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BINDING OF CIBACRON BLUE F3GA TO CHLOROPLAST COUPLING FACTOR

COMPETITIVE INHIBITION OF ATP SYNTHESIS AND HYDROLYSIS

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Photophosphorylation in spinach chloroplasts and the Ca^{2+}-ATPase activity of soluble coupling factor 1 (CF_1) were inhibited by Cibacron blue F3GA. The inhibition of the heat-activated ATPase of CF_1 was competitive with ATP while an allosteric effect was seen in dithioerythritol-activated CF_1 with a Hill coefficient of 1.6. The inhibition of photophosphorylation was competitive with ADP but not with P_i. The interaction between CF_1 and Cibacron F3GA induced a red shift in the spectrum of the dye. The stoicheiometry of the CF_1-dye complex calculated from the difference spectra was nearly 2 for native CF_1 and nearly 3 for activated CF_1. The difference spectra of the dye-CF_1 complex changed with time (hours). The changes with native CF_1 were larger in the trough (575–578 nm) and the dye became more tightly bound to CF_1 with time.

Introduction

ATP synthesis in photosynthetic cells of green plants is catalyzed by a H^+ ATPase complex associated with thylakoid membranes. This complex contains an extrinsic protein with latent ATPase, coupling factor 1 (CF_1), that can be detached from the membrane and purified to homogeneity [1]. The ATPase activity of CF_1 may be unmasked by treatment with heat [1,2], sulphydryl compounds [1,3] or trypsin [2].

Multiple adenine nucleotide-binding sites have been described in CF_1. All authors agree that CF_1 has two or three nucleotide-binding sites with dissociation constants in the micromolar range. Two tight binding sites for adenine nucleotide were found in CF_1 while a third site became available after heat or dithioerythritol activation [4,5]. It is not clear if any of these sites is the catalytic site. Recently, the systematic studies of Bruist and Hammes [6] clarified the picture. They reported the presence of three nucleotide sites in CF_1, one of which has a 'non-dissociable' tightly bound ADP, a second site binds MgATP and the third site has similar affinity for ADP or ATP.

Cibacron blue F3GA and other triazine dyes were suggested as site-specific probes for nucleotide-bindng sites and the 'Rossmann fold', since they specifically inhibited many dehydrogenases and kinases [7,8]. Their structures resemble that of nucleotides. Immobilized on gels they have been successfully used for affinity chromatography [9]. However, the specificity of the binding of Cibacron blue to nucleotide sites has been questioned, since other proteins lacking the dinucleotide fold such as interferon [10] and human serum albumin [11] have been found to bind the dye.

In this paper we studied the effects of Cibacron

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Abbreviation: Tricine, *N*-tris(hydroxymethyl)methylglycine.

blue F3GA on the enzymatic activities involving soluble and membrane-bound CF_1. We found that the dye is a competitive inhibitor of ATP synthesis and hydrolysis and the stoicheiometry of complex formation between CF_1 and Cibacron blue was determined by difference spectroscopy.

Materials and Methods

CF_1 was purified from spinach leaves (*Spinacea oleracea*) obtained from the local market as described previously [12] except that Tricine-NaOH (pH 7.8) was used as buffer in all steps. The enzyme was stored precipitated in 2 M ammonium sulphate, 50 mM Tricine-NaOH (pH 8), 1 mM EDTA and 1 mM ATP.

CF_1 was activated by heat [1] or dithioerythritol [3] and its ATPase activity determined [13] as described. P_i [14] and protein [15] concentrations were measured as described.

Spinach chloroplasts were obtained from fresh leaves by homogenization with 20 mM Tricine-NaOH (pH 8.0), 250 mM sucrose, 5 mM $MgCl_2$ and 10 mM NaCl at 4°C. The homogenate was filtered through a nylon cloth and centrifuged for 5 min at $3000 \times g$. The pellet was resuspended in the same buffer at a chlorophyll concentration of 1 mg/ml. Chlorophyll concentration was determined as described earlier [16].

Non-cyclic electron transport from ascorbate to methyl viologen was measured by O_2 consumption as described previously [17].

Cyclic photophosphorylation was measured in a reaction mixture (0.5 ml) containing 40 mM Tricine-NaOH (pH 8.0), 40 μM phenazine methosulphate, 5 mM $MgCl_3$, 10 mM NaCl, chloroplasts (1 μg Chl) and the stated substrate concentrations plus $^{32}P_i$ (10^6 cpm). The reaction was stopped after 1 min at 28°C by addition of 0.1 ml of 20% trichloroacetic acid. P_i incorporated into ATP was determined in a Beckman LS 8100 liquid scintillation counter after phosphomolybdate extraction [18]. In order to obtain reasonable quantities of phosphomolybdate complex, carrier phosphate was added to obtain a minimal concentration of 10 mM.

Cibacron blue F3GA was purified before use from Blue reactive 2 (CI 61211) by chromatography on Whatmann 3 MM paper [19]. The major band was eluted with water and concentrated in a rotatory evaporator. The purity was checked with the same system or by thin-layer chromatography on gel plates of 250 μM [20]. In both systems only one spot with $R_f = 0.68$ and 0.69, respectively, was observed. The dye concentration was measured spectrophotometrically in 10 mM Tris-HCl (pH 7.5) with $\epsilon = 13.6 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ at 610 nm [20].

Difference spectra and difference spectral titrations were performed in an Aminco DW-2a UV-VIS spectrophotometer.

ATP, ADP, Tricine, phenazine methosulphate, methyl viologen and Blue reactive 2 were obtained from Sigma (S. Louis). All other chemicals were of analytical grade.

Results

Effects of Cibacron blue F3GA on the enzymatic activities of soluble and membrane-bound CF_1

Fig. 1 shows a double-reciprocal plot of the kinetics of inhibition of the Ca^{2+}-ATPase of heat-activated CF_1 by Cibacron blue F3GA. The inhibition was competitive with ATP. In the inset the apparent K_m values are plotted against different dye concentrations giving a straight line and a K_i of 1.2 μM. With concentrations of Cibacron blue

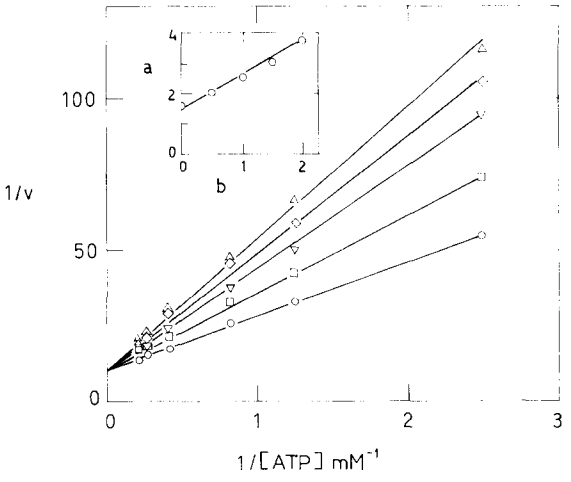


Fig. 1. Competitive inhibition kinetics of Ca^{2+}-ATPase of CF_1 by Cibacron blue. Dye concentrations were 0 (○), 0.5 (□), 1.0 (▽), 1.5 (◇) and 2.0 μM (△). Experimental conditions are described in the text. Inset: a, apparent K_m values; b, dye concentration (μM).

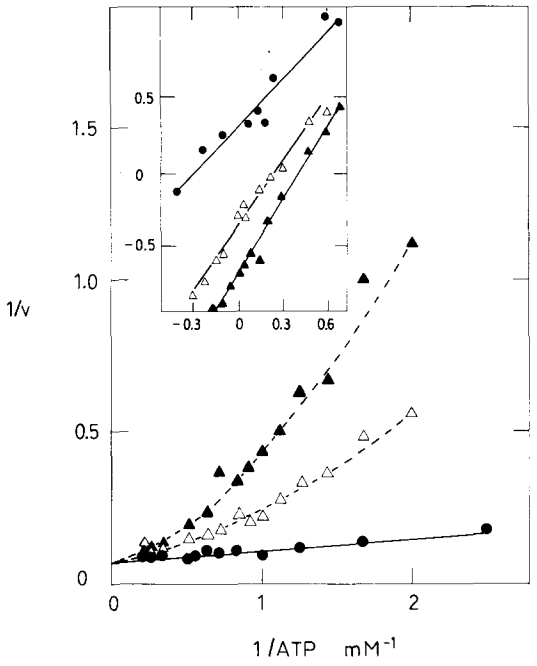


Fig. 2. Effect of Cibacron blue and ADP on the Ca^{2+}-ATPase of dithioerythritol-CF_1. The symbols correspond to control (●), and 0.5 mM ADP (△) or 2 μM Cibacron blue (▲) present in the assay medium. The inset shows a Hill plot of the same data. (Inset) Abscissa, log S; ordinate, $\log \frac{v}{V-v}$.

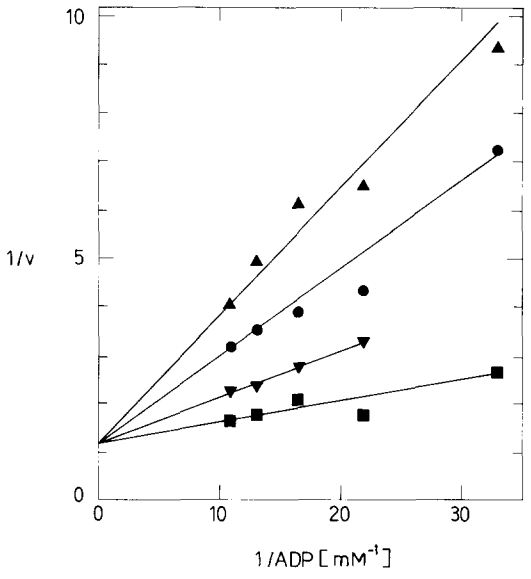


Fig. 3. Competitive inhibition kinetics with respect to ADP of cyclic photophosphorylation by Cibacron blue. The effect of Cibacron blue on cyclic photophosphorylation of spinach chloroplasts was assayed as described in the text with 3 mM P_i and the stated concentations of ADP. Dye concentrations were 0 (■———■), 0.75 (▼———▼), 1.5 (●———●) and 2.25 μM (▲———▲).

F3GA higher than 2 μM slightly upwardly directed curves were obtained in the double-reciprocal plot (not shown). This cooperative effect was clearly seen with dithioerythritol-activated CF_1 as shown in Fig. 2. For comparative purposes the inhibition pattern by 1 mM ADP is included. The Hill coefficients for ADP and Cibacron blue F3GA were 1.4 and 1.6, respectively.

Since CF_1 is part of the H^+-ATPase that catalyzes the synthesis of ATP in spinach chloroplasts the study of the effect of Cibacron Blue F3GA on this reaction was interesting. As expected, the dye is a potent inhibitor of cyclic photophosphorylation (Fig. 3). For instance, the I_{50} was 1.5 μM for a reaction medium with 150 μM ADP and 0.6 mM P_i. Fig. 3 shows that the inhibition of photophosphorylation by Cibacron blue F3GA was competitive with ADP but it was non-competitive with P_i (not shown). The inhibition of ATP synthesis competitive with ADP clearly suggests that Cibacron blue is acting on the H^+-ATPase at the active site.

Moreover, the dye inhibited coupled electron transport and slightly stimulated basal electron transport in spinach chloroplasts, thus behaving like a mixed-type inhibitor (not shown).

Binding of Cibacron blue F3GA to CF_1

The results reported above suggest that Cibacron blue F3GA interacts with CF_1 at the adenine nucleotide-binding site(s). The interaction between the dye and CF_1 was confirmed by the complex formation shown by a red shift in the spectrum of the dye (Fig. 4). The corresponding difference spectrum is also depicted in Fig. 4c. The latter has a positive band between 630 and 750 nm with a maximum at 673 nm and a nagative trough with a minimum at 575 nm. Addition of increasing concentrations of Cibacron blue F3GA to CF_1, either native or dithioerythritol-activated, resulted in larger increases of the main positive band of the latter (not shown, but see Fig. 5 and below). Thus, the absorbance at 673 nm is higher with the complex of dye with CF_1 activated by dithioerythritol than with the complex with native CF_1. This dif-

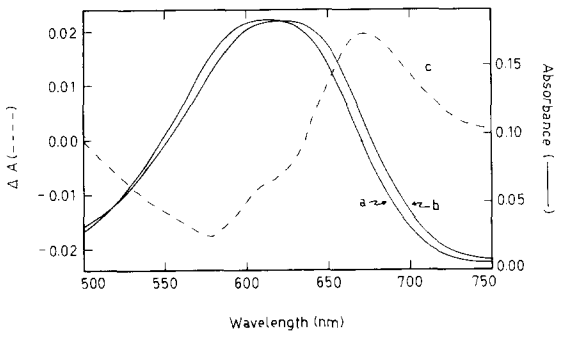


Fig. 4. Absorbance spectrum of 13.2 μM Cibacron blue in the absence (curve a) or presence (curve b) of 4 μM native CF_1. Curve c, corresponding difference absorbance spectrum.

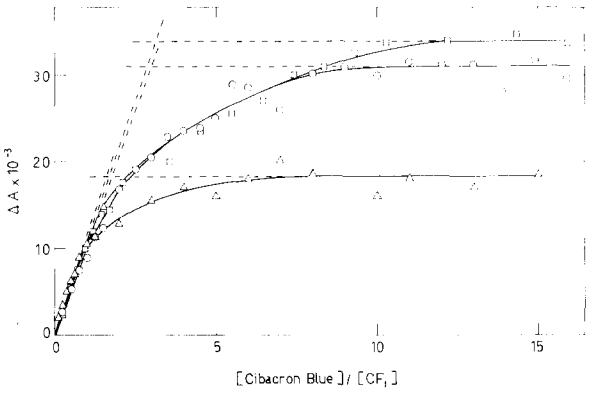
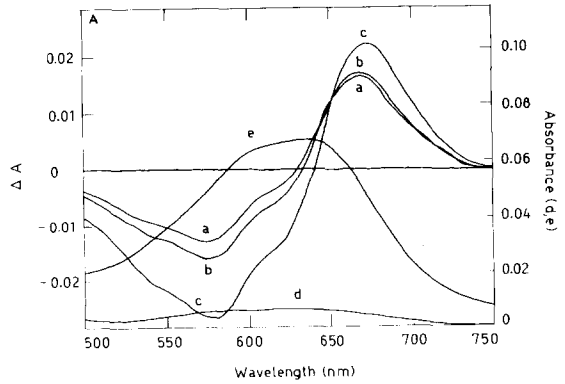


Fig. 5. Spectrophotometric titrations of CF_1-Cibacron blue complexes. Latent (△——△), dithioerythritol-activated (□——□) and heat-activated (○——○) CF_1 (4 μM) were titrated with increasing concentrations of Cibacron blue F3GA. The difference spectral absorbance at 673 nm is reported.

ference in absorbance may reflect an increase in the stoicheiometry of the activated CF_1-dye complex or a change in the ϵ of the complex or a combination of both. Since the affinity of Cibacron blue for CF_1 is high, the stoicheiometry of the complex may be determined from a plot of its absorbance at 673 nm against the ratio of the dye concentration to CF_1 concentration. Fig. 5 shows such a plot for native CF_1 and CF_1 activated by either heat or dithioerythritol. CF_1 (4 μM) was saturated with low concentrations of the dye and ΔA_{673} maxima were achieved that were clearly different for native and activated CF_1. The calculated stoicheiometries are 1.7 for native CF_1, 2.8 for heat-activated CF_1 and 2.9 for dithioerythritol-activated CF_1. Thus, activated CF_1 seems to have an extra binding site for the dye.

Since it has been suggested [4,5] that a new site for tightly bound adenine nucleotide appears in CF_1 after activation, it was tempting to suppose that the new site for Cibacron blue may be the same one for adenine nucleotide. We observed that ATP diminished the ΔA at 673 nm of the CF_1-Cibacron blue complex (data not shown). However, there is a strong interaction of Cibacron blue with ATP, ADP or AMP. Difference spectra obtained with mixtures of 30 μM of Cibacron blue and 3–20 mM adenine nucleotides were similar to that of Fig. 4 with ΔA maxima at 679–684 nm and ΔA minima at 579–583 (data not shown) resembling also the spectra obtained with the complex of the dye with several proteins [21–24]. To our



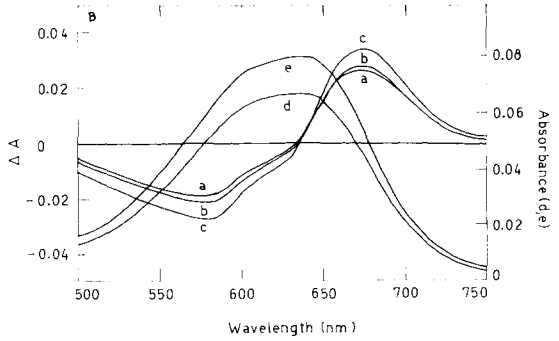


Fig. 6. Difference spectra of the complex CF_1-Cibacron blue as a function of time. Dye concentration, 23 μM. (A) Native CF_1. (B) Dithioerythritol-activated CF_1 (4 μM). Difference spectra were recorded at 1 (a), 6 (b) and 255 min (c). After 5 min (d) or 4 h (e) of incubation the CF_1-Cibacron blue complex was filtered twice through a G-50 Sephadex column [28] and the absorbance spectrum of the complex recorded.

knowledge these interactions have not been previously reported although difference spectra of enzyme/dye mixtures have been recorded in the presence of nucleotides.

The dye remained bound to CF_1 even after precipitation by ammonium sulphate, several filtrations through Sephadex G-25 or extensive dialysis against 20 mM Tricine-NaOH, pH 8, 2 mM EDTA. However, when the CF_1-Cibacron blue complex was dialyzed against 2.5 mM ATP for 18 h only 56% of the dye remained bound.

The interaction of Cibacron blue with CF_1 changed with time. When the difference spectrum of the dye-CF_1 complex was periodically recorded after mixing, increasing ΔA values were observed even after 18 h. Fig. 6A shows only three difference spectra, for simplicity, of the complex of dye with native CF_1 at 1, 6 min and 4 h. The increments of ΔA were larger in the trough than in the maximum and small shifts of the ΔA min (from 575 to 578 nm) and ΔA max (673 to 675 nm) were observed after 4 h. With dithioerythritol-activated CF_1 the increments of ΔA in the trough and in the maximum were similar (Fig. 6B).

When the free dye was eliminated from the mixture with native CF_1 by filtration through Sephadex columns after 5 min or 4 h of incubation and the spectra of the protein recorded, it was observed that very little dye remained bound after 5 min but the amount was larger after 4 h (Fig. 6A, spectra d and e). On the other hand, with dithioerythritol-activated CF_1 the amount of dye tightly bound to the protein was large after 5 min (Fig. 6B, spectrum, e) and practically the same after 4 h (Fig. 6B, spectrum d).

Discussion

A high-affinity, reversible interaction of Cibacron blue F3GA with the adenine nucleotide-binding site at the catalytic center of CF_1 is suggested by (i) the competitive inhibition with respect to ADP of photophosphorylation (Fig. 3) and (ii) the competitive inhibition with respect to ATP of the Ca^{2+}-ATPase activity of soluble CF_1 and its low K_i (Fig. 1).

However, with concentrations of Cibacron blue higher than 2 μM the double-reciprocal plots for ATP hydrolysis were not linear and a Hill coefficient of 1.6 was obtained (Fig. 2) for 2.5 μM Cibacron blue, suggesting an allosteric effect similar to that of ADP (Fig. 2) already described for the Mg^{2+}-ATPase of CF_1 [25]. Thus, in addition to interacting with the catalytic site, Cibacron blue seems to interact with the 'regulatory' or 'allosteric' site(s) like ADP.

Strotmann et al. [26] recently reported that 5 μM Cibacron blue, among other anthraquinone dyes, is a competitive inhibitor of trypsin-activated CF_1. The lack of an allosteric effect compared with our results may be due to a desensitization of the enzyme by the trypsin treatment. They also reported a mixed-type inhibition of photophosphorylation instead of the clear competitive inhibition with respect to ADP by purified Cibacron blue shown in Fig. 3 but the dye commercially available used by them may be a mixture of at least two compounds [27].

The binding of Cibacron blue to CF_1 is associated with a red shift in the spectrum of the dye and with a difference spectrum of the complex (Fig. 4) similar to those found with other enzymes [20–22]. A stoicheiometry of 2 or 3 mol dye per mol native or activated CF_1, respectively, was calculated. The higher stoicheiometry for the complex of Cibacron blue with CF_1 activated by either heat or dithioerythritol (Fig. 5) suggests the appearance of a new binding site for the dye in significant coincidence with the described availability of a new binding site for ADP [4,5]. Moreover, it may also be related to the exchange of nucleotides [29,30] induced by activation in membrane-bound CF_1.

According to the kinetics of inhibition of the Ca^{2+}-ATPase (Figs. 1 and 2) at least one of the three dye-binding sites of CF_1 may be the catalytic site and another the regulatory site.

The competitive inhibition with respect to adenine nucleotides and the high-affinity binding to CF_1 of Cibacron blue are in agreement with the proposed probing capability for the dye for nucleotide-binding sites.

With either native or activated CF_1 there was a rapid interaction with Cibacron blue but the formation of a tightly bound complex with native CF_1 required hours while a similar complex was observed with dithioerythritol-activated CF_1 after 5 min (Fig. 6). The slow tightening of the complex

with native CF_1 may be caused by a slow change in affinity or a slow displacement of tightly bound adenine nucleotides.

Recently, Subramanian [31] reported the spectral changes induced in Cibacron blue by salts or organic solvents, and analyzed the spectral characteristics of published dye-protein complexes in terms of electrostatic or hydrophobic interactions. The difference spectrum of CF_1-dye complex (Fig. 4) resembles that of the dye in high salt [31]. Thus, the interaction of CF_1 with the dye may be electrostatic.

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