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Catalytic and Regulatory Sites in CF1

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The Ca^{2+}-ATPase activity of the trypsin-activated CF1 presented a monophasic pattern, indicating that the active centres of the enzyme were acting with the same kinetic properties. The study of the effect of the anions cianate (OCN^-) and thiocyanate (SCN^-) on the ATPase activity showed the existence of cationic regulatory sites, capable of binding these modulators in a competitive way, resulting in the inhibition of the ATPase activity. Nucleotides ADP and ATP, at high concentrations, were competitive inhibitors for the substrate Ca^{2+}-ATP. ATP, at low concentrations, presented an activating effect. The study of the combined effects of ATP (at low concentrations) and SCN^- on ATPase activity revealed the existence of a non-competitive relationship between anions and nucleotides. The modification of CF1 with fluorescein isothiocyanate, a specific reagent that binds to amino groups of nuclotide binding centres, yielded a molar relationship FITC/CF1 = 4, both with the trypsin-treated and non treated enzyme. This specific incorporation took place on the α and, β subunits of CF1, and resulted in a decrease of about 30% of the ATPase activity. These results are consistent with the existence of either three catalytic and three regulatory sites or four catalytic and two regulatory sites on CF1.

Key words: CF1, Regulatory site, Catalytic site, FITC.

CF1 is the soluble portion of the ATP synthase of chloroplast, the enzyme complex responsible for the ATP synthesis during the photophosphorylation. Treatment of chloroplasts with EDTA releases CF1 from the thylakoid membrane as a soluble protein (4). Purified CF1 has Ca^{2+}-ATPase activity after being activated by different treatments, including incubation with reductant reagents (19, 20) and limited proteolysis with trypsin (18).

CF1 complex contains five different subunits named α, β, δ and ε, according to their increasing mobility in SDS-polyacrylamide gels. The stoichiometry of the subunits is probably α_3, β_3 γδε (25). The γ, δ and ε subunits are involved in the regulation of the activity of the enzyme (7, 21, 26). The larger subunits, α and β, have

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been reported to contain the catalytic and regulatory sites of the complex.

Three clearly defined nucleotide binding sites in CF_1 have been characterized (13, 14, 22). Site 1 contains tightly bound ADP, which readily exchanges with medium nucleotides during catalysis; it has ATPase and ATP synthesis capabilities. Site 2 is non-catalytic, binds Mg^{2+}-ATP with high affinity and does not exchange its nucleotide even during catalysis. Site 3 binds nucleotides reversibly and is believed to be catalytic and involved cooperatively with site 1. Evidence of additional sites on CF1 have appeared (23, 27) and the presence of six nucleotide binding sites with catalytic and regulatory activity has been suggested (11, 28).

The nucleotide binding sites seem to be present on 13-polypeptides, probably close to a/B interfaces (1, 3).

Although the binding of nucleotides to CF_1 has been extensively investigated, it is difficult to discriminate between nucleotides bound at catalytic and non-catalytic sites, and the number of sites involved in catalysis remains controversial. Kinetic measurements consistent with two (5, 13), three (8, 28) and up to four (23) catalytic sites have been obtained.

In order to gather more information about the catalytic and regulatory sites of CF_1, the effect of different anions and nucleotides on the Ca^{2+}-ATPase activity of the trypsin-activated enzyme has been studied. Modification of CF_1 with fluorescein iso-thiocyanate (FITC), which is known to bind covalently to primary amino groups located at nucleotide binding sites, was also carried out.

Materials and Methods

CF_1 was prepared from fresh market spinach by the method of BINDER *et al.* (4). Protein concentration was determined as described by LOWRY *et al.* (15). Ca^{2+}-ATPase activity was calculated from the inorganic phosphate released in 0.1 M Tris-acetate pH 7.5 and 3 mM substrate concentration. The reaction mixture was incubated at 37 °C for 5 minutes and then stopped by the addition of 0.05 ml of 50 % trichloroacetic acid. Inorganic phosphorus was determined according to FISKE and SUBBAROW (10). Specific activity has been expressed as µmoles of Pi released x min^{-1} x mg^{-1}.

To carry out partial proteolysis of CF_1, 0.25 ml of CF_1-containing solution (1 mg/ml) was incubated at room temperature with 0.25 ml trypsin solution containing 50 µg bovine pancreas trypsin. After 30 minutes, the reaction was interrupted by the addition of 175 µl trypsin inhibitor solution containing 300 µg soy bean trypsin inhibitor in distilled water.

CF_1 was modified with FITC by the addition of this labelling agent dissolved in dimethyl sulfoxide to a 40 mM CF_1 in 100 mM Tris-HCl, 4 mM EDTA pH 8.5, to reach a final concentration of 400 mM. The reaction was carried out at room temperature in the dark and stopped with the addition of ATP at 2 mM final concentration and transfer of the samples to an ice water bath. The unreacted FITC was eliminated passing the protein solution through Sephadex G-50 columns. The stoichiometry of FITC bound to CF_1 was determined by spectrophotometry using a molar extinction coefficient of ε_{492} = 80.000 M^{-1}x cm^{-1}.

Protein electrophoresis was carried out according to LAEMMLI and FAVRE (12) and gels were stained with Coomassie Blue. Fluorescein isothiocyanate labelled proteins were viewed under u.v. light and gels photographied through an u.v. filter.

The activation and inhibition constants have been determined as follows: using a constant concentration of the substrate Ca^{2+}-ATP, plots of $1/v-v_o$ or $1/v_o-v$ vs 1/ activator or inhibitor (where v is the velocity in the presence and v_o velocity in the absence of the activator or inhibitor) were constructed from the data using statistical regression; slope/intercept has been de-

fined as the K_a for the activator or the Ki for the inhibitor (6).

Results

Kinetics of the Ca^{2+}-ATPase activity. — In order to study the kinetic behaviour of trypsin-activated CF1, the representation described by Eadie-Hofstee was used. The Eadie-Hofstee plot of Ca^{2+}-ATPase activity at substrate concentrations between 0.5 and 15 mM is shown in figure 1. The straight line obtained suggests a monophasic kinetics.

Kinetic studies of the effect of SCN^-, OCN^-, ADP and ATP on ATPase activity of CF1 activated by proteolysis with trypsin. — Effect of anions on Ca^{2+}-ATPase activity of CFI. The effect of the anions on the Ca^{2+}-ATPase activity was determined by adding different amounts of the modulators to the incubation medium at 3 mM substrate concentration. Figure 2 shows the inhibitor effect of the anions SCN^- and OCN^-. The nucleotide ATP was found to have a biphasic effect, acting as an activator at low concentrations and as an inhibitor at concentrations higher than 6 mM; however, ADP acts as an inhibitor at all the concentrations tested (fig. 3).

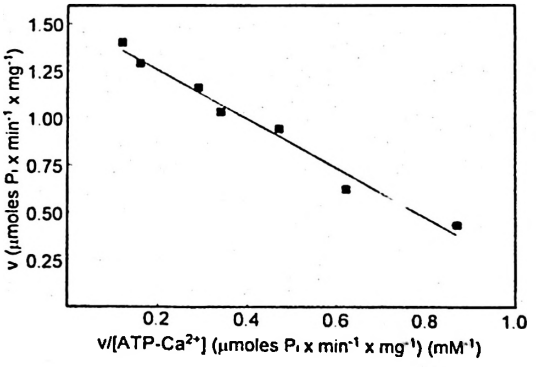

Fig. 1. *Eadie-Hofstee plot of Ca^{2+}-ATPase activity of trypsin-treated CF1.*

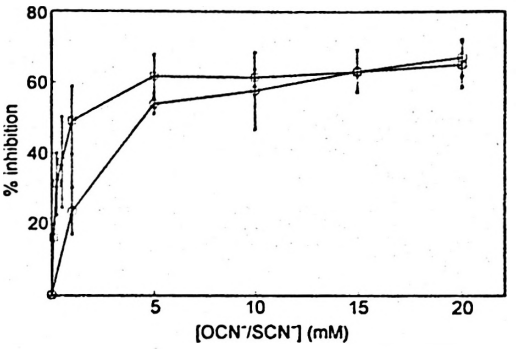

Fig. 2. *Effect of OCN^- (□) and SCN^- (O) on the Ca^{2+} ATPase activity of CF1 activated with trypsin.*

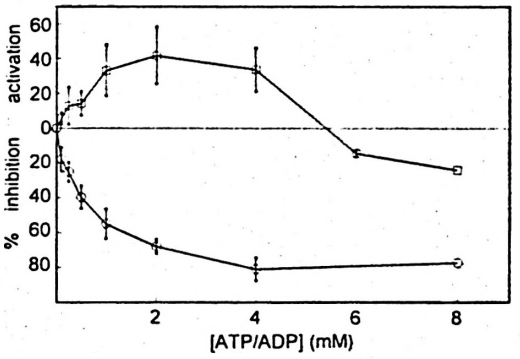

Fig. 3. *Effect of ATP (□) and ADP (O) on the Ca^{2+}-ATPase activity of CF1 activated with trypsin.*

Kinetic studies of the reaction catalyzed by CF1 in the presence of anions. To establish whether the inhibitor anions, SCN^-, OCN^-, ADP and ATP, at high concentrations, compete for the catalytic sites of the enzyme, the kinetics of the hydrolytic reaction was studied at several fixed concentrations of anions and at different substrate concentrations. The Lineweaver-Burk representation of the inhibition caused by SCN^- and OCN^- reflected an inhibitory effect of the non-competitive type, since the straight lines obtained do not intersect with the y-axis (fig. 4A).

The inhibitory effect of both ADP and ATP, at high concentrations, follows a competitive pattern with lines coinciding

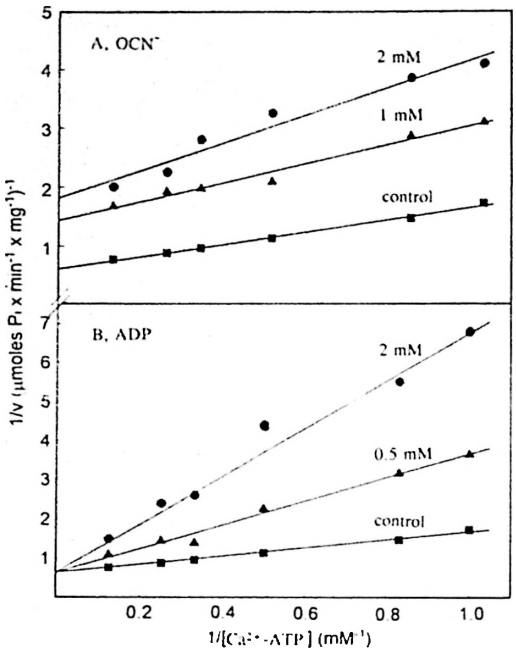

Fig. 4. *Lineweaver-Burk plots of the hydrolytic activity of CF1 in the presence of different concentrations of OCN⁻ (A) and ADP (B).*

at one point at the y-axis. Figure 4B shows the double reciprocal representation of ADP effects.

The value of Ki of these anions as well as the Ka value for ATP, were also determined. The inhibition constants for ADP and ATP were calculated from the Dixon diagrams (Ki for ATP= 0.64, Ki for ADP= 0.44). However, the Ki values of OCN^- and SCN^- were determined from Ebel and Lardy plots, since the Dixon diagrams for the effect of these anions were found to be non linear (Ki for OCN^-= 0.04, Ki for SCN^-= 1.66). The same double reciprocal plots were used in order to determine the Ka value of ATP at low concentrations (K_a = 1. 84).

Effect of the anions on the Ca^{2+}-ATPase activity in the presence of SCN^- on CFI activated by trypsin treatment. To study whether the different modulators compete for the same sites of the enzyme, the hydrolytic activity of CF1 in the presence of different concentrations of OCN^- and ADP, both in the presence and absence of SCN^-, was determined. The results have been plotted as double reciprocal diagrams.

Fig. 5A shows the effect of SCN^- on the Ca^{2+}-ATPase activity of CF1 at different concentrations of OCN^-; the plot obtained suggests that SCN^- and OCN^- bind at the same sites. However, with or without two ATP concentrations the different straight lines do not intersect at the same point, indicating a non-competitive relationship between SCN^- and ATP at low concentrations (fig. 5B).

CF1 modification with fluorescein isothiocyanate (FITC). — Kinetics of FITC binding to latent CF1. Fig. 6A shows the kinetics of binding of FITC with latent CF1, reflecting an approximate stoichiometry of 4:1.

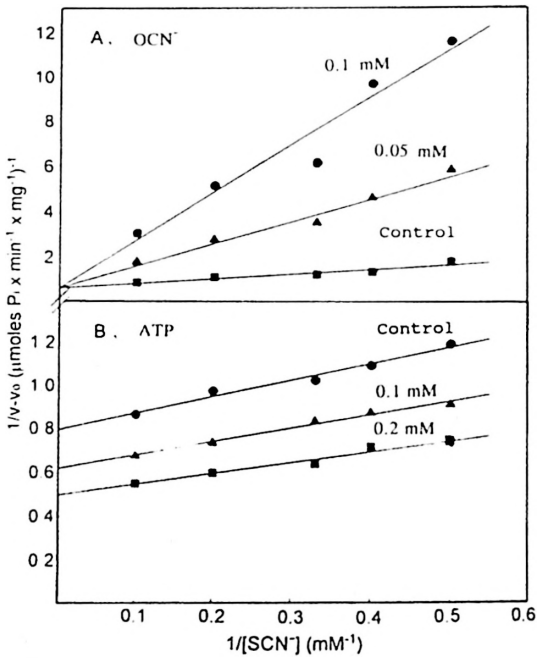

Fig. 5. *Inhibition by OCN^- (A) or ATP (B) of Ca^{2+}-ATPase activity of CFI at 3 mM Ca^{2+}-ATP and varying SCN^- concentrations.*

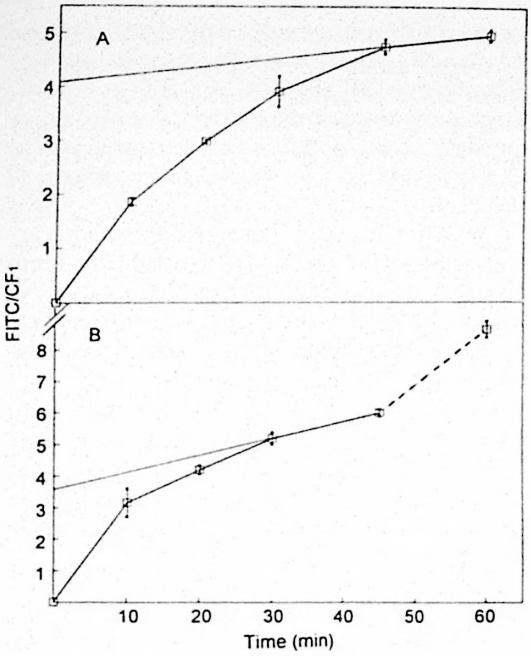

Fig. 6. *Time course of binding of FITC with CF1.* A: CF1. B: trypsin-treated CF1. The experimental conditions were as described under Material and Methods.

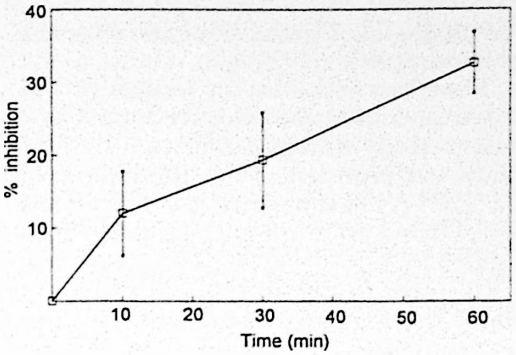

Fig. 7. *Effect of binding of FITC on Ca^{2+}-ATPase activity of CF1 activated with trypsin.*

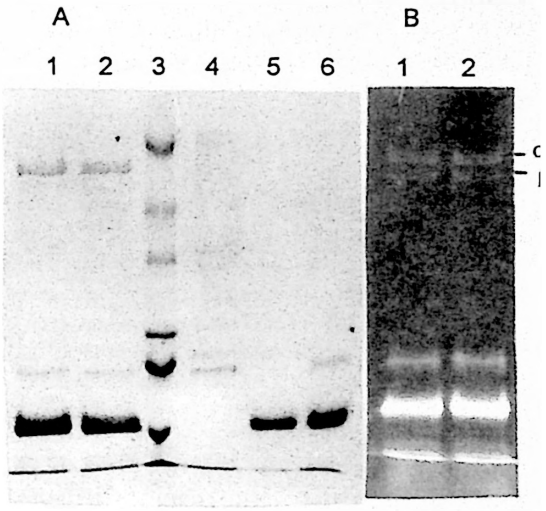

Fig. 8. *SDS-polyacrylamide gel electrophotograms of trypsin digested CF1 modified with FITC.* A: gel stained with Coomassie Blue. B: gel illuminated with u.v. light. 1-2: trypsin-digested CF1 modified with FITC. 3: molecular weight standard. 4: trypsin. 5: trypsin inhibitor. 6: trypsin and trypsin inhibitor.

Kinetics of FITC binding to trypsin-treated CF1. Similarly, trypsin activated CF1 treated with FITC allowed us to determine the effects of the FITC treatment on the Ca^{2+}-ATPase activity of CF1. In the trypsin treated enzyme the same relationship between FITC and CF1 was observed (fig. 6B). Figure 7 shows the percentage inhibition of Ca^{2+}-ATPase activity. At 30 minutes FITC treatment a maximum inhibition of 20 % was obtained.

SDS/PAGE of CF1 treated with FITC. The comparison of electrophoretical patterns of the CF1 treated with FITC and either stained with Coomassie brilliant blue or under u.v light, shows the incorporation of this reactant into the α and β subunits. An unspecific incorporation of FITC both in the reagent trypsin and trypsin inhibitor, was also observed in the trypsin-activated CF1 (fig. 8).

Discusion

The Ca^{2+}-ATPase activity of trypsin-activated CF1, showed a monophasic pattern (fig. 1). We asume that this kind of diagram was due to the activity of several active centres acting with the same kinetic properties, since the existence of more than one active centre on CF1 has been

shown (5, 23, 28) and confirmed by the present results.

The effect of anions on regulatory and active sites has been effectively used as a tool to study different ATPases. In CF1, azide was reported to be an inhibitor of the Ca^{2+}-ATPase activity (2). The effects of ATP and ADP on CF1 have also been studied (5, 17).

In order to obtain more information about the regulatory and active sites in trypsin treated CF1, studies employing different kinds of anions and nucleotides were carried out. The inhibition produced by SCN^- and OCN^- might be due to interactions with both regulatory or active sites. The determination of the mode of action of these anions showed a non-competitive pattern, suggesting the binding of SCN^- and OCN^- to regulatory sites. To establish whether these anions bind to the enzyme through the same or different regulatory sites, the study of their combined effects was carried out. The results show the existence of competition between them, indicating that they bind to CF1 through the same regulatory sites. Both OCN^- and SCN^- were found to have non linear Dixon plots. The non-linearity on this kind of diagram could be explained through the existence of a partial inhibition, in which the substrate-enzyme-inhibitor complexes still have some activity. In this case Ki value can be obtained from a double reciprocal plot according to EBEL and LARDY (6).

ADP has been reported to bind to sites 1 and 3 of CF1, and also to have an inhibitory effect on the ATPase activity of CF1 activated by heat (14). On the other hand ATP has been found to be an inhibitor of the ATPase activity of CF1. MILGROM *et al.* (17) have suggested that the binding of an ATP to a non-catalytic site (Site 2) of CF1 affects the ATPase activity of the active sites.

The results here reported show that ADP acts as a competitive inhibitor with respect to the substrate ATP-Ca^{2+}. Free ATP presents a biphasic behavior acting as an inhibitor at high concentrations and as an activator at low concentrations. The Lineweaver-Burk plot of ATP at inhibitory concentrations shows a competitive pattern. Thus, at these concentrations free ATP binds to the same active sites as substrate.

Ir order to establish if ATP, at activating concentrations, acts through the same regulatory site as the other anions, SCN^- and OCN^-, the study of the combined effect of ATP and SCN^- was carried out. The diagram obtained shows that no interactions between the two modulators were observed. Thus, soluble CF1 has not only regulatory sites that bind nucleotides, but also other cationic sites that might be implied in some regulatory processes affecting the activity of catalytic sites.

Fluorescein isothyocyanate (FITC) binds covalently to primary NH_2 groups and has been found to be more specific for nucleotide binding sites of proteins (9). The kinetics of incorporation of FITC into nonmodified CF1 reflects an approximate stoichiometry of 4FITC/1CF1, suggesting that the modification of 4 nucleotide binding sites is taking place. The existence of 6 nucleotide binding sites on CF1, probably three of them catalytic and three regulatory has been suggested (8, 27). Different studies with substrate analogs yielded a specific incorporation of 2.5 (3), 3 (16) and 4 (24) moles per mol of CF1.

The same experiment was repeated with CF1 previously digested with trypsin. The specific incorporation on these samples was similar to that obtained with non treated CF1 (FITC/CF1 = 4). However, the unspecific labelling of the trypsin-treated enzyme for between 30 and 45 minutes with FITC showed a drastic increase.

The comparison between the kinetics of FITC binding to CF1 and the inhibition of its Ca^{2+}-ATPase activity shows that only 20 % of the activity was lost with the incorporation of 4 FITC/CF1. These results suggest that not all the active sites of CF1 had been labelled and that

several regulatory sites could be implied. On the other hand the loss of activity cannot be due to the modification of only regulatory sites, since a maximum of 3 such seems to be present. Thus, among the different hypotheses proposed, our results are consistent either with the suggestion proposing the existence of 4 catalytic and 2 regulatory sites (23), or that suggesting the existence of three catalytic and three regulatory sites in CF_1 (27).

The treatment with FITC of the trypsin-activated enzyme, resulted in an incorporation of the reactant into both α and β subunits, suggesting that the two of them were implied in the binding of nucleotides. The unespecific incorporation observed in the reagent trypsin and trypsin inhibitor could explain the increase of the FITC/CF_1 relationship in the trypsin-activated enzyme.

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La cinética de la actividad ATPásica de la enzima CF_1 activada con tripsina se ajusta a un patrón de tipo monofásico. El estudio del efecto de los aniones cianato (OCN^-) y tiocianato (SCN^-) sobre dicha actividad, pone de manifiesto la existencia en CF_1 de centros reguladores de naturaleza catiónica a los que estos aniones se unen de manera competitiva produciendo inhibición. El ADP y el ATP (a altas concentraciones) presentan un efecto inhibidor sobre la actividad ATPásica de tipo competitivo respecto del sustrato ATP-Ca^{2+}. El ATP a bajas concentraciones se comporta como un activador. El estudio de los efectos combinados del ATP (a bajas concentraciones) y el anión SCN^- refleja una relación de tipo no competitivo entre aniones y nucleótidos. La modificación de CF_1 con fluoresceín isotiocianato (FITC), un agente específico de grupos amino de centros de unión de nucleótidos, da lugar a una relación molar específica FITC/CF_1 igual a 4, tanto en la enzima no activada como en la proteolizada con tripsina. Esta incorporación específca se produce en las subunidades α y β de CF_1, y conlleva una disminución de sólo un 30% en la actividad ATPásica de la enzima activada con tripsina. De estos resultados puede deducirse la existencia en CF_1 de tres o dos centros reguladores y de tres o cuatro centros catalíticos, respectivamente.

Palabras clave: CF_1, Sitios catalíticos, Sitios reguladores, FITC.

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