Vol. 86, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1979 Pages 440-446

INHIBITION OF CHLOROPLAST COUPLING FACTOR ATPASE BY 5'-p-FLUOROSULFONYLBENZOYL ADENOSINE[1]

Emanuele DeBenedetti and André Jagendorf
Biology Division, Cornell University, Ithaca, NY 14853

Received December 1, 1978

SUMMARY

Incubation of chloroplast coupling factor with 5'-p-fluorosulfonylbenzoyl adenosine in the 1 to 2 mM range inhibits subsequently measured ATPase activity. The inhibition is probably due to covalent binding since it survives ammonium sulfate fractionation and dialysis. The kinetics of the inhibited enzyme with respect to substrate show a decrease in V_{max} with no change in K_m for ATP. The presence of ATP or ADP together with the inhibitor provides some protection against inhibition. The results suggest a possible covalent attack at a nucleotide binding site, leading to inhibition of activity.

INTRODUCTION

Exploration of the function of chloroplast coupling factor, CF_1[2], will be aided by knowledge of the chemical nature of the active site. This site must bind nucleotides in the course of catalysis, and in addition the enzyme has at least two regulatory sites (1,2) at which adenylates are bound more tightly and probably with greater specificity than at the reaction center.

Of the covalent modifications described so far for this enzyme (3,4,5,6) only NBD-chloride binding to one tyrosine of the β subunit has been identified with a reasonable degree of certainty as being at the catalytic active site (3). Accordingly we have initiated studies with the chemical 5'-FSBA (7) which has the capacity to form covalent bonds due to the reactive sulfonyl halide, and whose specificity as an inhibitor of other enzymes (8,9) seems related to its adenyl moiety. This paper reports the inhibition of ATPase activity of CF_1 by 5'-FSBA, and protection from this inhibition by nucleotides.

MATERIALS AND METHODS

Enzyme preparation and activity measurement. CF_1 was isolated from chloroplasts of market spinach as described previously (10). Following purification on a sucrose density gradient, the enzyme was stored as an ammonium sulfate

[1]Supported by grant GM-14479 from the National Institutes of Health.

[2]Abbreviations: 5'-FSBA: 5'-p-fluorosulfonylbenzoyladenosine, NBD-chloride: 7-chloro-4-nitrobenzo-oxa-1,3-diazole, CF_1: coupling factor 1 from spinach chloroplasts.

0006-291X/79/020440-07$01.00/0

precipitate. This was desalted and equilibrated with 50 mM Na-tricinate and 2 mM EDTA at pH 8.0 either by filtration by centrifuging through Sephadex G-50 fine (11), or by dialysis.

Activation of ATPase by trypsin was performed by incubating for 50 min at 28°C with a ratio of CF_1/trypsin of 114 (w/w). The action of trypsin was stopped by addition of soybean trypsin inhibitor at a weight ratio of 10/1 to the trypsin present. These operations were performed in the same buffer as that used to dissolve the CF_1 which was present at 480 μg/ml, or occasionally at higher levels. ATP was omitted in order not to interfere with the subsequent attack by 5'-FSBA; the modified conditions for trypsin activation compared to those previously described (12) were needed to prevent excessive inactivation of CF_1 by trypsin in the absence of ATP. When used, heat activation of CF_1 was performed according to Farron and Racker (13).

The hydrolysis of ATP was assayed routinely by incubating 3.7 to 5 μg of activated enzyme in a solution containing 50 mM Tris-HCl at pH 8.5, 0.4 mg/ml bovine serum albumin, 10 mM $CaCl_2$ and 10 mM ATP for 14 min at 37°C. The reaction was stopped by addition of an equal volume of 5% trichloroacetic acid and inorganic phosphate assayed using the Taussky-Shorr procedure (14).

Routine determination of protein concentration depended on the absorbancy at 280 nm, using an E_{280} 1% of 5.2, close to the one previously reported (15). Other protein determinations used the Lowry procedure (16) with blank and standard in the same buffer.

Reaction with 5'-FSBA. Trypsin-activated CF_1 at 370 to 420 μg/ml was incubated with 5'-FSBA in a solution containing 50 mM Na-tricinate and 2 mM EDTA at pH 8.0 for varying lengths of time at 28°C. 5'-FSBA-reacted ATPase was assayed, removing directly from the reaction mixture aliquots containing 3.7 to 4.2 μg of CF_1. With the addition of 1 ml of the buffer used for the ATPase assay the reaction with 5'-FSBA was stopped by a 100-fold dilution of the inhibitor and by the addition of 10 mM ATP (within 5 min after the addition of the buffer). 5'-FSBA (final concentration 1.74 mM unless otherwise specified) was dissolved in dimethylsulfoxide prior to use (final concentration 8% in the incubation mixture). If latent ATPase was used, it was incubated with 5'-FSBA for 30 min, centrifuged to remove precipitated 5'-FSBA (under the incubation conditions 5'-FSBA precipitates slowly at concentrations higher than 1mM), then dialyzed against incubation buffer containing 8% dimethylsulfoxide to remove excess 5'-FSBA, prior to activation and ATPase assay.

Reagents. TPCK-treated trypsin was from Worthington, soybean trypsin inhibitor type I-S and 5'-FSBA hydrochloride were from Sigma.

RESULTS

In preliminary experiments the 5'-FSBA was dissolved in dimethylformamide. However, this solvent was found to inactivate CF_1 during prolonged incubation at 28°C, up to 50% in 2 hr using 9% dimethylformamide. By contrast, no inhibition has been observed with 9% dimethylsulfoxide under the same conditions.

As obtained commercially, 5'-FSBA contains equimolar amounts of dimethylformamide in its crystalline form. These concentrations of dimethylformamide were found not to inhibit CF_1 in early control experiments, hence the usual control incubations contained only dimethylsulfoxide.

The time course of inhibition of CF_1 by 5'-FSBA is approximately linear for the first 30 min when plotted on a semilogarithmic scale (Fig. 1). Over longer

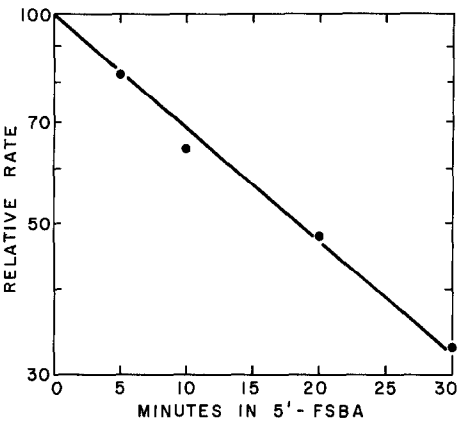

Fig. 1. Semilogarithmic plot of the time course for 5'-FSBA inhibition of CF_1. Trypsin-activated CF_1 370 μg/ml was incubated with 1.74 mM 5'-FSBA. 10 μl samples were withdrawn at the times indicated and immediately diluted 100 times with 50 mM Tris-HCl, pH 8.5, 0.4 μg/ml BSA and 10 mM $CaCl_2$. The samples were immediately assayed for the ATPase activity, adding 10 mM ATP (see Methods).

periods of time the rate of onset of inhibition slows down, for reasons not yet determined. The extent of the inhibition was the same using latent ATPase (data not shown) as when using trypsin-activated ATPase. Following exposure of latent ATPase to 5'-FSBA, the extent of inhibition was the same whether it was activated by heat or by trypsin.

The kinetics for ATP were examined using native CF_1 and CF_1 inhibited 50% by exposure to 1.98 mM 5'-FSBA for 17 min. In these experiments the CF_1 solutions containing 5'-FSBA were diluted 100-fold with the assay medium in which the $CaCl_2$ concentration was 1.5 times that of ATP. An excess of Ca^{2+} had been found, in earlier experiments, to be slightly inhibitory at the lower levels of ATP, consistent with previous observations (17). The control enzyme hydrolyzed 7.4 μmoles of ATP mg^{-1} min^{-1} (ATP at 4.8 mM), and both enzymes had a K_m of 2.7 mM for ATP.

Substrates prevented a considerable part of the inhibition due to 5'-FSBA (Table I). All the ligands tested afforded some protection, with ATP and ADP being the most effective. At 1 mM, AMP has essentially no protective effect (Table II), although at 4 mM some is evident. In order to obtain equivalent

Table I. Effect of substrates on the inactivation of CF_1 by 5'-FSBA.

Addition to reaction mixture	Residual activity (%)
none	34
ATP (4 mM)	79
ADP (4 mM)	83
AMP (4 mM)	58
GTP (4 mM)	66
ITP (4 mM)	63

CF_1 0.5 μg/ml was incubated with 1.74 mM 5'-FSBA as described in Methods. Nucleotides were added at 4 mM. Aliquots were assayed for ATPase activity after 30 minutes. Control activity was 7.4 μmoles ATP/mg·min.

Table II. Protection against 5'-FSBA inhibition.

Experiment 1		Experiment 2	
Additions	Activity	Additions	Activity
None	35[a]	None	44
ATP (1 mM)	61	ATP (1 mM)	74
ATP (4 mM)	79	ATP (4 mM)	82
PP_i (1 mM)	45	AMP (1 mM)	46
PP_i (4 mM)	53	AMP (4 mM)	55
Adenosine (1 mM)	38		
Adenosine (4 mM)	49		

[a] % of control activity, which was 11.6 μmoles ATP/mg min in experiment 1 and 9.0 μmoles ATP/mg min in experiment 2.

protection, AMP must be used at levels 16 times higher than those needed with ATP or ADP (data not shown). The AMP used did not contain ADP or ATP, as seen by thin-layer chromatography on PEI-cellulose. Adenosine and PP_i also afforded some protection, although less than ATP or ADP (Table II). Inhibition by 5'-FSBA was the same with or without 10 mM $CaCl_2$ (data not shown).

No discontinuities were seen in the plot of % protection vs ATP concentration. These data, replotted as % of control rate/ATP concentration vs ATP concentration fall along a straight line (Fig. 2). This result is consistent with a single action of ATP, preventing inhibition by 5'-FSBA at one site.

The % inhibition of the latent enzyme by 5'-FSBA was not changed by two successive precipitations of the enzyme by ammonium sulfate (Table III). Unaltered

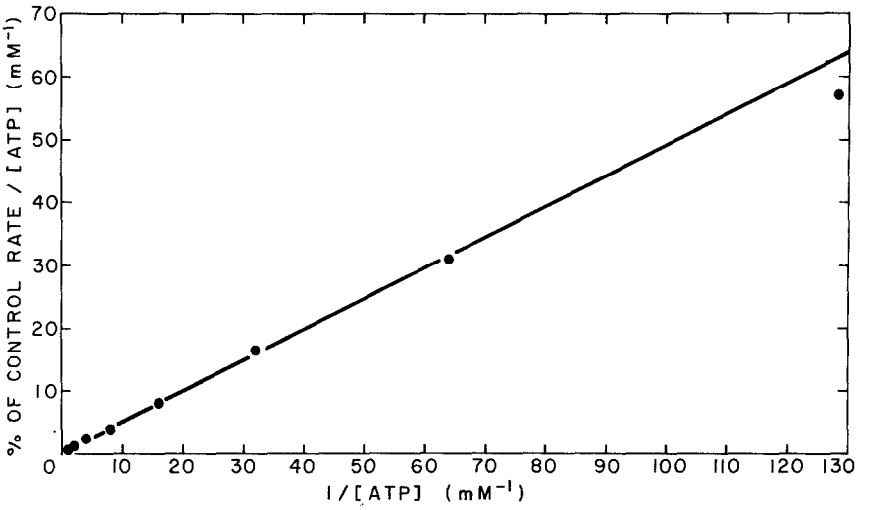

Fig. 2. Residual activity/ATP vs ATP concentration during the inhibition of CF_1 by 5'-FSBA. The experiment was performed as described in Table I. The lowest concentration of ATP used was 7.8 μM. Control activity was 8.8 μmoles ATP/mg·min.

Table III. Survivial of inhibition by 5'-FSBA through ammonium sulfate precipitations.

Ammonium sulfate precipitations	Specific activity		% of
	Control	Inhibited	Control
no.	μmoles ATP/mg/min		
0	4.9	1.23	25
1	8.0	1.12	14
2	7.0	1.47	21

Latent CF_1-ATPase at 2 mg/ml was reacted with 5'-FSBA for 30 min as described in Methods, precipitated 5'-FSBA removed by centrifuging, and an aliquot taken for activation and assay. The rest of the enzyme was precipitated by 50% ammonium sulfate, then dialyzed, and an aliquot taken for activation and assay. The rest of the enzyme was precipitated and dialyzed again. Control enzyme was subject to the same procedures except for absence of 5'-FSBA. The dialysis buffer was 50 mM Tricine-NaOH, 2 mM EDTA at pH 8.0. Activation of the original enzymes was performed by incubating with 1/15 weight ratio of trypsin plus 10 mM DTT for 10 min at 20°C; for the enzymes after ammonium sulfate activation was performed with the same concentration of trypsin but with the addition of 1 mM ATP, which accounts for apparent higher activity than in the original enzyme.

inhibition after ammonium sulfate precipitation was found for enzyme pre-activated with trypsin before exposure to 5'-FSBA as well. Also, the UV absorption spectrum of CF_1 was altered by exposure to 5'-FSBA, and this alteration persisted after

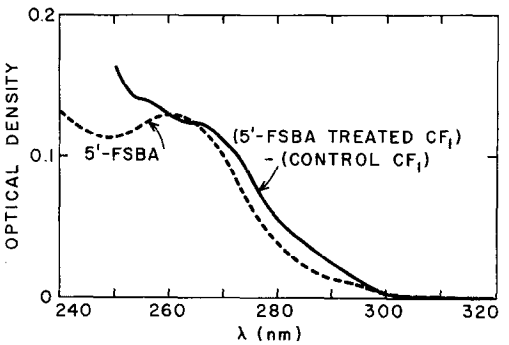

Fig. 3. Continuous line:difference spectrum between 5'-FSBA-reacted and control CF_1 of experiment 2 described in Table III. CF_1 was precipitated with 5% $HClO_4$ and resuspended in 6M guanidine buffered with 250 mM Tris-HCl, pH 8.5. Protein concentration was 1.18 mg/ml. Broken line:5'-FSBA spectrum in 50 mM tricine-NaOH, EDTA 2 mM, pH 8.0 plus dimethylsulfoxide 1%. The curve has been normalized at 260 nm with the difference spectrum of CF_1.

the enzyme was precipitated with perchloric acid and redissolved in 6 M guanidine (Fig. 3). These results, using either latent or activated CF_1, indicate the probable covalent nature of 5'-FSBA binding and consequent inhibition.

Fig. 3 shows the spectral difference between control and 5'-FSBA-treated CF_1. As this difference spectrum is not identical to the absorption spectrum of 5'-FSBA by itself (Fig. 3), the absorbance change of CF_1 cannot be used at present to determine accurately the stoichiometry of binding.

DISCUSSION

The inhibition of CF_1 by 5'-FSBA is probably due to covalent binding (Table II, Fig. 3) at a site that ordinarily binds adenylates. This is likely in view of the structure of 5'-FSBA, and because of the protection afforded by adenylates and other nucleotides. This protection might be the result of direct competition with 5'-FSBA at the same site, but might also result from modifying the enzyme structure by binding at a different site. The weak protection afforded by AMP, adenosine and PP_i is not entirely surprising. A possible role for AMP in the mechanism of catalysis has not been ruled out (18,19). PP_i is known to protect CF_1 from cold inactivation (20) and from TNBS attack (12). Adenosine has rarely been tested, and it might have some affinity where the adenosine moiety of ADP or ATP bind.

The fact that the K_m for ATP is not affected by 5'-FSBA, but the V_{max} is decreased, is consistent with complete inhibition of individual CF_1 molecules in the affected population. This could be due to reaction of 5'-FSBA at either the catalytic active site or at a regulatory site. Possible complete inhibition due to ADP binding to a regulatory site was discussed by Cantley and Hammes (1). However it is a little more likely that the catalytic site was affected, because our preliminary data indicate that the tightly bound regulatory nucleotides were not displaced by the 5'-FSBA binding (data not shown).

Further work is in progress to explore inhibition in thylakoid-bound CF_1 and to define the site(s) of binding of 5'-FSBA.

ACKNOWLDEGEMENT

Thanks are due to Dr. Roberta Colman for the initial sample of 5'-FSBA and encouragement to explore its effect on CF_1.

REFERENCES

1. Cantley, L.C., Jr., and Hammes, G.G. (1975) Biochemistry 14, 2968-2975.
2. Banai, M., Shavit, N., and Chipman, D.M. (1978) Biochim. Biophys. Acta 504, 100-107.
3. Deters, D.W., Racker, E., Nelson, N., and Nelson, H. (1975) J. Biol. Chem. 250, 1041-1047.
4. Schmid, R., Jagendorf, A., and Hulkower, S. (1977) Biochim. Biophys. Acta 462, 177-186.
5. Andreo, C.S., and Vallejos, R.H. (1977) FEBS Lett. 78, 207-210.
6. Sugiyama, Y., and Mukohata, Y. (1978) FEBS Lett. 85, 211-214.
7. Colman, R.F., Pal. P.K., and Wyatt, J.L. (1977) in Methods in Enzymology (Jakoby, W.B., and Wilchek, M., eds.), vol. XLVI, pp. 240-249.
8. Wyatt, J.L., and Colman, R.F. (1977) Biochemistry 16, 1333-1342.
9. Pal, P.K., Wetcher, W.J., and Colman, R.F. (1975) J. Biol. Chem. 250, 8140-8147.
10. Binder, A., Jagendorf, A., and Ngo, E. (1978) J. Biol. Chem. 253, 3094-3100.
11. McGhee, J.D., and Hippel, P.H. (1977) Biochemistry 16, 3267-3276.
12. Oliver, D., and Jagendorf, A.T. (1976) J. Biol. Chem. 251, 7168-7175.
13. Farron, F., and Racker, E. (1970) Biochemistry 9, 3829-3836.
14. Taussky, H., and Shorr, E. (1953) J. Biol. Chem. 202, 675-685.
15. Farron, F. (1970) Biochemistry 9, 3823-3828.
16. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
17. Hochman, Y., Lanir, A., and Carmeli, C. (1976) FEBS Lett. 61, 255-259.
18. Vambutas, V. and Bertsch, W. (1976) Biochem. Biophys. Res. Commun. 73, 686-693.
19. Roy, H., and Moudrianakis, E.N. (1971) Proc. Nat. Acad. Sci. USA 68, 464-468
20. Posorske, L., and Jagendorf, A. (1976) Arch. Biochem. Biophys. 177, 276-283.