Proton coupling is preserved in membrane-bound chloroplast ATPase activated by high concentrations of tentoxin

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Abstract The effect of tentoxin at high concentrations was investigated in thylakoids and proteoliposomes containing bacteriorhodopsin and CF_0CF_1. Venturicidin-sensitive ATP hydrolysis, ATP-generated ΔpH and ATP synthesis were practically 100% inhibited at 2 μM tentoxin, and restored to various extents beyond 50 μM. With respect to the native enzyme, tentoxin-reactivated ATPase had the following properties: (i) a higher ΔpH requirement to synthetise ATP; (ii) a decreased futile proton flow through CF_0CF_1 (without ADP), which remains 100% blocked by ADP. It is concluded that despite its altered kinetic performances, tentoxin-modified CF_0CF_1 preserves its mechanism and remains a tightly coupled proton pump.

Key words: F_0F_1 H^+-ATPase; Proton pump; Tentoxin; Inhibitor; Thylakoid; Proteoliposome

1. Introduction

Tentoxin (TTX) is a cyclic tetrapeptide, *cyclo*-(L-MeAla[1]-L-Leu[2]-MePhe[(Z)Δ][3]-Gly[4]) [1], produced by the fungus *Alternaria alternata* (or *Alternaria tenuis*). Its main target is the F_1 moiety of some photosynthetic H^+-ATPases [2–4]. The site of inhibition seems to be the β subunit [5], although the α subunit is probably required for TTX binding [6,7]. One TTX molecule per CF_1 would be tightly bound [7]. The amino acids involved in TTX-sensitivity are disputed [7,8].

At high concentrations (0.05–1 mM), TTX has the fascinating property to stimulate ATP hydrolysis by soluble CF_1 from plants [7,9] or from cyanobacteria [3]. The activity so recovered may be 2–3 times higher than that of the control [7,9]. It was reported that TTX at 60 μM also limited the AMP-PNP-induced release of tightly-bound ADP in isolated CF_1, an effect attributed to the loss of interactions between the different nucleotide sites [6]. This could be related to the inhibition of multisite catalysis, but not of unisite catalysis by 10 μM TTX [10]. On the other hand, TTX at 400 μM induces the exchange of tightly bound ADP, on isolated as well as membrane-bound CF_1, in a way which is thought to mimick the effect of membrane energisation [11].

Very little was known about a possible reactivation of membrane-bound CF_1 by high TTX concentrations. We have focussed this study on the effect of high concentrations of TTX, largely beyond the inhibitory range, on CF_1 bound to thylakoids and liposomes. CF_1 so modified by TTX recovered a significant activity, tightly coupled to the proton transport.

2. Materials and methods

2.1. Thylakoids preparation, storage and assay

Envelope-free chloroplasts from lettuce (*Lactuca sativa* L.) leaves with thiol-reduced ATPase were prepared and stored as previously [12], and assayed at 10 μM [Chl] in 0.1 M sorbitol, 50 mM KCl, 5 mM $MgCl_2$, 2 mM K_2HPO_4, 2 mM Tricine, pH 8.0, 50 μM pyocyanine, 4 μM 9-aminoacridine (9-AA), at 20°C, as in [13]. Other additions are indicated. Thylakoids were energised by red light (maximum intensity, 1.5 $kW \cdot m^{-2}$). ATP synthesis or hydrolysis was monitored from scalar H^+ consumption or production [14], as in [12].

ATP synthesis experiments were as following: (1) 2 min incubation with TTX, in the dark; (2) light on; (3) 90 s later, 250 μM ADP added; (4) 2 or 3 min later, 1 μM venturicidin added; (5) 1 min later, light off. ATP synthesis was linear for at least 30 s. In some experiments, carried out in the presence of 10 mM Tricine and 10 μM diadenosine pentaphosphate, ATP was titrated using the luciferin–luciferase technique [13,15]. Aliquots were taken up each 30 s, over at least 3 min, and quenched by tri-*n*-butyltin (5 μM) before titration. For ATP hydrolysis, the timing was: (1) 2 min with TTX in the dark; (2) light on; (3) 45 s later, 1 mM ATP added; (4) 45 s later, light off and addition of 0.15 μM nigericin plus 50 nM valinomycin; (5) 45 s later, 1 μM venturicidin added. The initial rate of ATP hydrolysis was estimated over a period from 5 s to 30 s, just after the $\Delta\tilde{\mu}_{H^+}$ collapse. ΔpH was monitored with 9-AA fluorescence [16], simultaneously with pH. ΔpH-induced fluorescence quenching was corrected for the quenching due to nucleotides additions. TTX itself did not affect the fluorescence.

2.2. Preparation and assay of the co-reconstituted system

Large unilamellar liposomes were made by reverse phase evaporation from a mixture of egg phosphatidyl-choline (20 mM), egg phosphatidic acid (2 mM) and cholesterol (10 mM) in 50 mM Na_2SO_4, 50 mM K_2SO_4 and 25 mM KH_2PO_4, pH 7.3. Proteoliposomes containing BR [17] and CF_0CF_1 [18] were co-reconstituted [19–21] as follows: 30 μl Triton X-100 (20% w/v) were mixed at 25°C with 10 μl CF_0CF_1 (3 mg protein/ml) and 100 μl BR (4 mg protein/ml in 25 mM KH_2PO_4, pH 7.3 and 2% (v/v) Triton X-100), and 860 μl liposomes at a lipid concentration of 4.7 mg/ml were added under vortexing. Detergent was removed by SM-2-Bio beads.

The assay samples, stirred and thermostatted at 40°C, contained proteoliposomes diluted 3- to 10-fold in the reconstitution buffer with TTX and 10 μM diadenosine-pentaphosphate. Proteoliposomes were energised by yellow light (filter bandpass 500–650 nm, intensity 1.2 $kW \cdot m^{-2}$). Once steady state $\Delta\tilde{\mu}_{H^+}$ was reached, ATP synthesis was initiated by adding 0.5 mM ADP and 2 mM $MgSO_4$. Small aliquots were quenched by trichloroacetic acid 2% and titrated for ATP using luciferin–luciferase technique [15].

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Abbreviations: 9-AA, 9-aminoacridine; BR, bacteriorhodopsin; Chl, chlorophyll; $\Delta\tilde{\mu}_{H^+}$, transmembrane difference in proton electrochemical potential (electrochemical proton gradient); ΔpH, transmembrane pH difference; DTT, dithiothreitol; CF_0CF_1, chloroplast ATP synthase (H^+-ATPase); (C)F_0, membranous sector of the H^+-ATPase; (C)F_1, extrinsic, catalytic sector of the H^+-ATPase; MES, 2(*N*-morpholino)ethanesulfonic acid; PS1, photosystem 1; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TTX, tentoxin; VTCD, venturicidin. *Enzyme:* ATP synthase (EC 3.6.1.3).

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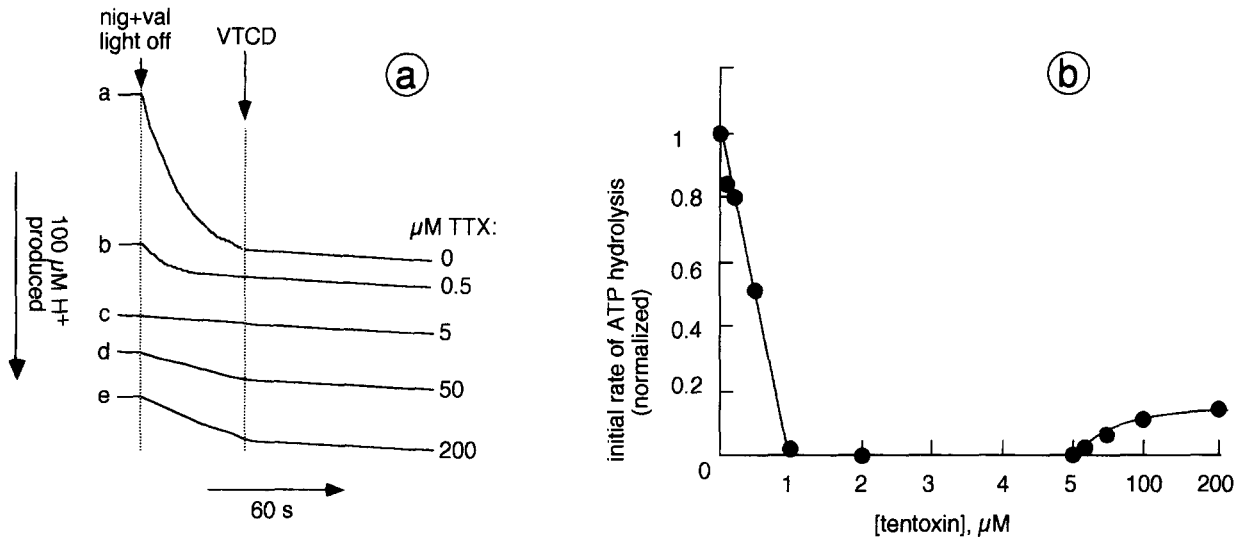

Fig. 1. Effect of tentoxin on the uncoupler-triggered ATP hydrolysis by DTT-treated thylakoids. Conditions as described in section 2. (a) pH traces obtained with different concentrations of tentoxin (TTX), converted into proton-equivalents. Injection of nigericin (nig), valinomycin (val) and venturicidin (VTCD) are indicated by arrows. (b) Initial rates of ATP hydrolysis as a function of TTX concentration. Activity of the control: 145 mmol ATP (mol Chl)$^{-1}\cdot s^{-1}$. Note the change in concentration scale at 5 μM.

3. Results

3.1. Tentoxin and ATP hydrolysis by thiol-reduced ATPase in thylakoids

Fig. 1a shows time courses of ATP hydrolysis by DTT-treated thylakoids, after illumination, addition of ATP, and fast collapse of $\Delta\tilde{\mu}_{H^+}$. Without TTX (trace a), the rate of ATP hydrolysis rapidly decreased and fell to zero, due to ATPase deactivation and ADP accumulation [12]. TTX 5 μM fully inhibited ATP hydrolysis (trace c). Higher concentrations of TTX induced the partial recovery of a permanent activity (traces d–e). The maximum restored activity (at [TTX] = 200 μM) was 15% of the control (Fig. 1b). This is clearly lower than the 2- to 3-fold stimulation generally observed on isolated CF_1 [7,9].

3.2. Tentoxin and proton flow through the CF_0CF_1 complex in thylakoids

TTX-induced ATP hydrolysis is coupled to proton pumping, because the reaction was triggered by uncoupling and inhibited by venturicidin (Fig. 1). This was also revealed by venturicidin-sensitive ΔpH generated by ATP hydrolysis in the dark. The

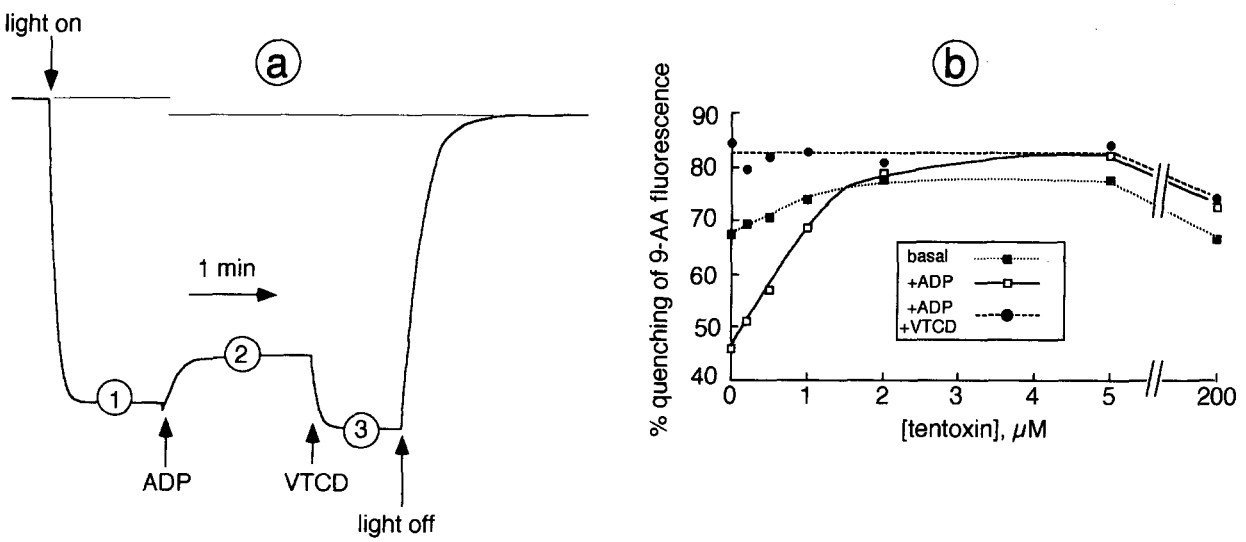

Fig. 2. Effect of tentoxin on the light-induced quenching of 9-aminoacridine fluorescence. DTT-treated thylakoids, conditions as described in section 2. (a) Light-induced 9-aminoacridine fluorescence quenching in the absence of TTX, under different regimes labeled by numbers on the trace. 1, basal conditions, i.e. without ADP; 2, phosphorylating conditions with 250 μM ADP added; 3, after addition of venturicidin (VTCD) 1 μM. The small instantaneous fluorescence drop upon ADP (250 μM) addition is a banal quenching effect; this quenching is more visible after complete relaxation of the proton gradient, when fluorescence is higher (note the lower position of 100% fluorescence level, as compared to the 100% initial level, indicated by horizontal straight lines). (b) Percentage of quenching vs. TTX concentration in basal condition (■), in the presence of ADP (□), and in the presence of ADP and VTCD (●). Breaks in abscissa axis indicate change in scale. Data corrected from the quenching effect of nucleotide molecules.

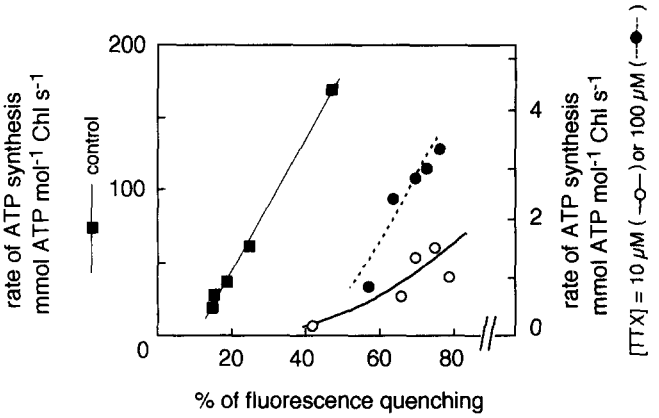

Fig. 3. Rate of ATP synthesis as a function of the light-dependent quenching of 9-aminoacridine fluorescence. Effect of high concentrations of tentoxin. DTT-treated thylakoids, conditions as described in section 2. (■), no TTX; (○), [TTX] = 10 μM; (●), [TTX] = 100 μM. Variable light intensity, adjusted by neutral density filters to 2, 3, 5, 10 and 100% of the maximum value. Activities in the presence of TTX are plotted on an expanded scale. All the activities were 100% inhibited by venturicidin.

9-AA fluorescence quenching induced by ATP hydrolysis was 32% without TTX, fell to 5% with 5 μM TTX, but rose again to 9% at 100 μM TTX (not shown). This further demonstrates the restoring of a coupled ATPase activity by a high concentration of TTX.

We have also characterized proton flows through CF_0CF_1 at different steady states, defined in Fig. 2a. Without TTX, ADP addition decreased the ΔpH, due to the outward of H^+ coupled to ATP synthesis (transition 1→2 on the figure). Venturicidin restored the ΔpH to a value which was even higher than before ADP addition (transition 2→3). This is due to a small proton leak through the ATPase complex in the absence of ADP [22]. Fig. 2b shows how TTX affects the ΔpH magnitude in these three different steady states. With venturicidin present, the ΔpH did not vary with TTX concentration up to 5 μM. This was expected if TTX does not disrupt the coupling between F_0 and F_1, as suggested [3,23]. In basal state, the ΔpH increased with [TTX], indicating that TTX decreased the futile H^+ leak through the ATPase. However, this internal leak was not fully blocked by TTX, since the basal ΔpH never reached the same level as that maintained in the presence of venturicidin (Fig. 2b, compare closed circles and squares). With ADP, the ΔpH dramatically increased with [TTX], as expected from an energy-transfer inhibitor. At 200 μM TTX, the three ΔpH values were somewhat lowered, probably due to a non-specific effect of TTX on the membranes.

Interestingly, at TTX concentrations above 2 μM, the ΔpH became higher in the presence than in the absence of ADP, reaching practically the same value as in the presence of venturicidin. Actually, ADP blocks the futile H^+-flow through the ATPase [22,24], presumably by binding onto catalytic site(s) [25]. This effect, normally masked by the high H^+ flow coupled to ATP synthesis, became visible here, due to the severe inhibition by TTX. Therefore, TTX did not prevent ADP binding responsible for the blocking of the internal leak. This pattern was conserved at 200 μM TTX, that is in the range of recovery of ATPase activity (see Fig. 1 and below).

3.3. Tentoxin and ATP synthesis in thylakoids

In DTT-treated membranes, TTX inhibited ATP synthesis with the same efficiency as ATP hydrolysis (half-inhibition at 0.5 μM TTX, full inhibition at 5 μM). A very small recovery (0.5% of the control) appeared at 10 μM TTX; this recovery reached about 2% at 100 μM TTX and 3–6% at 200 μM TTX (results not shown; ATP was titrated by the luciferine–luciferase technique as well as by pH-metry, and the same venturicidin-sensitive activities were found). Although the extent of recovery (a few percents) is here lower than for ATP hydrolysis (15%), it makes no doubt that TTX at high concentrations restores ATP synthesis.

Since steady state ΔpH depends on TTX concentration (Fig. 2b), we have studied how the rate of ATP synthesis varied with the light-induced quenching of 9-AA fluorescence, adjusted to different values by the actinic light input. This is shown on Fig. 3. The activity in presence of 100 μM TTX, higher than the activity at TTX 10 μM, seems not to obey the same law as the native activity. The flux vs. force curve indeed is shifted to higher values of ΔpH. The driving force requirement seems then higher for the TTX-modified form of the enzyme than for the native form.

3.4. Tentoxin and ATP synthesis by CF_0CF_1 co-reconstituted with bacteriorhodopsin into liposomes

We have investigated the effect of TTX on the light-driven ATP synthesis by CF_0CF_1 co-reconstituted with BR into liposomes. Fig. 4a shows typical time-courses of ATP synthesis (100% inhibited by venturicidin), and Fig. 4b shows the rate of ATP synthesis versus TTX concentration. As in thylakoids, the activity was fully abolished in the micromolar range, and restored for TTX concentrations beyond 10 μM. The TTX-in-

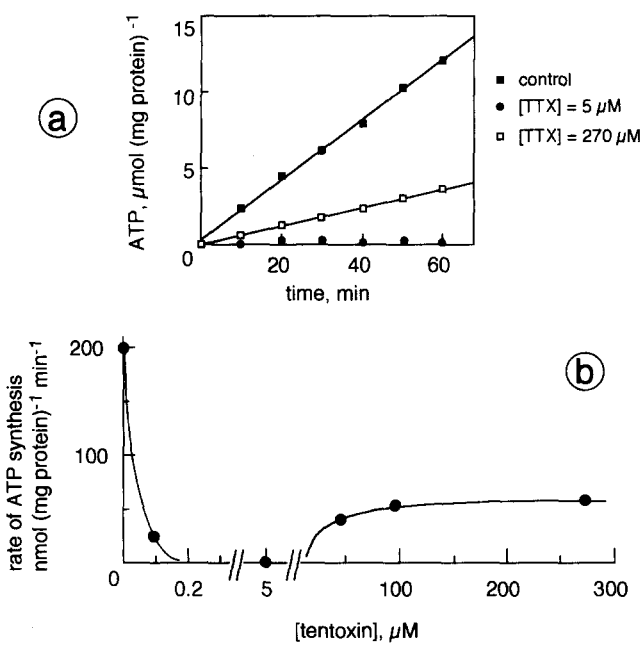

Fig. 4. Light-driven ATP synthesis by co-reconstituted proteoliposomes with bacteriorhodopsin and CF_0CF_1. Effect of tentoxin. Conditions as described in section 2. (a) ATP produced as a function of time, without TTX (■), with [TTX] = 5 μM (●), and with [TTX] = 270 μM (□). (b) Rate of ATP synthesis as a function of TTX concentrations. Values calculated from the slopes in (a). Changes in abscissa scale indicated by breaks on the axis.

duced recovery of ATP synthesis was 25–30% of the control, much more higher than in thylakoids. This more firmly proves that TTX-modified CF_0CF_1 preserves its coupling mechanism.

4. Discussion

In this paper, we were essentially interested in the recovery of the activity of the membrane-bound enzyme at high TTX concentration. Previous investigators had reported that this phenomenon did not exist in thylakoid membranes [11]. Actually, they did observe a significant Mg-dependent ATP hydrolysis in the presence of 400 μM TTX (about 35% of the control). But, probably because they expected a 2- to 3-fold stimulation of the rate, as in isolated CF_1, they neglected to explore intermediate TTX concentrations and missed the recovery process. Although less pronounced than in isolated CF_1, this recovery, which occurs in the same range of TTX concentration, is probably of the same nature. In our hands, less than 5% of ATP synthesis was recovered in thylakoids, but 25–30% were recovered in proteoliposomes. Without speculating about the different conditions (pH, ΔpH magnitude, absolute activities), one could suspect a role of temperature: experiments with thylakoids were made at 20°C, and experiments with proteoliposomes at 40°C; in [11], temperature was 37°C, and ATP hydrolysis by thylakoids treated with 400 μM TTX was 35% of the control. Anyway, the membrane-bound enzyme is less reactivated by TTX than isolated CF_1. All is likely specific constraints limit the turnover of the TTX-reactivated, membrane-bound CF_0CF_1.

It was shown, indirectly [23] or directly [3], that TTX-inhibited ATPase does not become leaky for protons. It is shown here for the first time that the TTX-reactivated form of CF_0CF_1 keeps intact its coupling properties. Even the nucleotide-dependent blocking of the futile proton flow is not affected. The TTX-induced form of CF_0CF_1 differs from the native form in two main aspects: (i) a low turnover rate, especially in the direction of ATP synthesis; (ii) the absence of deactivation of the thiol-reduced enzyme after collapse of the proton gradient (Fig. 1a, compare traces d–e to trace a). It has been known that the rate of F_0F_1 ATPase deactivation depends on the enzyme turnover (mitochondrial enzyme [26,27], CF_0CF_1 [12]). Since the TTX-modified CF_0CF_1 hydrolyses ATP 5–6 times slower than the native form, this could explain why the TTX-modified enzyme does not deactivate after $\Delta\tilde{\mu}_{H^+}$ collapse. Moreover, TTX at high concentration makes exchangeable ADP tightly bound to CF_1 [11].

It seems reasonable to think that TTX-reactivated CF_0CF_1 bears at least two TTX molecules. Unfortunately, the binding sites of TTX, and the aminoacids responsible for the specificity [7,8] have not yet been precisely characterized. Progress in this field is necessary to correlate the TTX effects reported here to structural changes.

Several reports suggest that TTX, at inhibitory concentrations [10] or higher concentrations [6] disrupts the interactions between the nucleotide binding sites. This could lead to the idea that such interactions are not strictly required for the proton pumping process. Intensive study of TTX effects should then bring new insights into the very molecular mechanism of F_0F_1 ATPase.

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