 4.72 + 1.15 = 5.87 for the second one.

On the other hand, previous experiments have shown that an increase of the ionic strength leads to a decrease of the retention time of the nucleotides on DEAE column and of their binding to $CF_1$. The choice of the pH and of the ionic concentration of the buffer results from a compromise between good binding properties and a reasonable retention time (10 mn at 1.5 ml/mn), with a sufficient ADP/ATP separation. On the filtration column, the protein and the protein-nucleotide complex are eluted before the nucleotides. On the contrary, they are retained on the

anion exchange column, under the conditions used. About thirty injections (15–20 mg $CF_1$) can be carried out before the protein comes out. The retention time and the resolution of nucleotides are not modified with time, which means that the fixation sites for nucleotides are still accessible on the column, even if it is overloaded with protein. The amount of $CF_1$ previously fixed on the column does not perturb the equilibrium between nucleotides and $CF_1$, injected hereafter, since, as baseline is stable between two injections, the immobilized $CF_1$ is in equilibrium with the eluent and equivalent amounts of nucleotides are bound on it and released from it. Indeed, there is no influence of the order in which the $\bar{r}$ values are determined : the points of curve 1 have been obtained first by successively increasing ADP concentration, then by decreasing it and they are intercalated on the same curve.



It must be noted that, on the filtration column, the peak of nucleotide is sometimes followed by another positive peak, more especially as the ionic strength of the injected solution and of the solvent are different. This artifact does not occur on anion exchange column.

The same comparison between gel filtration and anion exchange has been performed with ATP. Scattering of the data precludes a precise determination of the affinity constant but it is clear that it is smaller than that corresponding to ADP.

There is no ADP positive peak when $CF_1$ is injected on a DEAE column equilibrated with ATP. This shows that endogenous ADP initially present on $CF_1$ (1 to 2 mole/mole of $CF_1$) is not exchanged with ATP.

Measurement by anion exchange chromatography of simultaneous ADP and ATP binding

When the eluent contains a mixture of ADP and ATP (here in approximately equal concentrations) the injection of $CF_1$ alone leads to the observation of two negative peaks. This is interpreted as the simultaneous binding of both nucleotides.. The extent of binding of each nucleotide is measured by adding known amounts of one of them. One negative peak decreases and eventually becomes positive. It is null

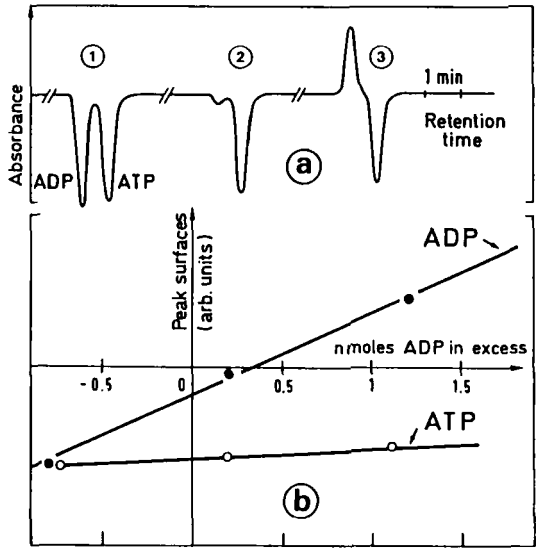


Figure 3 a) : Chromatographic profile of $CF_1$-ADP mixture on anion exchange column equilibrated with 1.59 $10^{-5}$M ADP and 1.44 $10^{-5}$M ATP.

Injected $CF_1$ 1.55 nmole, (1) alone, (2) with 1.01 nmole ADP and (3) with 2.02 nmole ADP. Buffer : 0.075M Tris pH 8.5, 1 mM $MgCl_2$. Temperature 22°C.

b) : Nucleotide peak surfaces in function of ADP excess over the quantity contained in the same volume of solvent. Same conditions as in a).

when the bound quantity is equal to the excess of injected nucleotide over that of the same volume of solvent (fig. 3 and 4).

The average numbers of bound ADP and ATP per molecule of $CF_1$ have been determined as a function of free nucleotides concentrations. Fig. 5 shows that the increase of ADP binding is equal or slightly greater than that of ATP. This indicates that the affinity of $CF_1$ for both nucleotides is nearly the same, up to a total occupancy greater than four sites. However, the dispersion of the results has not allowed to calculate the binding constants by Scatchard plots.

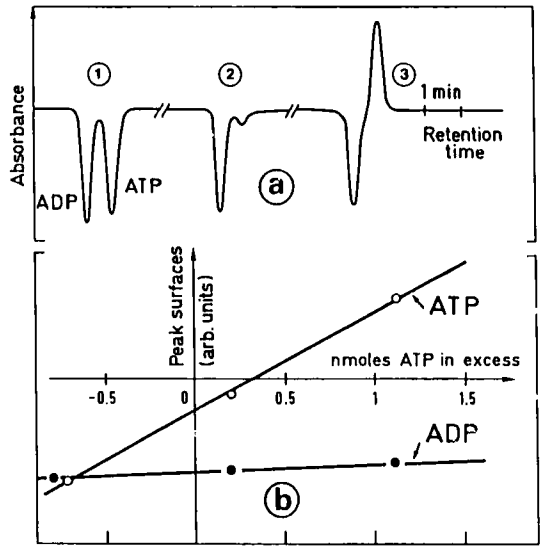


Figure 4 a) Chromatographic profile of $CF_1$-ATP mixture on anion exchange column equilibrated with 1.59 $10^{-5}$M ADP and 1.44 $10^{-5}$M ATP.

Injected $CF_1$ 1.55 nmole, (1) alone, (2) with 0.92 nmole ATP and (3) with 1.84 nmole ATP. Same conditions as in Figure 3.

b) Nucleotide peak surfaces in function of ATP excess over the quantity contained in the same volume of solvent. Same conditions as in a).

On the other hand, the endogenous ADP which is initially bound to $CF_1$ does not exchange with ATP, so that the total amount of bound nucleotides includes more ADP than ATP.

These results can be compared to those precedently found by other methods (18–23). The authors generally agree upon the irreversibility of ADP binding on the first high affinity site of $CF_1$, but not for that of ATP on the second one. The same dissociation constant has been found for ADP and ATP binding on the third high affinity site (22). However, the methods which are employed did not allow to study the low affinity sites, which have been characterized afterwards (12).

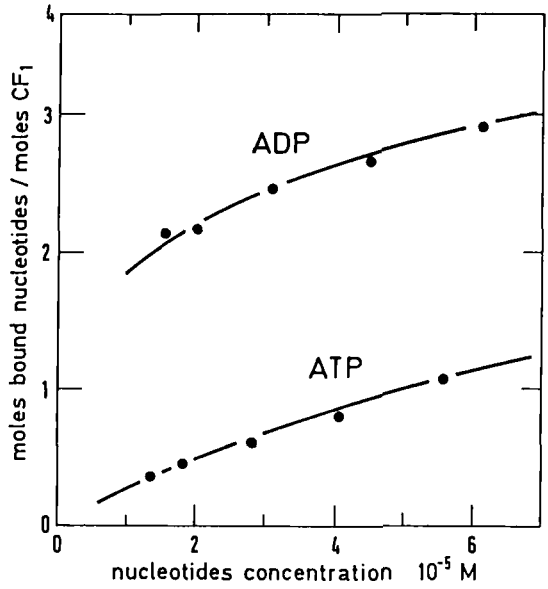


Figure 5 : Simultaneous ADP and ATP binding on $CF_1$, in function of free nucleotide concentration. Eluent and free ADP and ATP concentrations as in Fig. 3 and 4. The contents of $CF_1$ in endogenous ADP (1.88 mole/mole) and traces of endogenous ATP (0.19 mole/mole) have been included. Injected $CF_1$ : 1.55 nmole in 50 $\mu$l.

The utilisation of the Hummel and Dreyer method implies that binding is reversible and that no ulterior modification of the ligand could occur. $CF_1$, prepared as described, is a latent ATPase, which can be activated by different treatments (heat, dithiothreitol, trypsin) (24). We have determined by HPLC, using the same anion exchange column, but with 70 mM $PO_4H_2K$ and 300 mM NaCl as eluent, that 1.1 nmole of our $CF_1$ in the presence of 8.8 nmole ATP (contained in 50 $\mu$l Tris 0.075 M, pH 8.5, 1 mM $MgCl_2$) hydrolyse ATP into ADP, at the rate of 0.13 nmole/min. Mixture and injection last for at most 30 sec. In these conditions, the quantity of hydrolysed ATP is negligible in front of that which is added.

Hydrolysis which could occur during chromatography, due to $CF_1$ bound on the column, could modify the ADP/ATP ratio at the outlet of the column, but cannot

influence the extent of nucleotides bound by the injected amount of $CF_1$ at the inlet of the column, thus the size of the negative peaks.

On the contrary, formation of ATP + AMP from ADP, due to slight adenylate kinase activity, in the same conditions as above, occurs at the rate of 0.023 nmole/min. This value is much too low to modify significantly the quantity of added ADP.

Consequently, the enzymatic activity of $CF_1$ seems to have no influence on the binding of ADP and ATP when measured in these conditions. However, it would be different if $CF_1$ and nucleotides were incubated several minutes before injection on the column.

Is the chromatographic separation sufficient to measure the two nucleotides with accuracy ? The resolution $R_S$ (sum of the widths of the peaks at half height, divided by the difference of the retention times), is currently around 1.1. The overlapping of two peaks of same height is included between 2% (for $R_S$=1.0) and 0.6% (for Rs=1.25) (25). The error due to incomplete separation is thus negligible.



Fig. 3 and 4 show that when one nucleotide is mixed with $CF_1$, before injection on the column the negative peak corresponding to the other one decreases slightly, although it should be constant and give an horizontal line. Several explanations could occur for this phenomenon :

a) enzymatic activity of $CF_1$ : the above-mentioned rate of hydrolysis of ATP (0.13 nmole/min) could account for the slight decrease of the negative peak of ADP seen in fig. 4. However, the formation of ATP from ADP in the same conditions is too slow (0.005 nmole/min) to explain the phenomenon of fig. 3.

b) nucleotide impurities : ADP used for this study contained about 1% ATP and 3 to 4% AMP. Different batches of ATP contained between 2.3 and 4.5% ADP. These impurities can also account for a part of the phenomenon, but not for its totality, especially in the case of ATP.

c) The most probable explanation seems to be the following one. Reversible fixation takes place on the three low affinity sites. There is a rapid change of the fraction of these occupied sites on $CF_1$ when it goes from the mixing tube, where the

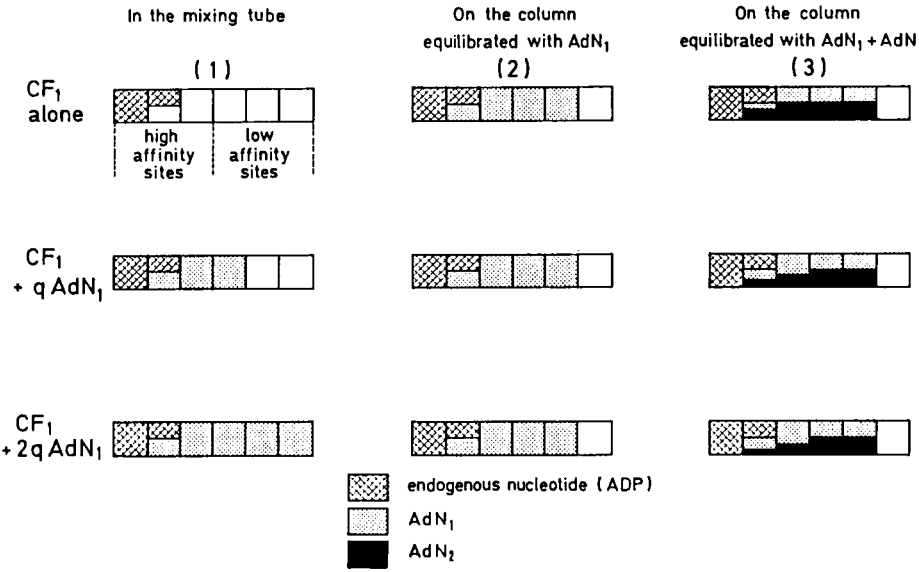


Figure 6 : Schematic representation of the nucleotide binding sites occupancy in different conditions.

1) $CF_1$ is mixed with increasing amounts of the first nucleotide $AdN_1$.

2) After injection on a column equilibrated with the same nucleotide, the equilibrium is rapidly reached at the level of the low affinity sites, determined by solvent concentration of Ad $N_1$.

3) When the column is equilibrated with a mixture of the two nucleotides $AdN_1$ and $AdN_2$, the equilibrium is always rapid on low affinity sites, but the fixation of $AdN_2$ on tight binding sites is slightly prevented by previous incubation with $AdN_1$.

In fact, when time of contact of nucleotides with $CF_1$, before injection, is limited to 30 sec., tight binding sites are not fully occupied and the results of binding are slightly underestimated.

added nucleotides are in large excess, to the column, where a lower concentration of free nucleotide is imposed (if it were not the case, no definite negative peak could be observed, but only a broad trough). On the other side, fixation on the first tight binding site, and perhaps on the second one, has been shown to be irreversible (18,19). If the rate of exchange on the other high affinity site is relatively slow, the mixing of $CF_1$ with an excess of one nucleotide before injection can partly prevent the fixation of the other one, when the mixture is applied on the column, and decrease the size of the corresponding negative peak (Fig. 6).

It must be noted that relative slowness of the fixation is not incompatible with tightness of binding on these sites, if a free enthalpy activation is needed to pass from the free state to the bound state.

In fact tight binding sites are not completely filled during the rapid contact of $CF_1$ with the excess of nucleotides (less than 30 sec). When incubation time is increased to 5 min, the total binding of nucleotides slightly increases. The results are thus underestimated if there is no preliminary incubation. However, this treatment is not possible, in the case of ATP, because of the enzymatic activity of $CF_1$.



## CONCLUSION

In conclusion, the modification of the Hummel and Dreyer method presented here, based on anion exchange chromatography, allows to measure the simultaneous binding of ADP and ATP on the low affinity sites of $CF_1$.

Several experiments conditions are required : the pH and the molarity of the eluent must allow sufficient binding of the nucleotides on $CF_1$, and a good chromatographic separation, in a reasonable time. Incubation time must be sufficiently short, in order to limit hydrolysis of ATP before injection on the column.

We have verified that the extent of binding of ADP alone, as measured by anion exchange chromatography, is identical to that determined by the gel filtration, used in the original method.

When ADP and ATP are both present in equal concentration, ADP is bound to $CF_1$ with a slightly higher affinity than ATP. Since endogenous ADP is not exchanged with ATP, total bound ADP is predominant.

It is proposed that the rate of fixation of nucleotides on unoccupied tight binding sites is relatively slow, compared to that on low affinity sites : the incubation with one nucleotide partly prevents the fixation of another one on these sites.

This example can be extended to any combination of two or more ligands, binding with rapid equilibrium and not modified by enzymatic activity. All types of columns could be utilized (ion exchange, normal or reversed phase columns), depending on the properties of the ligands.



**The abbreviations used are :**

$CF_1$ : soluble chloroplast coupling factor

$TF_1$ : thermophilic bacterium coupling factor

HPLC : high performance liquid chromatography

SDS : sodium dodecyl sulfate

PAGE : polyacrylamide gel electrophoresis

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