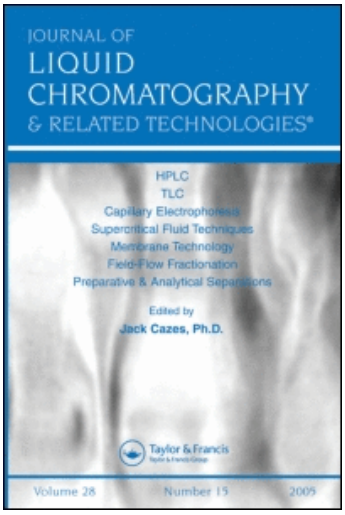

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Application of HPLC to the Study of the Chloroplast Atpase Mg^2 Dependent Mechanism

G. Berger[a]; G. Girault[a]; J. M. Galmiche[a]
[a] Service de Biophysique Département de Biologie, Cellulaire et Moléculaire CEN, Gif-sur-Yvette Cedex, France

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APPLICATION OF HPLC TO THE STUDY OF THE CHLOROPLAST ATPASE Mg^{2+} DEPENDENT MECHANISM

G. BERGER, G. GIRAULT, AND J. M. GALMICHE
Service de Biophysique
Département de Biologie Cellulaire et Moléculaire
CEN Saclay
91191 Gif-sur-Yvette Cedex, France

ABSTRACT

The determination by HPLC of released ADP from ATP by chloroplast ATPase (CF_1) is described.

The enzymatic rate measured by this method is well defined, over several minutes.

In the case of ϵ subunit-depleted CF_1 or activated CF_1, the rate is proportional to the enzyme concentration, the steady state theory is followed and the K_m and V_m constants have been calculated.

The enzymatic activity of CF_1 is inhibited by endogenous ϵ subunit and the inhibition constant has been measured.

The influences of ionic strength, pH, magnesium ion, phosphate and ADP concentrations have been studied and the results obtained by this method have been compared to previously reported data based on rate determination of released phosphate.

INTRODUCTON

ATP synthases (F_o-F_1) present in membranes of chloroplasts, bacteria and mitochondria synthesize ATP coupled with a gradient of protons generated by the electron transfer chain. On the other hand, the soluble part (F_1) of these enzymes hydrolyzes ATP into ADP and phosphate. The mechanism of F_1 ATPases has been extensively studied and different kinetic methods have been used to determine the rate constants of the reaction. These methods include :

a) those in which the phosphate released from ATP was measured (1) without separation from other reaction products, (2) after solvent extraction of the

complexed Pi, (3) precipitation of this complex, or (4) after adsorption of the reaction products on charcoal.

b) Those in which the acidification of the medium due to hydrolysis of ATP was determined by a pH indicator (5) or using a pH-Stat (6).

Several authors have used an ATP regenerating system such as phosphoenol pyruvate and pyruvate kinase to maintain a constant substrate concentration. The reaction was followed by the release of phosphate (7) or by the decrease of absorbance of NADH in the presence of lactate dehydrogenase (8).

We report here a method where the product of ATP hydrolysis, ADP, is chromatographically determined using HPLC. This method has the advantage of being simple and allows the analysis of complex mixtures of solutes.

MATERIALS AND METHODS

ATPase activities are measured at 37°C in 0.075 M Tris sulfate buffer pH 8.5, $MgSO_4$, 1mM, with ATP concentrations ranging between 10^{-5} and 10^{-3}M.

Soluble chloroplast ATPase (CF_1), ε or δ subunit depleted CF_1 (CF_1-ε or CF_1-δ) are purified by HPLC (9). They are stored at 5°C in ammonium sulfate 50% saturation, dialyzed overnight before use against adequate buffer and centrifuged.

Assays are also performed with activated CF_1 (1 mg in Tricine 33 mM, pH 8, EDTA 1.6 mM, ATP 8.4 mM, dithiothreitol 12.5 mM, heated 4 min at 60°C).

Aliquots of the reaction mixture are directly injected on a TSK DEAE 2SW column (30 x 0.4 cm). The nucleotides are separated by isocratic elution with PO_4H_2K 0.1M, NaCl 0.25 M, at the rate of 1.5 ml/min. The reaction is immediately stopped after injection, by the separation of the enzyme from the substrate and by the drop of pH (4.3). The concentration of ADP is measured by the height of the peak, a calibration curve is established in the same conditions.

ATP and ADP concentrations of standards are calculated from the absorption at 260 nm, with $E^{M}_{1cm} = 15{,}400$. They are corrected from presence of AMP and ATP in ADP and from ADP in ATP, determined by HPLC.

The chromatographic apparatus consists in a M510 pump, U_6K injector and M 480 or 490 E detector, at 260 nm (Waters).

Protein dosages are performed with Bio Rad protein assay (10), using bovine serum albumin as a standard, or by u.v. spectrum, assuming for CF_1 or derivatives an $E^{1‰}_{1cm} = 0.48$ at 280 nm.

Endogenous ADP or ATP bound to CF_1 are measured by the same chromatographic method (usually 1 to 1.5 mole ADP/mole CF_1, 0.3 mole ATP/mole CF_1, in the conditions of preparation).

RESULTS

As shown in Fig. 1, the procedure described allows rapid separation of the nucleotides in the mixture in less than five minutes and the resolution between

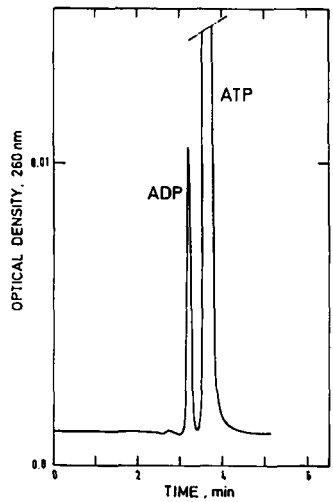

Fig. 1 : Separation of ADP from ATP by HPLC. Conditions described in the text.

ADP and ATP is greater than 2. Aliquots of the reaction mixture can be injected at intervalls of 2 ½ min without overlapping of the peaks. CF_1 is eluted under these conditions at the exclusion volume of the column and inorganic phosphate is not detectable.

The amounts of ADP carried along with CF_1 are negligible and the amount in ATP carried as an impurity (≈ 2%) does not interfere with the measurement of the reaction rate. The separation between ATP and ADP is not perturbed by changing the conditions in the reaction mixture (pH or molarity), or by the volumes of aliquots injected.

The amount of released ADP increases linearly with time, up to a degree of hydrolysis of about 10% (Fig. 2). The initial enzymatic rate is thus well defined. Beyond, the rate decreases, because of the consumption of ATP and of inhibition of the reaction by the released ADP. No lag time was observed under these conditions : the initial concentration of ADP (at zero time) corresponds to that of ATP. The precision of the injection time is about 10 seconds.

The concentrations of enzyme were chosen to produce a linear release of ADP with time for about 10 min of reaction.

Inhibition of CF_1 ATPase activity by ε subunit

The equations of the steady state theory imply proportionality between the enzymatic reaction rate and the total concentration of enzyme.

$$v = \frac{k\, e_t}{1 + \frac{K_m}{s}} \qquad \text{(equation a)}$$

where e_t = total enzyme concentration

s = substrate concentration

We did not verify this relation with CF_1. As indicated in Fig. 3, the specific activity decreased as the concentration of CF_1 increased. This phenomenon could be due to inhibition by partial dissociation of ε subunit.

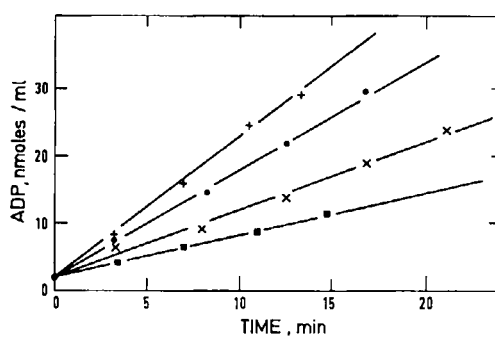

Fig. 2 : Released ADP concentrations versus time. Buffer : Tris sulfate 0.075 M, $MgSO_4$ 1 mM, ATP 218 µM
CF_1 concentration ■ 0.045, × 0.089, ● 0.178, + 0.349 mg/ml.

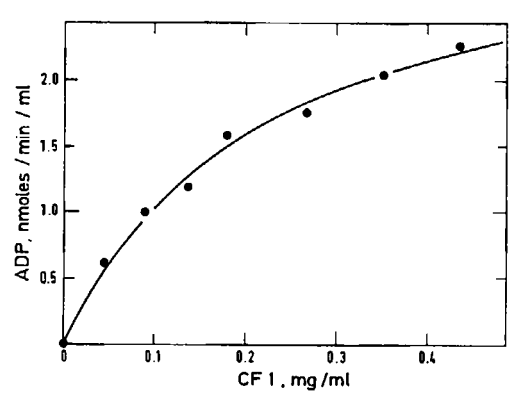

Fig. 3 : ATPase activity versus CF_1 concentration. Same conditions as in Fig. 2.

Inhibition by endogenous ε subunit has been shown with CF_1 (11-14) and with E. coli F_1 ATPase (15-20), and this subunit is easily dissociable, as shown by washing adsorbed CF_1 on a DEAE column with low ionic strength alkaline buffer (9).

Regardless of the mechanism of inhibition, equations of Henderson (21) for the tight-binding inhibitors are applicable :

$$\frac{i_t}{1 - \frac{v}{v_o}} = e_t + K\left(\frac{v_o}{v}\right) \qquad \text{(equation b)}$$

where i_t and e_t are the total inhibitor and enzyme concentrations, v and v_o are the rates with and without inhibitor, and K is a function of s, K_m and K_i, the inhibitor dissociation constant. In our particular case, CF_1 dissociates partially into CF_1- ε (the active enzyme, concentration e) and ε (inhibitor, concentration i), and since there is one ε subunit per CF_1,

$$e_t = i_t = (CF_1)_{total} = c$$

Applying the Henderson equation gives :

$$c = c + K\frac{v_o}{v} - c\frac{v}{v_o} - K$$

v_o is the rate without inhibitor and is proportional to the concentration of total CF_1- ε (see below)

$$v_o = k'e_t = k'c \qquad \text{with } k' = \frac{k}{1 + \frac{Km}{s}}$$

$$\text{then, } K\left(k'\frac{c}{v} - 1\right) = \frac{v}{k'} \qquad \text{(equation c)}$$

As can be seen on Fig. 4, a plot of v versus c/v is linear. The intersection with the x axis is 1/k' and with the y axis is -Kk'. The product of these two values is then -K.

K is roughly independent of substrate concentration (10.4, 7.8, 8.8 10^{-8}M for respective concentrations of ATP of 0.0482, 0.194 and 0.97 10^{-3}M). The inhibition is thus non competitive and K represents the inhibition constant K_i (21). However, a value of 4.2 10^{-8}M has been found with a different preparation of CF_1.

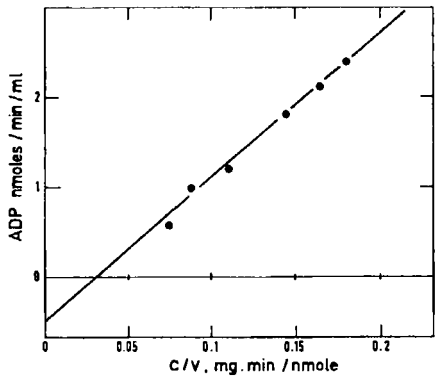

Fig. 4 : Determination of ε inhibition constant. The intersections with the x and y axis are respectively 1/k' and -Kk'. The product is -K. Same conditions as in Fig. 2.

Different values of the inhibition constant of this system have been obtained with other methods : less than $5\ 10^{-11}$M (14), $1.4\ 10^{-10}$M (13) for CF_1, and values ranging between $0.2\ 10^{-9}$M and 1.10^{-8}M for E. coli ATPase (16, 17, 19, 20). These discrepancies could be due either to differences in rate measurement or the conditions used to attain equilibrium, or to the method of calculation.

Rate of hydrolysis with CF_1- ε , activated CF_1 and CF_1-δ

The rate of hydrolysis is practically proportional to the concentration of CF_1- ε (Fig. 5), (which justifies the use of $v_o = k\ e_t$). The same linear relationship is also observed with higher CF_1- ε concentrations (comparable to those of CF_1 in Fig. 3) provided that sufficient ATP concentration is used (10 mM). In comparison with CF_1, at same substrate concentration, a specific activity of 10 to 20 fold higher was observed.

In the case of activated CF_1, the activity varies also linearly with the amount of enzyme, except at very low concentrations. This could be explained by the presence in the mixture of a fraction of non activated CF_1. The specific activity of activated CF_1 is slightly lower than that of CF_1- ε at similar concentrations of substrate. As judged by polyacrylamide gel electrophoresis, the ε subunit is still present, although its binding is weakened (13,14) and therefore the inhibition is almost abolished.

The activity of CF_1-δ complex is comparable to that of CF_1, for the same concentration of substrate and enzyme, which confirms that inhibition is due to the ε subunit.

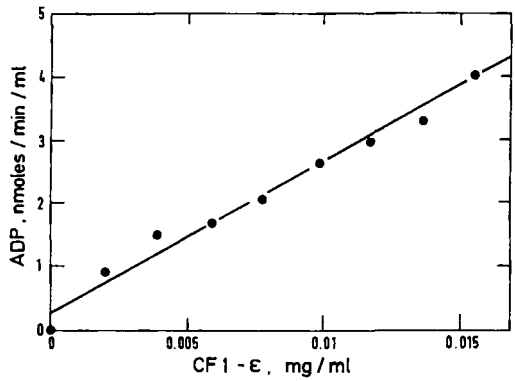

Fig. 5 : ATPase activity versus CF_1- ε concentration. ATP 417 µM. Other conditions as in Fig. 2.

The equations of the steady state theory can be applied to CF_1- ε and to activated CF_1. The Lineweaver-Burk plots (1/v versus 1/s, from equation a) are linear in the range of ATP concentrations between 5 10^{-5}M and 5 10^{-4}M for CF_1- ε (Fig. 6) and for activated CF_1 (Fig. 7). The respective K_m values are 0.9 10^{-3}M and 1.4 10^{-3}M. These values are similar to those already reported for activated CF_1 : 1.1 with Tris-maleate buffer (22), 1 10^{-3}M and 0.21 10^{-3}M with Tricine-NaOH (23,24). However, the V_m values found here (0.4 and 0.3 µmoles/min/mg) are between one and two orders of magnitude lower than previously published values (22,24,25).

A part of this discrepancy can be ascribed to differences in the conditions of the measurement, eg : molarity, pH, nature of the buffer, concentration of magnesium ion (22) and in some cases to the presence of known stimulatory agents such as octylglucoside (26) or organic solvents such as methanol (27,28) or dimethylsulfoxide (29). Nevertheless, under identical conditions, some discrepancy still remains. We have verified that our preparation of CF_1- ε had no adenylate kinase activity which might explain a difference between rates of release of ADP and phosphate. On the other hand, Zhou and coll. reported that under certain conditions, the rate of phosphate release was not linear with time: "A rapid burst of P_i formation is followed by a much slower constant steady state rate" (30). Thus, the rate of P_i release was time dependent.

In the presence of octylglucoside (12.5 mM), the rate of ADP released with CF_1- ε increased extensively, when determined chromatographically (17 µmoles/min/mg, for an ATP concentration of 5 10^{-4}M, Mg SO_4 1 mM), without reaching the very high levels obtained by other methods (120 µmoles/min/mg (26).

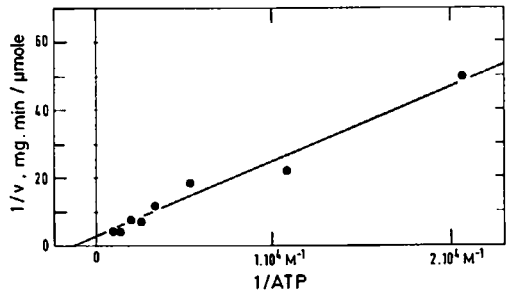

Fig. 6 : Lineweaver-Burk plot of 1/v versus 1/s, with CF_1- ε (0.089 mg/ml). Other conditions as in Fig. 2.

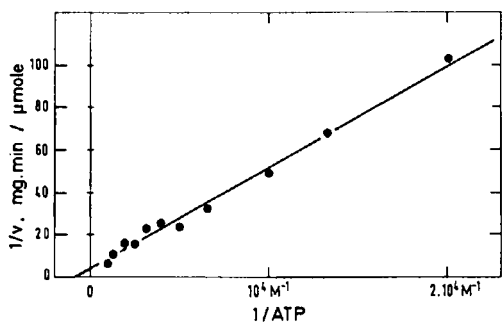

Fig. 7 : Lineweaver-Burk plot of 1/v versus 1/s, with activated CF_1 (0.122 mg/ml). Other conditions as in Fig. 2.

However detergents as organic solvents surely modify normal binding and velocity constants and they have not been used throughout this study.

Influence of measurement conditions

ATPase activity of CF_1- ε increases slightly by increasing the Tris concentration, between 25 and 100 mM, at pH 8.5.

The role of magnesium ions is rather complex : at low concentrations, Mg^{2+} stimulates the rate of the reaction by virtue of the formation of the Mg ATP complex which acts as the hydrolysable substrate. At higher concentrations, it binds to the enzyme at a specific inhibitory site (31). Assuming that Mg ATP is the only hydrolysable substrate and that the enzyme-magnesium ion complex is inactive, the steady state theory leads to the equation (32).

$$V = \frac{k\, e_t}{1 + \frac{K_m^{MgATP}}{(Mg\ ATP)}\left(1 + \frac{Mg^{2+}_{free}}{K_i^{Mg^{2+}}}\right)} \qquad \text{(equation d)}$$

where $K_i^{Mg^{2+}}$ is the dissociation constant of the enzyme-magnesium ion complex. In the range of ATP concentrations between 5 10^{-4}M and 5 10^{-5}M and for a constant total magnesium ion concentration of 10^{-3}M (as used in this study), most of the ATP is in the Mg ATP form and the inhibition is rather negligible : indeed the dissociation constant of Mg ATP, at temperatures and ionic strengths similar to ours, is $10^{-4,7}$M (33). The fraction of complexed ATP is 0.963 and 0.979 for concentrations of 5 10^{-4}M and 5 10^{-5}M respectively. $K_i^{Mg^{2+}}$ has been evaluated to be 15 10^{-3}M with heat activated CF_1 (1/v versus Mg^{2+}_{free} plot, data not shown), which agrees with the value of 20.8 10^{-3}M precedently reported (23). The error brought by using the simple steady state equation a can be evaluated to 5% at 5 10^{-4}M and 7.6% at 5 10^{-5}M. Equation d is thus practically equivalent to equation a, the above indicated K_m being related to Mg ATP.

At pH 6.5, ATP is partially in the protonated form (pK = 6.63, (33)) and the complex with magnesium ion has a dissociation constant higher than that of MgATP ($10^{-2,79}$M, (33)). Although the fraction of available hydrolysable substrate is lower, the activity of CF_1- ε is about twofold higher at pH 6.5 than at pH 8.5. The enhancement in activity is probably due to the effect exerted on the enzyme.

Phosphate ion has been shown to bind to CF_1 (34), in particular to the β subunit (35). Under the conditions of our measurements and in the range 0-15 mM, phosphate has a slight inhibitory effect on CF_1- ε activity, and this effect is enhanced at low concentrations of ATP (data not shown), which is consistent with the competition of binding observed between phosphate and nucleotides (34,35). However, a stimulatory effect of phosphate, in the same range of concentrations, has been reported with membrane bound CF_1 (36). The stimulation by phosphate, in this case, could be due to prevention of inhibition by magnesium ion (reduction of Mg^{2+}_{free} , increase of $K_i^{Mg^{2+}}$ (31), which does not occur significantly in our conditions (1 mM Mg^{2+}).

Inhibition by ADP

It is well known that ADP inhibits ATPase activity of activated CF_1 (22,30,37,38). Its formation, as well as ATP depletion, explain the decrease of the enzymatic reaction rate when hydrolysis proceeds beyond around 10%.

Whether ADP inhibits by binding to catalytic sites (30) or to other sites (38) was not fully established. Dixon plots of 1/v versus ADP concentration (39) are linear and the intersection of 3 straight lines obtained with different ATP concentrations gives a K_I, the inhibition constant, of about 0.75 10^{-5}M (Fig.

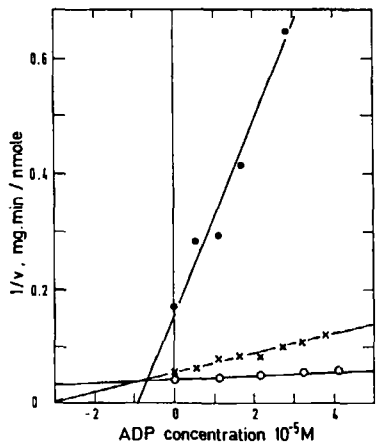

Fig. 8 : Dixon plot of 1/v versus ADP concentration, with activated CF_1 (0.122 mg/ml). ATP concentrations : O 0.978 10^{-4}M, × 0.489 10^{-4}M, ● 0.193 10^{-4}M.

8). This point is above the x axis, which means that the inhibition may be either competitive or mixed. However, this does not allow to discern between different sites of binding of ADP on CF_1.

DISCUSSION

Although HPLC has already been used for the determination of nucleotides (40) or of ATP-AMP phosphotransferase of tonoplasts (41,42), it had not yet been applied to steady state catalysis of purified chloroplast ATPase.

The determination by HPLC of ADP released by the ATPase reaction described here is simple and rapid. Under these conditions, the rate is well defined, over several minutes, as long as the percentage of hydrolysis is below 10% of the maximum value. The conditions during the enzymatic assay do not perturb the chromatographic separation so that modifications of the amounts of ADP released can be directly ascribed only to the modifications of the enzymatic rate.

In the range of ATP concentrations between 5 10^{-5}M and 5 10^{-4}M, and under the conditions used (Tris buffer 0.075 M, pH 8.5, Mg^{2+} 10^{-3}M), CF_1- ε and activated CF_1 follow steady state kinetics, with no apparent cooperativity. Their K_m are around 10^{-3}M, similar to values already reported. However, the V_m values obtained by chromatographic determinations are 20 to 50 times smaller than those reported for phosphate release. The difference in the conditions of measurement used (concentration of Mg^{2+}, pH, presence of detergents or organic

solvents) can account for a part of this discrepancy. On the other hand, under certain conditions, phosphate release is not proportional with time and the enzymatic reaction rate is different, when measured initially or after a longer period.

Mg^{2+} ions act both as an activator, by complexing to ATP, and as an inhibitor by binding to enzyme. With 1 10^{-3}M Mg^{2+}, all the ATP is under the Mg ATP form and inhibition by Mg^{2+} is negligible.

The inhibition by ADP measured by the chromatographic method is competitive or mixed (K_i around 0.75 10^{-5}M), which is consistent with previously described data.

The activity of CF_1 is inhibited by the endogenous ε subunit, so that the hydrolysis rate is not proportional to the concentration of enzyme as for CF_1- ε or activated CF_1, and the steady state theory formulas cannot be applied directly. The inhibition constant found by our method (5 10 10^{-8}M) is greater than those found by others (13,14). The discrepancy may be due to differences in rate measurement, to equilibrium conditions (especially pH), or to the method of calculation.

No assumption has been made in this study with regard to the nature of the catalytic sites on the enzyme. 6 nucleotides binding sites (ADP and ATP) have been shown, by the chromatographic method of Hummel and Dreyer applied to HPLC (43,44), to exist on CF_1, CF_1- ε , CF_1-δ. They can be shared into high affinity sites (3 sites with K_D in the micromolar range) and low affinity sites (3 sites with K_D^{ADP} around 20 µM and K_D^{ATP} around 100 µM, under the same conditions as those used here). With ATP concentrations between 5 10^{-5}M and 10^{-3}M, low affinity sites are partially to totally filled. The question is raised whether and how they could participate, with the high affinity sites, in the hydrolytic reaction.

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