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Use of the Hummel and Dreyer method for the study of nucleotide binding on chloroplast ATPase CF1

G. Berger*, G. Girault

Section de Bioenergétique, DBCM, CEA Saclay, F 91191 Gif sur Yvette, France

Abstract

The study of the binding of the nucleotides ADP and ATP on the exchangeable sites of chloroplast ATPase CF1 has been carried out by the Hummel and Dreyer method applied to HPLC. It has been shown that this method was well fitted to the problem: rapidity of exchange, absence of noticeable modification after binding, presence of a constant concentration of ligand during the chromatography, which stabilizes these low affinity complexes. The dissociation constants of binding of ADP, ATP and of their magnesium salt complexes have been determined. In order to measure the simultaneous binding of ADP and ATP when present in mixture, we have modified the method by using an anion-exchange column in place of the gel filtration column: the two nucleotides were easily separated, while the binding on the protein was unchanged. The extension of this method to the reversed-phase chromatography could also be considered for the binding of hydrophobic ligands.

Keywords: Hummel and Dreyer method; Nucleotides; ATPase CF1

1. Introduction

The binding of a ligand on a macromolecule can be measured either in the presence of all the components of the reaction mixture or after separation of the complex. In the later case, the separation can be performed by different means (centrifugation, filtration, chromatography, etc.) and it is generally assumed that the equilibrium is not modified during the separation step. This condition is not necessarily fulfilled, 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 10% in 0.1 s for a dissociation constant $K_D=10^{-6}\ M$ or in 1 s for $K_D=10^{-7}\ M$ [1]. Separation techniques must then be extremely rapid in the case of loosely bound complexes. In the particular case of the study of the binding of nucleotides on ATPase, separation by size-exclusion chromatography on small Sephadex columns has been widely used and elution was accelerated by centrifugation [2–5]. However, rapidity involved a decrease of the efficiency of the chromatography, and several operations were then needed, during which dissociation could occur.

The methods which do not require separation are not subject to this constraint. In the case of ATPase, they include physical methods, such as fluorescence emission of the tetracyline–CF1 complex [6], or of ethenoadenine bound to CF1 [7], 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 [8–10], or circular

*Corresponding author.

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dichroism of ADP, which is changed when fixed on CF1 [11]. The UV–visible spectral changes induced by binding of analogs of nucleotides (TNP ADP, TNP ATP) have also been studied [12,13] as well as the modification of fluorescence of the tryptophane probes incorporated by site-directed mutagenesis near the binding site [14–16], or the steady state fluorescence intensity of maleimide attached to the cysteine at position 63 on β CF1 [17]. 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 ligand is measured by the difference of concentrations between two compartments. Moreover, the results can be disturbed by the ligand adsorption on the dialysis membrane.

For our part, we have studied the binding of nucleotides on the chloroplast ATPase by the chromatographic method of Hummel and Dreyer [18]. It is based on the following protocol: a known quantity of protein is injected into a gel filtration column equilibrated with a constant concentration of a ligand of that protein. In the simplest case (one kind of equilibrium, characterized by an unique dissociation constant K_D), the amount of ligand that binds is proportional to the quantity of protein injected and depends only on K_D and on the ligand concentration. This amount is withdrawn from the eluent and migrates with the protein, while a trough in the ligand concentration profile runs with the ligand rate. If r represents the fraction of the n exchangeable sites of the protein which are filled, nr is the number of molecules of ligand bound per molecule of protein and is determined by the negative peak. According to the multiple equilibrium theory, $nr/(s)$ is linear versus nr, where (s) is the free ligand concentration. The slope of the straight line is $1/K_D$ and the extrapolation on the x-axis gives the number n of sites by the Scatchard plot [19]. The advantage of this method is that there is no dissociation of the complex during the chromatographic separation, since the complex is always in equilibrium with a constant concentration of ligand. It must permit one to measure the K_D of low-affinity complexes.

The bound ligand quantity can 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 to measure the negative peak by the means of an internal calibration: 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 ligand in the injected volume over the quantity contained in the same volume of eluent. The intersection with the x-axis occurs when the bound ligand quantity compensates exactly what has been added to the solvent.

This method implies that the equilibrium between the protein and the ligand is sufficiently rapid: it must be achieved in a time inferior to the passage of the chromatographic peak [in the order of a few seconds, in the case of high-performance liquid chromatography (HPLC)]. The measurements require a large amount of ligand. They cannot be applied to rare and expensive components. They are also time-consuming, even with HPLC: the number of determinations is limited to 20–30/day. However, it has the valuable advantage to allow low affinity binding measurements. The initial method of Hummel and Dreyer used conventional gel filtration chromatography. It has then been extended to HPLC by Sebille et al. [20,21], for the study of the binding of different drugs on albumin. In precedent works, we have applied it to the determination of the fixation of ADP and ATP on the chloroplast coupling factor CF1 (F_1 ATPase). This protein is a part of a proton translocating multisubunit enzyme found in photosynthetic membranes, which is able to hydrolyze and synthetize ATP.

In the original method, the separation between the free ligand and the complex was based on the difference of size. When there is simultaneous fixation of two ligands of similar molecular mass, 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 case of an ADP–ATP mixture, the situation is unfavorable: same optical spectra, similar sizes. In order to avoid to handle 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.

2. Materials and methods

CF1 was extracted by EDTA, sucrose, and chloroform treatment from spinach chloroplasts, then passed on a DEAE cellulose column, equilibrated with 50 m*M* Tris–HCl, 2 m*M* 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 [22]. Crude extracts were purified by HPLC (Waters apparatus consisting of two pumps M510, a solvent programmer M660, an injector U6K and a multi-wavelength detector M490), on Protein Pak DEAE 5 PW columns 150×21.5 mm equilibrated with 20 m*M* Tris buffer, pH 8.5. A linear ammonium sulfate gradient 0 to 1 *M* was applied during 30 min at 4 ml/min [23]. The purity of the different protein fractions was checked by slab sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (8 *M* urea, 12% polyacrylamide) according to the method of Piccioni et al. [24] and by the fluorescence emission ratio at 308 and 340 nm, when excited at 280 nm. The purified fractions used for nucleotide binding experiments had a fluorescence ratio between 1.6 and 1.8. Protein content was obtained from the UV absorption spectrum, assuming an $E^{1\%}_{1\ cm}$ of 4.8 at 280 nm for CF1 and by the Bio-Rad protein assay [25], using bovine serum albumin as a standard. Molecular mass of 400 000 was used to calculate the molar concentration of CF1.

1 to 2 mol ADP/mole of CF1 were found to be firmly bound and resist to ammonium sulfate precipitation and dialysis. They were released by acidic treatment (0.5 *M* HCl) which precipitates the protein, separated, after neutralisation, by HPLC on an anionic TSK DEAE 2SW column, with 70 m*M* KH_2PO_4 and 300 m*M* NaCl as eluent, and measured by absorption at 260 nm.

ADP and ATP concentrations of standard or eluent solutions were calculated from the absorption at 260 nm, with $E^{M}_{1\ cm}=15\ 400$.

Nucleotide binding to CF1 was measured according to Hummel and Dreyer, with the Waters apparatus described above,with a gel filtration TSK SW 2000 (300×7.5 mm) column, and in the modified method, with an anion-exchange TSK DEAE 2SW (250×4.6 mm) column. Eluents were ADP or ATP solutions, from 0.5 to $10{\cdot}10^{-5}$ *M* in 0.075 *M* Tris sulfate, pH 8.5, $1{\cdot}10^{-3}$ *M* $MgCl_2$. A constant volume (50 μl) containing 1 to 2 nmol of CF1 and variable quantities of ADP or ATP was injected on the column immediately after mixing.Elution was carried out at 1 ml/min, at room temperature. Nucleotide concentration of the eluent was recorded by absorption spectrophotometry at 260 nm and the areas of the negative or positive peaks were determined by weighing.

Nucleotides and chemical reagents were purchased from Sigma. Water used in preparation of the solutions was purified by a Milli-Q Water system (Millipore).

HPLC columns were purchased from Beckman.

3. Results

3.1. ADP binding

The problem of the binding of ADP is simple, because this nucleotide does not undergo modification after binding: the adenylate kinase activity of CF1, measured in our conditions, was of 0.023 nmol/min (for 1.5 nmol CF1 injected), this value is thus too low to modify significantly the quantities of ADP during the preparation of the mixture and the injection on the column (max 15 s). The area of the peak of ADP varies linearly with the excess of ADP in the injected volume over the quantity contained in the same volume of eluent, (Fig. 1). Under these conditions,the intersection with the *x*-axis corresponds to the quantity of ligand bound by the protein and a blank subtraction is not necessary, as in Pinkerton and Koeplinger representation [26]

We have verified that this intersection with the *x*-axis was independent on the injected volume (Fig. 2). We have also verified that this value was proportional to the quantity of protein injected (data not shown).

The equilibrium between the protein and the nucleotide contained in the injected volume and in the eluent was sufficiently rapid, since the width of

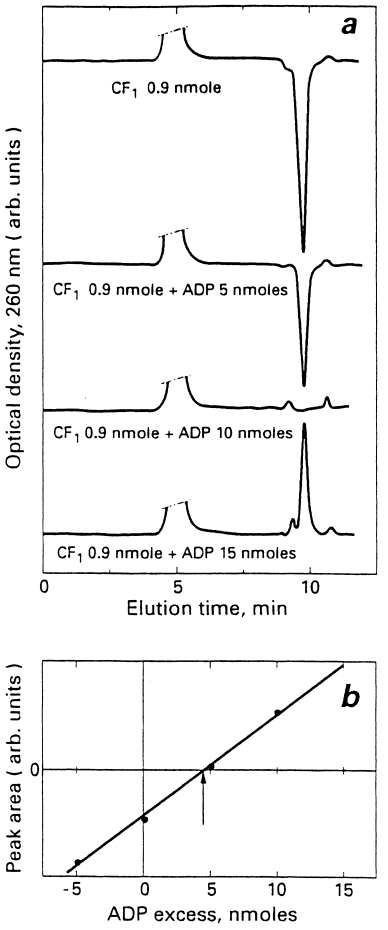

Fig. 1. ADP–CF1 binding measurements by the chromatographic method of Hummel and Dreyer. (a) Chromatographic profiles. Conditions: column: TSK 2000 SW (300×7.5 mm). Eluent: Tris buffer $75\cdot10^{-3}$ *M*, pH 8.5, ADP $0.99\cdot10^{-4}$*M*, Mg^{2+} 10^{-3} *M*. (b) ADP peak area versus ADP excess. The arrow indicates the quantity of ADP bound by the injected CF1. Reprinted by courtesy of Marcel Dekker Inc. from Ref. [27].

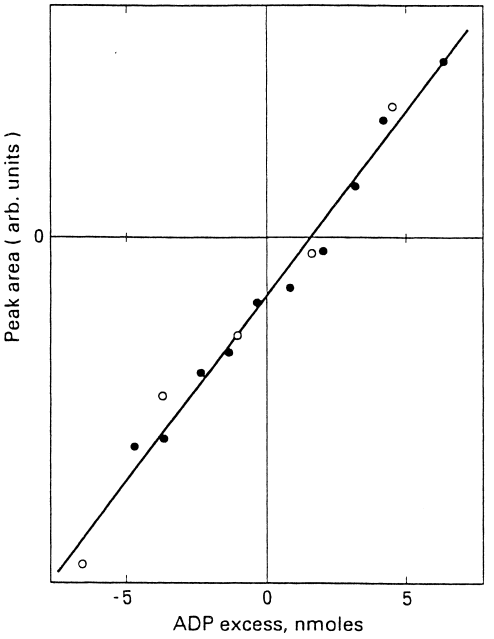

Fig. 2. ADP peak area versus ADP excess. Influence of injection volume. ● 80 μl, ○ 160 μl. Conditions: column TSK 2000 SW (300×7.5 mm). Eluent: Tris buffer $25\cdot10^{-3}$*M*, pH 8.5, ADP $0.57\cdot10^{-4}$ *M*.

the nucleotide peak was comparable to that when no protein was added. However, besides the rapidly exchangeable sites, the ATPase has irreversibly or slowly exchangeable sites. In our experiments, one to two sites of the protein were filled with firmly bound ADP, which did not intervene in the filling measurements of the exchangeable sites. The remainder of the slowly binding sites (total number=3) did not reach equilibrium with the eluent sufficiently rapidly to modify the size of the negative peak. They were filled slowly during the chromatography, giving rise to small trailing in certain cases, or not filled at all. Only the rapidly exchangeable sites were in equilibrium with the nucleotides of the solvent, were able to bind them rapidly, at the time of injection, and to produce the trough of the nucleotide concentration.

If an unique category of sites is implied in this phenomenon, the Scatchard plot [19], must be linear. In fact we have shown that ADP (as ATP), and its magnesium complex in equilibrium with it, were each able to bind to ATPase. The theory is then more complicate, the fraction *r* of the sites which are filled is given by the relationship [27]:

$$1 - r = r/s \cdot 1/\left(1/K_{\mathrm{D}}^{\mathrm{EMg\ ADP}} + a/K_{\mathrm{D}}'^{\mathrm{EMg\ MgADP}} K_{\mathrm{D}}^{\mathrm{MgADP}}\right)$$

where $K_{\mathrm{D}}^{\mathrm{EMg\ ADP}}$ and $K_{\mathrm{D}}'^{\mathrm{EMg\ MgADP}}$ are, respectively, the dissociation constants of the complexes of the sites of CF1 with the magnesium free and the magnesium complexed ADP, and $K_{\mathrm{D}}^{\mathrm{MgADP}}$ is the dissociation constant of MgADP.

Under our conditions of measurement, *a*, the

concentration of the magnesium salt is quite constant (1 m*M*) and the plot of r/s versus r must be linear. It must be the same for nr/s versus nr, when n sites are present on the protein. nr represents then the quantity of ligand bound per mol of protein. Fig. 3 shows that this representation is linear. By extrapolation on the x-axis, the total number of exchangeable sites has been found to be 3 ± 0.3.

From the slope of the Scatchard diagram for different magnesium salt concentrations, $K_D'^{EMg\ MgADP}$ can be calculated with sufficient accuracy ($K_D'^{EMg\ MgADP}=64\ \mu M$) [27]. However the dispersion of the results did not allow the determination of K_D^{EMgADP}, which has been calculated from the inhibition of the enzymatic rate of ATPase by ADP ($K_D^{EMg\ ADP}=5–15\ \mu M$) [27]. These values of the dissociation constants showed that in the conditions of the measurements used here, the nucleotide was bound on the protein mainly on the MgADP form [27].

We have seen that the non or slowly exchangeable sites were not visible by this method. However, if the additional nucleotide used to calibrate the negative peak was incubated several minutes with the protein

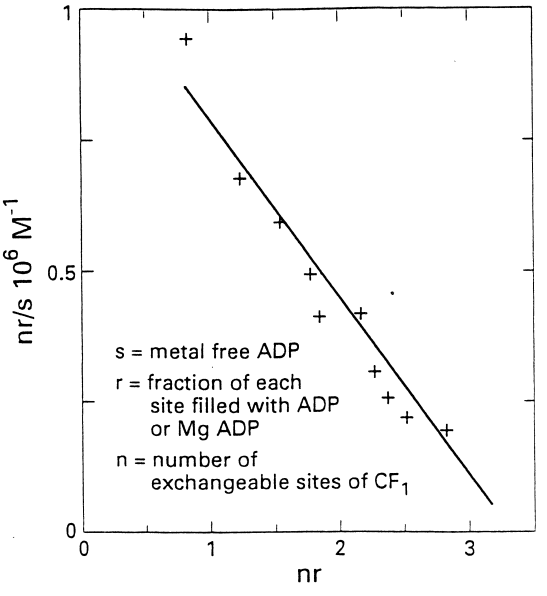

Fig. 3. Scatchard plot of CF1–ADP binding measurements by the Hummel and Dreyer method. Same conditions as in Fig. 1, ADP variable. Reprinted by courtesy of Marcel Dekker Inc. from Ref. [27].

before the injection on the column, allowing the slowly exchangeable sites to be filled, another kind of Scatchard plot was obtained, with two slopes, corresponding to two kinds of sites (but the slope corresponding to the slowly exchangeable sites did not correspond exactly to an equilibrium). The total number of sites obtained by extrapolation was then 6 ± 0.5 sites [28].

3.2. ATP binding

As with ADP, the injection of CF1 on a column equilibrated with ATP gave rise to an uptake of nucleotide and the same type of chromatogram was obtained (Fig. 4).

The validity of the Hummel and Dreyer method requires that no ulterior modification of the ligand could occur. CF1, prepared as described, is a latent ATPase, with very low hydrolytic activity. We have determined by HPLC, using a TSK DEAE 2SW column, with 70 m*M* KH_2PO_4 and 300 m*M* NaCl as eluent, that in the conditions used here, CF1 hydrolyzed ATP at the rate of 0.13 mol/min (for 1.5 nmol CF1 injected). In the short time needed for mixing and injecting (less than 15 s), the quantity of hydrolyzed ATP was negligible in front of that which was added. Hydrolysis could occur afterwards, during the chromatography, but it would not modify the initial uptake of ATP by CF1, from the solvent, which determined the size of the negative peak (however, it would be different if CF1 and ATP were incubated several minutes before injection on the column).

Thus the theory of Hummel and Dreyer can be applied to the study of the binding of ATP by CF1. Assuming that both metal free ATP and MgATP complex are able to bind to CF1, the same type of relationship as with ADP was obtained. The Scatchard plot of nr/s versus nr was linear (Fig. 5). A plot of the slopes of the Scatchard diagrams as a function of the magnesium salt concentration gave the dissociation constants corresponding to the metal free and metal complexed forms $K_D^{EMg\ ATP}=14\ \mu M$, $K_D'^{EMg\ MgATP}=180\ \mu M$ [29]. From these values, the respective fractions of ATP and MgATP bound on CF1 could be calculated in different conditions. Under our conditions of the binding measurements ($Mg^{2+}\approx1$ m*M*, $ATP_{total}<10^{-4}\ M$), ATP was bound

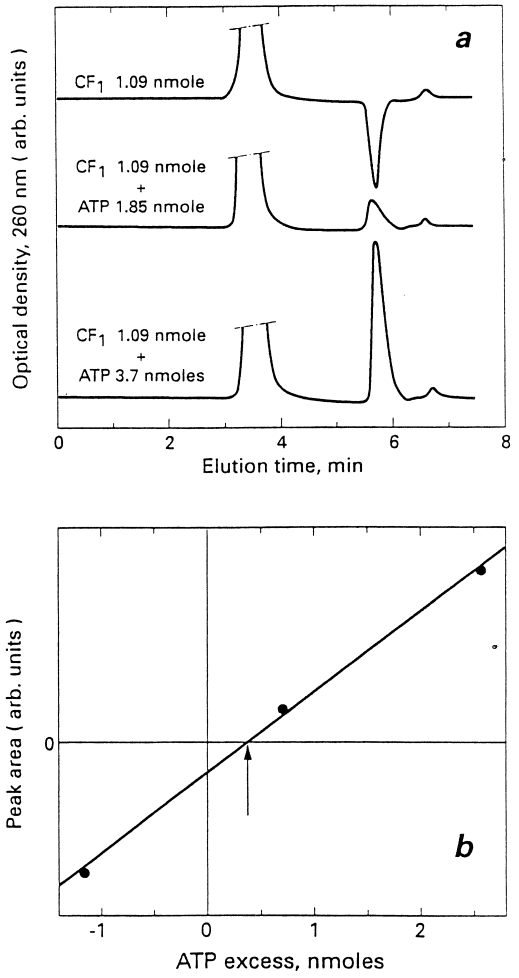

Fig. 4. ATP–CF1 binding measurements by the chromatographic method of Hummel and Dreyer. (a) Chromatographic profiles. Conditions: column: TSK 2000 SW (300×7.5 mm), eluent: Tris buffer $75\cdot10^{-3}$ *M*, pH 8.5, ATP $2.28\cdot10^{-4}$*M*, Mg^{2+} $0.4\cdot10^{-3}$ *M*. (b) ATP peak area versus ATP excess. The arrow indicates the quantity of ATP bound by the injected CF1. Reprinted by courtesy of Marcel Dekker Inc. from Ref. [29].

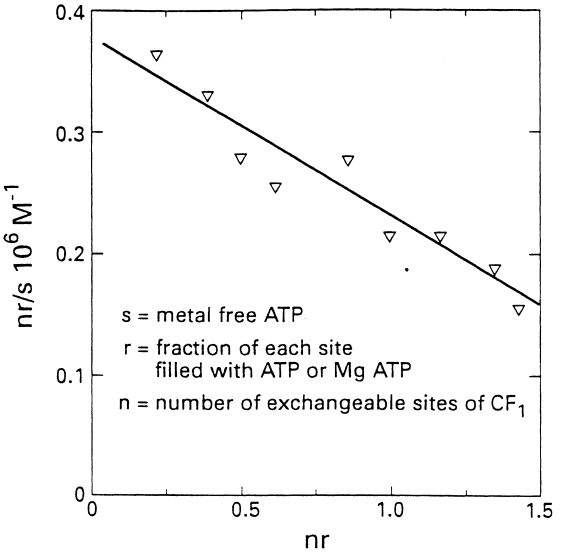

Fig. 5. Scatchard plot of CF1–ATP binding measurements by the Hummel and Dreyer method. Same conditions as in Fig. 4, ATP variable. Reprinted by courtesy of Marcel Dekker Inc. from Ref. [29].

on the enzyme predominantly under the MgATP form (>70%).

3.3. Simultaneous binding of ADP and ATP

3.3.1. Comparison of the gel filtration and the anion-exchange methods

When ADP and ATP are simultaneously present, the method of Hummel and Dreyer cannot be used: these two ligands have similar molecular masses and absorption spectra. They cannot be separated or differentiated by a gel filtration chromatography. We have thus tried to extend the method to anion-exchange chromatography, which allows easy separation of the two nucleotides. When the column was equilibrated with the nucleotide, the injection of the protein gave rise to a negative peak, showing that a fraction of the nucleotide has been bound by the protein. The difference with the gel filtration chromatography was that the protein–nucleotide complex was not eluted and remained at the head of the column. It was not necessary to elute this complex before performing the following assay: when the baseline was constant: the complex protein–nucleotide did not bind nor release any nucleotide and did not intervene in the following binding measurements. The determinations of the *r* values of the curve of Fig. 6 were independent on the order they were performed. With a TSK DEAE 2SW column 250× 4.5 mm, eluted by 0.075 Tris sulfate, pH 8.5 containing nucleotides and $MgCl_2$ 1 m*M*, more 30 assays with 1 to 2 mg of CF1 can be performed successively before the protein began to be eluted. We have compared the binding of ADP alone,

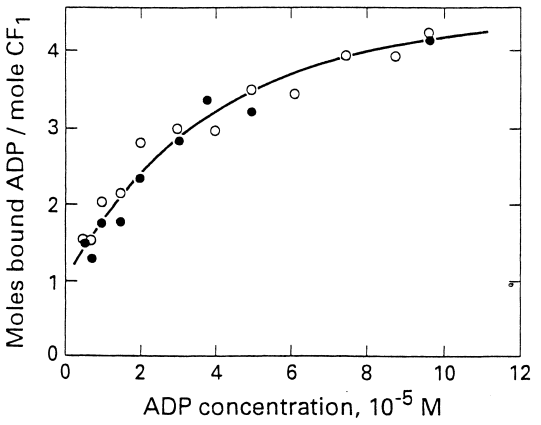

Fig. 6. Comparison of ADP binding on CF1, versus ADP concentration. ● Measured by gel filtration chromatography; ○, measured by anion-exchange chromatography. Conditions: eluent as in Fig. 1. Two different batches of CF1 have been used. The contents of endogenous ADP have been included. Reprinted by courtesy of Marcel Dekker Inc. from Ref. [30].

measured by the gel filtration method and the anion-exchange separation. Fig. 6 shows that is was quite identical, with two different batches of CF1. $K_D'^{EMg\ MgADP}$ values calculated from the Scatchard plots were 45 and 52 μM, respectively, for the gel filtration and the anion-exchange chromatographies. This means that the binding of CF1 on the DEAE column did not modify the equilibrium between the protein and the nucleotide [30].

The same comparison between gel filtration and anion-exchange has been performed with ATP. There was no ADP positive peak when CF1 was injected on a DEAE column equilibrated with ATP. This shows that endogenous ADP present on CF1 (1 to 2 mol/mol of CF1) was not exchanged with ATP and that the catalytic activity of CF1 was negligible under our conditions.

3.3.2. Measurement by anion-exchange chromatography of simultaneous ADP and ATP binding

When the eluent contained a mixture of ADP and ATP (here in approximately equal concentrations), the injection of CF1 led to the formation of two negative peaks, due to the simultaneous binding of the two nucleotides. The amount of each nucleotide bound by CF1 was measured as above by adding known quantities of one of them: one negative peak decreased, was null when the bound quantity was equal to the excess of nucleotide in the injected volume over that contained in the same volume of solvent, and became positive (Figs. 7 and 8). Fig. 9 shows the number of moles of each nucleotide, alone or in equimolecular mixture, bound per mole of CF1. The binding of ADP was larger than that of ATP, alone or in competition, which is consistent with the K_D values ($K_D'^{EMg\ MgADP} = 64\ \mu M$, $K_D'^{EMg\ MgATP} = 180\ \mu M$). The competition with ATP reduced the binding of ADP, as expected, but the reverse for ATP was not evident [27].

Remark: Figs. 7 and 8 show that when one nucleotide was mixed with CF1 before injection on the column, the negative peak corresponding to the other one decreased slightly, although it should be constant and give an horizontal line. This phenomenon, which is minor, could be due partly to the above mentioned low activities of the CF1 batches (ATPase activity and adenylatekinase activity) or to

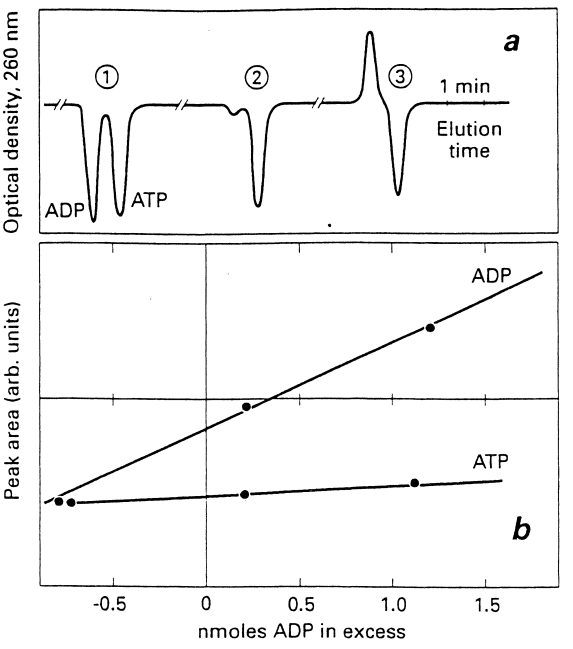

Fig. 7. CF1–ADP binding measurements in the presence of ATP. (a) Chromatographic profile of CF1–ADP mixture on an anion-exchange column equilibrated with $1.59 \cdot 10^{-5}$ M ADP and $1.44 \cdot 10^{-5}$ M ATP. Conditions: buffer, as in Fig. 1; injected CF1 1.55 nmol. (1) Alone, (2) with 1.01 nmol ADP, (3) with 2.02 nmol ADP. (b) ADP peak area versus ADP excess. Reprinted by courtesy of Marcel Dekker Inc. from Ref. [30].

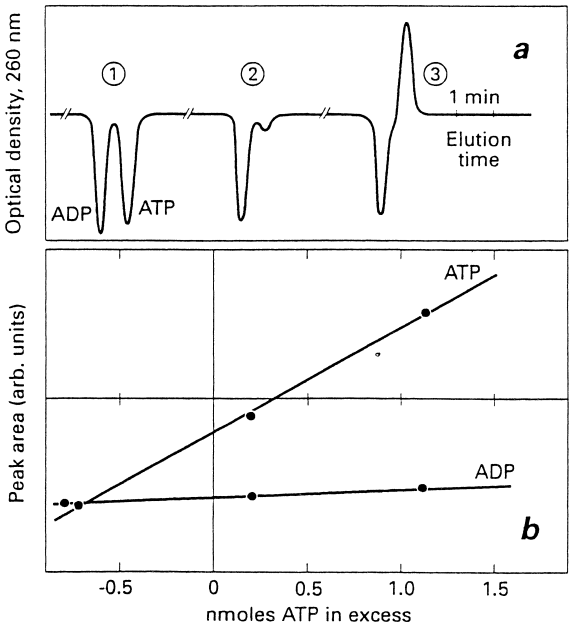

Fig. 8. CF1–ATP binding measurements in the presence of ADP. (a) Chromatographic profile of CF1–ATP on an anion-exchange column equilibrated with $1.59 \cdot 10^{-5}$ *M* ADP and $1.44 \cdot 10^{-5}$ *M* ATP. Conditions: buffer as in Fig. 1; injected CF1 1.55 nmol. (1) Alone, (2) with 0.92 nmol ATP, (3) with 1.84 nmol ATP. (b) ATP peak area versus ATP excess. Reprinted by courtesy of Marcel Dekker Inc. from Ref. [30].

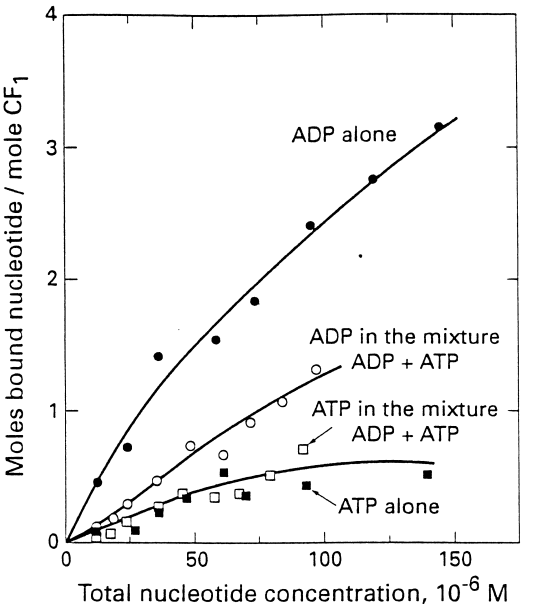

Fig. 9. Number of mol of ADP and ATP, alone or in mixture, bound on CF1, measured by anion-exchange chromatography, versus concentration. Conditions: buffer as in Fig. 1, equimolar concentrations of ADP and ATP. Reprinted by courtesy of Marcel Dekker Inc. from Ref. [27].

impurities of the added nucleotides. It also could be due to the slowly exchangeable sites which retained a part of the nucleotide added for calibration and prevented the other one, present in the solvent, to be bound by the protein, which decreased the size of the corresponding negative peak.

4. Conclusion

We have shown here that the chromatographic method of Hummel and Dreyer, applied to HPLC, was a method of choice for the study of the exchangeable sites of the chloroplast ATPase CF1. The dissociation constants with ADP, ATP and their magnesium salt complexes have been determined. In order to measure the binding of each nucleotide when they are simultaneously present, we have modified the initial method by replacing the gel filtration by an anion-exchange chromatography. The binding of more than two, and of any kind of nucleotides can be studied, the only condition being that the buffer characteristics (pH and ionic strength) allowed the binding of the nucleotides on the protein and a good chromatographic separation (resolution and retention time). One could also envisage extension of the method to reversed-phase chromatography, in the case of hydrophobic ligands.

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References

[1] J. Benett, in: Y. Yamamura, S. Enna, M. Kuhar (Eds.), Neurotransmitter Receptor Binding, Raven Press, New York, 1978, p. 57.
[2] H. Penefsky, J. Biol. Chem. 252 (1977) 2891.
[3] C. Grubmeyer, R. Cross, H. Penefsky, J. Biol. Chem. 257 (1982) 12092.
[4] F. Kironde, R. Cross, J. Biol. Chem. 262 (1987) 3488.
[5] D. Cunningham, R. Cross, J. Biol. Chem. 263 (1988) 18850.
[6] G. Girault, J.M. Galmiche, FEBS Lett. 95 (1978) 135.
[7] G. Girault, J.M. Galmiche, Eur. J. Biochem. 77 (1977) 501.
[8] T. Hisabori, H. Kobayashi, C. Kaibara, M. Yoshida, J. Biochem. 115 (1994) 497.
[9] T. Hisabori, M. Mochizuki, in: N. Murata (Ed.), Research in Photosynthesis, Vol. 2, Kluwer, Dordrecht, 1992, p. 725.
[10] T. Hisabori, M. Yoshida, H. Sakurai, J. Biochem. 100 (1986) 663.
[11] G. Girault, J.M. Galmiche, M. Michel Villaz, J. Thiery, Eur. J. Biochem. 38 (1973) 473.
[12] T. Hisabori, E. Muneyuki, M. Okada, K. Yokoyama, M. Mochizuki, M. Yoshida, J. Biol. Chem. 267 (1992) 4551.
[13] J. Geczi Digel, R. Mc Carty, Biochemistry 34 (1995) 14482.
[14] J. Weber, S. Wilke Mounts, E. Grell, A. Senior, J. Biol. Chem. 269 (1994) 11261.
[15] J. Weber, S. Wilke Mounts, R. Lee, E. Grell, A. Senior, J. Biol. Chem. 268 (1993) 20126.
[16] J. Weber, C. Bowman, S. Wilke Mounts, A. Senior, J. Biol. Chem. 270 (1995) 21045.
[17] D. Mills, S. Seibold, T. Squier, R. Richter, Biochemistry 34 (1995) 6100.
[18] H. Hummel, W. Dreyer, Biochim. Biophys. Acta 63 (1962) 530.
[19] G. Scatchard, Ann. N.Y. Acad. Sci. 51 (1949) 660.
[20] B. Sebille, N. Thuaud, J.P. Tillement, J. Chromatogr. 167 (1978) 159.
[21] B. Sebille, N. Thuaud, J.P. Tillement, J. Chromatogr. 180 (1979) 103.
[22] A. Binder, A. Jagendorf, E. Ngo, J. Biol. Chem. 253 (1958) 3094.
[23] G. Berger, G. Girault, F. André, J.M. Galmiche, J. Liq. Chromatogr. 10 (1987) 1507.
[24] R. Piccioni, G. Bellemare, N.H. Chua, in: M. Edelman (Ed.), Methods in Chloroplast Molecular Biology, Elsevier Biomedical Press, 1982, p. 985.
[25] M. Bradford, Anal. Biochem. 72 (1976) 248.
[26] T. Pinkerton, K. Koeplinger, Anal. Chem. 62 (1990) 2114.
[27] G. Berger, G. Girault, J.L. Zimmermann, J. Liq. Chromatogr. Rel. Technol. 23 (2000) 1639.
[28] G. Girault, G. Berger, J.M. Galmiche, F. André, J. Biol. Chem. 263 (1988) 14690.
[29] G. Berger, G. Girault, S. Pezennec, J.L. Zimmermann, J. Liq. Chromatogr. Rel. Technol. 21 (1998) 1925.
[30] G. Berger, G. Girault, J.M. Galmiche, J. Liq. Chromatogr. 12 (1989) 535.