Effect of Tentoxin on the Activation and on the Catalytic Reaction of Reconstituted H^+-ATPase from Chloroplasts

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The proton-translocating ATPase from chloroplasts, CF_0F_1, was isolated, purified and reconstituted into asolectin liposomes. The effect of the energy transfer inhibitor, tentoxin, on different functions of the enzyme was investigated. Tentoxin does not inhibit the nucleotide release during energization by a pH/$\Delta\Psi$ jump, *i.e.* the activation of the enzyme is not influenced. ATP synthesis driven by a pH/$\Delta\Psi$ jump and multi-site ATP hydrolysis are completely inhibited by tentoxin, whereas uni-site ATP hydrolysis is not influenced.

Introduction

Membrane-bound H^+-ATPases of the F_0F_1-type catalyze ATP synthesis/hydrolysis coupled with a transmembrane proton transport. These ATPases have a hydrophilic part, F_1, containing the nucleotide-binding sites and a hydrophobic membrane-integrated part, F_0, functioning as a proton channel. The F_1 part has six nucleotide-binding sites. Three of them are supposed to have catalytic properties, *i.e.* they can hydrolyze ATP [1–4]. The phytotoxin, tentoxin [cyclo(L-leucyl-N-methyl-(*Z*)-dehydrophenylalanyl-glycyl-N-methyl-L-alanyl)], produced by the fungus *Alternaria alternata* (= *A. tenuis;* [5, 6]), binds to a site on the α and/or β subunits of the CF_1 part [7]. At low concentrations between 0.1 and 1 μM (at a tentoxin : enzyme ratio of 10 : 1) tentoxin is a non-competitive inhibitor of the enzyme [8]; at high concentrations (10 μM to 1 mM) it is able to stimulate ATP hydrolysis activity of the isolated CF_1 [9–11]. It has been shown by Junge *et al.* [12] that inhibition of photophosphorylation by tentoxin is accompanied by the cessation of the phosphorylation-coupled proton efflux through ATPase. This explains the slower relaxation of light-induced transmembrane electric potential difference and the enhanced delayed fluorescence which were observed with tentoxin-treated chloroplasts [13–15]. The specificity of tentoxin action prompted us to investigate its effect on the isolated, reconstituted CF_0F_1.

Abbreviation: CF_0F_1, H^+-ATPase (ATP synthase) from chloroplasts.

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Materials and Methods

Isolation and reconstitution of CF_0F_1

CF_0F_1 was isolated from spinach chloroplasts, purified and reconstituted into asolectin liposomes by cholate dialysis as described earlier [16–18]. The proteoliposomes finally contained approximately 1 μM CF_0F_1 and 30 g/l asolectin, 10 mM Na-Tricine, pH 8.0, 0.2 mM EDTA, 2.5 mM $MgCl_2$ and 0.25 mM dithiothreitol. Prior to every set of experiments the enzyme was reduced in the presence of dithiothreitol and then activated by a pH/$\Delta\Psi$ jump as described earlier [19].

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Reduction of CF_0F_1 in proteoliposomes

The proteoliposomes were incubated for 1.5 h with 50 mM dithiothreitol at pH 8.0 at room temperature.

Incubation with tentoxin

The reduced proteoliposomes ($[CF_0F_1] = 1$ μM) were incubated with tentoxin before every set of experiments: an aqueous solution of tentoxin (1 mM) was stored at −20 °C and freshly diluted to appropriate concentrations. Then 2 μl of this solution were added to the five-fold volume of reduced proteoliposomes and incubated for the time indicated.

Activation of reduced CF_0F_1

After preincubation of proteoliposomes with tentoxin, CF_0F_1 was activated by a pH/$\Delta\Psi$ jump ($\Delta pH = 3.2$, $\Delta\Psi = 60$ mV) as follows: 10 μl of the proteoliposome solution were added to 50 μl of the acidic solution (buffer 1) containing 30 mM Na-succinate (pH 4.9), 5 mM NaH_2PO_4, 2 mM $MgCl_2$, 0.5 mM KCl and 1 μM of freshly added valinomycin. The pH during incubation was 5.0. After 30 s of incubation, 50 μl of an alkaline solution (buffer 2) consisting of 200 mM Na-Tricine (pH 8.7), 120 mM KCl, 5 mM NaH_2PO_4 and 2 mM $MgCl_2$ were added. The final pH was 8.2.

The acidic and the alkaline solutions both contained the same tentoxin concentration as was used for the preincubation, because tentoxin effect is reversible [9]. Fifteen seconds after the pH/$\Delta\Psi$ jump, 75 μl of the reaction sample were used for the determination of ATP and for the sum of ADP and ATP by luciferin/luciferase in two separate assays as described elsewhere [19]. In order to obtain samples without activation of CF_0F_1 the acidic and alkaline solution were mixed before addition of proteoliposomes.

Synthesis of ATP in a pH/$\Delta\Psi$ jump (ATP yield)

The acid-base transition was carried out as described for activation except that 200 μM ADP were present in the alkaline solution (buffer 3). Fifteen seconds after the acid-base transition, the reaction was stopped by the addition of trichloroacetic acid (final concentration 2% w/v). Ten μl of this solution was used for the determination of ATP with luciferin/luciferase as described elsewhere [19]. The ATP background was determined by mixing the acidic and alkaline solutions before the addition of proteoliposomes; fifteen seconds after this addition the sample was denatured and ATP content was determined as previously described. This background was always subtracted.

Uni-site ATP hydrolysis

The enzyme was incubated with tentoxin (1 μM CF_0F_1, 4 μM tentoxin) for 10 min and then activated as described above. Fifteen seconds after the pH/$\Delta\Psi$ jump, 40 nM Mg-ATP and 10 mM NH_4Cl were added in a solution identical to the reaction medium. At different reaction times 75 μl samples were taken to determine the free concentration of ATP or the sum of ATP and ADP with luciferin/luciferase.

Multi-site ATP hydrolysis

The enzyme was activated as described above. Mg-ATP labeled in the γ-position with ^{32}P (30 kBq; final concentration 10 μM to 1 mM) was added fifteen seconds after the pH/$\Delta\Psi$ jump in a solution identical with the reaction medium but containing in addition 10 mM NH_4Cl. At reaction times between 10 and 30 seconds samples were taken and denatured with 3% trichloro-acetic acid (final concentration). The $^{32}P_i$ content of the samples was determined as described elsewhere [19].

Results and Discussion

CF_0F_1 can exist in four different states, oxidized and reduced and both active or inactive. After reconstitution the enzyme is in the oxidized, inactive state. The inactive enzyme is unable to catalyze ATP hydrolysis or ATP synthesis. The isolated, reconstituted enzyme contains 1 ADP and 1 ATP per CF_0F_1. The ATP can be released only by denaturating the enzyme. The ADP is released during activation. If the enzyme is activated by a pH/$\Delta\Psi$ jump in the presence of phosphate, the ADP is partly phosphorylated to ATP when the membrane is energized (about 1−2 sec; [20]). Therefore, the total amount of nucleotides (ADP + ATP) released indicates the amount of the active enzyme. About 20−30% of the total CF_0F_1 can be activated [19].

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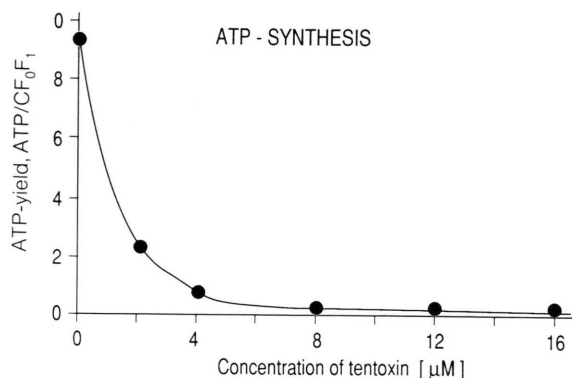

Fig. 1. ATP synthesis by CF_0F_1 (1 μM) reconstituted into proteoliposomes: The ATP yield in a pH/ΔΨ jump is shown as a function of tentoxin concentration. Preincubation medium (5 min) and reaction medium contain the same concentration of tentoxin.

Fig. 1 shows ATP synthesis by proteoliposomes in a pH/ΔΨ jump as a function of the tentoxin concentration. The proteoliposomes were preincubated for 5 min with the indicated tentoxin concentration and then the pH/ΔΨ jump was carried out (see Materials and Methods). The ATP yield decreased rapidly and at 8 μM tentoxin (at a tentoxin:CF_0F_1 ratio of 8:1), no further ATP synthesis was measured. It should be mentioned that the inhibition of ATP hydrolysis catalyzed by trypsin-treated CF_1 occurred at a ratio of tentoxin:enzyme of 10:1 [10].

Here the question is addressed in which step of catalysis is tentoxin involved. Does it inhibit the activation of the enzyme, or one step in the catalytic turnover of the enzyme (substrate binding, catalytic reaction ATP↔ADPP$_i$ on the enzyme, product release) or the cooperativity between the sites?

For this purpose the fraction of active CF_0F_1 was measured as a function of the preincubation time with tentoxin. The experiment was carried out as follows: the proteoliposomes were incubated with 2 μM tentoxin for different times. Then, CF_0F_1 was activated by a pH/ΔΨ jump in the absence of exogenous ADP. In two separate experiments free ATP and the sum of free ADP and ATP was determined. As the control the same experiments were carried out without a pH/ΔΨ jump. The result is shown in Fig. 2:

(A) About 0.2 free nucleotides (ATP + ADP) per CF_0F_1 were found after pH/ΔΨ jump and this amount does not depend on the incubation time with tentoxin.

(B) The amount of free ATP per CF_0F_1 after the pH/ΔΨ jump decreases from 0.06 to 0.01 ATP per CF_0F_1 during incubation with tentoxin, *i.e.* the ratio between free ADP and free ATP changed although the total amount of nucleotides remained constant.

(C) Without an activating pH/ΔΨ jump the amount of free nucleotides (ATP + ADP) is 0.05 per CF_0F_1 and does not depend on incubation time with tentoxin.

(D) Almost no free ATP is detected without pH/ΔΨ jump.

(E) For comparison the ATP yield in the presence of 100 μM ADP was measured with the same pH/ΔΨ jump as a function of incubation time with tentoxin (dashed curve, scale on the right side). The ATP yield decreased continuously with increasing incubation time.

These results lead us to the following conclusions:

(1) The nucleotide release induced by energization of the membrane is not influenced by incubation with tentoxin. Since the ADP release from CF_0F_1 upon energization is related to the activa-

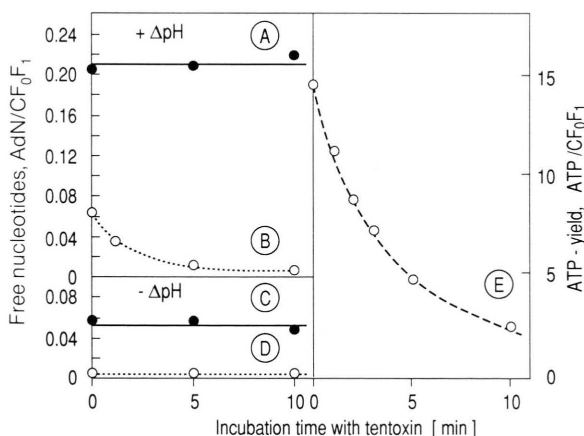

Fig. 2. Influence of tentoxin on ATP synthesis and nucleotide release during activation. The proteoliposomes ([CF_0F_1] = 1 μM) were incubated with 2 μM tentoxin for the time indicated. Then the following measurements were carried out in the presence of 2 μM tentoxin: (A) The sum of free (ATP + ADP)/CF_0F_1 after a pH/ΔΨ jump. (B) Free ATP/CF_0F_1 (after a pH/ΔΨ jump). (C) The sum of free (ATP + ADP)/CF_0F_1 (without pH/ΔΨ jump). (D) Free ATP/CF_0F_1 (without pH/ΔΨ jump). (E) ATP yield in a pH/ΔΨ jump.

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tion, this result shows that tentoxin does not change the fraction of the activated enzyme.

(2) A part of the released ADP is re-bound and phosphorylated to ATP during the pH jump. This part decreases during incubation with tentoxin indicating that the catalytic process is inhibited. The ATP yield measured in the same type of experiment decreases in parallel and supports this conclusion.

(3) The ATP yield (curve E) was measured in the presence of 100 μM ADP and therefore, all accessible ADP-binding sites are involved (multi-site ATP synthesis). The amount of ATP generated in the experiment (B) was measured under uni-site conditions since the concentration of free ADP was much lower than the enzyme concentration (uni-site ATP synthesis). Therefore, multi-site and uni-site ATP synthesis are inhibited by tentoxin.

(4) Tentoxin (2 μM; tentoxin : CF_0F_1 = 2 : 1) does not itself induce a release of tightly bound nucleotides (Fig. 2C and D). Therefore, we conclude that at this low concentration no tentoxin-induced activation of the enzyme occurs.

At high tentoxin concentration (400 μM) the ATPase activity of isolated CF_1 was stimulated and can be correlated to the release of tightly bound nucleotides [9, 21]. This stimulatory effect by high tentoxin concentration can, therefore, be explained by the release of tightly bound ADP from the regulatory site activating the CF_1.

In the next experiment the effect of tentoxin on uni-site ATP hydrolysis was measured. CF_0F_1 was activated by a pH/ΔΨ jump and ATP was added, so that the initial concentration of ATP was 50 nM. The concentration of ATP and that of (ADP + ATP) was measured. Fig. 3 shows the result: ATP was bound to the enzyme and with almost the same kinetics ADP was released.

From this measurement the rate constant for ATP binding was determined to be $1 \times 10^6\ M^{-1}\ s^{-1}$. This is the same number as found in the absence of tentoxin. In addition, the ADP release is the same in the presence and absence of tentoxin [19]. Therefore, we have to conclude that neither ATP-binding, nor ATP hydrolysis on the enzyme nor release of ADP is affected by tentoxin.

In the next experiments the influence of tentoxin on the rate of ATP hydrolysis was measured at concentrations of ATP from 10 μM to 1 mM where multi-site hydrolysis occurs. First, the rate of ATP

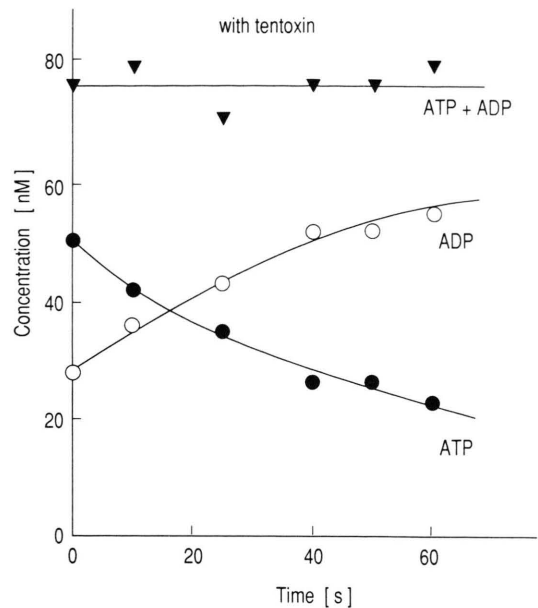

Fig. 3. Single-site ATP hydrolysis: The proteoliposomes ($[CF_0F_1]$ = 1 μM) were preincubated with 4 μM tentoxin for 10 min. Then, the enzyme was activated by a pH/ΔΨ jump and ATP was added. During the reaction 4 μM tentoxin was present. The concentration of free ATP and the sum of free (ATP + ADP) were measured as a function of reaction time. The concentration of free ADP was calculated from the difference between both measurements.

hydrolysis was measured without tentoxin. The results are shown in Fig. 4. The rate increases with increasing ATP concentration. A Lineweaver-Burk plot of these data gives a K_M = 180 μM and V_{max} = 58 ATP/(CF_0F_1 × s). For investigation of

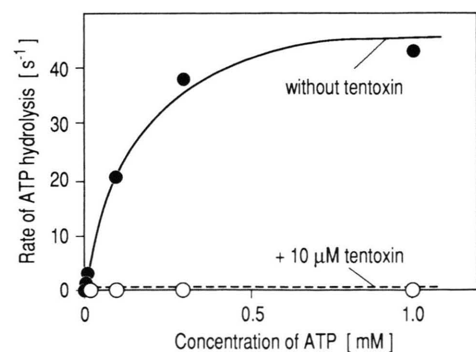

Fig. 4. Rate of ATP hydrolysis as a function of the ATP concentration. CF_0F_1 (1.4 μM) was preincubated either without or with 10 μM tentoxin for 10 min, then the enzyme was activated by a pH/ΔΨ jump and the reaction was started by addition of γ-[^{32}P]-ATP and 10 mM NH_4Cl (final concentration).

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the tentoxin effect the enzyme ($[CF_0F_1] = 1.4$ μM) was preincubated for 10 min with 10 μM tentoxin. At this concentration ATP synthesis is completely blocked (Fig. 1). In the presence of tentoxin no ATP hydrolysis could be detected, *i.e.* tentoxin completely inhibits multi-site ATP hydrolysis.

These results can be summarized as follows. (1) Tentoxin does not change the fraction of activated CF_0F_1. (2) In the presence of tentoxin neither multi-site nor uni-site ATP synthesis occurs. (3) Under uni-site conditions ATP binding, hydrolysis of ATP to enzyme-bound $ADPP_i$ and the release of ADP are not inhibited by tentoxin. (4) Tentoxin completely inhibits multi-site ATP hydrolysis.

The differential inhibition of uni-site and multi-site ATP hydrolysis can be explained in two ways. (1) The P_i release could be inhibited by tentoxin. In this case all reaction steps before the P_i release can occur, however, only one turnover of the reaction is possible since the free enzyme is not regenerated. Therefore, several turnovers under uni-site conditions and multi-site ATP hydrolysis are inhibited. (2) Uni-site ATP hydrolysis is not inhibited but the cooperativity between different catalytic sites is prevented by tentoxin. This suggestion is supported by measurements of the differential effect of acid inactivation on uni-site and multi-site ATP hydrolysis [22].

Fig. 2 and Fig. 3 show that also uni-site ATP hydrolysis and uni-site ATP synthesis are differently affected by tentoxin. There are several possibilities to interpret this observation.

1) Uni-site ATP synthesis and uni-site ATP hydrolysis occur at different sites, *e.g.* the ATP synthesis is coupled with proton translocation, the ATP hydrolysis is not (as for example in CF_1-catalyzed ATP hydrolysis).

2) Uni-site ATP synthesis and hydrolysis occur at the same site but tentoxin inhibits the conformational change which is necessary to couple proton transport through CF_0 with the chemical reaction in CF_1. Also in this case, uncoupled uni-site hydrolysis can occur but synthesis is inhibited.

3) If uni-site ATP synthesis and ATP hydrolysis occur *via* the same reaction pathway (microreversibility), both directions must be inhibited to the same extent. The measurements in this part (with the exception of Fig. 2E) are carried out under conditions where only a part of the enzymes carries out one turnover ("single turnover" conditions). If *e.g.* tentoxin inhibits the conformational change connected with proton transport (this is the first step of the ATP synthesis direction), then ATP synthesis will be completely blocked. However, the first steps of proton transport-coupled ATP hydrolysis can occur (*e.g.* ATP binding etc.) up to that step where coupling to proton transport must take place. In fact the enzyme is completely inhibited, however, this does not influence the first steps. In order to show this, the enzyme must carry out at least one turnover under uni-site conditions. As yet, we cannot decide between these possibilities.

The dependence of the fraction of activated CF_0F_1 and the catalytic reaction of CF_0F_1 on $pH/\Delta\Psi$ [23] showed that both processes are driven by the transmembrane electrochemical potential difference of protons. Our results demonstrate that ATP synthesis was completely blocked by tentoxin, whereas the fraction of activated enzymes was not affected. Therefore, we have to assume that for activation of CF_0F_1 and the catalytic reaction different pathways for protons should exist.

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