TENTOXIN-INDUCED ADENINE NUCLEOTIDE EXCHANGE WITH SOLUBLE AND THYLAKOID MEMBRANE-BOUND CHLOROPLAST COUPLING FACTOR 1

Bruce R. SELMAN and Susanne SELMAN-REIMER

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, WI 53706, USA

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1. Introduction

Membrane-bound chloroplast coupling factor 1 (CF_1) contains 1–3 'tightly bound' adenine nucleotides [1–9]. These adenine nucleotides exchange with medium ADP upon energization of the thylakoid membrane [2,4,10,11]. Although neither the function of the bound adenine nucleotides nor the significance of the exchange reaction is understood, the exchange reaction per se has been interpreted as a 'conformational change' of the membrane-bound protein [12,13].

We have recently shown that tentoxin induces an adenine nucleotide exchange with both 'non-energized' thylakoid membranes [14] and soluble CF_1 [15]. We demonstrate here that the adenine nucleotide binding sites revealed by tentoxin with both the membrane-bound and soluble CF_1 are identical to the 'exchange site' revealed by the energization of CF_1-containing thylakoid membranes.

2. Methods

CF_1 [16], tentoxin [17] and chloroplasts [3] were prepared as described. The specific activity of the protein used for this investigation was 23 μmol ATP hydrolyzed/mg protein/min, assayed as the Ca^{2+}-dependent ATPase after trypsin activation [18]. In all experiments in which the binding of ADP to CF_1 was measured, the protein was first desalted by chromatography on Sephadex G-50 as in [15].

The light-induced binding of [^{3}H]ADP to thylakoid membranes was measured as in [10]. Tentoxin-induced binding of ADP to thylakoid membranes was measured under similar conditions. The release of either tentoxin or light-induced bound [^{3}H]ADP was measured as in [14].

Chloroplast thylakoids, depleted ~50% in CF_1, were prepared by washing the membranes in buffer containing 2.0 mM Tris–tricine (pH 7.8), 0.3 M sucrose and 10 μM $CaCl_2$ [19]. The reaction conditions for the binding of [^{3}H]ADP to soluble CF_1, the reconstitution of CF_1 with partially-depleted thylakoid membranes, and the release of bound [^{3}H]ADP from the membranes are described in the legend to table 4.

All experiments reported here were repeated a minimum of 4 times.

3. Results

3.1. *Tentoxin-induced exchange of ADP with thylakoid membranes*

Table 1 compares the effect of light and tentoxin on inducing the binding of ADP to thylakoid membranes. After 1.0 min illumination at room temperature, ~0.9 nmol ADP/mg chlorophyll are bound, in good agreement with [2,4,13]. If, however, thylakoid membranes are incubated in the dark with 400 μM tentoxin, 0.4–0.5 nmol ADP/mg chlorophyll are bound or ~50% of the light-induced binding.

Abbreviations: CF_1, chloroplast coupling factor 1; PMS, phenazine methosulfate

Table 1
Adenine nucleotide binding to spinach thylakoid membranes

	nmol ADP bound/ mg chlorophyll
Light	0.90
Dark	0.08
Dark + tentoxin (400 μM)	0.40

Chloroplasts (1.0 mg chlorophyll/ml) were incubated in the dark for 30 min at room temperature in a reaction mixture containing 25 mM tricine–NaOH (pH 8.0), 50 mM NaCl, 1.0 mM $MgCl_2$, 50 μM PMS and, where indicated, 400 μM tentoxin. After the addition of 25 μM [^{3}H]ADP (containing ~5 × 10^6 dpm/ml), the chloroplasts were illuminated or kept in the dark for 1.0 min. Unbound [^{3}H]ADP was removed by repeated washing and the bound ADP determined

Table 2 demonstrates that the tentoxin-induced binding site is identical to the light-induced binding site. In these experiments, the ADP binding sites were first filled or partially filled with [^{3}H]ADP by incubating the chloroplasts either in the light (part A) or in the dark plus tentoxin (part B). Subsequently, the bound [^{3}H]ADP was released either in the dark plus tentoxin (part A) or in the light (part B). Part A

Table 2
Reversibility of energy-dependent and tentoxin-induced binding of ADP to thylakoid membranes

	nmol ADP released/ mg chlorophyll
Part A: light loaded[a]	
Light	0.65
Dark	0.10
Dark + tentoxin (400 μM)	0.37
Part B: tentoxin loaded[b]	
Light	0.26
Dark	0.09

Controls after loading were [a]1.14 and [b]0.41 nmol ADP bound/mg chlorophyll

Part A: Chloroplasts were loaded with [^{3}H]ADP in the light and washed as in table 1. The release of bound, labeled ADP was induced by either light (1.0 min), dark, or dark plus tentoxin. The released ADP was measured as in section 2. Part B: Chloroplasts were loaded with [^{3}H]ADP as in table 1 (+ 400 μM tentoxin). The release of bound ADP was measured as in part A

Table 3
Tentoxin-induced binding of ADP to soluble CF_1

	nmol ADP/nmol CF_1
– Tentoxin	0.33
+ Tentoxin	0.95

Coupling factor 1, 0.5 μM, was incubated for 30 min at 37°C in a mixture containing 20 mM tricine–NaOH (pH 8.0), 20 μM [^{3}H]ADP with or without the addition of 400 μM tentoxin. The protein was separated from unbound [^{3}H]ADP [20] and the bound ADP determined as in section 2

shows that 400 μM tentoxin causes 55% (cf. the light control) of the light-induced bound [^{3}H]ADP to be released in the dark. Concomitantly, light induces the release of 63% of the tentoxin-induced bound [^{3}H]ADP (part B).

3.2. *Tentoxin-induced binding of ADP to soluble CF_1*

Table 3 shows the results of an experiment in which soluble CF_1 was incubated with [^{3}H]ADP at 37°C for 30 min and then the amount of ADP bound to CF_1 was determined [15]. In the absence of tentoxin, after a 30 min incubation, only a relatively low amount of ADP is bound to CF_1 (0.2–0.3 ADP/CF_1). This contrasts with 2 binding sites/molecule [21,22] probably because equilibrium has not been attained. In the presence of tentoxin, the binding increases about 3-fold over the level obtained in the absence of tentoxin. The $t_{1/2}$ for the binding of ADP to CF_1 in the presence of tentoxin at 37°C is about 5 min (data not shown). The maximal binding that we have observed is ~1.0 ADP/CF_1. We have shown [15] that this is not simply due to tentoxin exposing new binding sites for ADP on CF_1, but rather represents a tentoxin-induced exchange of ADP already bound to the enzyme for medium ADP.

3.3. *Energy-dependent release of ADP bound to CF_1 after reconstitution with thylakoid membranes depleted in CF_1*

In order to determine whether the adenine nucleotide binding site exposed by tentoxin on soluble CF_1 and the tight binding site observed upon illumination of thylakoid membranes in the presence of ADP are identical, soluble CF_1 was first loaded with labeled ADP (by incubating the protein in the presence of

Table 4
Reconstitution of soluble CF_1 with CF_1-depleted thylakoid membranes and exchange of CF_1-bound ADP

CF_1 plus tentoxin (nmol ADP bound/mg protein)	1.66
Reconstituted chloroplasts (nmol ADP bound/mg chl.)	0.242
Dark + ADP (nmol ADP released/mg chl.)	0.021
Light + ADP (nmol ADP released/mg chl.)	0.053

CF_1 was loaded with [^{3}H]ADP by incubating the protein in a reaction mixture containing 18 μM CF_1, 25 μM [^{3}H]ADP (4×10^{-16} mol ADP/cpm), 5.0 mM tricine–NaOH (pH 8.0), and 400 μM tentoxin at 37°C for 30 min. The protein was then desalted [20] and used directly for reconstitution experiments. Incubation mixtures for reconstitution contained in 1.5 ml, chloroplasts, 1.0 mg chlorophyll/ml, 1.2 mg CF_1/ml, 10 mM tricine–NaOH (pH 8.0) and 20 mM $MgCl_2$. The suspensions were incubated for 5.0 min at room temperature and the chloroplasts were washed twice in buffer containing 50 mM NaCl and 25 mM tricine–NaOH (pH 8.0) to remove all CF_1 not bound to the thylakoid membranes. The membranes were resuspended in the same buffer containing, in addition, 1.0 mM $MgCl_2$. Release of bound [^{3}H]ADP was measured as in table 2

tentoxin and [^{3}H]ADP as above) and then reconstituted with CF_1-depleted thylakoid membranes*. The reconstituted membranes were incubated in the presence of unlabeled ADP either in the light or in the dark, and the release of labeled ADP into the supernatant was measured. These results are shown in table 4. In the dark ~8% of the bound label comes off of the thylakoid membranes, whereas in the light the fraction that exchanges with medium ADP increases to about 20–30%. Exchange in both the light and dark is dependent upon the presence of unlabeled ADP (data not shown). A very similar experiment has been reported [19] with almost identical results using a CF_1 that had been labeled with [^{14}C]ADP by illuminating thylakoid membranes prior to the isolation of the protein.

* It has been reported [19] that when thylakoid membranes are washed in 0.3 M sucrose, 2.0 mM Tris–tricine (pH 7.8) buffer containing 10 μM $CaCl_2$, ~50% of the total CF_1 is removed from the membranes based on the total remaining membrane-bound ATPase activity. The data in table 4 confirm this observation. The specific activity of the ADP-loaded CF_1 during reconstitution was 2.43×10^6 cpm/mg protein. After reconstitution, the chloroplasts had spec. act. 3.56×10^5 cpm/mg chlorophyll. Assuming that no label was lost during the reconstitution, this would correspond to 0.15 mg protein bound/mg chlorophyll. If the (w/w) ratio of CF_1 to chlorophyll is 0.42 (Strotmann, H., Hesse, H. and Edelmann, K. (1973) Biochim. Biophys. Acta 314, 200–210), 0.15 would represent a 36% reconstitution

4. Discussion

Tentoxin induces an exchange of bound ADP for medium ADP both with soluble and thylakoid-bound CF_1 [14,15]. We had observed some differences in the specificity of the binding site exposed by tentoxin that depended on the state of the protein, i.e., membrane-bound versus soluble CF_1. The most notable discrepancy was the high affinity of the tentoxin-induced ADP binding site of soluble CF_1 for the ATP analogue, adenylyl imidodiphosphate (AMP-PNP). Recent results have shown that tentoxin increases both the affinity and number of binding sites of CF_1 for AMP-PNP (S.S.R., B.R.S., unpublished results). On the other hand, AMP-PNP is a poor competitor of ADP for the energy-dependent [4] and tentoxin-induced [14] ADP tight-binding site of membrane-bound CF_1. Because of this discrepancy, it was not clear that the ADP binding sites (exchange sites) that we were measuring were identical.

Clearly, in the case of thylakoid membranes the data presented in tables 1 and 2 show that the tentoxin-induced adenine nucleotide binding site and the energy-dependent adenine nucleotide exchange site are identical. Thylakoid-bound labeled ADP, obtained by incubating the membranes either with tentoxin or in the light, can be released from the thylakoid membranes by either light or tentoxin. It is not clear to us why the maximal binding or release of ADP to or from the thylakoid membranes induced by tentoxin is only ~50% of that observed upon illumination of the thylakoid membranes (tables 1 and 2A). There are several possibilities:

(i) Tentoxin might interact with only ~50% of the total membrane-bound CF_1 pool;
(ii) In the presence of saturating levels of tentoxin, the maximum ratio of CF_1 containing bound ADP to CF_1 without ADP might be about 1 (thus, in order to measure a binding ratio higher than 0.5–0.6 nmol ADP/mg chlorophyll, it would be

necessary to remove tentoxin without removing the labeled ADP before washing the thylakoid membranes);

(iii) The light-induced binding site might be a mixture of (two) sites, only one of which responds to high concentrations of tentoxin.

At the present time, we cannot distinguish between these possibilities.

The interaction of tentoxin with soluble CF_1 leads to both the induction of the ATPase activity [15,23] and the binding of ADP (table 3). The tentoxin-induced binding of ADP is an exchange of CF_1-bound ADP for medium ADP [15]. The mechanism by which tentoxin induces this exchange, however, is not known. We do know that under similar conditions, tentoxin induces a 5-fold increase in the binding of *N*-ethylmaleimide to CF_1, presumably by altering the conformation of the protein (S.S.R., B.R.S., unpublished results). The interaction between soluble CF_1 and tentoxin is currently under investigation.

The exchange site exposed by tentoxin with the soluble CF_1 is apparently the same site revealed by illuminating thylakoid membranes containing bound CF_1 (table 4). The observation that only ~30% of the ADP bound to the CF_1 that reconstitutes with CF_1-depleted thylakoid membranes exchanges in the light probably reflects a partial inactivation of both CF_1 and the CF_1-depleted thylakoid membranes [19].

5. Conclusions

Tentoxin interacts with both membrane-bound and soluble CF_1 to induce an exchange of the 'tightly bound' adenine nucleotides. The exchange site induced by tentoxin is identical to the site exposed upon energization of the thylakoid membranes.

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