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TENTOXIN-INDUCED BINDING OF ADENINE NUCLEOTIDES TO SOLUBLE SPINACH CHLOROPLAST COUPLING FACTOR 1

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Summary

The effect of tentoxin on the binding of adenine nucleotides to soluble chloroplast coupling factor (CF_1) has been studied and the following results have been obtained:

1. Tentoxin (400 μM) increases the maximum attainable tight binding of ADP to CF_1. In the absence of tentoxin, the maximal binding observed by the method employed is about 0.3 nmol ADP/mg protein, whereas in the presence of tentoxin this ranges from 1.5 to 2.0 nmol ADP/mg protein.

2. Tentoxin-induced binding of ADP to CF_1 is severely inhibited by divalent cations (50% inhibition at about 2 mM) but only weakly inhibited by monovalent cations (less than 50% inhibition at 100 mM).

3. The binding of ADP to CF_1 induced by tentoxin is inhibited by ATP and adenylyl imidodiphosphate but is not inhibited by other nucleotides including AMP, GDP, CDP, IDP, or β,γ-methylene ATP.

4. The ADP-CF_1 complex induced by tentoxin is quite stable. 75% remains bound to CF_1 even after passage of the complex through a gel filtration column. An additional 25% can be removed by incubation in the presence of ADP, and all of the bound ADP can be removed only after incubation in the presence of both tentoxin and ADP. The latter result is interpreted as a tentoxin-induced exchange of bound ADP for medium ADP.

Introduction

Coupling factor 1 proteins isolated from a wide variety of organisms contain 1—3 mol tightly bound adenine nucleotides per mol protein (see ref. 1 for a

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Abbreviations: CF_1, chloroplast coupling factor 1; AMP-PNP, adenylyl imidodiphosphate.

recent review). Although the function of these nucleotides with respect to the role of the coupling factor in phosphorylation is uncertain, it is clear that they do not directly participate in the catalytic activity of the coupling factors [2—5]. Adenine nucleotides tightly bound to membrane-bound coupling factors have been shown in some systems to undergo energy-dependent exchange with free adenine nucleotides [6—10].

The most complete studies on the exchange of tightly bound adenine nucleotides have been performed with the thylakoid membrane-bound ATPase complex. Exchange can be induced by illumination which generates a protonmotive force [6—8] or by an artificially induced transmembrane pH gradient [11,12]. It is inhibited by uncouplers [6—8] and energy transfer inhibitors of the type that block proton flux through the hydrophobic proton channel part of the ATPase complex (e.g. dicyclohexylcarbodiimide and triphenyl tin chloride) [13]. Exchange is not inhibited by energy transfer inhibitors that directly block the catalytic activity of CF_1 (e.g. Dio-9 and phloridzin) [6,7].

Recently, we found that the exchange of adenine nucleotides bound to coupling factor 1 (CF_1) is induced by tentoxin under conditions in which the thylakoid membrane is not energized [13]. At that time, however, we found no effect of tentoxin on the binding and/or exchange of adenine nucleotides with the soluble CF_1. We have re-examined this problem and in this paper we report conditions where tentoxin greatly alters the binding of adenine nucleotides to the soluble protein. In addition, we show that the tentoxin-induced binding of ADP to CF_1 is very tight, and that once the adenine nucleotide is bound, it can only be exchanged.

Methods

Spinach CF_1 was isolated as described by Younis et al. [14]. The protein was stored at —20°C in 20 mM tricine-NaOH (pH 8.0) buffer containing 2.0 mM dithiothreitol, 1.0 mM ATP, 1.0 mM EDTA, and 50% glycerol. The specific activity of the preparation used for these experiments was 23 μmol ATP hydrolyzed/mg protein per min assayed as the Ca^{2+}-dependent ATPase after trypsin activation as described by Lien and Racker [15]. No noticeable loss of activity was observed upon storage. Prior to being used for binding studies, the protein was desalted by passing 0.2 ml (containing approx. 1 mg protein) through a Sephadex G-50 (fine) column (1.0 × 25 cm) equilibrated with 5.0 mM Tricine-NaOH (pH 8.0). Protein concentration was determined as described by Lowry et al. [16] using bovine serum albumin as the standard. Tentoxin was prepared as previously described [17].

The binding of [^{3}H]ADP to CF_1 was measured as described by Penefsky [18]. The Sephadex G-50 (fine) used to separate ADP bound to CF_1 from free ADP was equilibrated with 5.0 mM Tricine-NaOH (pH 8.0) and 1.0 ml bed volumes were packed in 1.0 ml disposable syringes by centrifugation. Incubation mixtures contained in a total volume of 70 μl: 20 μg CF_1, 5.0 mM Tricine-NaOH (pH 8.0), 0—5.0 μM [^{3}H]ADP (containing approx. $1.5 \cdot 10^7$ dpm/ml incubation mixture), and other reagents as indicated in the legends. Protein solutions were routinely incubated at 37°C for 30 min prior to the determination of bound ligand to insure that equilibrium had been established (see Fig. 2

for a time course for binding). After incubation, the samples were loaded onto the 1.0 ml packed gel bed and centrifuged for 2.0 min at about 1200 X *g*. 50 μl of the effluent were counted in 5.0 ml scintillation cocktail containing toluene (666 ml/l), triton X-100 (334 ml/l, PPO (5.0 g/l), and POPOP (0.1 g/l).

Results

Tentoxin-induced binding of ADP to CF_1

The latent ATPase of thylakoid membranes has been shown to have several binding sites for adenine nucleotides [19—22]. The binding constants for adenine nucleotides at these sites are sensitive to mono- and divalent cations [20], phosphate [22], pH [20], and the activation state of the protein [19]. Fig. 1 shows the results of an experiment in which the binding of [^{3}H]ADP to latent spinach CF_1 was measured as a function of the ADP concentration in the presence (upper curve) and absence (lower curve) of 400 μM tentoxin. In the absence of tentoxin, the maximal binding observed is about 0.3 nmol ADP per mg protein (approx. 0.1 ADP/CF_1). At saturating tentoxin concentrations, the maximal binding of ADP increases approximately 4—6-fold to about 1.3—2.0 nmol ADP/mg protein (approx. 0.4—0.7 ADP/CF_1).

The rate of ADP binding to CF_1 induced by tentoxin is relatively slow. At room temperature, no increase in binding is observed even after 1 h incubation (data not shown). At higher temperatures, however, the rate of binding increases and Fig. 2 shows the time course for the tentoxin-induced binding of ADP to CF_1 at 37°C. Apparent equilibrium is established after about 15 min incubation. Thus, to insure that maximal binding of ADP to CF_1 had occurred,

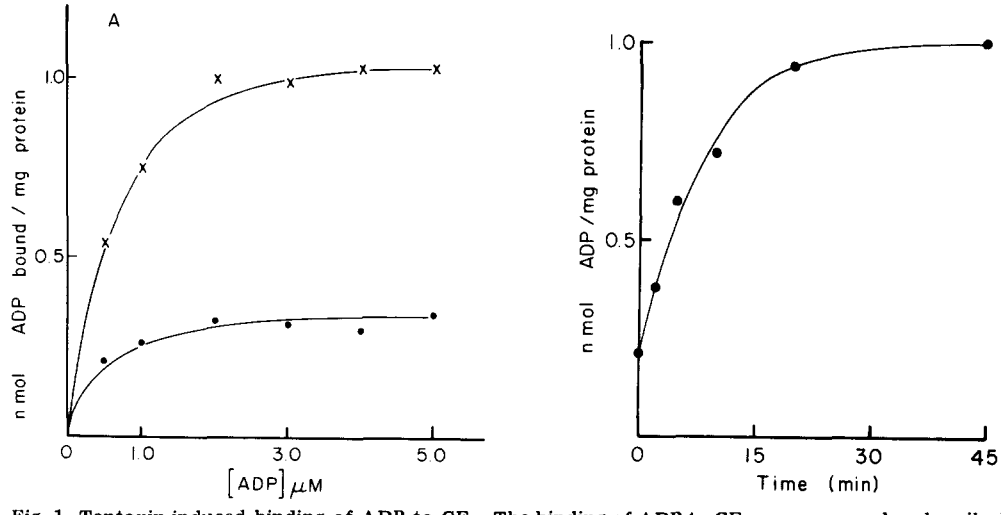

Fig. 1. Tentoxin-induced binding of ADP to CF_1. The binding of ADP to CF_1 was measured as described in Methods. Incubation mixtures containing 20 μg protein, 5.0 mM Tricine-NaOH (pH 8.0) and variable amounts of [^{3}H]ADP were incubated for 30 min at 37°C. Binding was measured either in the absence (●———●) or in the presence (X———X) of 400 μM tentoxin.

Fig. 2. Time course for the tentoxin-induced binding of ADP to CF_1. Conditions as in Fig. 1. Incubation mixtures contained 5.0 μM ADP and 400 μM tentoxin.

all subsequent experiments were performed with a 30 min incubation at 37°C.

The effect of tentoxin on the binding of ADP to CF_1 saturates at about 300 μM. This is the same concentration of tentoxin required to saturate the induction of the latent ATPase activity of soluble CF_1 and the incubation of the energy-independent exchange of the tightly bound adenine nucleotides of membrane-bound CF_1 [13].

Divalent cation inhibition of the tentoxin-induced binding of ADP to CF_1

Divalent cations alter the affinity of CF_1 for ADP [19,20]. Calcium (5 mM) effectively suppresses the tentoxin-induced binding of ADP to CF_1 at all concentrations of tentoxin. Fig. 3 shows a concentration curve for the calcium inhibition of the binding of ADP to CF_1 both in the absence and presence of (400 μM) tentoxin. The inhibition by Ca^{2+} saturates at about 5 mM and 50% inhibition is achieved at about 2—3 mM. This effect is not specific for Ca^{2+} (Table I, A). Ba^{2+}, Sr^{2+}, Mn^{2+}, and Mg^{2+} are equally effective as Ca^{2+} in suppressing the tentoxin-induced binding of ADP to CF_1. Monovalent cations, on the other hand, are far less effective in suppression the tentoxin-induced binding of ADP to CF_1 (Table I, B). The most potent monovalent cation tested was Li^+, and even at 100 mM the tentoxin-induced binding was only inhibited about 40%.

Specificity of the tentoxin-induced nucleotide binding site

The nucleotide specificity of the tentoxin-induced binding site was determined by measuring the ability of different nucleotides to inhibit the binding of [^{3}H]ADP to CF_1. Typical results are shown in Table II. Of the various nucleotides examined, at equimolar concentrations the most pronounced inhibition is obtained with ATP and the ATP analogue, adenylyl imidodiphosphate (AMP-PNP). GTP inhibits about 10%, whereas GDP, IDP, AMP, CDP, CMP, and the methylene analogue of ATP, β,γ-methylene ATP, have no

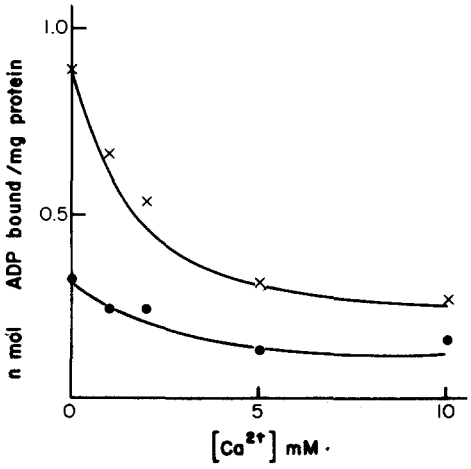

Fig. 3. Calcium inhibition of the binding of ADP to CF_1. Conditions as in Fig. 1. Incubation mixtures contained 5.0 μM ADP: ●———●, in the absence of tentoxin; and X———X, in the presence of 400 μM tentoxin.

TABLE I

CATION SUPPRESSION OF ADP BINDING TO CF_1

The binding of ADP to CF_1 was measured as described in Methods. Concentrations were: 5 μM [^{3}H]ADP and 400 μM tentoxin. All cations were added as the Cl^- salts and the concentrations of di- and monovalent cations were 5.0 and 100 mM, respectively.

Addition	nmol ADP bound per mg protein
(A) Divalent cations:	
none	1.14
Ca^{2+}	0.46
Sr^{2+}	0.37
Ba^{2+}	0.37
Mn^{2+}	0.34
Mg^{2+}	0.34
(B) Monovalent cations:	
none	1.10
Li^+	0.66
Na^+	0.73
K^+	0.70
Cs^+	0.77

apparent effect. Fig. 4 demonstrates that the inhibition by ATP, AMP-PNP, and GTP is competitive with ADP. The tentoxin-induced binding site is relatively specific for adenine nucleotides although AMP does not bind to it. Apparently, a significant distortion in the bond angle between the β and γ phosphates (as in the case with β,γ-methylene ATP but not with AMP-PNP) also leads to a large decrease in the affinity of the nucleotide binding site for the nucleotide.

Stability of the ADP-CF_1 complex induced by tentoxin

To determine the stability of the ADP-CF_1 complex, CF_1 was loaded with [^{3}H]ADP by incubating the protein with tentoxin, the CF_1-ADP complex was centrifuged through the 1.0 ml Sephadex G-50 column as usual, and the eluate

TABLE II

EFFECT OF VARIOUS NUCLEOTIDES ON TENTOXIN-INDUCED BINDING OF ADP TO CF_1

The binding of ADP to CF_1 was measured as described in Methods. The concentration of [^{3}H]ADP was 5.0 μM during incubation. Final concentration of all other nucleotides was 5.0 μM.

Nucleotide	nmol ADP bound per mg protein
— Tentoxin control	0.37
+ Tentoxin (400 μM) control	1.24
+ AMP	1.20
+ ATP	0.69
+ AMP-PNP	0.30
+ β,γ-methylene ATP	1.18
+ GDP	1.28
+ GTP	1.11
+ CMP	1.28
+ CDP	1.36
+ IDP	1.22

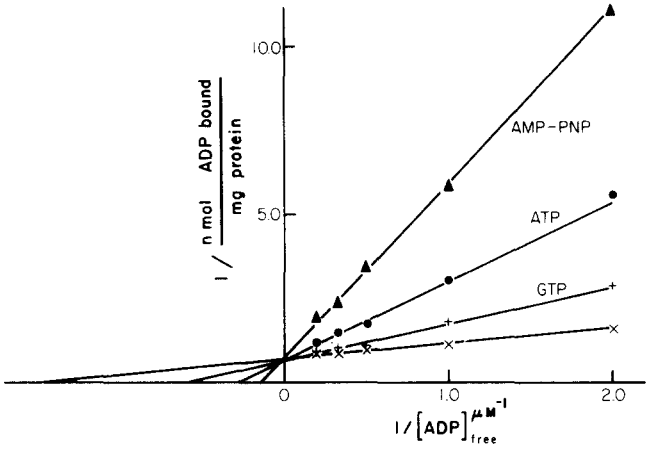

Fig. 4. Double reciprocal plot for the inhibition of tentoxin-induced binding of ADP to CF_1 by other nucleotides. Conditions as in Fig. 1 with variable concentrations of ADP. The concentration of free ADP was calculated assuming that all of the ADP in the incubation mixture was capable of binding. Incubation mixtures contained either (X———X) no further additions, (+———+) 15 μM GTP, (●———●) 2.5 μM ATP, or (▲———▲) 2.5 μM AMP-PNP.

was chromatographed on a Sephadex G-50 column (1.0 X 30 cm) to separate ADP bound to CF_1 from free ADP. 75% of the ADP that comes through the original 1.0-ml column with CF_1 remains tightly bound to the protein. Identical results are obtained when the sample is recentrifuged throug a second 1.0 ml Sephadex G-50 column.

Conditions necessary for the removal of ADP from CF_1 were investigated. CF_1 was loaded with [^{3}H]ADP and the complex was separated from free ADP by centrifugation through Sephadex G-50. The eluate was then incubated a second time at 37°C and the amount of [^{3}H]ADP remaining bound to the pro-

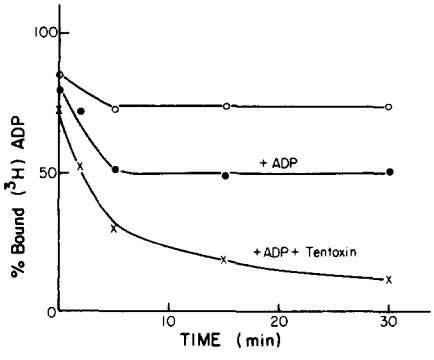

Fig. 5. Time course for the release of ADP bound to CF_1. CF_1 was incubated with 5.0 μM [^{3}H]ADP in the presence of 400 μM tentoxin and separated from free ADP and tentoxin as described in Methods. The control bound 1.9 nmol ADP per mg protein. Fractions were incubated a second time at 37°C for variable lengths of time and the CF_1 was again separated from free ADP by centrifugation through 1.0 ml Sephadex G-50 columns. The second incubation was performed (○———○) without any further additions in the presence of 400 μM tentoxin, (●———●) in the presence of 5.0 μM (unlabeled) ADP, or (X———X) in the presence of 5.0 μM (unlabeled) ADP plus 400 μM tentoxin.

tein after varying incubation times was determined by centrifuging the protein through a second Sephadex G-50 column. In the absence of added ADP (or the presence of tentoxin alone) only 25% of the bound labeled ADP is removed from the protein by the second incubation in agreement with the results obtained by using the longer gel filtration column (Fig. 5). Unlabeled ADP causes the release of an additional 25% of the bound labeled ADP within the first 5 min and this level remains constant over the next 25 min. However, tentoxin (plus ADP) induced a much more rapid loss of bound labeled ADP from the protein, and after 30 min only about 10% of the original amount of labeled ADP remains bound to CF_1. Thus, tentoxin induces an exchange of bound ADP with medium ADP.

Discussion

Tentoxin, a cyclic tetrapeptide, has a variety of effects on the thylakoid energy transfer transducing complex of 'tentoxin-sensitive' plants. These effects can be categorized with respect to the concentration of tentoxin and depend upon the physical state of CF_1, i.e. whether it is membrane-bound or soluble. Low concentrations of tentoxin (5—100 nM) inhibit the activated ATPase activity of both soluble and membrane-bound CF_1 [17,23]. Moderate concentrations of tentoxin (0.1—5 μM) inhibit photophosphorylation, an effect which can be directly related to its inhibition of the catalytic activity of CF_1 [17,23, 24]. High concentrations of tentoxin (5—500 μM) activate the latent ATPase activity of soluble but not membrane-bound CF_1 [13,25] and induce an energy-independent exchange of adenine nucleotides with membrane-bound [13] as well as with soluble CF_1.

Many investigators have measured the binding of adenine nucleotides to soluble CF_1, both to the latent [20—22] and to the activated [19] ATPase. Two tight binding sites for ADP having a dissociation constant between 2 and 10 μM have been reported [20—22]. This would appear to conflict with our data in which we find very little binding to the protein in the absence of tentoxin and a maximum of one site per molecule in the presence of tentoxin with a very low dissociation constant (less than 0.5 μM). This apparent discrepancy is not due to an inactivation of our protein as the specific activity (measured as the trypsin activated Ca^{2+}-ATPase) is fairly high. More likely the differences in the observed binding are a result of the different methods used to measure the binding (except ref. 21) and may reflect binding to different sites on CF_1. It is unlikely that we would be able to observe binding sites for ADP on the soluble protein that have a dissociation constant greater than 0.5 μM.

Roy and Moudrianakis [21] have measured the binding of ADP to CF_1 under similar conditions employed in our study. In agreement with their results, we find that the binding of ADP to CF_1 is quite slow even at 37°C. Their binding studies required a 2-h incubation time to obtain equilibrium. Tentoxin clearly accelerates the rate of ADP binding to CF_1. The binding of ADP to soluble CF_1 induced by tentoxin is an exchange of ADP already bound to the enzyme for medium ADP.

There are several similarities between the binding site exposed by tentoxin on soluble CF_1 and that revealed by energization of membrane-bound CF_1:

(1) the maximal binding observed is about 1 ADP/CF_1 (refs. 7—9, except ref. 6, where a higher light-induced exchange was reported); (2) in both cases the apparent free acid binds and not the metal chelate [9]; (3) they are both relatively specific for adenine nucleotides; and (4) the adenine nucleotides bound cannot be easily removed and must be exchanged [6—9]. On the other hand, there are some significant differences: (1) divalent cations repress the tentoxin-induced binding but enhance the energy-dependent binding of ATP [8]; and (2) AMP-PNP is a relatively poor inhibitor of energy-induced binding, whereas it is a very good inhibitor of the tentoxin-induced binding of ADP to CF_1 [8]. These difference may reflect that the binding sites on CF_1 revealed by an energization of the thylakoid membrane and by tentoxin with soluble CF_1 are completely different or they may reflect changes in the properties of the tight binding site when the protein is solubilized.

Because the tentoxin-induced binding of ADP to CF_1 and the tentoxin-induced back exchange of bound ADP are so slow ($t_{1/2} \approx 5$ min), it seems unlikely that the binding that we are measuring is at the active site of CF_1. Rather, it seems more likely that tentoxin is exposing some other adenine nucleotide binding site on the soluble protein that can then exchange bound ADP for medium ADP. The function of this is not known, but it may very well be the same binding site that is exposed upon energization of membrane-bound CF_1. If so, the use of tentoxin to study this binding site might eventually lead to the elucidation of its function.

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References

1 Harris, D.A. (1978) Biochim. Biophys. Acta 463, 245—273
2 Strotmann, H., Bickel-Sandkötter, S., Edelmann, K., Schlimme, E., Boos, K.S. and Lüstorff, J. (1977) in Structure and Function of Energy Transducing Membranes (van Dam, K. and van Gelder, B.F., eds.), pp. 307—317, Elsevier/North-Holland Biomedical Press, Amsterdam
3 Smith, D.J. and Boyer, P.D. (1976) Proc. Natl. Acad. Sci. U.S. 73, 4314—4318
4 Smith, D.J., Stokes, B.O. and Boyer, P.D. (1976) J. Biol. Chem. 251, 4165—4171
5 Shavit, N., Lien, S. and San Pietro, A. (1977) FEBS Lett. 73, 55—58
6 Harris, D.A. and Slater, E.D. (1975) Biochim. Biophys. Acta 387, 335—348
7 Bickel-Sandkötter, S. and Strotmann, H. (1976) FEBS Lett. 65, 102—106
8 Magnusson, R.P. and McCarty, R.E. (1976) J. Biol. Chem. 251, 7417—7422
9 Strotmann, H. and Bickel-Sandkötter, S. (1977) Biochim. Biophys. Acta 460, 126—135
10 Harris, D.A., John, P. and Radda, G.K. (1977) Biochim. Biophys. Acta 459, 546—559
11 Strotmann, H., Bickel, S. and Huchzermeyer, B. (1976) FEBS Lett. 61, 194—198
12 Huchzermeyer, B. and Strotmann, H. (1977) Z. Naturforsch. 32c, 803—809
13 Reimer, S. and Selman, B.R. (1978) J. Biol. Chem., in the press
14 Younis, H.M., Winget, G.D. and Racker, E. (1977) J. Biol. Chem. 252, 1814—1818
15 Lien, S. and Racker, E. (1971) Methods Enzymol. 23, 547—555
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
17 Steele, J.A., Uchytil, T.F., Durbin, R.D., Bhatnagar, P. and Rich, D.H. (1976) Proc. Natl. Acad. Sci. U.S. 73, 2245—2248

18 Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891—2899
19 Cantley, Jr., L.C. and Hammes, G.G. (1975) Biochem. 14, 2968—2975
20 Vandermeulen, D.L. and Govindjee (1977) Eur. J. Biochem. 78, 585—598
21 Roy, H. and Moudrianakis, E.N. (1971) Proc. Natl. Acad. Sci. U.S. 68, 464—468
22 Girault, G., Galmiche, J.-M., Michel-Villaz, M. and Thiery, J. (1973) Eur. J. Biochem. 38, 473—478
23 Selman, B.R. and Durbin, R.D. (1978) Biochim. Biophys. Acta 502, 29—37
24 Arntzen, C.J. (1972) Biochim. Biophys. Acta 283, 539—542
25 Steele, J.A., Uchytil, T.F. and Durbin, R.D. (1978) Biochim. Biophys. Acta 504, 136—141