THE PARTIAL CHARACTERIZATION OF FLUORIDE INHIBITION OF CHLOROPLAST COUPLING FACTOR

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SUMMARY

Exposure of isolated thylakoid membranes to sodium fluoride inhibited chloroplast ATPase activity. Fluoride incubation decreased the acidification of isolated thylakoid lumen when proton translocation was driven by reverse ATP synthase activity. The in vitro inhibition by fluoride is a complex interaction between the CF_1, fluoride, and required cofactor. The magnitude of the fluoride inhibitory action was shown to be related to the ratio of Ca^{2+} to ATP. The shape of the activity plots suggests that fluoride exposure inhibits the CF_1 directly rather than a reduction of the substrate levels due to binding.

INTRODUCTION

The inhibitory action of fluorides on phosphatase activity has been known for many years to the histochemist. High concentrations were used to prevent phosphatase

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activity in tissue slices. Increased industrial emission of fluorides into the atmosphere has renewed interest in the phytotoxic effects on vegetation. It has been estimated that 166,000 tons of industrial fluoride pollution is released into the North American environment annually [1]. Agriculture in areas surrounding fluoride-emitting industries has been adversely affected as growth rate, apparent photosynthesis, and total yield of plants are substantially reduced.

Even slightly elevated fluoride levels significantly increase fluoride concentrations in plant foliage. The effect on yield depends on the exposure level, duration of exposure, and the relative sensitivity of the plant species and varieties. An early visual symptom of fluoride toxicosis is the loss of chlorophyll. Woltz and Leonard [2] reported that photosynthetic activity in Valencia orange trees exposed to fluoride pollution was correlated with a decrease in pigment. The net carbon dioxide uptake (apparent photosynthesis) was inhibited in leaves of alfalfa and barley that were fumigated with hydrogen fluoride for 2 hours at 40 and 200 ppb [3]. Horvath et al. [4] described the destruction of Vicia faba chloroplast granal and stromal lamellar organization associated with 50 ppb fluoride fumigation. It has also been suggested that decreased yields may be correlated with the loss of leaf area resulting from fluoride-induced necrosis. These observations suggest that fluoride toxicity may be associated with biochemical changes in the chloroplast.

A recent investigation on the effects of fluoride on the biochemistry of chloroplasts exposed to soluble fluorides in vitro revealed that neither the Calvin cycle nor the Hill reactions are directly affected at physiological pHs [5]. Results of these studies suggest, however, that fluoride affects these processes by inhibiting photophosphorylative mechanisms. This suggestion is consistent with results obtained in the in vitro exposure of mitochondrial ATPases to comparable concentrations of NaF [6]. Results of these studies indicate that the inhibitory action of fluoride on the metabolic processes of these organelles is mediated through a direct effect on the ATPase activity.

Such observations prompted two questions: (1) does fluoride prevent translocation of the proton via the CF_0, or (2) does the fluoride affect the catalytic subunit (CF_1) of the chloroplast ATPase? To answer these questions we examined the effect of fluoride on sealed photoactive thylakoid vesicles. The effect of fluoride on the CF_0 was examined by employing vesicles which had been partially stripped of the CF_1 particles (phosphatase).

METHODS AND MATERIALS

Plant material

Soybeans (Glycine max) were germinated in vermiculite in a greenhouse. Plants were harvested after the primary leaves were completely expanded (approx. 20 days).

Thylakoid vesicle isolation

Soybean leaves were homogenated in an ice-cold blender containing the isolation medium in a 1:4 (tissue:solution) ratio. The isolation solution contained 330 mM mannitol, 30mM 3-[N-Morpholino] propanesulfonic acid. (MOPS), pH 7.2, 1mM ethylenediaminetetraaceticacid (EDTA), 1mM $MgCl_2$, and 0.2% bovine serum albumin (BSA). The homogenate was filtered through 8 layers of cheesecloth and then centrifuged at 3500g for 5 min at 0^oC. The pellet was resuspended in 5 ml of isolation buffer, then loaded on 5 ml of a 40% (v/v) step gradient of Percoll^ isolation buffer and centrifuged for 2.5 min at 2^oC. The pellet was osmotically shocked with 1/25 strength isolation solution, the suspension was allowed to sit 2 min and then centrifuged at 1500g for 10 min at 0^oC. The pellet was resuspended in a minimum volume of isolation solution to make a final chlorophyll concentration of 200 μg/ml.

pH gradient formation in thylakoid vesicles

One ml of the vesicle preparation was added to 3 ml of thylakoid activation buffer; (20 mM tricine, pH 8.0, 50 mM NaCl, 5mM $MgCl_2$, and 10 mM dithiothreitol (DTT). The mixture was placed under saturating illumination (40 W Sylvania cool white fluorescent bulbs at a distance of 2") for 5 min at 4^oC. Determination of pH in the vesicle resulting from ATPase activity was obtained by quinacrine fluorescence quenching in the presence of exogenously supplied ATP. The reaction mixture contained 900 μl thylakoid activation buffer mixture, 2.5 mM ATP, and 0.75 μM

quinacrine. Fluorescence quenching was measured by a Perkin-Elmer LS-5 spectrofluorometer with an emission wavelength of 500 nm and excitation wavelength of 430 nm.

CF_1 isolation

CF_1 particles were isolated according to the procedures of Strotmann et al. [7]. Purity of the preparation was measured according to the methods of Lien and Racker [8]. Preparations were excited with a wavelength of 280 nm. The fluorescence intensity ratio of emission at 300 and 350 nm of protein preparation was used as a purity measurement. A ratio of 1.85 was considered to be a purified preparation of CF_1. All preparations used had lower values than 1.85 and were considered to be enriched in CF_1. Preparations were devoid of activity as measured prior to trypsin activation by ATP hydrolysis.

Ca^{2+} dependent ATPase (CF_1) activity

Activation of CF_1 activity was accomplished by addition of 20 μl of 5mg/ml trypsin (pancreatic) to 1 ml of the CF_1 preparation with 25 μl of 80 mM ATP. The mixture was incubated at room temperature for 6 min, then 50 μl of 5mg/ml of trypsin inhibitor (chicken egg white) was added. ATPase activity was measured as release of inorganic phosphate [9]. The reaction mixture contained 200 μl of protein preparation, ATP, $CaCl_2$ and 40 mM tricine, pH 8.0. The reaction was conducted at $37^{o}C$ for 10 min and stopped by the addition of TCA at a final concentration of 5%.

CF_1 depleted membrane activity

CF_1 depleted membranes were obtained as the final pellet of the CF_1 isolation procedure. The degree of uncoupling for various treatments was measured as the rate of O_2 consumption in a medium containing CF_1 depleted membranes (50 μg chlorophyll) in 2 ml of 0.1M mannitol, 50 mM HEPES, pH 7.6, 3 mM $MgCl_2$, and 0.1 mM methylviologen. The rates were compared with the values of O_2 consumption in control preparations uncoupled by addition of 5 mM NH_4Cl.

Protein determination

Protein determinations were made by the methods of Lowry [10].

Chlorophyll determination

Chlorophyll concentrations were determined according to the procedures of Moran [11].

RESULTS

The hydrogen ion gradient, formed by reversed ATP synthetase activity in isolated thylakoid vesicles, was inhibited when exposed to 30 mM NaF compared with a similar concentration of NaCl (Table 1). The relative traces of quinacrine-quenching fluorescence demonstrate the inhibitory nature of NaF on the formation of a transmembrane Δ pH when uncoupled from electron transport (Fig. 1). These data suggest that either the hydrolytically active enzyme subunit (CF_1) or the proton translocating capacity via the proton pore (CF_0) are adversely affected by fluoride exposure.

TABLE 1

Fluoride effects on the chloroplast ATPase generated proton gradient in sealed thylakoid vesicles.

Treatment[1]	% Inhibition
30 mM NaCl	0.0
30 mM NaF	54.25±10

[1] The relative H^+ ion gradient is measured as a decrease in the quenching after the addition of gramacidin D. The % inhibition is the average of 4 assays ± S.D.

TABLE 2

Uncoupling in CF_1 depleted membranes with various treatments.

Treatment	Rate[1] (μmol O_2/mg chl/hr.)	% Uncoupled electon transport
Control	38±5	27
5 mM NH_4Cl	139±2	100
50 mM NaF	45±4	32
40 M DCCD	23±2	17

[1] The rates are reported as the average of 3 replications ± S.D.

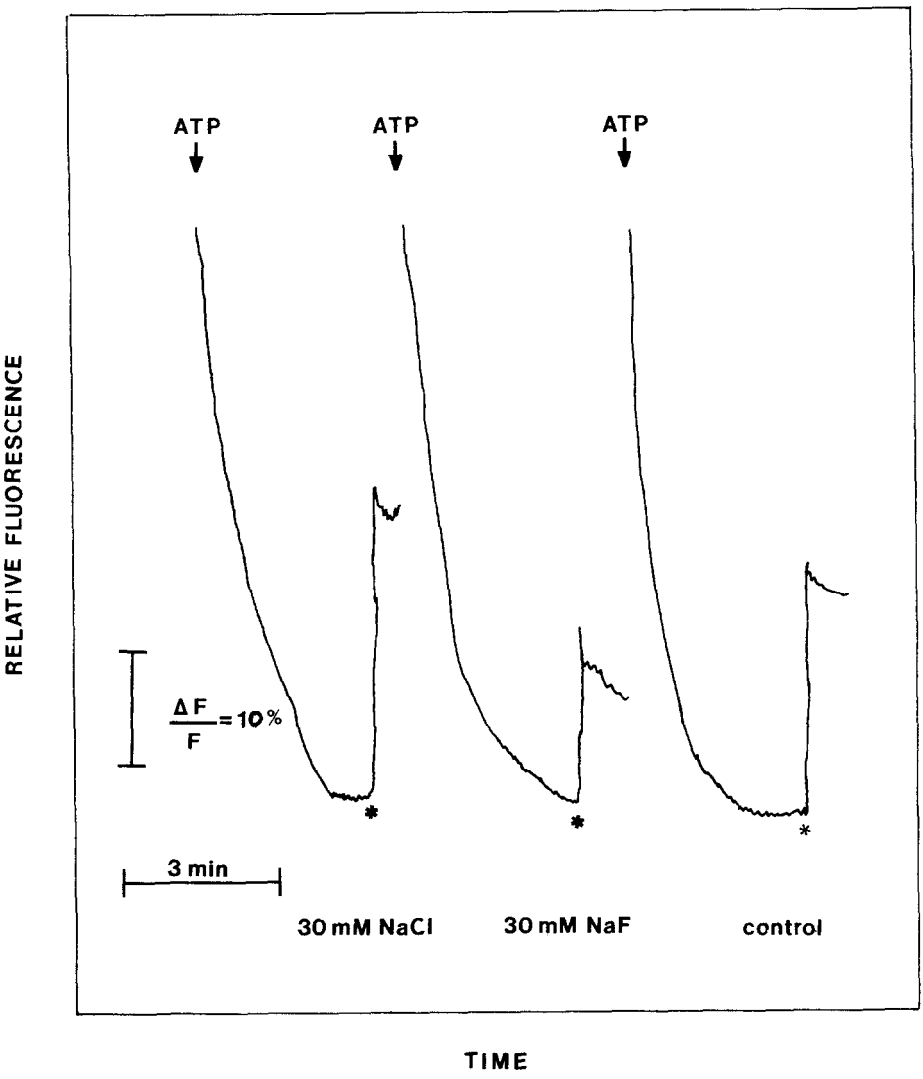

Fig. 1. Steady state H^+ gradient formation in thylakoid vesicles isolated from soybeans. Gradient formation was measured by quinacrine fluorescence quenching. The asterisk (*) indicates the addition of gramacidin D. Each assay contained 95 μg chl.ml.

The degree of free proton movement through the exposed CF_0 pore in photoactive membranes depleted of CF_1 is shown as the per cent of uncoupled electron transport rates with various treatments (Table 2). Treatment of membranes with NH_4Cl established the maximal level of uncoupling and was a baseline to compare the treatments. Membranes depleted of CF_1 but with no additional treatment displayed higher rates of photosynthetic electron transport than intact vesicles. This indicates that at least some CF_1 was removed from the ATPase complex. Addition of 40 μM DCCD to block the proton pore in the CF_0 substantially reduced photosynthetic electron transport rates. DCCD is known to bind with the CF_0 and prevent transmembrane proton movement which would result in a membrane potential inhibitory to further electron transport. The inclusion of a high concentration of NaF (50 mM) in the reaction mixture slightly stimulated photosynthetic O_2 consumption indicating that fluoride might act as a weak uncoupler of the pH gradient.

The rate of ATPase activity in a CF_1 enriched preparation was measured with increasing fluoride concentrations with different substrate and cofactor ratios (Table 3). It was demonstrated that a ratio of 1 and 1.5 Ca^{2+}/ATP were optimal for isolated CF_1 activity. Maximal activity was observed in the mixture containing 8 mM Ca^{2+} and 5.3 mM ATP. Activity decreased sharply when mixtures contained less calcium than ATP. Figure 2 shows the percentage activity of the CF_1 in the presence of NaF at various ratios of Ca^{2+}/ATP. Activity in a mixture

TABLE 3

CF_1 ATPase activity in the presence of various substrate concentrations.

Ratio Ca/ATP	Ca Conc. mM	ATP Conc. mM	Enzyme Activity (μg Pi/mg protein/min)
2.30	12.0	5.3	245±2
1.50	8.0	5.3	278±3
1.50	12.0	8.0	213±3
1.00	8.0	8.0	274±6
0.67	8.0	12.0	29±8
0.33	5.3	12.0	0

[1] Rates are reported as average of 3 replications ± SD.

containing 8 mM Ca^{2+} and 5.3 mM ATP(C) in the presence of NaF was identical to that in a mixture containing 12 mM Ca^{2+} and 8 mM ATP(B), indicating that the ratio of the Ca^{2+} to ATP is the important parameter in determining the amount of inhibition due to NaF exposure. The activity plots displayed a sigmoidal response to increasing NaF concentrations; increasing the ATP concentration relative to the Ca^{2+} concentration (e.g., 8 mM Ca^{2+} and 12 mM ATP) increased the inhibition due to NaF exposure. Increasing Ca^{2+} relative to ATP decreased sensitivity to NaF. The decrease was not related to free Ca^{2+} but to the ratio of Ca^{2+} and ATP, as indicated above. Control experiments were conducted with similar concentrations of NaCl; the maximum

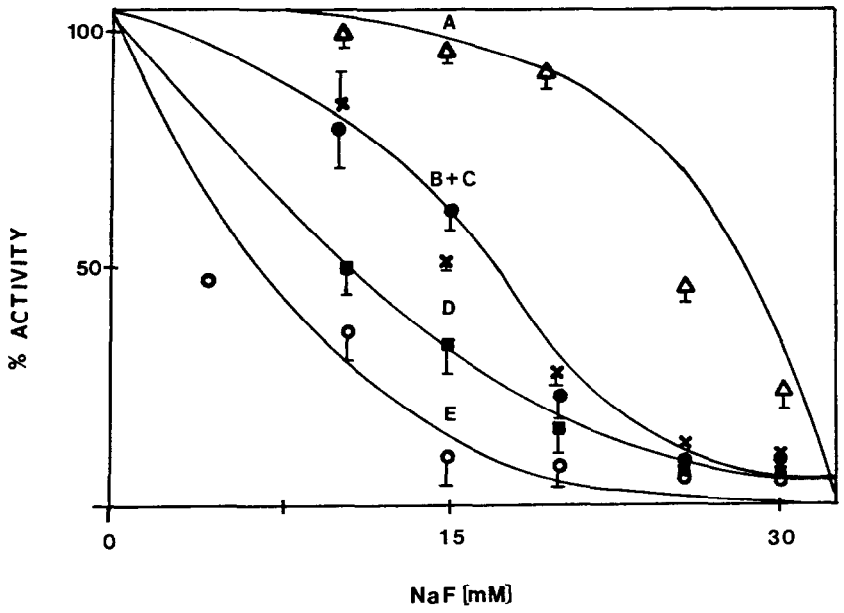

Fig. 2. CF_1 ATPase activity in the presence of NaF. Ca^{2+} and ATP concentrations were varied as indicated: (A)12mM Ca^{2+}/5.3mM ATP,(B) 12mM Ca^{2+}/8mM ATP,(C) 8mM Ca^{2+}5.3mM ATP,(D) 8mM Ca^{2+}/8mM ATP,(E) 8mM Ca^{2+}/12mM ATP. Data points represent the average of three assays ±SD.

observed salt inhibition was 20% at 30 mM. The effects of NaF inhibition were reversible. If the preparations were incubated in 30 mM NaF, then subsequently diluted to 6 mM NaF, holding all other factors constant, the resulting inhibition was similar to that observed at an initial 6 mM NaF exposure. Collectively, these data suggest that fluoride principally affects CF_1 activity and has little effect on the CF_0 proton channel.

DISCUSSION

The most significant response to NaF exposure was exhibited by the catalytic subunit of the ATPase (CF_1). The CF_1 particle is a complex multimeric enzyme with its mechanism of catalysis still under investigation. As the CF_1 particle was incubated in reaction mixtures containing higher concentrations of fluoride, enzyme inhibition increased. The inhibitory response appears to be related to the ratio of the *in vitro* Ca^{2+} concentration to the concentration of the ATP in the reaction mixture. The activity plots of CF_1 particles exposed to fluoride and varying ratios of ATP and Ca^{2+} exhibited sigmoidal shapes, indicative of a complex interaction among substrate, cofactor and inhibitor. The data presented here, although not answering all the questions about substrate, cofactor and inhibitor interaction, shed light on fluoride's effect on the CF_1.

Deviation from a 1:1 ratio of Ca^{2+} to ATP altered the fluoride sensitivity of the isolated CF_1. Increasing the ratio of Ca^{2+}:ATP (1.5) reduced the fluoride inhibitory response up to a concentration of 20mM. Decreasing the ratio (0.67) significantly enhanced fluoride inhibitions at concentrations as low as 5 mM. The increased inhibition of the CF_1 as the ratio of Ca^{2+} to ATP decreased appears to occur because free ATP acts as a competitive inhibitor of the CF_1 [12] which would compound the inhibition observed with fluoride.

Note that the absolute Ca^{2+}:ATP concentration did not contribute to the NaF inhibitory response as long as the ratio was not changed. The data indicate that the inhibition due to fluoride is not simply a F^- binding of the available Ca^{2+} to reduce substrate availability. This type of interaction (binding of cofactor by fluoride) has been implicated in other enzyme fluoride interactions [13, 14]. If this were the case, increasing the Ca^{2+} and ATP levels without changing the ratio would increase the absolute amount of available substrate and thus decrease observable inhibition. The activity plots then would have shown competitive inhibition patterns and not identical sigmoidal plots. Instead, fluoride appears to directly inhibit the CF_1, due to binding at the active site or an allosteric site, which reduces the catalytic ability of the enzyme. The inhibition may also be due to a non-specific salt effect on the enzyme. Fluoride is strongly negative and could interact with the positive charges on the CF_1 surface. A strong interaction could neutralize charges and cause conformational changes resulting in inhibition. The minor (20%) inhibition observed when 30mM NaCl is added to control solutions may indicate a non-specific salt interaction. Increasing the Ca^{2+}/ATP ratio seemed to reduce the inhibitory response of the CF_1 to fluoride. It again appears that this effect is a complex interaction between fluoride, CF_1 and cofactors, and not a simple CaF_2 binding phenomenon. Further study is necessary to determine the exact nature of the decrease in inhibition due to increased Ca^{2+}/ATP ratios.

The exact nature of the inhibitory response was not ascertained under these experimental conditions, but it appears to be a complex interaction among the CF_1, F^- ion, ATP and Ca^{2+}. Presumably, similar interactions would occur *in vivo* when the cofactor is Mg^{2+}. In this case, environmental exposure to fluoride could severely reduce the plant's energy production leading to necrosis and eventually to plant death. Some researchers conclude that plants accumulate excess Ca^{2+} and/or Mg^{2+} in response to fluoride exposure [15]. Our data do indicate a reduced inhibition in the presence of Ca^{2+}, but more research is necessary to determine if this response is found *in vivo*.

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