The mechanism of action of tentoxin on the soluble part (chloroplast F₁, H⁺-ATPase; CF₁₄) of chloroplast ATP synthase was analyzed in the light of new kinetic and equilibrium experiments. Investigations were done regarding the functional state of the enzyme (activation, bound nucleotide, catalytic turnover).

Dialysis and binding data, obtained with ¹⁴C-tentoxin, fully confirmed the existence of two tentoxin binding sites of distinct dissociation constants consistent with the observed $K_{\text{inhibition}}$ and $K_{\text{overactivation}}$. This strongly supports a two-site model of tentoxin action on CF₁. Kinetic and thermodynamic parameters of tentoxin binding to the first site ($K_i = 10 \, \text{ns}; K_\text{on} = 4.7 \times 10^4 \, \text{s}^{-1} \cdot \text{m}^{-1}$) were determined from time-resolved activity assays. Tentoxin binding to the high affinity site was found independent on the catalytic state of the enzyme.

The analysis of the kinetics of tentoxin binding on the low affinity site of the enzyme showed strong evidence for an interaction between this site and the nucleotide binding sites and revealed a complex relationship between the catalytic state and the reactivation process. New catalytic states of CF₁ devoid of $\epsilon$-subunit were detected: a transient overstimulated state, and a dead end complex unable to bind a second tentoxin molecule. Our experiments led to a kinetic model for the reactivation phenomenon for which rate constants were determined. The implications of this model are discussed in relation to the previous mechanistic hypotheses on the effect of tentoxin.

$F_0F_1$-ATP synthases are the purveyors of ATP in chloroplasts, mitochondria and bacteria. They are bound to energy-transducing membranes and couple the synthesis of ATP (through photophosphorylation of ADP) to the dissipation of a proton motive force (1, 2). The enzyme consists of two discrete parts, $F_0$ and $F_1$, interconnected by a stalk. $F_0$ is embedded in the membrane and behaves as a proton channel. The extrinsic part $F_1$ bears the six nucleotide binding sites; three of them are catalytic sites for ATP synthesis (for a review, see Ref. 3). Depending on species, three or more different subunits comprise the $F_0$ moiety: $a(1), b(2)$, and $c(9–12)$ in Escherichia coli; $a(1), b(1), b'(1)$, and $c(9–12)$ in chloroplasts. In mitochondria, the $F_0$ moiety bears additional subunits (4). The $F_1$ part consists of five different subunits: $\alpha$, $\beta$, $\gamma$, $\delta$, and $\epsilon$, with $\alpha(3), \beta(3), \gamma(1), \delta(1), \epsilon(1)$ stoichiometry. It is proposed that the ATP synthase acts as a proton-driven motor (5–7): in chloroplasts and bacteria, subunit $c$ and subunits $\gamma$ and $\epsilon$ (presumably linked to the $c$-crown) compose the rotor, while the extrinsic $\alpha\beta_g$ crown linked to the membranous $a$-subunit by the $\delta$ and $b$ subunits acts as a stator (8, 9). The rotation relayed by $\gamma$ would sequentially modify the three-dimensional structure of the three catalytic sites (10) and induce the ATP synthesis. Evidence for the rotation of $\gamma$ relative to the $\alpha\beta_g$ crown has been presented in the case of ATP hydrolysis (11–14) and ATP synthesis (15). As an essential breakthrough, this model strictly correlates the cooperative mechanism of the enzyme to the rotation of the $\gamma$-subunit and thus to the proton gradient dissipation.

Tentoxin (TTX) is a natural cyclic tetrapeptide (cyclo-$(\text{L-}$-methyl-Ala$^{1}$-$\text{L-Leu}^{2}$-$\text{ methyl-}$$\Delta^2\text{Phe}^{3}$-$\text{Gly}^{4}$)) produced by several phytopathogenic fungi of the Alternaria genus (16–17), which induces chlorosis of many sensitive higher plants (18). Chlorosis seems to be a consequence of the inhibition of photophosphorylation. TTX indeed specifically inhibits ATP synthesis in chloroplasts of sensitive species (19) as well as ATP hydrolysis in isolated CF₁ (20). In addition, TTX was shown to bind the extrinsic part $P_F$ of the $F_0F_1$-ATP synthase (19). An interesting feature of this toxin is its dual effect: in vitro and at low concentrations ($10^{-8}$ to $10^{-7}$ m), TTX inhibits ATP hydrolysis and synthesis either in isolated chloroplasts or in isolated CF₁, while at high concentrations ($10^{-8}$ to $10^{-4}$ m) it strongly stimulates ATPase activity of the isolated enzyme (21) and leads to a partial recovery of the coupled activity of membrane-bound ATP synthase (22).

The mechanism of TTX action, for the inhibition as well as for the reactivation, is still unknown. The number of binding sites involved in the reactivating effect remains controversial. It has been reported that CF₁ binds two molecules of TTX on distinct sites (23) presumably located on $\beta$-subunit, although this point remains obscure (24–26). Since the affinity of TTX for these two binding sites was found different, we suggested that they were respectively related to the inhibitory and stimulatory activities of TTX (23). Also, Mochimaru and Sakurai (27) have recently suggested the existence of a third very low affinity binding site, which would account for the reactivation process. The affinity of TTX for the inhibitory site has been measured in various forms of the ATP synthase (20, 28), with limited efforts to relate it to the functional states of the enzyme (27). Rare investigations have been carried out on the interactions

---

* This work was supported by Ministère de l’Enseignement Supérieur et de la Recherche Contract ACC-SV5 (interface Chimie-Physique-Biologie) no. 9505221 and by CNRS grant (Physique-Chimie du Vivant) no. 97N21/0122. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 33 1 69 08 44 32; Fax: 33 1 69 08 87 17; E-mail: santo@dsvidf.cea.fr.

This paper is available on line at http://www.jbc.org

---

1 The abbreviations used are: TTX, tentoxin or cyclo-$(\text{L-}$-methyl-Ala$^{1}$-$\text{L-Leu}^{2}$-$\text{Meth-}$$\Delta^2\text{Phe}^{3}$-$\text{Gly}^{4}$); $F_0F_1$, chloroplast $F_1$, H⁺-ATPase; $CF_1$, chloroplast $F_1$, H⁺-ATPase devoid of $\epsilon$-subunit; DTT, dithiothreitol; $^{14}$C-TTX, $^{14}$C-methyl-Phe($\Delta^2$)-tentoxin.
between the nucleotide binding sites and the TTX binding sites. TTX at inhibiting concentration does not interfere with the exchange of tightly bound nucleotide (29), while it seems to promote the ADP release at high concentrations (30). Nevertheless, the effect of the presence of nucleotides and, more generally, of the catalytic state of the enzyme on TTX binding to its low affinity site has never been studied.

In this report, we show strong evidence for the existence of only two TTX binding sites on chloroplast ATP synthase in the concentration range of in vitro assays, and we show that they directly account for the inhibitory and reactivating effects of TTX on the activity of CF₁ and CF₁-ε. In addition, thermodynamic and kinetic characteristics of TTX binding on the inhibitory site were thoroughly determined. No influence of the dynamic state of the enzyme on this inhibitory binding has been observed. As regards the reactivation process, kinetic experiments revealed the existence of two new dynamic forms of CF₁-ε related to the presence of ATP: a transient overactivated state and a dead end complex unable to bind a second TTX molecule. We show that TTX binding to its reactivating site is strongly dependent on the presence of nucleotide on the enzyme. We propose a kinetic model that accounts for all of our results, of which most of the constants were determined.

**EXPERIMENTAL PROCEDURES**

**Enzyme Preparation**—The soluble chloroplast ATPase (CF₁) was extracted and purified from spinach (*Spinacia oleracea* L.) leaves in the active form devoid of its inhibitory subunit ɛ, unless specified. The enzyme was stored at 5 °C in 34% ammonium sulfate buffer as described previously (31) at a protein concentration of 16 mg ml⁻¹. CF₁-ε was activated by preincubation at 20 °C in 20 mM Tricine, pH 8, 3 mM DTT for at least 3 h. When CF₁ was used, no activation was performed. Protein concentration was determined by UV absorption spectroscopy assuming for CF₁-ε an extinction coefficient of 0.48 cm⁻² mg⁻¹ at 278 nm (31).

**Binding Experiments**—Samples of 500 μl of DTT-activated CF₁-ε at various concentrations (10 nM to 10 μM) were dialyzed in SPECTRA/POR tubing (molecular weight cut-off = 6000–8000) for 24 h at 37 °C against 50 mM Tris-SO₄, pH 8, 40 mM KHCO₃, 4 mM MgSO₄, 1 mM sodium orthovanadate, and 0.1 mM sodium fluoride. Bound and free ATP were then rapidly separated using a PD 10 Amersham Pharmacia Biotech chromatography column. ATPase activity was measured as indicated above. Binding curve was fitted to Equation 1 using a two-independent site model (Fig. 2). The fit clearly shows the existence of two binding sites of 14C-TTX (ranging from 10 nM to 10 μM). 14C-TTX was obtained as described previously (31) at a protein concentration of 16 mg ml⁻¹. Equilibrium conditions were checked by measuring in small aliquots the inner and outer radioactivity at four different times during dialysis. Each counting was performed twice for 4 min using a Beckman LS 3801 Scintillation Counter. 14C cpm ranged from 500 ± 60 to 5 × 10⁵ ± 200. Free TTX concentration was deduced from the radioactivity measured outside the dialysis tubing, and bound TTX concentration was calculated from the difference between the radioactivity measured inside and outside the dialysis tubing. No significant loss of activity occurred within 24 h in these experimental conditions. For TTX concentrations higher than 10 μM, 14C-TTX and CF₁-ε were first equilibrated for 3 h at 37 °C. Bound and free TTX were then rapidly separated using a PD 10 Amersham Pharmacia Biotech chromatography column, and their concentrations were measured as indicated above. Binding curve was fitted using Microlor ORIGIN 5.0 (Microcal Software).

**Kinetics Dialysis**—500-μl samples of 14C-TTX at various concentrations were dialyzed at 37 °C in a low cut-off (M_w = 1000) SPECTRA/POR dialysis tubing against 20 ml of assay buffer (50 mM Tris-SO₄, pH 8, 40 mM KHCO₃, 0.18 mM MgSO₄). Small aliquots were withdrawn from the outer buffer at different times, and the radioactivity was measured as described above and converted into TTX concentration. The volume of the dialysis tube, and thus the exchange surface, was nearly constant in each experiment. The outer TTX concentration was plotted as a function of time and fitted to Fick’s law using Microlor ORIGIN 5.0. Control experiments were performed with 500 μM samples of ADP, diadenosine 5′-hexaphosphate, and Alcyan Blue, titrated by spectrophotometry.

**Steady-state Activity Assays**—DTT-activated CF₁-ε (10 nM) was incubated at 37 °C in 50 mM Tris-SO₄, pH 8, 8 μl buffer, in the presence of TTX at various concentrations for 4 h up to 12 h for the lowest concentrations. 5 min after the addition of 40 mM KHCO₃, and 0.18 mM MgSO₄, ATP hydrolysis was initiated by the addition of ATP (final concentration 1 mM). The reaction mixture was thermostatted at 37 °C, and aliquots of 10 μl were taken every 3.5 min (up to 15 min) and injected into a TSK DEAE 2SW 5-μµm analytical high pressure liquid chromatography column for nucleotide determination. The nucleotides were separated by isocratic elution with 0.1 M KH₂PO₄, pH 4.3, 0.25 M NaCl, at a rate of 1.0 ml min⁻¹. ADP concentration was determined from the area of the peak detected at 260 nm. The ATPase activities of CF₁-ε in the presence of various concentrations of TTX were deduced from the plots of ADP concentration as a function of time and normalized to the control activity without TTX (Fig. 4).

**Time-resolved ATPase Activity Assays**—For inhibition kinetic studies, DTT-activated CF₁-ε (10 nM) was incubated for 10 min at 37 °C in a stirred reaction buffer in a spectrophotometer cuvette. The reaction buffer (50 mM Tris-SO₄, pH 8, 40 mM KHCO₃, 4 mM MgSO₄, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 0.1 mg/ml lactate dehydrogenase, 0.1 mg/ml pyruvate kinase) allowed us to couple ATP hydrolysis to NADH oxidation. The time response of this system, checked by ADP addition in the absence of TTX, was always significantly shorter than a few seconds. ATP hydrolysis was started by adding MgATP (final concentration 2 mM) and monitored by absorbance decrease at 340 nm. In a first series of experiments (Fig. 3a), TTX was added at various inhibitory concentrations (from 5 nM to 5 μM) 3 min after the addition of MgATP. In another series of experiments (Fig. 3b), MgATP was added after the incubation of CF₁-ε for various times with 75 nM TTX. The resulting kinetics were fitted to Equation 4. For reactivation kinetic studies, DTT-activated CF₁-ε (10 nM) was incubated either in the presence or in the absence of inhibitory concentration of TTX (500 nM). ATP hydrolysis was started by the addition of MgATP (final concentration 2 mM) and continuously monitored at 340 nm. In a first series of experiments, TTX was added at various reactivating concentrations (from 5 to 150 μM) to inhibited CF₁-ε, 3 min after the addition of MgATP (Fig. 6a). Similar experiments were achieved by adding MgATP after the addition of reactivating concentrations of TTX (Fig. 6b). The decay phases of the kinetics of Fig. 5, a and b, were fitted to a primitive function of Equation 14. The resulting apparent rate constants k_app were fitted to Equation 13, which allowed the determination of k⁺, k⁻, and K. Other protocols are detailed in the text.

**RESULTS**

**Binding of 14C-Radiolabeled TTX to CF₁-ε**—Fig. 1 shows the amount of 14C-TTX bound to CF₁-ε, when equilibrated at 37 °C, as a function of the concentration of free 14C-TTX. The data were fitted to a model based on multiple independent binding sites. The fit clearly shows the existence of two binding sites of different affinities (K_d1 = 50 ± 20 nM; K_d2 = 80 ± 20 μM) in the concentration range investigated (between 10 nM and 1.2 μM). The two dissociation constants differ by about 3 orders of magnitude, and the binding of TTX to CF₁-ε can therefore be considered to occur in a sequential mode.

**Monomeric Behavior of TTX at High Concentrations**—To validate the present analyses, which imply that TTX is mono-
not diffuse at all. This shows that TTX does behave as predicted by Fick’s law only if molecules do not form concentration-dependent aggregates. In Fig. 2 (inset) shows exponential kinetics of diffusion of 14C-TTX, as expected from Fick’s law. The kinetic data, normalized to the equilibrium concentration, were identical for each initial concentration inside the dialysis tube between 1.8 and 500 \( \mu M \). The initial rate of diffusion of 14C-TTX was thus found proportional to its concentration. As expected from a unique binding site model, the derived rate constants plotted as a function of their concentration in the reaction medium, were identical for each initial concentration. The forward binding rate constant \( k_{on} \) of ATPase inhibition as a function of TTX concentration was directly determined from the slope of the graph (Equation 5). Inset, typical time courses of ATP hydrolysis after the addition of TTX at 50 nM (1), 300 nM (2), and 1500 nM (3). Every kinetic profile was fitted to Equation 4. b, initial ATPase activity \( (\kappa) \) of incubation time with TTX. Similar experiments are shown as in a but with 75 nM TTX added at various times before ATP. The activities were normalized to the control without TTX (5 ± 1 \( \mu M \) of ATP/mg). Data were fitted to Equation 2, which gave \( k_{on} = 4 \times 10^{-3} \pm 10^{-3} \text{s}^{-1} \) and then \( k_{off} = 6 \times 10^{-3} \pm 1.5 \times 10^{-3} \text{M}^{-1} \text{s}^{-1} \) (Equation 3 neglecting \( k_{off} \)). Inset, typical time courses of ATP hydrolysis after 1 min (1), 5 min (2), and 10 min (3) of incubation, fitted to Equation 4.

The rate of binding of TTX on the inhibitory site was also measured in the absence of ATP. CF1-\( e \) was incubated in presence of 75 nM TTX for various times. Then ATP was added, the corresponding kinetics were recorded (Fig. 3b, inset), and the initial rate of ATP hydrolysis was determined. This rate was plotted as a function of the time of incubation with TTX (Fig. 3b). The kinetics of inhibition so obtained were fitted with a monoeponential function (Equation 2). Taking into account the TTX concentration and assuming that the \( k_{off} \) is negligible, the derived \( k_{app} \) gave rise to a forward rate constant \( k_{on} = 6 \times 10^{-3} \text{M}^{-1} \text{s}^{-1} \). This value is close to that determined above in the presence of ATP.

Comparison of the Effects of TTX on the ATPase Activity of CF1 and CF1-\( e \)—Since the rate constant \( k_{on} \) of TTX for the inhibitory site is not very high, long times of incubations are required to observe the full effect of low concentrations of TTX (below 100 nM), even at 37 °C. For this reason, we investigated the inhibitory effect of low concentrations of TTX under conditions similar to those previously reported (32), except that the time of incubation with TTX was raised in order to reach the...
Overactivation Process of Chloroplast F$_1$-ATPase by Tentoxin

Fig. 4. ATPase activity of CF$_1$ and CF$_1$-e as a function of TTX concentration. Conditions were as described under “Experimental Procedures.” Activities were normalized to the control without TTX (5 ± 1 μmol of ATP/min/mg for native CF$_1$). a, experimental points (●) were determined at equilibrium after the incubation of 5 μM of DTT-activated CF$_1$-e with various concentrations of TTX in the inhibitory range. The right part of the curve corresponds to the nonlinear fitting previously determined in the reactivatory range (32). The experimental data were fitted to Equations 6 and 7; $K_{d1} = 8 ± 1$ nM; residual activity $V_1 = 5%$ of the control. b, experimental points (●) were obtained in the same conditions with 50 nM of untreated CF$_1$. The left part (up to 1 μM of TTX) was fitted to Equations 6 and 7; $K_{d1} = 13 ± 1$ nM; residual activity $V_1 = 3%$. The right part (from 1 μM to 1 mM TTX) was fitted with Equation 8. See text for details.

The rate constants of dissociation $k_{off}$ and $k_{on}$ were determined at equilibrium by using the method described in “Experimental Procedures.” The results are displayed in Fig. 4a. The data points were fitted to Equations 6 and 7, giving $K_{d1} = 3.7 × 10^{-4}$ s$^{-1}$. This value confirms that $k_{off}$ was negligible with respect to $k_{on}[T]$ above.

The same protocol was used to investigate the effect of TTX on ATPase activity of native CF$_1$ for concentrations ranging from 1 nM to 1 mM (Fig. 4b). The affinity of TTX for the inhibitory site of CF$_1$ ($K_{d} = 13$ nM) was found almost identical to the one determined for CF$_1$-e. The lack of the e-subunit and the DTT activation have no effect on the inhibition processes of TTX. Since the solubility of TTX is limited to 3.5 mM and the monomeric state of TTX is uncertain above 1 mM, the investigation of its effect on CF$_1$ above 1 mM would be of little interest.

Therefore, we only measured the reactivation of the ATPase for TTX concentrations up to 1 mM. Since no plateau of CF$_1$ activity was reached, we were not able to precisely determine the dissociation constant for the second, loose site. Whatever it may be, the $K_{d}$ varied from 700 μM to 2.7 mM for a reactivation level set from 5- to 20-fold the control. As a result, the binding of a second TTX molecule appears quite different between CF$_1$ and CF$_1$-e.

Kinetics of Reactivation of ATPase Activity of CF$_1$-e by TTX Critically Depend on the ATP Preincubation Time—We have investigated the kinetics of reactivation of CF$_1$-e by TTX. The enzyme was introduced in the spectrophotometric cuvette with TTX at 500 nM to fill the inhibitory site and in the presence of an enzymatic system coupled to NADH oxidation. In order to check the effect of nucleotides on the TTX-dependent reactivation process, the enzyme was incubated with ATP for various times (from 2 s to 10 min) before the addition of a reactivating concentration of TTX. As expected, the ATPase was inhibited by 500 nM of TTX (6–7% of the control). Reactivation was then initiated by adding 20 μM of TTX (Fig. 5). Surprisingly, a biphase profile was observed for short preincubation time with ATP. It consisted of a fast rise followed by a slow monoeponential decay. This behavior progressively disappeared with increasing ATP incubation time to finally give rise to a simple monoeponential reactivation.

Rate of Reactivation of CF$_1$-e by High Concentrations of TTX Added after ATP Preincubation—TTX-inhibited CF$_1$-e was incubated with ATP during 3 min (conditions leading to a monoeponential profile of reactivation; Fig. 5), and high concentrations of TTX (5–150 μM) were then added. A progressive recovery of ATPase activity was observed. The kinetics of recovery of activity could be satisfactorily fitted to a monoeponential function (Fig. 6a). Surprisingly, the rate constant $k_{app}$ was not a linear function of the TTX concentration. It was even found to decrease with TTX concentration (Fig. 6b). This behavior is not that expected from reactivation kinetically controlled by TTX binding on the low affinity site.

Rate of Reactivation of CF$_1$-e by High Concentrations of TTX without ATP Preincubation—We also checked the kinetics of reactivation obtained at different TTX concentrations, added together with ATP (Fig. 6b). The kinetics were this time clearly biphasic; the activity passed through a transient overactivation and then decayed to a plateau similar to the one obtained with long ATP preincubation (Fig. 6a). We did not try to analyze the fast rising phase, because its kinetic resolution was not good enough. However, the slow phase has been fitted to a monoeponential function. Contrary to what has been calculated in the kinetic analysis of Fig. 6a, the rate constant $k_{app}$ was here independent of TTX concentration (Fig. 6c). Identical results were obtained when reactivating TTX was added to inhibited CF$_1$-e at different times before ATP (data not shown).
Overactivation Process of Chloroplast F$_1$-ATPase by Tentoxin

If TTX is added several minutes after ATP (Fig. 6a), it is a monophasic rise of activity toward an equilibrium value. The second one is observed when ATP is added after or with TTX (Fig. 6b). It consists of a fast rise of activity followed by a slow monoexponential decay, leading to about the same equilibrium level as the first mode. Another major difference between these two modes of reactivation is the characteristic evolution of $k_{app}$ as a function of TTX concentration.

**DISCUSSION**

Two Binding Sites Completely Account for Inhibition and Reactivation of CF$_1$-$\epsilon$ by TTX—Two binding sites with different $K_d$ values account for the inhibitory and reactivatory effects of TTX. The $K_d$ values determined by $^{14}$C-TTX binding and kinetic experiments are compatible. They differ from those determined by Pinet et al. (23) through binding studies, probably because the conditions were critically different (buffer at 37 instead of 4 °C). We do not confirm or invalidate the existence of a third binding site of very low affinity recently proposed (27). However, we think that it should be considered very cautiously for different reasons. This third site was indeed revealed at TTX concentrations above 2 mM, where its monomeric character was not proved. Therefore, acquisition and modeling of few binding data in millimolar range, which led to an extrapolated $K_d$ of 6.3 mM, seem very questionable. The authors also attribute the very strong level of reactivation (almost 2000% of the control, instead of the 200–300% generally obtained) to the filling of a third site. However, they used a nonactivated CF$_1$, about 20-fold less active than DTT-treated CF$_1$-$\epsilon$. We confirmed this relative magnitude of reactivation of CF$_1$ (Fig. 4b), but at 1 mM TTX the absolute activity of CF$_1$ remains lower than the CF$_1$-$\epsilon$ activity. Thus, this huge reactivation value may be due to the combination of the regular reactivation with a TTX-induced overcoming of the latent character of CF$_1$ (21), and not to the existence of a third TTX-binding site on CF$_1$.

The Dynamic State of CF$_1$ Does Not Influence TTX Binding at the First Site—We have quantitatively shown that TTX binding on the inhibitory site is identical, whether CF$_1$ is activated or not. Mochimaru and Sakurai (27) also reported that partial trypsic digestion activation of CF$_1$ had no effect on the rate of TTX binding on the low affinity site. Moreover, our results directly demonstrate that ATP addition and the catalytic turnover do not change the rate of TTX binding at the high affinity site. Consequently, there is no direct influence of the dynamic state of CF$_1$ on the TTX binding on the first site. Conversely, it was previously shown that TTX binding on the inhibitory site modifies neither the affinity of isolated CF$_1$ for ATP, nor the exchange of tightly bound nucleotide (29). All of these results may indicate that TTX does not inhibit CF$_1$ by preventing its activation, normally achieved by the protonmotive force in the membrane-bound CF$_0$CF$_1$ and mimicked by various treatments in isolated CF$_1$.

In other experiments, the kinetics of ATPase reactivation were investigated as in Fig. 6 by adding high concentrations of TTX, but directly to the active, noninhibited enzyme. The kinetics of reactivation also followed the biphasic mode already observed in Fig. 6b. Contrary to what was observed in the previous experiments, preincubation with ATP several minutes before TTX addition did not change the kinetics of activation (data not shown).

From these experiments, two different modes of reactivation can be discriminated on a kinetic basis. The first one, observed when TTX is added several minutes after ATP (Fig. 6a), is a...
the active enzyme; therefore, nonsaturating TTX concentrations lead to a mixture of fully inhibited and active enzymes. This explains why in thylakoids, partial inhibition by a low concentration of TTX did not change the Michaelis constant of ADP for ATP synthesis (33).

ATP Interferes in a Complex Way with TTX Binding at Its Second Site—By contrast, ATP modifies the properties of the low affinity TTX binding site. We have developed a minimal model to account for all of our data. The main feature is the progressive change, during incubation with ATP, in the pattern of TTX-triggered reactivation (Fig. 5). It shifted from a biphasic kinetics with a transient overactivation (Fig. 6a) to a monotonic rise of activity (Fig. 6c). The simplest way to qualitatively explain this result is depicted in Scheme 1, where only enzymes bearing one or two molecules of TTX are represented.

In the presence of ATP, the ET form is slowly converted into a dead end complex, ET, unable to bind a second TTX molecule. The forward rate can be kinetically controlled either by ATP binding or by any further conformational change. TTX exchange at the low affinity site is much faster than the ATP-

When ATP is added to inhibited CF₁-ε with or after reactivating concentrations of TTX, all of the enzyme is initially in equilibrium between the ET and ETT states. Therefore, a burst of activity is produced followed by a slow decay of activity until the equilibrium, involving the three forms, is reached. When inhibited CF₁-ε is incubated with ATP a few minutes before the reactivating TTX addition, an equilibrium is reached between ET and ET'T; after the TTX addition, the activity monotonously rises to the same equilibrium as before without giving rise to a transient overaccumulation of the ETT state. The kinetics observed in Fig. 6, a–b, are well described by this model, which also predicts that the slow apparent rate constant of the exponential kinetics (case b) upon TTX concentration (Equation 13). This explains why in phylakoids, partial inhibition by a low concentration of TTX did not change the Michaelis constant of ADP for ATP synthesis (33).

ATP interferes in a complex way with TTX binding at its second site. a, general case leading to biphasic kinetics. b, simplified case assuming quasi-equilibrium for TTX binding on the low affinity site and leading to monoexponential kinetics. In both cases, the top curve describes the theoretical time course of ATPase activity without ATP preincubation, and the bottom curve describes the theoretical time course of ATPase activity after ATP preincubation. c, predicted dependence of the apparent rate constant of the exponential kinetics (case b) upon TTX concentration (Equation 13). See "Discussion" and "Appendix" for details.
From Fig. 6 and Equation 13, the true $k_{app}$ and inhibited enzymatic states is reached (Fig. 6). The TTX induces, as in Scheme 1, a monotonous rise of the ATPase rate constant $k_{app}$ and is about $0.5$ min$^{-1}$ for this decay (Fig. 6). Two unidirectional arrows with a box, quasiequilibrium; single unidirectional arrow, irreversible conversion. Thermodynamic and kinetic constants indicated have been determined from the experiments. The catalytic activities normalized to the control (activity without TTX) are displayed inside the different complexes. See “Discussion” and “Appendix” for details.

Scheme 2. Multistate model of TTX binding to the low affinity site. Tentoxin binding sites are represented by notches. The left notch represents the inhibitory (high affinity) site, and the right notch shows the reactivatory (low affinity) site. When filled, they are, respectively, shaded in gray and black. For the loose site, the notch is an open triangle when TTX is slowly exchangeable and an open rectangle when TTX is quickly exchangeable. $T$, tentoxin. Left column, states of CF$_1$-e in the absence of ATP; right column, states of CF$_1$-e in the presence of ATP. State A is the active form of the enzyme catalyzing ATP hydrolysis; states I$_1$, I$_2$, and D are inhibited forms of the enzyme bearing one TTX molecule; states O and S are reactivated states bearing two TTX molecules. Two unidirectional arrows with a box, quasiequilibrium; single unidirectional arrow, irreversible conversion. Thermodynamic and kinetic constants indicated have been determined from the experiments. The catalytic activities normalized to the control (activity without TTX) are displayed inside the different complexes. See “Discussion” and “Appendix” for details.

Let us consider the effect of TTX addition to inhibited CF$_1$-e. When ATP is added just before, simultaneously with, or after stimulatory concentrations of TTX to inhibited CF$_1$-e, the enzyme is first in rapid equilibrium between an overstimulated state (state O) and an inhibited state (state I). Then it experiences an irreversible and slow conversion into two states also in rapid equilibrium (named S and I$_2$, respectively, and corresponding to the ETT and ET states described in Scheme 1). State I$_2$ slowly equilibrates with the dead end state D (corresponding to ET$^+$ in Scheme 1). The global process gives rise to a slow decay of the activity, as observed in Fig. 6b, toward an equilibrium among three enzymatic forms. The $k_{app}$ apparent for this decay (Fig. 6c) is about $0.5$ min$^{-1}$ ($8 \times 10^{-3}$ s$^{-1}$). This value gives the forward rate constant of the $O \rightarrow S$ and $I_1 \rightarrow I_2$ transitions. The rate constants are assumed to be the same for both conversions, in agreement with the fact that $k_{app}$ does not depend on TTX concentration. Since the inhibited enzyme is subjected to the same slow conversion as the overstimulated one, this step does not seem to be directly correlated to the catalytic turnover.

After a long preincubation of the inhibited enzyme with ATP, the enzyme is in equilibrium between the inhibited state (state I$_2$) and the dead end state (state D). The addition of stimulatory TTX induces, as in Scheme 1, a monotonous rise of the ATPase activity until the equilibrium between the stimulated, dead end, and inhibited enzymatic states is reached (Fig. 6a). The $k_{app}$ of this monoeponential growth decreases with the TTX concentration (Fig. 6c, Equation 13). The data gave the $K_d$ of TTX for the loose site and the forward and backward rate constants $k^+$ and $k^-$ (Scheme 1). Due to the existence of the dead end complex, the $K_{d2}$ measured at equilibrium is only apparent and is a function of the true $K_{d2}$, $k^+$, and $k^-$ (Equation 15). From Fig. 6c and Equation 13, the true $K_{d2}$ is about $10\mu M$. Consequently, the apparent $K_{d2}$ should be about $30\mu M$ (Equation 15), which is in accordance with the previous estimate in equilibrium ($K_{d2} = 40\mu M$; Ref. 32).

When TTX at reactivating concentrations was added directly to the active complex (without TTX on the tight site), the kinetic profiles did not depend on the preincubation of the enzyme with ATP (data not shown). It consisted in all cases in a fast overstimulation followed by a slow decay. This is easily explained if we assume that the active enzyme (state A) does not experience a transition like states I$_1$ and O. After the addition of high concentrations of TTX, with or after ATP, the enzyme immediately goes, through the inhibited states, to the overstimulated state (state O). Then it goes slowly to the stimulated one (state S) and reaches an equilibrium between states D (dead end), S (stimulated), and C (inhibited).

Structural Considerations—The presently available data do not give a clear cut structural explanation of our results. First, a high resolution structure of CF$_1$ is still lacking. Second, there is only indirect evidence (24, 26) that $\beta$-Asp-832 takes part in the TTX binding site. However, for illustrating the TTX effects in relation to the CF$_1$ structure, we can take for granted that the TTX binding site is located in this region and that the structure of this well conserved domain is equivalent in CF$_1$ and MF$_1$, although MF$_1$ is TTX-insensitive. A survey of the structure of MF$_1$ (10) indicates that a region, located at an $\alpha/\beta$ interface in the vicinity of the upper $\beta$-barrel of the $\beta$-subunit, corresponds to a pocket whose opening critically depends on the nucleotide occupancy. The $\alpha/\beta$ pair comprising the adenine triphosphate-loaded $\beta$-subunit (chains B and F in Protein Data Bank structure 1cow) presents the most favorable conformation of this region for TTX binding. This domain is rich in aromatic residues. One of them, $\alpha$-Tyr-292, located in a homologous sequence, FYLH, is a good candidate to stack with the methyl-Phe$(Z)$ residue of TTX. We have shown that the characteristics of TTX binding on the first site do not depend on the catalytic state of the enzyme. This site is certainly not subjected to turnover-dependent changes of exposure. Considering a stepped rotative mechanism of CF$_1$ (34), the probability for TTX to bind the most favored site is indeed the same in resting and dynamic states of the enzyme.

*In CF$_1$ primary sequence numbering.*
The asymmetry of the complex, and thus of the location of bound TTX, could also explain the differential effect of TTX on ATP synthesis and hydrolysis under single site conditions (29). In these conditions, the enzyme does not run a complete revolution during the experiment, and thus the effect of TTX could depend on the direction of rotation. In the direction of ATP synthesis, TTX could block the first step(s) of the rotation and would therefore inhibit the unisite catalysis. In the other direction, TTX could block more distal step(s) of the rotation, allowing the release of the ADP molecule, without apparent effect on the unisite hydrolysis. This hypothesis could also account for the ATP-dependent reactivatory behavior of TTX. In the presence of ATP, inhibited forms of CF1-ε do not pass through the overstimulated form observed in the absence of ATP. This conformational variation would modify the binding properties of the second TTX molecule and explain why, in the presence of ATP, CF1-ε does not pass through the overstimulated form observed in the absence of ATP. The present discussion shows that the microscopic states, here revealed by kinetic experiments, should be taken into account in the further investigations of the catalytic mechanism of the ATP synthase.

Acknowledgments—Thanks are due to Véronique Mary for the extraction of the spinach chloroplast F1-ATPase. 14C-TTX was provided by Marqueés, Commissariat à l’Energie Atomique-Saclay.

APPENDIX

Kinetics of ATPase Inhibition by TTX

The binding of TTX to the high affinity site is described as follows,

\[ E + T \xrightarrow{k_{on}} ET \quad (\text{Eq. 1}) \]

where \( E \) is the active form, and \( ET \) is the inhibited one. When the toxin is in excess with respect to the enzyme, the rate of ATP hydrolysis as a function of time is as follows,

\[ V_t - V_{eq} = (V_0 - V_{eq})e^{-k_{off}t} \quad (\text{Eq. 2}) \]

with

\[ k_{app} = k_{off}[T] + k_{on} \quad (\text{Eq. 3}) \]

where \( V_0 \) and \( V_{eq} \) are, respectively, the initial and final rates of ATP hydrolysis.

The instantaneous concentration of ATP can be deduced from Equation 2

\[ [\text{ATP}]_t - [\text{ATP}]_0 = A(e^{-k_{off}t} - 1) + Bt \quad (\text{Eq. 4}) \]

where

\[ A = \frac{V_0 - V_{eq}}{k_{app}} \quad \text{and} \quad B = V_{eq} \quad (\text{Eq. 5}) \]

A, B, and \( k_{app} \) were derived by fitting the experimental data (Fig. 3a) to Equation 4. \( k_{on} \) can be derived from \( k_{app} \) using Equation 3.

Inhibition by the First TTX Molecule at Equilibrium and \( K_{d1} \) Determination

To fit the curve of activity (V) versus TTX concentration, we account here for the concentration of enzyme, which is not negligible, and for a possible activity of the ET complex. The activity is described as follows,

\[ V = V_{da} + V_1(1 - \alpha) \quad (\text{Eq. 6}) \]

where \( \alpha \) is the fraction of the noninhibited enzyme, \( V_0 \) is the activity of \( E \), and \( V_1 \) is the activity of \( ET \). In a noncompetitive model, \( \alpha \) can be expressed as follows,

\[ \alpha = \frac{[E]_0 - K_{d1} - [T]_0}{\sqrt{[E]_0^2 - K_{d1} - [T]_0^2} + 4[E]_0K_{d1}} \quad (\text{Eq. 7}) \]

where \( K_{d1} \) is the dissociation constant of the ET complex and \( [E]_0 \) and \( [T]_0 \) are the total concentrations of enzyme and TTX. For a noncompetitive inhibition, \( K_{d1} \) is not substrate concentration-dependent.

Reactivation by the Second TTX Molecule at Equilibrium

At high concentrations of TTX, the concentration of TTX-free enzyme is negligible. Therefore, the activity versus concentration curve is represented as follows,

\[ V = \frac{V_i[ET]}{[E]_0} + \frac{V_i[ET]}{[E]_0} - \frac{V_1}{1 + \frac{[T]}{K_{d2}}} + \frac{V_2}{1 + K_{d2}[T]} \quad (\text{Eq. 8}) \]

where \( V_1 \) is the activity of ET complex, \( V_2 \) is the activity of ET complex, and \( K_{d2} \) is its dissociation constant. In the presence of a large excess of toxin, \( [T] \) can be taken as \( [T] = [T]_0 \).

Three-state Kinetics of Reactivation by TTX at High Concentration (Scheme 1)

The following three states are considered: ET, the enzyme bearing one TTX molecule; ET, the enzyme bearing two TTX molecules; and ET, a dead end complex, reversibly formed from ET in the presence of ATP and unable to bind a second TTX molecule. This system is displayed in Scheme 1. Scheme 16 describes a rapid equilibrium between states ET and ET, which is formalized by the equation,

\[ K = \frac{[ET][T]}{[ETT]} \quad (\text{Eq. 9}) \]

where \( K \) is the equilibrium dissociation constant.

Scheme 16 also displays a slow equilibrium between states ET and ET characterized by the forward and backward rate constants \( k^+ \) and \( k^- \). The evolution of the whole system is described by the following differential equation.

\[ \frac{d([ET] + [ETT])}{dt} = -k^-[ET] + k^+ [ETT] \quad (\text{Eq. 10}) \]

Equation 10 may be written as follows,

\[ \frac{d([ET] + [ETT])}{dt} = \frac{k^+}{1 + \frac{[T]}{K}} [ET] + [ETT] - k^- [ETT] = 0 \quad (\text{Eq. 11}) \]

where \( [E]_{tot} \) is the total enzyme concentration, \([ET] + [ETT]\) is an exponential function of time, as follows,

\[ y_t - y_{eq} = [y_0 - y_{eq}]e^{-k_{off}t} \quad (\text{Eq. 12}) \]

with

\[ k_{off} = \frac{k^+}{1 + \frac{[T]}{K}} \quad (\text{Eq. 13}) \]

\( y_0 \) and \( y_{eq} \) are the initial and equilibrium values of the sum of \([ET] + [ETT]\), respectively. \( ET \) is here assumed to be the only active form. Since \([ETT]\) is a constant fraction of \([ET] + [ETT]\), it obeys the same exponential law. The rate of ATP hydrolysis
is proportional to \([E\text{T}]\) at any time. It can then be written as follows.

\[ [E\text{T}] - [E\text{T}]_\text{eq} = ([E\text{T}]_\text{b} - [E\text{T}]_\text{eq})e^{-k\alpha t} \quad (\text{Eq. 14}) \]

with

\[ [E\text{T}]_\text{eq} = \frac{[E\text{T}]_\text{tot}}{1 + \left(1 + \frac{k}{k_D}\right)/[T]} \quad (\text{Eq. 15}) \]

Equation 14 shows that the time course of the activity depends on the initial concentration of \([E\text{T}]\), which is itself determined by the time of \([A\text{T}]\) preincubation.

Two extreme situations can be distinguished in Scheme 1.

**Situation 1**—If \([A\text{T}]\) is added at reactivating concentrations a long time after \([A\text{T}]\), all forms are present upon \([A\text{T}]\) addition. The activity, proportional to \([E\text{T}]_\text{eq}\), is initially low and then increases to reach its equilibrium value.

**Situation 2**—Without incubation with \([A\text{T}]\), \([E\text{T}]\) and \([E\text{T}]_\text{b}\) are the only forms initially present. The activity starts at its maximal level and decreases to its equilibrium value.

Equation 13, which gives \(k_{\text{app}}\) as a function of \([A\text{T}]\) (Scheme 1c) applies to both situations.

The final multistate model (Scheme 2) will be developed from Scheme 1b, which is a simplified form of Scheme 1a in which the equilibrium between the \([E\text{T}]\) and \([E\text{T}]_\text{b}\) states is instantaneously reached.

**Multistate Kinetics of Reactivation at High Concentrations of \([A\text{T}]\) (Scheme 2)**

In Scheme 2, the enzyme may bear zero, one, or two \([A\text{T}]\) molecules. The left three \([A\text{T}]-\text{free}\) states will not be considered here. When \([A\text{T}]\) is added, the states initially present are forms \(I_1\) (active, no \([A\text{T}]\) bound), \(I_2\) (inhibited, one \([A\text{T}]\) molecule bound), and \(O\) (overstimulated, two \([A\text{T}]\) molecules bound). States \(I_1\) and \(O\) are in rapid equilibrium.

**Situation 1**—The incubation of the inhibited enzyme with \([A\text{T}]\) for a long time directly leads to the formation of states \(I_2\) and \(D\) (\([E\text{T}\]) and \([E\text{T}]_\text{b}\) in Scheme 1). The addition of reactivating \([A\text{T}]\) leads to a situation quite identical to that described in Scheme 1, with the same prediction, an activity initially low that increases to reach its equilibrium value, with a rate constant, \(k_{\text{app}}\), defined in Equation 13.

**Situation 2**—When \([A\text{T}]\) is added after reactivating \([A\text{T}]\) or, at the same time, the enzyme is initially under states \(I_1\) and \(O\) (state \(A\) can be neglected), the system then evolves toward the formation of states \(S\), \(I_2\), and \(D\). The quasi-equilibrium between states \(I_1\) and \(O\) is described by the dissociation constant \(K_\alpha\) as follows.

\[ K_\alpha = \frac{[I_1][T]}{[O]} \quad (\text{Eq. 16}) \]

If \([X] = [I_1] + [O]\) and if the irreversible conversion of states \(I_1\) and \(O\), respectively, into states \(I_2\) and \(S\) occurs with the same rate constant \(k_\alpha\), the evolution of \([X]\) is given by the equation,

\[ \frac{d[X]}{dt} = -k_\alpha[X] \quad (\text{Eq. 17}) \]

where

\[ [X] = [X]e^{-k_\alpha t} \quad (\text{Eq. 18}) \]

Likewise, if \([Y] = [I_2] + [S]\), where the following is true,

\[ [I_2] = \frac{[Y]}{1 + \frac{K}{[T]}} \quad (\text{Eq. 19}) \]

the evolution of \([Y]\) is given as follows.

\[ \frac{d[Y]}{dt} = -k_\alpha[I_1] + k_\alpha[D] + k_\alpha[X] \quad (\text{Eq. 20}) \]

Using Equation 19, Equation 20 becomes the following,

\[ \frac{d[Y]}{dt} = -\frac{k_\alpha}{1 + \frac{K}{[T]}}[Y] + k_\alpha[D] + k_\alpha[X] \quad (\text{Eq. 21}) \]

where \(K\), \(k_\alpha^+\), and \(k_\alpha^-\) have the same definitions as in Scheme 1. Since the following is true,

\[ [E\text{T}]_\text{eq} = [X] + [Y] + [D] \quad (\text{Eq. 22}) \]

Equation 21 becomes the following.

\[ \frac{d[Y]}{dt} = -\frac{k_\alpha}{1 + \frac{K}{[T]}}[Y] + k_\alpha[I_2] + (k_\alpha - k_\alpha) [E\text{T}]_\text{eq}e^{-k\alpha t} \quad (\text{Eq. 23}) \]

The solution of this equation is as follows,

\[ [Y] = [Y]e^{-k\alpha t} + [Y]e^{-k\alpha t} + [Y]_1 \quad (\text{Eq. 24}) \]

with

\[ k_\alpha^- + k_\alpha^+ \frac{[Y]}{1 + \frac{K}{[T]}} \quad (\text{Eq. 25}) \]

and

\[ [Y]_1 = \frac{[E\text{T}]_\text{tot}}{1 + \left(1 + \frac{K}{[T]}\right)/[T]} \quad (\text{Eq. 26}) \]

\[ [Y]_2 = \frac{[E\text{T}]_\text{tot}}{k_\alpha^- + k_\alpha^+ (1 + \frac{K}{[T]})/1} \quad (\text{Eq. 27}) \]

\[ [Y]_3 = \frac{[E\text{T}]_\text{tot}}{(k_\alpha^-)(1 + \frac{K}{[T]})} \quad (\text{Eq. 28}) \]

\[ [Y]_4 = \frac{[E\text{T}]_\text{tot}}{[K]} \quad (\text{Eq. 29}) \]

and

\[ [Y]_5 = -[Y]_1 - [Y]_2 \quad (\text{Eq. 30}) \]

If \(V_{\text{Overs}}\) is the activity of state \(O\) and \(V_{\text{Stim}}\) is the activity of state \(S\), the global ATPase activity \(V\) as a function of time is \(V_t = V_{\text{Overs}}[O] + V_{\text{Stim}}[S]\), which gives the equation,

\[ V_t = \frac{V_{\text{Overs}}[X]}{K_\alpha^+} + \frac{V_{\text{Stim}}[Y]}{1 + \frac{K}{[T]}} \quad (\text{Eq. 31}) \]

or

\[ V_t = \frac{V_{\text{Overs}}[E\text{T}]_\text{tot} + V_{\text{Stim}}[Y]_1}{1 + \frac{K}{[T]}} e^{-k\alpha t} + \frac{V_{\text{Stim}}[Y]_2}{1 + \frac{K}{[T]}} + \frac{V_{\text{Stim}}[Y]_3}{1 + \frac{K}{[T]}} + \frac{V_{\text{Stim}}[Y]_4}{1 + \frac{K}{[T]}} \quad (\text{Eq. 32}) \]

The model is described by the sum of two exponential decays characterized by two distinct rate constants: \(k_1\), which is \([A\text{T}]\)-dependent (Equation 24), and \(k_2\), which is not (Equation 25).

In Situation 2, we took into account the kinetic data collected
after 1 min in order to focus on the slow component of the decay (Fig. 6b). The rate constant that characterized this decay proved to be [TTX]-independent (Fig. 6c) and was thus identified as $k_a$.

REFERENCES

Kinetic Analysis of Tentoxin Binding to Chloroplast F₁-ATPase: A MODEL FOR THE OVERACTIVATION PROCESS

Jérôme Santolini, Francis Haraux, Claude Sigalat, Gwénaëlle Moal and François André

doi: 10.1074/jbc.274.2.849

Access the most updated version of this article at http://www.jbc.org/content/274/2/849

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 14 of which can be accessed free at http://www.jbc.org/content/274/2/849.full.html#ref-list-1