Three kinds of binding site for tentoxin on isolated chloroplast coupling factor 1

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Abstract Tentoxin binding on chloroplast coupling factor 1 (CF_1) was studied using a centrifugation column method followed by HPLC analysis. From non-linear regression analysis of the results, the presence of three types of binding site with the following K_d values was deduced: 6.9×10^{-8} M (first site), 1.4×10^{-5} M (second site), and 6.3×10^{-3} M (third site). The binding of one tentoxin inhibits, that of two tentoxins moderately restores, and that of three tentoxins greatly stimulates the ATPase activity of CF_1. The forward rate constant of the binding of tentoxin on the first site was 6.3×10^{3} M^{-1} s^{-1}.

Key words: Chloroplast ATP synthase; Chloroplast coupling factor 1; F_1-ATPase; Energy-transfer inhibitor; Tentoxin

1. Introduction

Tentoxin, a cyclic tetrapeptide (cyclo[L-leucyl-*N*-methyl-*trans*-dehydrophenylalanyl-glycyl-methyl-L-alanyl]) produced by the fungus *Alternaria alternata* causes species-specific chlorosis in sensitive higher plants [1]. One of its main targets is the CF_1 (chloroplast F_1-ATPase) [2] where it acts as an energy-transfer inhibitor in photophosphorylation [3]. It was recently found that an F_1 from the cyanobacterium *Anacystis nidulans* is also sensitive to tentoxin [4].

F_1, a hydrophilic portion of F_oF_1-ATP synthase, is composed of five different subunits, α–ε, in order of decreasing molecular weight, with a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ [5–7]. The tentoxin binding sites on CF_1 seem to be located at least on the β-subunit, since *Rhodospirillum rubrum* chromatophore, itself insensitive to tentoxin, became sensitive after the β-less F_1 was reconstituted with the heterologous β-subunit of tentoxin-sensitive CF_1 from spinach [8]. Avni et al. [9] reported that tentoxin sensitivity was determined by codon 83 of the β-subunit in several species of the genus *Nicotiana*. When codon 83 was aspartic acid, it became sensitive to tentoxin, and when it was glutamic acid, it became resistant to tentoxin. Recently, Hu et al. [10] confirmed the importance of codon 83 by site-directed mutagenesis in *Chlamydomonas reinhardtii* CF_1. Residue 83, however, is located in the β-barrel domain [11], far from nucleotide and Pi binding sites. No function has been assigned to it yet.

Tentoxin has the peculiar property of inhibiting ATPase activity in isolated sensitive CF_1 at low concentrations ($<10^{-6}$ M) but stimulating it at high concentrations ($>10^{-5}$ M) [12]. Although it was reported that photophosphorylation inhibited by low concentrations of tentoxin did not recover in high concentrations of tentoxin [12], recent studies indicated that light-driven ATP synthesis was slightly restored at high concentrations of tentoxin [13]. These results indicate the existence of at least two types of tentoxin-binding sites on CF_1. From the stimulating effects of tentoxin on ATPase, the K_d of the low-affinity site for tentoxin was estimated to be $>1.6\times10^{-4}$ M [12]. Direct measurements of K_d values for tentoxin-binding sites have also been reported. For the first binding site (high-affinity site), Steele et al. [14] determined the K_d of lettuce CF_1 to be 5.5×10^{-9} M by ultrafiltration. Dahse et al. [15] determined the K_d of spinach CF_1 to be 8.7×10^{-8} M using HPLC techniques. Recently, Pinet et al. [16] extended these studies using isotopically labeled tentoxin, and found two types of binding site on spinach CF_1 at 4°C: 1.3 sites/CF_1 with $K_{d1}=1.2\times10^{-6}$ M and 1.5 sites/CF_1 with $K_{d2}=6.7\times10^{-5}$ M. Their K_{d1} value seems to be considerably larger than those of Steele et al. [14] and Dahse et al. [15], and than those estimated from the inhibition of ATPase activity of isolated CF_1 [12]. Their K_{d2} value seems to be rather small compared with those estimated from the activation of ATPase activity of isolated CF_1 [12].

As the β-subunit is involved in tentoxin binding and as each CF_1 has three β-subunits, the existence of three binding sites for tentoxin is plausible but has not been experimentally shown yet. If CF_1 has three tentoxin-binding sites, it would be interesting to see if the ATPase activity of $C{\cdot}T_2$ (CF_1 which binds two molecules of tentoxin) and that of $C{\cdot}T_3$ is the same or not. However, this has not been studied. As there are large differences among the reported K_d values (e.g. [14] vs. [16]) and the possible existence of a third type of binding site has not been carefully studied, we studied the binding of tentoxin on CF_1 using column centrifugation over tentoxin concentrations ranging from 10^{-8} to 4×10^{-3} M. Our results strongly suggest that CF_1 actually has three tentoxin-binding sites. This conclusion was reinforced by the results of tentoxin concentration on ATPase activity.

2. Materials and methods

2.1. Preparation of CF_1

CF_1 was extracted from spinach chloroplasts by the chloroform method of Younis et al. [17], and purified in a buffer containing 10 mM Tricine-KOH (pH 8.0), 1 mM EDTA, and 2 mM ATP by Sepharose CL-4B and HiPrep Sephacryl S-300 (Pharmacia) columns, followed by sucrose density-gradient (15–35%, w/v) ultracentrifugation ($140\,000\times g$, 22 h). The purified CF_1 was precipitated by 50% saturated $(NH_4)_2SO_4$ and stored at 4°C. The CF_1 preparation contained substoichiometric amounts of δ-subunits on SDS-PAGE analysis as reported by McCarty and Moroney [5] (data not shown). Before use, CF_1 precipitate was dissolved in 1 mM Tricine-KOH

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Abbreviations: CF_1, chloroplast coupling factor 1; $C{\cdot}T_n$, CF_1 which binds *n* molecules of tentoxin; K_d, dissociation constant; PCA, perchloric acid

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(pH 8.0) unless otherwise indicated, and the solution was passed slowly through a Sephadex G-50 (1.0 cm×20 cm) column equilibrated with 1 mM Tricine-KOH (pH 8.0) at room temperature. It took about 45 min before CF_1 was eluted from the column. The concentration of CF_1 in the effluent from a typical column was determined by the absorbance at 278 nm assuming $A_{278nm} = 0.53$ for CF_1 solution at 1 mg/ml (Hisabori and Sakurai, unpublished). Trypsin-activated CF_1 was obtained by incubation with 0.1 mg/ml trypsin in 2 mM ATP, 1 mM EDTA, and 30 mM Tricine-NaOH (pH 8.0) at room temperature for 5 min. The activation was stopped by adding soybean trypsin inhibitor to 0.25 mg/ml.

2.2. Tentoxin binding on CF_1

Solutions of native or trypsin-activated CF_1 were incubated at room temperature with various concentrations of tentoxin (either purchased from Sigma Chemical Co., St. Louis, MO, or kindly donated by Mr. T. Fukushima of JT Co., Yokohama) for the time as indicated. For the determination of tentoxin bound on CF_1, a portion of this mixture, containing CF_1, was centrifuged through a Sephadex G-50 column [18] to remove free tentoxin. The effluent was treated with PCA to 2.6% (w/v), and neutralized with 0.35 M KCl, 0.18 M Tricine-KOH (pH 9.0), and 0.18 mM EDTA, and centrifuged. Tentoxin in the neutralized supernatant was separated on a reverse phase HPLC column (Merck, LiChrospher 100 RP-18(e), 4.6 mm×25 cm), with 40% (v/v) acetonitrile-0.06% (v/v) trifluoroacetic acid as the elution buffer (0.5 ml/min) and analyzed at 285 nm with UV detector (SPD-6A, Shimadzu). Recovery of CF_1 from the centrifugation column was estimated using the protein determination method of Bradford [19] (>0.2 mg/ml CF_1) or from the recovery of CF_1-bound ADP (bound ADP/CF_1 = 1.13) after column centrifugation. The effluent was treated with PCA and neutralized with 175 mM K_2CO_3. ADP was determined as ATP by luciferase-luciferin assay after incubation with pyruvate kinase and phosphoenolpyruvate.

2.3. Effects of tentoxin on ATPase activity of CF_1

For concentrations of tentoxin less than 1.0×10^{-6} M, solutions of native CF_1 (2.3×10^{-9} M) were pre-incubated with various concentrations of tentoxin, 1.3 mM ATP, 1.3 mM EDTA and 0.7 mM Tricine-KOH (pH 8.0) for 60 min at room temperature. The ATPase assay (at 37°C) was initiated by adding the assay solution containing the same concentrations of tentoxin as in the pre-incubation mixture, and $MgCl_2$ to a final concentration of 1.4 mM. The reaction was terminated by injecting a portion of the reaction mixture into an HPLC column (Shodex AXpak WA-624, 6.0 mm×15 cm, Showadenko), and the amount of ATP hydrolyzed was determined from ADP at 259 nm essentially according to the HPLC method of Pinet et al. [20]. For concentrations of tentoxin greater than 1.0×10^{-6} M, solutions of CF_1 (2.3×10^{-7} M) were incubated as above, and the ATPase activity was determined as above except that the concentration of ATP was 4 mM, and the reaction times ranged from 2 to 15 min. The reaction was stopped by adding trichloroacetic acid to 2.7% (w/v). Pi liberated in the reaction mixture was determined by colorimetry after reducing the phosphomolybdate with $SnCl_2$, according to the methodology developed for the Autoanalyzer of Technicon Co. (1966).

Trypsin-activated CF_1 (1.27×10^{-7} M) was incubated with 5.0×10^{-7} M tentoxin at room temperature for the times indicated. ATPase activity was then assayed at 37°C in a reaction mixture containing 1 mM ATP, 0.75 mM $MgCl_2$, and 15 mM Tricine-NaOH (pH 8.0). After 3 min, the reaction was stopped by adding trichloroacetic acid to 2.7% (w/v), and Pi in the acidified reaction mixture was determined as described above.

3. Results and discussion

Fig. 1 shows the binding of tentoxin to CF_1 studied over a range of tentoxin concentrations from 10^{-8} M to 4×10^{-3} M, the latter being the solubility limit of tentoxin in water [20]. The binding curve clearly indicates several types of binding site. We analyzed the results, assuming two or three types of saturable (Michaelis-Menten type) binding site on CF_1, with a non-linear regression procedure using the computer program 'GraFit' (Erithacus Software Ltd., Staines). Assuming three

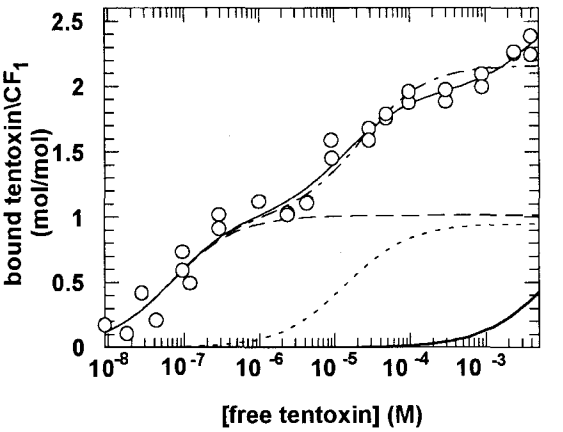

Fig. 1. Relation between free tentoxin concentration and the amount of tentoxin bound on native CF_1. Binding of tentoxin (initial concentration: 10^{-9}–4×10^{-3} M) by concentrations of CF_1 ranging from 5.8×10^{-9} M to 1.2×10^{-6} M incubated for 60 min was measured as described in Section 2. Free tentoxin concentration was calculated by subtracting the bound tentoxin from the total added. ○, Experimentally obtained values. Theoretical curves assuming three kinds of binding site with different affinities: (– – –), deduced binding to first binding site; (- - -), deduced binding to second binding site; (bold solid line), deduced binding to third binding site, (solid line): the best-fit curve (sum of the total tentoxin bounds). Theoretical curve (—·—) assuming two kinds of binding site with different affinities. For details, see text.

types of binding sites, we obtained deduced kinetic values ($\chi^2 = 1.20\times10^{-2}$) of 1.01±0.10, 0.95±0.11, and 0.95±1.95 binding sites/CF_1, with K_d values of 6.92×10^{-8} M (first site, K_{d1}), 1.35×10^{-5} M (second site, K_{d2}), and 6.31×10^{-3} M (third site, K_{d3}), respectively. The number of the third type of binding site per CF_1 and its K_d value were difficult to determine very accurately because only about 0.4 mol of tentoxin binds to this third site even at the solubility limit of tentoxin. Assuming two types of binding site, we obtained deduced kinetic values ($\chi^2 = 1.58\times10^{-2}$) of 1.01 and 1.15 binding sites/CF_1 with K_d values of 6.92×10^{-8} M and 2.23×10^{-5} M, respectively. Holding the kinetic values for the first site steady at 1.01 site/CF_1 with a K_d value of 6.92×10^{-8} M, we then subtracted the deduced tentoxin binding on the first site from the experimental value to obtain binding value(s) on the other binding site(s). Analysis of the results at a concentration range of 2×10^{-6}–4×10^{-3} M tentoxin gave χ^2 values of 9.29×10^{-3} and 1.52×10^{-2}, respectively, for the three- and two-site assumptions. Comparison of these χ^2 values shows that the three-site assumption gives a better fit than the two-site assumption. This is also suggested by a comparison of the two theoretical binding curves with the data obtained experimentally at $>10^{-4}$ M tentoxin (Fig. 1).

The effects of tentoxin concentration on the ATPase activity of native CF_1 are shown in Fig. 2A. At $<10^{-6}$ M tentoxin, activity was inhibited, and at $>10^{-6}$ M, it was recovered or stimulated as reported previously [12]. The activity was not saturated by concentrations of tentoxin higher than 10^{-3} M, consistent with the results of Steele et al. [12]. The tentoxin concentration required for 50% of maximum activity was clearly higher than 10^{-3} M. Using K_d values deduced from the three-site model in Fig. 1, we calculated the ratio of four states of CF_1 with respect to number of tentoxin bound as a function of free tentoxin concentration: C·T_0, C·T_1, C·T_2, and

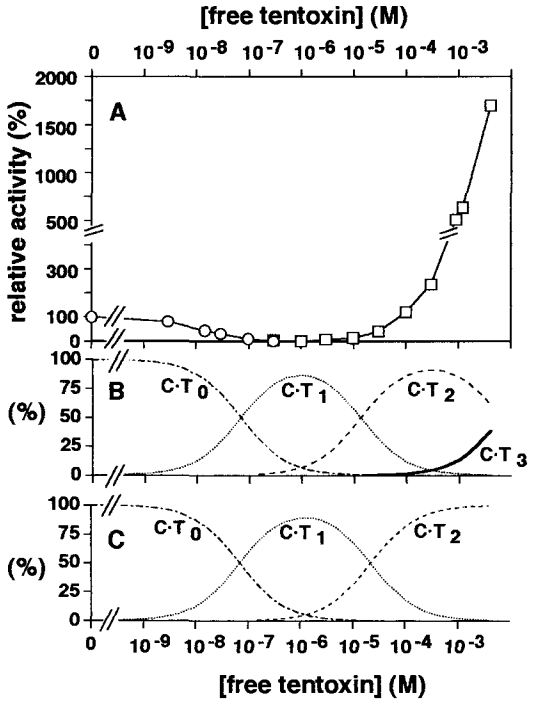

Fig. 2. A: Effects of tentoxin on ATPase activity of native CF_1. ○: The concentration of CF_1 was 2.3×10^{-9} M and ATPase activity was determined from the product ADP by the HPLC method. □: The concentration of CF_1 was 2.3×10^{-7} M and ATPase activity was determined by colorimetry of Pi. ATPase activity of CF_1 in the absence of tentoxin was 0.29 μmol/mg/min. Free tentoxin concentration was calculated from the kinetic values obtained in Fig. 1, assuming three binding sites. B: Relative proportions of $C\cdot T_n$ deduced by assuming three tentoxin-binding sites. C: Relative proportions of $C\cdot T_n$ deduced by assuming two tentoxin-binding sites.

$C\cdot T_3$ (Fig. 2B). Our data, at concentrations of tentoxin less than 10^{-6} M, showed the activity of $C\cdot T_1$ to be almost zero. At 10^{-5} M tentoxin, we calculated that about 60% of CF_1 was $C\cdot T_1$, and about 40% $C\cdot T_2$. At this concentration, the total activity was less than 40% that of free CF_1 ($C\cdot T_0$), indicating that $C\cdot T_2$ was less active than $C\cdot T_0$. At 4×10^{-3} M tentoxin, about 60% of CF_1 was $C\cdot T_2$, and about 40% $C\cdot T_3$. The activity was then about 1700% of $C\cdot T_0$. If we assume that the activity of $C\cdot T_2$ is 50% that of $C\cdot T_0$, the activity of $C\cdot T_3$ would be about 4000% that of $C\cdot T_0$. If we assume that CF_1 has only two kinds of binding site (Fig. 2C), the deduced K_{d2} is 2.2×10^{-5} M (Fig. 1). This is very different from the tentoxin concentration required to stimulate ATPase activity to half maximum, as shown in Fig. 2A ($>10^{-3}$ M). The results shown in Fig. 2 clearly favor the three-site model. The value of K_{d1} was estimated to be in the order of 10^{-8} M (Fig. 2A), not very different from values deduced from the inhibition of ATPase (1×10^{-8} M [12], $<10^{-8}$ M [15], 3×10^{-7} M [21]).

Fig. 3 shows the time course of the binding of a low concentration of tentoxin (5.0×10^{-7} M) to native or trypsin-treated CF_1. We analyzed the results on the basis of Equation 1 using the computer program 'KaleidaGraph' (Synergy Software, Reading), neglecting the binding on the second and third sites at this low concentration of tentoxin:

$$CF_1 + \text{tentoxin} \underset{k_-}{\overset{k_+}{\rightleftharpoons}} CF_1\cdot\text{tentoxin} \quad (1)$$

where $k_- = K_{d1}\times k_+$ (K_{d1} being 6.9×10^{-8} M). We thus obtained values of $k_+ = 6.3\times10^3$ M^{-1} s^{-1} and $k_- = 4.3\times10^{-4}$ s^{-1}. This k_- value indicates that less than 1% of tentoxin bound on the first site dissociates from CF_1 in 20 s. This slow dissociation assures that the column centrifugation method is suitable for analyzing tentoxin binding at least on the first site. Trypsin treatment greatly activates ATPase activity [22], but did not significantly affect the binding of tentoxin on the first site of CF_1 (Fig. 3).

We also estimated the value of k_+ by analyzing the time course of inhibition of ATPase on the basis of Eq. 1 and $K_{d1} = 6.9\times10^{-8}$ M, assuming that $C\cdot T_1$ has no activity (Fig. 4). For this purpose, we used trypsin-activated CF_1, because it has much higher activity than native CF_1, while its tentoxin-binding properties are similar (Fig. 3). We thus obtained values of $k_+ = 6.9\times10^3$ M^{-1} s^{-1} and $k_- = 4.8\times10^{-4}$ s^{-1}. These values agreed well with those obtained from the direct binding assay (Fig. 3), suggesting the accuracy of our estimated k values.

The value of K_{d1}, estimated from the results shown in Fig. 1, was 6.9×10^{-8} M, not very different from some previously reported values (5×10^{-9} M [14], 8.7×10^{-8} M [15]), though Pinet et al. [16] reported the much larger value of 1.2×10^{-6} M. Our CF_1 preparation contained substoichiometric amounts of a δ-subunit. Recent results suggest that this δ-subunit touches the β-barrel domain of the β-subunit [23,24], which binds tentoxin. However, a low δ content did not seem to affect the K_{d1} value of our CF_1 significantly. This is suggested by the following: (1) the value of K_{d1} of a five-subunit CF_1 (8.7×10^{-8} M [15]) was similar to ours; and (2) competition experiments for tentoxin binding between a five-subunit CF_1 and a CF_1 lacking the δ- and ε-subunits, as estimated by inhibition of ATPase activity by tentoxin in the latter, indicated that they did not much differ from each other in their binding affinities (affinities of the CF_1 lacking the δ- and ε-subunits for tentoxin were higher by a factor of less than 3, as shown in Fig. 2 of [25]). Our results shown in Fig. 3 indicate that the trypsin treatment of CF_1, which would eliminate the δ- and ε-subunits and partially digest the γ-subunit, did not significantly affect the affinity of CF_1 for tentoxin. The cause for the much larger K_{d1} value reported by Pinet et al. [16] is not clear, but it might be due to the low temperature (4°C) which they chose for incubation.

We have shown that CF_1 has three kinds of binding site for tentoxin, with different affinities, and that binding of tentoxin

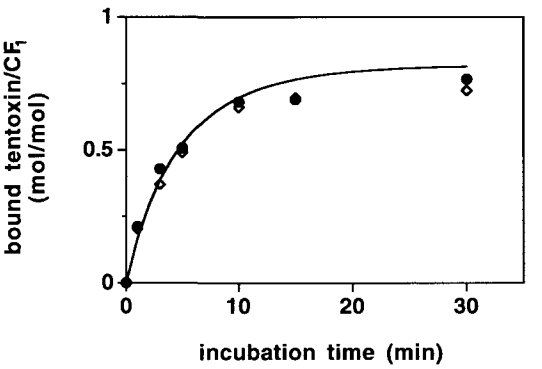

Fig. 3. Time course of the binding of a low concentration of tentoxin to native or trypsin-treated CF_1. Initial concentrations of CF_1 and tentoxin were 2.3×10^{-7} M and 5.0×10^{-7} M, respectively. Experimentally obtained value: ●, native CF_1; ◇, trypsin-treated CF_1; line: the deduced theoretical curve (see text).

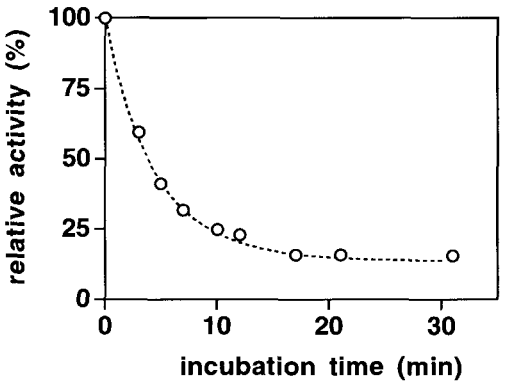

Fig. 4. Time course of inhibition of ATPase activity of trypsin-treated CF_1 by a low concentration of tentoxin. 1.27×10^{-7} M of trypsin-treated CF_1 and 5.0×10^{-7} M tentoxin were mixed at time zero, at room temperature. A portion of the mixture was then transferred to an assay mixture at specified times, and ATPase activity was assayed for 3 min. Except for the zero time control which was assayed in the absence of tentoxin (activity: 7.5 μmol/mg/min), the time indicated was 1.5 min after the onset of each assay. ○: Experimentally obtained value; (---): the deduced theoretical curve (see text).

on these sites differentially affects the ATPase activity of CF_1. This three-site model finds its counterpart in nucleotide binding on CF_1, as revealed by structural studies of mitochondrial F_1 (pseudo-threefold symmetry) [11], and by functional studies of CF_1 [5,26]. The nucleotide binding sites not only have different affinities, but the binding of nucleotides also differentially affects ATPase kinetics in a complex manner. The relationship between the β-subunits concerned in the high-affinity binding of tentoxin and in tight ADP binding remains to be investigated.

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