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EVIDENCE FOR A CATALYTIC FUNCTION OF THE COUPLING FACTOR 1 PROTEIN RECONSTITUTED WITH CHLOROPLAST THYLAKOID MEMBRANES

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Summary

The effects of tentoxin on the ATPase activities of coupling factor 1 proteins (CF_1) and photophosphorylation with isolated chloroplasts and chloroplasts reconstituted with coupling factor proteins have been examined.

1. The calcium-dependent ATPase activities of coupling factors isolated from spinach, lettuce and *Nicotiana otophora* are completely inhibited by tentoxin. The ATPase activities of coupling factors isolated from *Nicotiana tabacum* and *Nicotiana knightiana* are not affected by tentoxin.

2. Phenazine methosulfate-catalyzed cyclic photophosphorylation with chloroplasts isolated from spinach, lettuce and *N. otophora* is completely inhibited by tentoxin, whereas chloroplasts isolated from *N. knightiana* and *N. tabacum* are relatively insensitive to tentoxin.

3. Spinach chloroplasts, partially depleted in CF_1, can be reconstituted with coupling factors isolated from a wide variety of plants including lettuce, radish, *N. tabacum*, *N. knightiana* and *N. otophora*.

4. Spinach chloroplasts reconstituted with spinach, lettuce and *N. otophora* CF_1 retain their sensitivity to tentoxin; however, when reconstituted with *N. knightiana* and *N. tabacum* coupling factor proteins, a significant fraction of the reconstituted rate remains tentoxin insensitive.

These data are interpreted as evidence that coupling factors that reconstitute with spinach thylakoid membranes have both a catalytic and structural function.

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Abbreviation: CF_1, chloroplast coupling factor 1.

Introduction

It has long been known that isolated chloroplasts can be partially depleted of endogenous coupling factor 1 (CF_1) and that this partial depletion leads to a total loss of coupled photophosphorylation (see ref. 1 for a recent review). Hesse et al. [2] and Berzborn and Schröer [3] have recently shown that if less than 50% of the total pool of CF_1 is removed from thylakoid membranes, complete activity can be recovered upon reconstitution with the purified protein. However, the question still remains as to the function the reconstituted protein has with respect to phosphorylation, i.e., whether it acts in a similar manner to dicyclohexylcarbodiimide by simply plugging the proton channels exposed by the removal of CF_1 from the membrane [4] or whether it is actually catalytically active in ATP synthesis.

Hesse et al. [2] have shown that coupling factor 1, preloaded with radioactive adenine nucleotides, regains the ability to exchange these nucleotides with medium nucleotides when reconstituted with partially depleted thylakoid membranes. This clearly demonstrates that re-bound coupling factor 1 can undergo conformational changes under conditions where the membranes are energized, but does not explain the catalytic nature of the re-bound protein. On the other hand, Berzborn and Schröer [3] have argued that from the difference in the amount of ATPase activity remaining on stripped membranes and the amount of catalytic activity regained upon reconstitution, the re-bound CF_1 is catalytically active. Their argument, however, requires that the ATPase activity of the membrane-bound and soluble coupling factors must be identical. This has, to date, not been demonstrated.

Recently, it has been shown that a small cyclic tetrapeptide, tentoxin, produced by the fungus *Alternaria alternata* is a potent and highly specific energy transfer inhibitor which interacts with the CF_1 part of the energy-transducing mechanism of chloroplasts [5—7]. Furthermore, the sensitivity of CF_1 to tentoxin has been found to be species specific [6]. In this paper, we show that when CF_1, isolated from a plant species relatively insensitive to tentoxin, is reconstituted with partially CF_1-depleted chloroplast membranes from a plant species sensitive to tentoxin, these membranes not only regain photophosphorylation but the reconstituted photophosphorylation is partially insensitive to tentoxin. This is taken as direct evidence demonstrating that the reconstituted coupling factor not only fulfils a structural role but has catalytic function as well.

Methods

Spinach and lettuce chloroplasts were prepared as previously described [8]. Chloroplasts from *Nicotiana tabacum*, *Nicotiana knightiana*, and *Nicotiana otophora* were prepared similarly in a medium containing 50 mM Tris · HCl (pH 7.6)/10 mM KCl/5 mM $MgCl_2$/20 mM sodium ascorbate/2.0 mg/ml bovine serum albumin/1.0% polyethylene glycol (M_r 4000) (v/v).

Coupling factors from spinach, *N. tabacum*, *N. knightiana*, and *N. otophora* were isolated essentially as described by Hesse et al. [2]. Chloroplasts, after being washed twice in 10 mM sodium pyrophosphate (pH 7.8), were suspended

in a medium containing 0.3 M sucrose/2 mM Tris-tricine (pH 7.8)/0.1 mM ATP, and chloroplasts equivalent to 0.1 mg chlorophyll/ml. The suspensions were gently stirred at room temperature for 20 min and the chloroplasts were pelleted by centrifugation at 48 000 × *g* for 30 min. The supernatants (100—500 ml) were concentrated to approximately 5 ml by pressure dialysis using an Amicon PM10 filter and centrifuged at 144 000 × *g* for 60 min to pellet any remaining debris. Radish and lettuce coupling factors were isolated by the procedure described by Lien and Racker [9] and equilibrated in 2 mM Tris-tricine (pH 7.8) containing 0.1 mM ATP. The purity of the coupling factor preparations ranged from 40 to 80% as judged by disc gel electrophoresis. All coupling factors were stored frozen in 2 mM Tris-tricine (pH 7.8)/0.1 mM ATP without any noticeable loss of coupling activity.

The calcium-dependent ATPase activities of all the coupling factors were measured as previously described [6].

Spinach chloroplasts, partially depleted in CF_1, were prepared according to Hesse et al. [2] using either 5 or 10 μM $CaCl_2$ during the final washing procedure.

Protein concentrations were determined by the method of Lowry et al. [10] using bovine serum albumin as a standard.

Reconstitution of spinach chloroplasts, partially depleted of CF_1, with various coupling factors was performed in a 0.255 ml mixture containing chloroplasts, equivalent to about 0.12 mg chlorophyll per ml/10 mM tricine-NaOH (pH 8.0)/2 mM KCl/20 mM $MgCl_2$/0.24 M sucrose and varying amounts of CF_1. The suspensions were stirred in the dark for 1.0 min and the temperature was maintained at 19°C. The suspensions were then diluted to 1.5 ml and contained the following: chloroplasts, equivalent to about 20 μg chlorophyll per ml/50 mM tricine-NaOH (pH 8.0)/10 mM KCl/8 mM $MgCl_2$/40 mM sucrose/5 mM ADP/3.3 mM [^{32}P]phosphate (containing approximately $3 \cdot 10^6$ cpm/ml reaction mixture)/0.1 mM sodium ascorbate/30 μM phenazine methosulfate. Reaction mixtures were illuminated with heat-filtered, saturating intensity white light for 1.0 min in a water-jacketed cell maintained at 19°C. Incorporation of [^{32}P]phosphate into ATP was measured as previously described [8]. Preliminary experiments had shown that a 1.0 min pre-incubation of CF_1-depleted spinach chloroplasts with coupling factor was sufficient to obtain maximal reconstitution for any given coupling factor concentration. All coupling factor preparations were titrated to determine the maximal amount of coupling factor that had to be added to yield the maximal rate of photophosphorylation upon reconstitution.

Tentoxin was isolated as previously described [11].

Spinach, lettuce and *N. otophora* were chosen as examples of "tentoxin-sensitive" plants, whereas *N. knightiana*, *N. tabacum* and radish are examples of "tentoxin-insensitive" plants (see fig. 1, A and B).

Results

(1) *Reconstitution of spinach chloroplasts partially depleted in CF_1 with coupling factors isolated from various sources*

Table I shows typical results obtained when spinach chloroplasts, partially

TABLE I

ABILITY OF COUPLING FACTORS FROM VARIOUS SPECIES TO RECONSTITUTE WITH SPINACH CHLOROPLASTS DEPLETED IN CF_1

Spinach chloroplasts were treated with 2.0 mM Tris-tricine (pH 7.8)-0.3 M sucrose/10 μM $CaCl_2$ to remove a portion of the coupling factor. The concentration of protein during reconstitution (in mg/ml) was: 0.39, spinach; 0.82. *N. tabacum*; 1.27, *N. otophora*; 0.86, lettuce; 0.65, radish; 0.78, *N. knightiana*. All suspensions included chloroplasts, equivalent to 0.11 mg chlorophyll/ml. Reconstitution was performed and photosphorylation was measured as described in Methods.

Source of CF_1	μmol ATP/mg chlorophyll per h	
	Expt. I	Expt. II
None	36	19
Spinach	353	495
Radish	200	—
Lettuce	253	—
N. tabacum	288	504
N. otophora	248	460
N. knightiana	—	424

depleted in CF_1 by washing in a low-salt buffer [2], are reconstituted with coupling factor preparations isolated from a wide variety of plants. Preparations of coupling factors from all plants tested are capable of restoring between 70 and 100% of the photophosphorylation activity obtained with the spinach CF_1, with the exception of the radish preparation. The failure of the radish CF_1 preparation to yield a higher activity upon reconstitution may be related to a loss of an integral factor from the multi-subunit complex required for binding as described by Nelson and Karny [12] for lettuce CF_1; however, preliminary experiments (data not shown) have indicated the presence of an inhibitor of phosphorylation in this and similar radish preparations.

The data shown in Table I can be interpreted in several ways. It is possible that all of the coupling factors tested are capable of binding to the CF_0 part of the chloroplast energy-transducing mechanism and merely prevent the leak of protons through these channels in an analogous manner to the energy transfer inhibitor dicyclohexylcarbodiimide [4] or that, once bound, the coupling factors actually participate catalytically in the synthesis of ATP. In order to discriminate between these two possibilities, we have made use of the unique properties of the energy transfer inhibitor, tentoxin.

(2) *Inhibition by tentoxin of the calcium dependent ATPase activity of isolated coupling factors and phenazine methosulfate catalyzed cyclic photophosphorylation*

Tentoxin has been shown by Arntzen [5] and Steele et al. [6] to be an effective energy transfer inhibitor of photophosphorylation with chloroplasts isolated from lettuce. In addition, Steele et al. [6] have shown that tentoxin interacts directly with CF_1 (probably with the $\alpha\beta$ subunit complex (see ref. 13)) and inhibits the ATPase activity of the isolated coupling factor 1. Steele et al. [6] have also found that the effect of tentoxin on both photophosphorylation and the ATPase activities of isolated coupling factor varies with the plant species; lettuce and spinach being examples of plants that are "tentoxin-sensi-

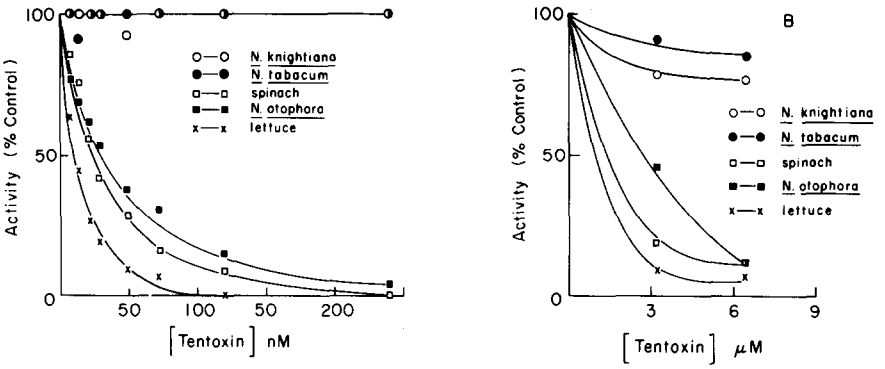

Fig. 1. A. Species specificity for the tentoxin inhibition of the coupling factor ATPase reaction. Coupling factors were isolated as described in Methods and the calcium-dependent ATPase activity was measured as previously described [6]. Reaction mixtures were pre-incubated for 1 h at room temperature with tentoxin and contained 25 μg protein per ml. Note that the coupling factors were not activated. Control rates were (μmol ATP hydrolyzed/mg protein per h): 13.9, lettuce; 19.4 spinach; 7.8, *N. otophora;* 9.0, *N. knightiana;* 4.8, *N. tabacum.* B. Species specificity for the tentoxin inhibition of phenazine methosulfate-catalyzed cyclic phosphorylation. Chloroplasts were prepared as described in Methods. Reaction mixtures contained, in 1.5 ml total volume chloroplasts, equivalent to 25 μg chlorophyll/ml, 50 mM tricine-NaOH (pH 8.0)/10 mM KCl/5 mM $MgCl_2$/5.0 mM ADP/3.3 mM [^{32}P]phosphate (containing approximately $3.5 \cdot 10^6$ cpm/ml)/0.1 mM sodium ascorbate/30 μM phenazine methosulfate and tentoxin as indicated. Reaction mixtures were pre-incubated with tentoxin for 1.0 min at 19°C before being illuminated for 1.0 min. Control rates were (μmol ATP/mg chlorophyll per h): 669, spinach; 191, *N. otophora;* 517, *N. knightiana;* 356, lettuce; 207, *N. tabacum.*

tive", whereas radish is "tentoxin-insensitive".

Fig. 1A shows the effect of tentoxin on the calcium-dependent ATPase activities of (non-activated) coupling factors isolated from spinach, lettuce, *N. otophora*, *N. tabacum* and *N. knightiana.* Clearly, the sensitivities to tentoxin of the ATPase activities of the various coupling factor proteins is strikingly different. The ATPase activities of coupling factors from *N. knightiana* and *N. tabacum* are not affected by tentoxin, whereas the activities of coupling factors isolated from spinach, lettuce and *N. otophora* are extremely sensitive to the inhibitor, the activities of all three of these three proteins being almost completly inhibited at 0.1 μM tentoxin.

A similar pattern of inhibition by tentoxin is also seen for phenazine methosulfate-catalyzed cyclic photophosphorylation with the chloroplasts isolated from these five plant species (Fig. 1B). Tentoxin, at about 2—3 μM, completely inhibits photophosphorylation with spinach, lettuce and *N. otophora* chloroplasts, whereas photophosphorylation with chloroplasts from *N. tabacum* and *N. knightiana* is hardly affected at these low concentrations of this inhibitor. Higher concentrations of tentoxin do cause a partial inhibition of photophosphorylation even with chloroplasts isolated from "tentoxin-insensitive" plants, as shown in Fig. 2A for *N. tabacum* chloroplasts; however, the inhibition is only about 50% of the control rate and further increasing the concentration of tentoxin does not result in increased inhibition of photophosphorylation.

The partial sensitivity of photophosphorylation with *N. tabacum* chloroplasts to tentoxin is not, as yet, understood; however, it should be pointed that these experiments (in contrast to the experiment show in Fig. 1A) were not been performed under equilibrium conditions. Tentoxin has a rather slow for-

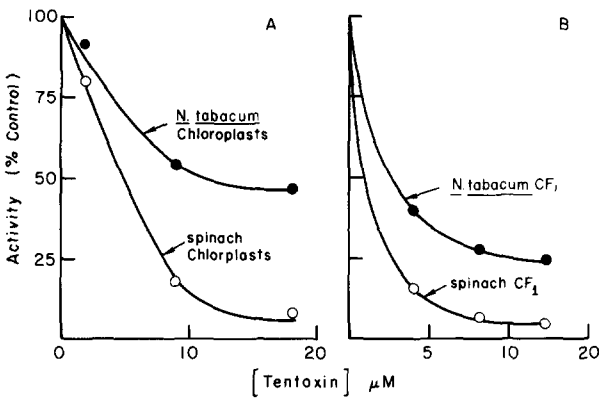

Fig. 2. A. Differential inhibition by tentoxin of phosphorylation with spinach and *N. tabacum* chloroplasts. Spinach and *N. tabacum* chloroplasts were prepared as described in Methods. Reaction mixtures and assay were as in Fig. 1B. Control rates were 744 and 563 μmol ATP/mg chlorophyll per h for spinach and *N. tabacum* chloroplasts, respectively. B. Partial insensitivity to tentoxin of photophosphorylation with spinach chloroplasts reconstituted with *N. tabacum* coupling factor. Spinach chloroplasts were partially depleted in CF_1 by washing in 2.0 mM Tris-tricine (pH 7.8/0.3 M sucrose/5 μM $CaCl_2$. Reconstitution with spinach coupling factor (0.32 mg protein/ml) and *N. tabacum* coupling factor (0.82 mg protein/ml) was performed as described in Methods. The concentration of chloroplasts during reconstitution was equivalent to 0.10 mg chlorophyll/ml. Control rates were 213 and 199 μmol ATP/mg chlorophyll per h for chloroplasts reconstituted with spinach and *N. tabacum* coupling factor, respectively, and in the absence of added coupling factor was 25.

ward rate constant for binding [13] and it takes an hour to establish equilibrium. In order to decrease the time necessary to observe inhibition of photophosphorylation with tentoxin, relatively high concentrations have been used throughout these experiments. The partial inhibition of photophosphorylation observed with *N. tabacum* chloroplasts (Fig. 2A) may be the result of secondary effects of tentoxin on the *N. tabacum* thylakoid membrane and may not reflect a direct effect of tentoxin on the energy-transducing mechanism of the chloroplasts.

(3) *Reconstitution of photophosphorylation and sensitivity of the reconstituted rate to inhibition by tentoxin*

Fig. 2A and B compares the sensitivities of phenazine methosulfate-catalyzed cyclic phosphorylation to tentoxin with either intact spinach and *N. tabacum* chloroplasts or spinach chloroplasts partially depleted in CF_1 reconstituted with either spinach or *N. tabacum* coupling factors. As already noted in the previous section, a substantial fraction of the rate of photophosphorylation catalyzed by *N. tabacum* chloroplast is insensitive to the phytotoxin (Fig. 2A). Fig. 2B shows that when spinach chloroplasts, which have been washed in low salt (5 μM $CaCl_2$) buffer to remove a portion of the total coupling factor 1 pool, are reconstituted with *N. tabacum* coupling factor 1, the reconstituted rate of phosphorylation is partially insensitive to tentoxin. Furthermore, the difference in rates between spinach chloroplasts reconstituted with *N. tabacum* and spinach coupling factor protein remains about constant at all concentrations of tentoxin (in the experiment shown in Fig. 2B this fraction is about 25% of the control rate or 50 μmol ATP/mg chlorophyll per h). Other experi-

TABLE II

N. TABACUM AND *N. KNIGHTIANA* CF_1-INDUCED INSENSITIVITY OF RECONSTITUTED SPINACH CHLOROPLASTS TO TENTOXIN

Spinach chloroplasts were treated as in Fig. 2B. The concentration of protein during reconstitution was (in mg/ml): 0.39, spinach; 0.82, *N. tabacum*; 0.78, *N. knightiana*. The concentration was equivalent to 0.08 mg chlorophyll per ml. The suspensions were pre-incubated for 1.0 min at 10°C with 1.12 μM tentoxin before being illuminated.

Source of CF_1	μmol ATP/mg chlorophyll per h	
	— Tentoxin	+ Tentoxin
None	19	—
Spinach	495	129
N. tabacum	504	263
N. knightiana	424	225

ments (data not shown) have shown that the fraction of activity that remains insensitive to tentoxin is a function of the amount of spinach coupling factor 1 that can be replaced by *N. tabacum* coupling factor. Similar results have also been obtained with the radish coupling factor preparation (data not shown) but the presence of a phosphorylation inhibitor in the radish coupling factor preparation has made that data more difficult to interpret. Table II compares the tentoxin sensitivity of photophosphorylation for spinach chloroplasts reconstituted with coupling factors isolated from spinach, *N. tabacum* and *N. knightiana*. Both species of *Nicotiana* are "tentoxin-insensitive" (see Fig. 1A and 1B) and, indeed, whereas 1.2 μM tentoxin inhibits the reconstituted rate with spinach coupling factor about 75%, the reconstituted rates with *N. tabacum* and *N. knightiana* coupling factors are only inhibited 48% and 47%, respectively.

Fig. 3 shows the results for a similar reconstitution experiment comparing spinach and *N. otophora* coupling factor proteins. *N otophora* is a "tentoxin-sensitive" plant (Fig. 1, A and B) and, as seen in Fig. 3, the reconstituted rates

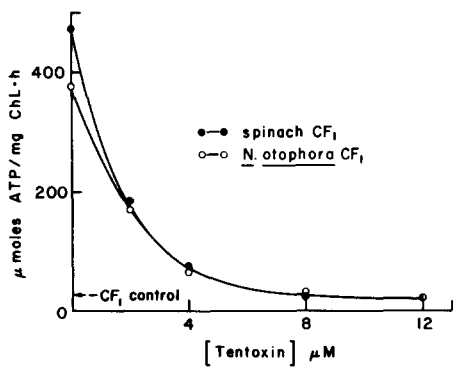

Fig. 3. Lack of a differential sensitivity to tentoxin of photophosphorylation with spinach chloroplasts reconstituted with spinach and *N. otophora* coupling factors. Spinach chloroplasts were prepared as in Table I and reconstituted with spinach (0.32 mg protein/ml) and *N. otophora* (1.27 mg protein/ml) coupling factors in a suspension containing 0.12 mg chlorphyll/ml. Reaction mixtures with tentoxin were pre-incubated for 1.0 min at 19°C before being illuminated.

of photophosphorylation with both coupling factors are equally sensitive to inhibition by tentoxin. Identical results have been obtained when using the lettuce coupling factor for the reconstitution (data not shown).

Discussion

A number of attempts have been made to determine whether or not the coupling factor reconstituted with thylakoid membranes actually has catalytic activity; however, these attempts have been complicated by the observation that when a large fraction of the coupling factor is removed from the thylakoid membrane, the rate of phosphorylation with reconstituted membranes is very low [1]. Recently, Hesse et al. [2] have offered an explanation for this phenomenon and have proposed that the low salt conditions necessary to remove a large fraction of membrane-bound CF_1 has secondary effects leading to the disruption and eventual creation of large holes in the membrane.

In order to avoid this disruptive phenomenon and yet be able to discriminate between the CF_1 remaining on the membrane after partial removal and the CF_1 that re-binds to the membrane upon reconstitution, we have used heterologous reconstitutions with coupling factors that have different sensitivities to the energy transfer inhibitor, tentoxin. The ability of CF_1 from a variety of plants to reconstitute with spinach chloroplasts is clearly demonstrated in Table I. Indeed, we have screened coupling factors isolated from many other plant species and have found that all coupling factors tested so far are capable of restoring between 60 and 100% of the activity obtained using spinach CF_1. Of importance, however, is the observation that only those CF_1 isolated from plants that are relatively insensitive to tentoxin, e.g. *N. tabacum* and *N. knightiana*, confer a partial tentoxin insensitivity to spinach chloroplasts when reconstituted with spinach chloroplasts partially depleted in CF_1.

This data could be interpreted as indicating that, when reconstituted with spinach membranes, other coupling factors are catalytically active but not spinach CF_1; however, because the reconstituted rates of photophosphorylation with most coupling factor proteins approach the rate obtained with spinach CF_1, we feel that this alternative is unlikely. Indeed, we believe that our data provides the first direct evidence showing that upon reconstitution, the reconstituted CF_1 has both a structural function in re-sealing the proton channels created by the previous removal of CF_1 and catalytic activity in the synthesis of ATP.

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