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MODIFICATION OF CHLOROPLAST CF_1 BY FLUORESCEINISOTHIOCYANATE

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Summary

Incubation of the chloroplast coupling factor with fluorescein isothiocyanate inhibits the Ca-ATPase activity of the enzyme and results in incorporation of fluorescein into the α and β subunits. High concentrations of ATP prevent the inhibition and reduce the incorporation of fluorescein into the α and β subunits. Ca and Mg ions increase fluorescein isothiocyanate inhibition and fluorescein incorporation into the α, β and γ subunits. It is suggested that fluorescein isothiocyanate modifies the catalytic site of the enzyme by blocking lysine residues in the α and β subunits which are involved in the binding of ATP.

1. Introduction

Chemical modifications of essential tyrosine (1), arginine (2) and cysteine (1, 3) residues in the active site of chloroplast coupling factor (CF_1) were previously reported. Modifications of lysine residues in CF_1 by two fluorescent reagents: fluorescamine (4) and eosin isothiocyanate (EITC, ref. 5) was previously used to follow conformational changes in CF_1 which take place during light activation of chloroplast thylakoid membranes.

This paper describes the modification of ATP binding sites in CF_1 by fluorescein isothiocyanate (FITC). The results suggest that a lysine group in the active site of the enzyme is essential for ATP binding and hydrolysis.

Abbreviations

FITC isomer I - Fluorescein 5' isothiocyanate
isomer II - Fluorescein 6' isothiocyanate
EITC - Eosin 5' isothiocyanate
ØITC - Phenyl isothiocyanate
TNBS - 2,4,6 trinitrobenzene sulphonate
CF_1 - Chloroplast coupling factor

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2. Materials and Methods

Chloroplast thylakoid membranes were prepared from lettuce leaves essentially as previously described (6) with the following modifications: the protease inhibitor p-chloro-mercuribenzoate (100 μM) was included in the grinding medium and two washing steps were added according to Nelson et al. (7): 0.15N NaCl + 10 mM Na-tricine, pH 8.0, and finally 10 mM Na-tricine pH 8.0. The extraction of CF_1 by the EDTA procedure and the purification on DEAE Sephadex columns was done as previously described (8) and the enzyme was precipitated in 50% ammonium sulphate and kept at 2°C. The enzyme was desalted before use on a Sephadex G-50 column by centrifugation (9) and suspended in a medium of 50 mM tris Cl, pH 8.0 and 0.1 mM EDTA at a concentration of 1 mg/ml. Samples of fluorescein 5'-isothiocyanate (FITC, isomer 1) desolved in dry dimethylformamide were added to the CF_1 suspension and the mixture was incubated for 1 h at 37°C. The incubation was terminated by passing 100 μl samples through Sephadex G-50 columns contained in 1 ml tuberculin syringes and preequilibrated with 40 mM Na-tricine pH 8.0, 20 mM ATP and 5 mM DTT (activation medium) by the centrifugation procedure (9). The enzyme was activated 4 min at 60°C as previously described (8), cooled to 23°C and diluted with 4 volumes of the activation medium.

Ca ATPase was assayed by the release of ^{32}P from γ-^{32}P-ATP according to (7). For spectral measurements samples of 30-50 μg protein were suspended in 2.5 ml containing 50 mM Tris Cl, pH 7.5 and 0.1 mM EDTA. Absorption measurements were carried out in a Cary 16 spectrophotometer and the amount of bound fluorescein was calculated using $\varepsilon = 8 \times 10^4\ M^{-1} \times cm^{-1}$ for fluorescein at 492 nm (10). Fluorescence measurements were performed on a Perkin-Elmer MPF 44 spectrofluorimeter. The excitation and emission wavelength were 492 and 518 nm respectively.

Separation on 12.5% polyacrylamide gels (11) and photographs of fluorescein labeled proteins (10) were done as previously described.

Chemicals including FITC isomers I and II, nucleotides and 2,4,6 trinitrobenzene sulphonate were obtained from Sigma Chemicals Inc., acrylamide and other chemicals used for the preparation of the gels from Bio-Rad and Sephadex G-50 (fine) from Pharmacia. γ-^{32}P-ATP was prepared by photophosphorylation of ADP in the presence of ^{32}Pi (obtained from the Negev Radiochemical Center in Israel) essentially as described before (7) and purified on a polyethylene-iminocellulose column according to Magnusson et al. (12).

3. Results and Discussion

Figure 1 shows that incubation of the chloroplast coupling factor (CF_1) with FITC for 1 h at 37°C irreversibly inhibited Ca-ATPase activity and that the presence of ATP during the incubation protected against the inhibition. The 5' isomer (isomer 1) was more effective than the 6' isomer (isomer II). The protection by ATP became less effective at increasing FITC concentrations, probably indicating a competition between ATP and FITC for a common binding site. The inhibition by FITC was not due to interference in the process of activation of CF_1 which was routinely done after the incubation with FITC since

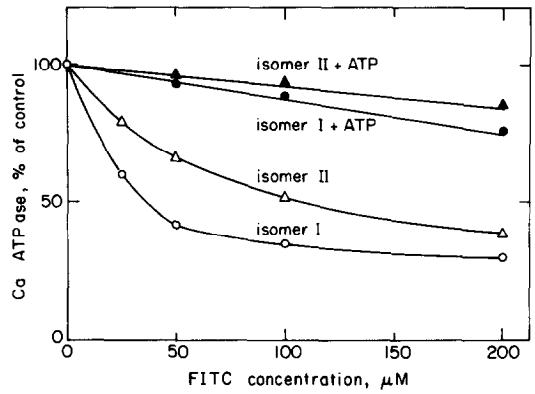

Figure 1: Inhibition of the ATPase activity of CF_1 by two FITC isomers. Chloroplast coupling factor (CF_1) was incubated for 1 h at 37° with different concentrations of the FITC isomers (F-5'-ITC = isomer 1, F-6'-ITC = isomer II) in the presence or absence of 5 mM ATP and the Ca ATPase activity was assayed as described under Materials and Methods. The control Ca-ATPase activity was 9.2 μmoles Pi released x mg protein^{-1} x min^{-1}.

the same results were obtained in control experiments in which CF_1 was heat activated before incubation with FITC (data not shown).

Table 1 demonstrates the effect of nucleotides and divalent metal ions on the FITC inactivation and binding to CF_1. Effective protection against 50 μM

Table 1: Effect of ligands on FITC inactivation and labeling.[a]

Additions to incubation medium	Ca ATPase % of control[b]	Bound Fluorescein[c] fluorescence, relative units	Bound Fluorescein[c] fluorescein/CF_1 mol/mol
-	100		
FITC	32	100	2.15
FITC + 10 μM ATP	35		
FITC + 100 μM ATP	37		
FITC + 1 mM ATP	85		
FITC + 5 mM ATP	90		
FITC + 10 mM ATP	97	50	0.95
FITC + 5 mM ADP	87		
FITC + 5 mM GTP	88		
FITC + 5 mM $CaCl_2$	2	150	3.05
FITC + 5 mM $MgCl_2$	15		

[a] CF_1 was incubated with 50 μM FITC (isomer 1) and the indicated ligands and the Ca-ATPase activity, the fluorescence and the absorption spectrum were determined as described under Materials and Methods.

[b] The control Ca-ATPase activity was 9.8 μmols Pi released x mg^{-1} protein x min^{-1}.

[c] The amount of bound fluorescein was calculated from the absorption at 492 nm as described under Materials and Methods.

FITC required high (mM) concentrations of ATP. ADP and GTP were also effective. Table I shows also that Ca and Mg largely increase the inactivation by FITC. The third and fourth columns in the table show that ATP decreased while Ca increased the binding of fluorescein to the enzyme. Assuming an extinction coefficient $\varepsilon = 8 \times 10^4\ M^{-1} \times cm^{-1}$ at 492 nm (10) for bound fluorescein and a molecular weight of 360,000 for CF_1 we calculated binding ratios of 2 and 3 fluorescein equivalents per CF_1 in the absence or presence of Ca respectively.

Most of the fluorescein is incorporated into the α and β subunits of CF_1 as is demonstrated in figure 2, well 1. The presence of a 20 fold excess ϕITC did not decrease fluorescein incorporation into α and β subunits. The presence of ATP (well 2) markedly decreased the fluorescein incorporation into both α and β subunits. The results are consistent with the observations that the ATP binding sites in CF_1 and in similar enzymes are located in the α and β subunits (13, 14). Ca increased the fluorescein incorporation into subunits

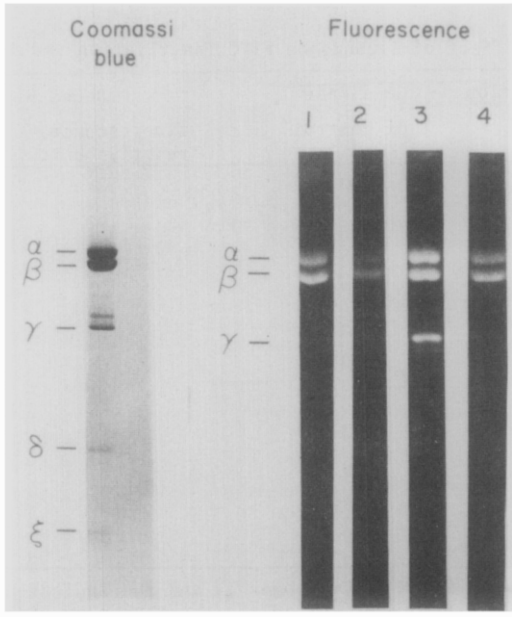

Figure 2: Fluorescein incorporation into CF_1 subunits. CF_1 samples were incubated with 50 μM FITC (isomer 1) in the presence of no additions (1) 10 mM ATP (2), 10 mM $CaCl_2$ (3) or 2 mM ϕITC as described under Materials and Methods. The unbound FITC was removed on Sephadex G-50 columns and samples of labeled enzyme containing 25 μg protein were applied to the gel. Other details are described under Materials and Methods.

α, β and particularly in γ (well 3). This may indicate that binding of divalent cations induce a conformational change in the enzyme, which increases the accessibility of lysine groups to FITC.

Table II demonstrates the effect of 2,4,6 trinitrobenzene sulphonate (TNBS), a lysine specific reagent, and phenylisothiocyanate (ϕITC) on FITC inhibition and on fluorescein incorporation. TNBS strongly inhibited the activity of CF_1 and the inhibition was largely prevented by ATP. TNBS also markedly lowered the incorporation of fluorescein into all the subunits both in the presence or absence of Ca. These results are consistent with competition of TNBS and FITC for the same lysine groups. ϕ ITC consistently decreased fluorescein incorporation into the γ subunit without affecting the labeling of α and β or the inhibition of the ATPase activity. Taken together, these results suggest that the inactivation is mainly due to a modification of two or more lysine equivalents in the α and β subunits which can be protected by ATP, and a third

Table II: Protection against FITC by lysine specific reagents.[a]

Additions to incubation medium	Ca ATPase, % of control[b]	Fluorescein fluorescence, relative units	Fluorescein labeling of CF_1 subunits		
			α	β	γ
-	100				
TNBS	21				
ϕITC	102				
FITC	32	100	+++	+++	+
FITC + TNBS	14.5	38	+	++	-
FITC + ϕITC	36.5	90	+++	+++	-
FITC + ATP	96	52	+	++	-
FITC + ATP + TNBS	70	-	+	++	-
FITC + ATP + ϕITC	98	-	+	++	-
FITC + Ca	3	152	++++	++++	++
FITC + Ca + TNBS	0	73	++	+++	-
FITC + Ca + ϕITC	5	150	++++	++++	+

[a] CF_1 was incubated for 1 h at 37° with the additions indicated in the table. FITC (50 μM) was added 1 min after TNBS (1 mM) or ϕITC (1 mM). The concentrations of Ca and ATP were 5 mM. Samples from each treatment were taken for determination of Ca ATPase activity, fluorescence measurements or analysis by polyacrylamide gel electrophoresis as described under Materials and Methods.

[b] The control ATPase activity was 9.5 μmol Pi released x mg^{-1} protein x min^{-1}.

lysine group in the γ subunit which is modified by FITC only in the presence of divalent metal ions and is probably not essential for ATPase activity.

The FITC modification differs from EITC modification of CF_1 described by Wayner et al. (5) since: (a) EITC modification did not inhibit significantly the Ca ATPase activity (b) in situ activation by light increased the eosin incorporation and the inhibition of cyclic photophosphorylation by EITC. In contrast fluorescein incorporation into CF_1 was unaffected by such an activation (not shown) (c) Eosin is incorporated mainly into the γ subunit (W. Junge, private communication).

The observations described in this paper namely that (1) FITC inhibits ATP hydrolysis in activated CF_1 by blocking the α and β subunits, (2) ATP protects against FITC inactivation, (3) lysine specific reagents decrease fluorescein incorporation into CF_1 and (4) a large excess of ϕ ITC is ineffective in the protection against FITC inactivation suggest that FITC acts as an affinity probe to the ATP binding sites in CF_1 blocking a lysine group essential for ATP binding.

This conclusion is also consistent with similar inhibitions of the ATP binding sites of the sarcoplasmic reticulum Ca-ATPase (10, 15) and of the mammalian Na-K-ATPase (16) by FITC. This surprising similarity in the sensitivity to FITC in widely different ion trasnporting ATPases suggest a conservative structure of the ATP binding sites in these enzymes and the presence of a lysine residue probably involved in ATP binding.

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