Eight synthetic analogues of tentoxin (cyclo-(L-N-MeGlu1-L-Leu2-N-MeA2Phe3-Gly4)) modified in residues 1, 2, and 3 were checked for their ability to inhibit and reactivate the ATPase activity of the activated soluble part of chloroplast ATP synthase. The data were consistent with a model involving two binding sites of different affinities for the toxins. The occupancy of the high affinity site (or tight site) gave rise to an inactive complex, whereas filling both sites (tight + loose) gave rise to a complex of variable activity, dependent on the toxin analogue. Competition experiments between tentoxin and nonreactivating analogues allowed discrimination between the absence of binding and a nonproductive binding to the site of lower affinity (or loose site). The affinity for the loose site was not affected significantly by the modifications of the tentoxin molecule, whereas the affinity for the tight site was found notably changed.

Increasing the size of side chain 1 or 2 and introducing a net electrical charge both resulted in a decrease of affinity for the tight site, but the second change dominant the first one. The activity of different ternary complexes enzyme-tentoxin-analogue depended on the nature of the toxin bound on each site and not only on that bound on the loose site. This demonstrates that the reactivation process results from an interaction, direct or not, between these two binding sites. Possible molecular mechanisms are discussed.

F0F1 proton ATPases (or ATP synthases) are bound to energy-transducing membranes and couple the phosphorylation of ADP into ATP to the dissipation of a protonotive force. They consist of a transmembrane proton channel (F0) and an extrinsic part (F1) bearing six nucleotide binding sites, catalytic and noncatalytic. The F0 moiety is composed of five different subunits named α, β, γ, δ, and ε (stoichiometry α[3]β[3]γ[1]δ[1]ε[1]). Subunits α and β bear the nucleotide binding sites and are disposed as a crown, the γ subunit being located in the center of this structure (1–4). The F0 moiety basically consists of three or four different subunits (Escherichia coli: α[1]β[2]c[9–12]; chloroplast: α[1]β[1]β'[1]c[9–12]), the mitochondrial enzyme having additional subunits (5, 6). It is proposed that the F0 moiety would work as a rotative proton-driven motor, the rotor consisting of the c subunits (7), presumably arranged in a crown (8). The rotation would be transmitted to the γ subunit of the F1 moiety (9), which should modify sequentially the three catalytic sites located on β subunits (4) to induce ATP synthesis (10). Experimental arguments have been presented against (11, 12) and for (9, 13–15) the rotation of γ. An essential feature of this model is that the cooperative functioning among the three catalytic sites is strictly related to the rotation of the γ subunit and thus to the proton pumping activity.

Tentoxin (TTX) is a natural cyclic tetrapeptide (cyclo-(L-MeAla1-L-Leu2-MeA2Phe3-Gly4)), produced by several phytopathogenic fungi of the Alternaria genus (16, 17). Under special conditions, this toxin induces a chlorosis in some higher plants (18). It specifically inhibits ATP synthesis in isolated chloroplasts (19). In vitro and at low concentrations (10−6–10−7 M), TTX inhibits the isolated chloroplast F0F1-ATPase (19–22), but at higher concentrations (10−5–10−4 M), it strongly stimulates ATPase activity (21–23). At these same concentrations, the effect observed on membrane-bound ATPase (F0F1 complex) is restricted to a partial release of inhibition, but the reactivated F0F1 complex recovers the ability to couple proton transport to ATP synthesis (24). TTX dramatically disturbs the interactions among different nucleotide sites of ATPase, whatever the toxin concentration range (25, 26). Simultaneous perturbation of these interactions and preservation of proton coupling in the TTX-reactivated form are intriguing in the context of rotational catalysis. Understanding the inhibitory and reactivating properties of TTX is therefore one of the elements that may contribute to the elucidation of the mechanism of energy coupling.

It has been demonstrated (27) that CF1 binds two molecules of TTX on two sites of different affinities, which could be related to the inhibitory and reactivating effects of this molecule. These binding sites have not yet been identified, and the reasons for the specificity of TTX for the CF1-ATP synthase of some higher plants remain obscure (23, 28). TTX stabilizes and enhances the ATPase activity of an αβδ complex from spinach CF1 (29), which proves that the γ, δ, ε subunits are not required for the stimulation effect of TTX but suggests that they could be necessary for the inhibition.

We have shown recently (30) that a very limited change in the molecule of TTX (replacement of l-MeAla1 by l-MeSer1)

---

1 The abbreviations used are: TTX, tentoxin or cyclo-(l-N-MeAla1-l-Leu2-N-MeA2Phe3-Gly4); CF1, chloroplast F0 F1 -ATPase; CF1, l-chloroplast F1, H+ -ATPase devoid of ε subunit; FTIR, Fourier transformation infrared spectroscopy; Lys2/TTX, cyclo-(l-N-MeAla1-l-Lys2-N-MeA2Phe3-Gly4); Lys3/TTX, cyclo-(l-N-MeAla1-l-Lys3-N-MeA2Phe3-Gly4); MeGlu2/TTX, cyclo-(l-N-MeGlu1-l-Leu2-N-MeA2Phe3-Gly4); MeGlu3/TTX, cyclo-(l-N-MeGlu1-l-Leu2-N-MeA2Phe3-Gly4); Lys3/TTX, cyclo-(l-N-MeAla1-l-Lys3-N-MeA2Phe3-Gly4); MeSer1/TTX, cyclo-(l-N-MeSer1-l-Leu2-N-MeA2Phe3-Gly4); MeSer2/TTX, cyclo-(l-N-MeSer2-l-Leu2-N-MeA2Phe3-Gly4); MeSer3/TTX, cyclo-(l-N-MeSer3-l-Leu2-N-MeA2Phe3-Gly4); MeSer2/TTX, cyclo-(l-N-MeSer2-l-Leu2-N-MeA2Phe3-Gly4).

2 This work was supported by the Ministère de l’Enseignement Supérieur et de la Recherche Contract ACC-SVS (interface Chimie-Physique-Biologie) 9505221. 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.: 33-01-6908-4432; Fax: 33-01-6908-8717; E-mail: andre@davidf_cea.fr

This paper is available on line at http://www.jbc.org/ Downloaded from http://www.jbc.org/ by guest on July 23, 2018
resulted in a dramatic loss of the reactivating effect at high concentrations, although the inhibitory effect at lower concentrations was unaffected. This led to the idea that it was possible to discriminate inhibitory and activating effects by an appropriate set of molecules derived from TTX. Because high concentrations of MeSer\textsuperscript{1}-TTX were able to prevent CF\textsubscript{1}-ATPase reactivation by high concentrations of TTX, we proposed that MeSer\textsuperscript{1}-TTX could bind the reactivating site competitively with TTX, giving rise to a poorly active form of the enzyme. However, it cannot be excluded that MeSer\textsuperscript{1}-TTX prevents reactivation simply by chasing TTX from the high affinity site. The question was whether the stimulation by TTX only involves the low affinity binding site or the two binding sites of CF\textsubscript{1}. To get information about possible cooperation among TTX binding sites, we have used a kinetic approach consisting of studying the catalytic properties of ternary complexes formed by CF\textsubscript{1}-e and different TTX analogues. This approach involves various combinations of analogues of different affinities for the inhibitory site and able or not to reactivate the enzyme at high concentrations. The results suggested that both binding sites participate in the formation of the reactivated state. At the same time, we were able to characterize the binding and effector properties of the set of TTX analogues modified in various positions. This allowed us to make hypotheses about the domains of the TTX molecule which are important for binding, inhibition, and stimulation.

MATERIALS AND METHODS

Preparation and Assay of Solubilized, Activated CF\textsubscript{1}-e—The soluble chloroplast ATPase (CF\textsubscript{1}) was extracted and purified from spinach (Spinacia oleracea L.) leaves in the active form, devoid of the inhibitory ε (31). Storage conditions and determination of the concentration of CF\textsubscript{1}-e were described previously (31). The assays were modified slightly with respect to previous conditions (30). The enzyme (80 μg ml\textsuperscript{-1}) was activated by incubation for 3 h, at room temperature, in a medium containing 20 mM Tricine and 3 mM dithiothreitol, pH 8. Its activity, measured as described below, was constant for the entire experiment (5–8 h), ranging between 4 and 6 μmol of hydrolyzed ATP/mg of protein/min. For assays of ATP hydrolysis, the activated enzyme was diluted 40-fold in the reaction medium containing 50 mM Tris-SO\textsubscript{4}, 0.18 mM MgSO\textsubscript{4}, 40 mM KHCO\textsubscript{3}, pH 8.0. This medium was supplemented with toxins at the indicated concentrations. After 5 min of incubation at 37 °C, the reaction was triggered by adding 1 mM ATP (final concentration). Aliquots were taken up at different time intervals and analyzed for nucleotide contents by high performance liquid chromatography, as described (30). The ADP concentration increased linearly with time, which allowed measurement of the rate of ATP hydrolysis. All of the rates displayed in the figures were normalized to that of the control (not toxin-treated). A rates versus concentration plot was fitted by a nonlinear iterative algorithm using Microcal Origin 3.54 (Microcal Software). With respect to our previous experimental conditions (30), the main change consisted in replacing the 1-h preincubation stage with toxin at 80 μg ml\textsuperscript{-1}, at room temperature, by a direct 5-min incubation in the reaction medium, at 37 °C. This time was sufficient to get the maximal effect of toxins. Suppression of the preincubation stage with TTX or derivatives had the advantage of controlling the toxin/enzyme concentration ratio strictly.

Synthesis of TTX and Derivatives—All toxins, including TTX, were synthesized by Drs. Florine Cavaliere and Jean Verducci, Laboratoire des Amino acides, Peptides et Protéines, Université Montpellier II, Montpellier, France. The synthesis and structural properties of TTX and MeSer\textsuperscript{1}-TTX have been reported already (30, 32). Synthesis and structural features of the other derivatives will be published elsewhere.

RESULTS

The Different Analogues of TTX—Fig. 1 displays the different analogues of TTX which were assayed for the inhibition and reactivation of CF\textsubscript{1}-e ATPase activity. In four of them, the residue methylalanine\textsuperscript{1} (MeAla\textsubscript{1}) was replaced, respectively, by methylyserine (MeSer\textsuperscript{1}), by the benzyl ester of methylyserine (MeSer(Bu)\textsubscript{1}), by methyl glutamate (MeGlu\textsubscript{1}), and by the terbutyl ester of methyl glutamate (MeGlu(tBu)\textsubscript{1}). Two of them had their leucine replaced by lysine (Lys\textsuperscript{2}) or Z-protected lysine (Lys(Z)\textsuperscript{2}), respectively. Two others were modified on the α,β-dehydrophenylalanine residue, which was replaced either by an α,β-dehydrotyrosine (Tyr\textsuperscript{3}) or by the methyl ester derivative (Tyr(Me)\textsuperscript{3}).

Inhibitory and Reactivating Effects of TTX and l-MeSer\textsuperscript{1}-TTX—We have shown previously that MeSer\textsuperscript{1}-TTX inhibits CF\textsubscript{1}-ATPase with the same efficiency as TTX at low concentrations, but it reactivates CF\textsubscript{1}-ATPase poorly at high concentrations. This analogue also prevents the reactivation of ATPase by TTX (30). We have reinvestigated the effects of these two toxins to estimate quantitatively the binding and catalytic parameters. Fig. 2 shows the effect of TTX and MeSer\textsuperscript{1}-TTX on the ATPase activity of CF\textsubscript{1}-e. Although much less pronounced than with TTX, the reactivation by MeSer\textsuperscript{1}-TTX at high concentrations was effective. It was possible to fit the data with a simple model involving two independent binding sites for the toxin, the high affinity site being responsible for the inhibitory effect and the low affinity site being responsible for the reactivation (see Equation 12 under “Appendix”). TTX and MeSer\textsuperscript{1}-TTX were found to have exactly the same affinity for the first site (K\textsubscript{d1} = 0.038 μM) and also for the second site (K\textsubscript{d2} = 39 μM for TTX, K\textsubscript{d2} = 41 μM for MeSer\textsuperscript{1}-TTX). The only difference between the two toxins was the ATPase activity of the complex having its two sites occupied: 220% of the control in the case of TTX but only 27% in the case of MeSer\textsuperscript{1}-TTX.

Effect of l-MeSer\textsuperscript{1}-TTX in the Presence of TTX at Reactivating Concentrations—To know whether the activity of these ternary complexes is governed by the nature of the toxin bound to the low affinity site, on the high affinity site, or both sites, we have carried out the following experiment. MeSer\textsuperscript{1}-TTX at various concentrations was first mixed with TTX at a constant concentration (30 μM) in the reaction medium, then CF\textsubscript{1}-e was added. After incubation, MgATP was added and the ATPase activity measured. Fig. 3 (closed squares) shows the continuous decrease of the rate of ATP hydrolysis caused by the addition of increasing concentrations of MeSer\textsuperscript{1}-TTX. The reaction rate actually depends on the proportions and on the catalytic activities of the following four ternary complexes (see “Appendix”): that bearing two molecules of TTX (ET\textsubscript{1}T\textsubscript{2}), that bearing two molecules of MeSer\textsuperscript{1}-TTX (EX\textsubscript{1}X\textsubscript{2}), that bearing TTX on the
tight site and MeSer\(^{-1}\)-TTX on the loose site (ET\(_1\)X\(_2\)), and that bearing MeSer\(^{-1}\)-TTX on the tight site and TTX on the loose site (EX\(_1\)T\(_2\)). The activities of ET\(_1\)T\(_2\) and EX\(_1\)X\(_2\) (respectively, 220 and 27% of the control) were known from the data of Fig. 2 fitted with Equation 12 under “Appendix” as well as the dissociation constants \(K_{d1}\) and \(K_{d2}\). Dashed curve, theoretical values of the rate \(V\) calculated from the same equation but assuming that \(V_{TX} + V_{XT} = 2.6\). For details, see “Results.”

For reactivating toxins (TTX, MeSer\(^{-1}\)-TTX, Lys\(^{Z}\)-TTX, Tyr\(^{3}\)-TTX, and Tyr(Me)\(^{3}\)-TTX), the data were fitted satisfactorily using the same two-site model, with the assumption that the complex bearing a single molecule of toxin was fully inactive (\(V_1\) set to zero, see Equation 12 under “Appendix”). When no reactivation occurred (MeSer(Bn)\(^{-1}\)-TTX, MeGlu\(^{-1}\)-TTX, and (MeGlu(tBu)\(^{1}\)-TTX), a simpler model, involving only one binding site (Equation 5 under “Appendix”), could fit easily the data. In this latter model, to account for possible incomplete inhibition, the enzyme-toxin complex was allowed to have an activity different from zero, which was derived from the fit. In the case of Lys\(^{2}\)-TTX, the two models (two sites and single site) have been used to fit the data because this toxin did not reactivate the ATPase significantly at high concentration but was nevertheless shown to bind to the reactivating site (see below). In all cases, the dissociation constant for the inhibitory site (\(K_{d1}\)) could be determined. The values are summarized in Table I, first column.

Effect of l-Lys\(^{2}\)-TTX, a Nonreactivating Compound, in the Presence of TTX at Reactivating Concentrations—The question is now to know whether the absence of reactivation by MeSer(Bn)\(^{-1}\)-TTX, MeGlu\(^{-1}\)-TTX, MeGlu(tBu)\(^{1}\)-TTX, and Lys\(^{2}\)-TTX results from a default of binding or a nonproductive binding on the high affinity site. The experiments carried out to address this question were based on the same principle as that used in Fig. 3 for the poorly reactivating analogue MeSer\(^{-1}\)-TTX. The effect of high concentrations of nonreactivating analogues on the ATPase activity was checked in the presence of reactivating concentrations of TTX.

Fig. 5 shows the effect of Lys\(^{2}\)-TTX on the ATPase activity in the presence of three different concentrations of TTX in the reactivating range. The data show that the addition of Lys\(^{2}\)-TTX decreases the activity, by limiting the reactivation by TTX, and that the concentration of Lys\(^{2}\)-TTX needed to prevent the enzyme reactivation increases with the concentration of TTX (compare the three curves of inhibition). This effect is consistent with a competition on the loose site. To determine the binding parameters of Lys\(^{2}\)-TTX and the activity of the ternary complexes, we have fitted the data of Fig. 5 with the two-sites model described above (see also Equation 26 under “Appendix”). In this model, the enzyme can exist under the following states: \(E\) (without toxin), ET\(_1\) (with TTX bound at the high affinity site), ET\(_1\)T\(_2\) (with TTX bound at both sites), ET\(_1\)X\(_2\) (with TTX bound at the high affinity site and Lys\(^{2}\)-TTX bound at the low affinity site), and EX\(_1\)X\(_2\) (with Lys\(^{2}\)-TTX bound at both sites). These states are the only ones present at significant levels, when one considers the values of \(K_{d1}\) for TTX and for Lys\(^{2}\)-TTX. Fitting of the competition data allowed a refined
determination of the binding constant of Lys²-TTX for the high affinity site and the estimation of its binding constant for the low affinity site. The corresponding values of $K_{d1}$ and $K_{d2}$ have been found close to 2 $\mu$M and 55 $\mu$M, respectively. We also determined the ATPase activities of the ternary complexes $E_X X_2$ and $E_T X_2$ (called, respectively, $V_{XX}$ and $V_{TX}$). There were found almost negligible (about 5% of the control).

Effect of Lys(Z)²-TTX in the Presence of TTX at Inhibitory Concentrations—Contrary to the enzyme bearing two molecules of Lys(Z)²-TTX, the enzyme bearing two molecules of Lys(Z)²-TTX (EX,X₂ complex) exhibited a significant ATPase activity, about 30% (Table I, last column). The affinity of Lys(Z)²-TTX for the high affinity site is low compared with TTX ($K_{d1}$ = 1 $\mu$M instead of 0.04 $\mu$M), but its affinity for the low affinity site is somewhat better than that of TTX ($K_{d2}$ = 11 $\mu$M versus 39 $\mu$M). This means that, starting from the complex where the high affinity site is occupied by TTX, one can fill specifically the low affinity site with Lys(Z)²-TTX, forming the ET,X₂ complex. This was achieved by adding various concentrations of Lys(Z)²-TTX to an enzyme sample already containing TTX at a micromolar concentration. Fig. 6 shows the ATPase activity measured in such conditions. Two different concentrations of TTX were used. The addition of Lys(Z)²-TTX to CF₁-e inhibited by TTX did not restore any significant activity. The theoretical curve displayed on Fig. 6, drawn using the same activity for ET,X₂ and EX,X₂, i.e. 30% of the control, does not fit the experimental data. The activity of the ET,X₂ complex can indeed be estimated to be less than 10% of the control.

### Table I

| Toxin       | $K_{d1}$ | $V_{relative}$ | $K_{d2}$ | $V_{relative}$ |
|-------------|----------|----------------|----------|----------------|
| TTX         | 0.038    | 0              | 0.038    | 2.22           |
| MeSer¹-TTX  | 0.038    | 0              | 0.045    | 0.27           |
| MeSer(Bn)¹-TTX | 0.50 | 0.02           | 0.05     | 0.05           |
| MeGlu¹-TTX  | 7.2      | 0.02           | 0.05     | 0.05           |
| MeGlu(Bu)¹-TTX | 1.7  | 0.02           | 0.05     | 0.05           |
| Lys²-TTX    | 2.5      | 0.07           | 0.05     | 0.05           |
| Lys(Z)²-TTX | 1.0      | 0              | 1.0      | 1.0            |
| Tyr³-TTX    | 0.040    | 0              | 0.040    | 0.75           |
| Tyr(Me)³-TTX | 0.053   | 0              | 0.053    | 2.89           |

**Fig. 5. Rate of ATP hydrolysis as a function of Lys²-TTX concentration in the presence of three different concentrations of TTX.** Conditions are as described under “Materials and Methods.” [TTX] = 9 $\mu$M; [TTX] = 18 $\mu$M; [TTX] = 32 $\mu$M. Fitted curves were obtained with Equation 26 of the “Appendix” (competition between two toxins). The dissociation constants $K_{d1}$ and $K_{d2}$, and the activity of the CF₁,e-TTX-TTX complex were known from data of Figs. 2 and 4b and fixed at their values: $K_{d1}$ = 0.038 $\mu$M; $K_{d2}$ = 38 $\mu$M; $V_{XT} = 2.22$. The parameters of Lys(Z)²-TTX were obtained from the fit. The three fits gave: $K_{d1}$ = 2 $\mu$M, $K_{d2}$ = 55 $\mu$M, $V_{X1} = 0.05$, $V_{XX} = 0.05$. 

**Fig. 4. Effect of TTX and analogues on the ATPase activity of CF₁-e.** Conditions are as described under “Materials and Methods.” Panel a, analogues modified on position 1 (MeAla); ▲, MeSer(Bn)¹-TTX; ■, MeGlu¹-TTX; □, MeGlu(Bu)¹-TTX. Panel b, analogues modified on position 2 (Leu); ●, Lys²-TTX; ◯, Lys(Z)²-TTX. Panel c, analogues modified on position 3 (ΔPhe); ◇, Tyr³-TTX; □, Tyr(Me)³-TTX. Solid curves, fitting with Equation 12 of the “Appendix” (the same two-sites model as in Fig. 2). Dashed curves, fitting with Equation 5 of the “Appendix” (single-site model). The parameters obtained from the fits are displayed in Table I. The fitted curve obtained with TTX in Fig. 2 was redrawn (dots) in panels a, b, and c for comparison.

**Fig. 3.** Effect of TTX and analogues on the ATPase activity of chloroplast F₁-ATPase. Conditions are as described under “Materials and Methods.” a, TTX. b, analogues modified on position 1 (MeAla); ▲, MeSer(Bn)¹-TTX; ■, MeGlu¹-TTX; □, MeGlu(Bu)¹-TTX. Panel b, analogues modified on position 2 (Leu); ●, Lys²-TTX; ◯, Lys(Z)²-TTX. Panel c, analogues modified on position 3 (ΔPhe); ◇, Tyr³-TTX; □, Tyr(Me)³-TTX. Solid curves, fitting with Equation 12 of the “Appendix” (the same two-sites model as in Fig. 2). Dashed curves, fitting with Equation 5 of the “Appendix” (single-site model). The parameters obtained from the fits are displayed in Table I. The fitted curve obtained with TTX in Fig. 2 was redrawn (dots) in panels a, b, and c for comparison.
The value of stimulation processes. More, the binding parameters have been binding sites and their direct connection to the inhibition and fully confirms the existence of these two independent TTX

ments similar to those carried out with Lys2-TTX and depicted

5–10 M. The present study, using TTX synthetic analogues,

was drawn from data of Figs. 2 and 4.

ments (70 M) does not depend on the toxin (TTX or Lys(Z)2-TTX)

 Effects of Modifications of the TTX Molecule on Its Inhibitory Properties—All the analogues of TTX studied in the present work have retained their inhibitory properties. In all cases, the ATPase activity of CF1-ε was completely lost when a single molecule of toxin was bound. The only effect of the substitutions was to decrease to various extents the affinity of the molecule for the tight site, moderately for certain modifications (MeAla1 → MoSer1, Phe3 → Tyr3, Phe3 → Tyr(Me)3) and more drastically (up to 200 times) for others (in the increasing Kd1...
order: MeAla\(^1\) → MeSer(Bn)\(^1\), Leu\(^2\) → Lys(Z)\(^2\), MeAla\(^1\) → MeGlu(tBu)\(^1\), Leu\(^2\) → Lys\(^2\), MeAla\(^1\) → MeGlu\(^1\). Different molecular factors can account for this \(K_{f1}\) increase, such as the introduction of longer chains, giving rise to additional steric hindrance (MeAla\(^1\) → MeSer(Bn)\(^1\), MeAla\(^1\) → MeGlu(tBu)\(^1\), Leu\(^2\) → Lys(Z)\(^2\), or the introduction of a net electrical charge on the molecule (MeAla\(^1\) → MeGlu\(^1\), Leu\(^2\) → Lys\(^2\)). The introduction of charges seems to be more determining, since the replacement of a charged residue by a neutral residue, even larger (MeGlu\(^1\) → MeGlu(tBu)\(^1\), Lys\(^2\) → Lys(Z)\(^2\)), led to some recovery of the affinity.

A first conclusion of our work is that the nature of residues 1 and 2 (\(N\)-MeAla and Leu in the natural molecule) is not so critical for the inhibitory power of the molecule once it is bound to its site. This can be related to previous structural results obtained by NMR (30, 32). Indeed, TTX and MeSer\(^1\)TTX exhibited the same conformation of the cyclic backbone (cis-trans-cis-trans configuration of the amide bond sequence) and the same interconversion among four conformers. These structural properties have also been observed for all of the analogues of the residue 1. The conservation of the conformational features of the molecules can account for the ability of the molecule to inhibit the enzyme (\(V_0 = 0\)) totally, and the variations of side chain can account for the changes in the affinity for the tight site. However, changes in residue 3 did not result in an increase in \(K_{f1}\). In fact, these modifications were confined to a substitution of the para proton of the benzyl group, without any consequence on the rigid configuration of the double bond of the \(\alpha,\beta\)-dehydroamino acid. Despite the introduction of polar groups on this side chain, there was no repercussion on the affinity of the molecule for the tight site. This result is not so unexpected if the binding of the molecule inside the hydrophobic site involves a stabilization by an aromatic ring stacking.

Effects of Modifications of the TTX Molecule on Its Activating Properties—Whereas the only change in the inhibitory properties of toxin derivatives is a variation of their affinity for the tight site, the situation is quite different with regard to reactivating properties. Noteworthy is that with the exception of MeGlu\(^1\)TTX and MeGlu(tBu)\(^1\)TTX, all the analogues were found to bind to the low affinity site with a \(K_{f2}\) comparable to that of natural TTX, whether they were reactivating or not. The differences lie in the effect of the molecule once bound to this site. It is possible that the loose site has a more open configuration than the tight one, which makes it less sensitive to steric hindrance variations and also to electrical charges (that can be shielded by water molecules). The exceptions of MeGlu\(^1\)TTX and MeGlu(tBu)\(^1\)TTX, however, remain to be explained.

In the absence of a structural model of the chloroplast ATPase species, which differs from the mitochondrial species in its sensitivity to TTX and in various structural features (notably regarding the \(\gamma\) subunit), it is still difficult to explain the various levels of reactivation obtained with the different analogues bound on the two sites. For the complexes bearing two molecules of the same toxin (homogeneous complexes), a change of hydrophobicity of the molecule (30) can result in a significant change in the reactivation level. Thus, the replacement of a proton by a hydroxyl group (Ala → Ser; Phe → Tyr) dramatically decreases the activity of the ternary complex when made in position 1 and significantly when made in position 3. In the latter case, this activity is restored when the labile proton is replaced again by a more hydrophobic group (Tyr → Tyr(Me)). The comparison of the reactivating properties of MeSer\(^1\)TTX and Tyr\(^1\)TTX, two molecules slightly modified with unchanged affinities for both sites, suggests that the nature of residue 1 is more important than that of residue 3 in conferring to TTX its reactivating properties. But also, as discussed below, the level of reactivation was found to be dependent on the combination of the two toxins bound (hybrid ternary complexes).

Importance of the Two TTX Binding Sites in the Reactivation—It is tempting to speculate whether the two TTX binding sites are homologous domains of two different \(\alpha\beta\) pairs, put into different states by the asymmetry of the ATPase complex. Such a situation has already been stated in the case of the binding of two molecules of aurovertin to the bovine heart MF\(_1\) complex (33), with the noteworthy difference that aurovertin is never reactivating. If the two binding sites of TTX were located on different \(\alpha\beta\) pairs, the TTX molecule bound to the first \(\alpha\beta\) pair could block the enzyme, and the TTX molecule bound to the second pair could unlock it.

An original result of this work deals with hybrid ternary complexes, bearing natural TTX on the tight site and a synthetic derivative on the loose site. By applying a simple model, with two independent binding sites, we could estimate the activities of some of these hybrid complexes and compare them with the activities of ternary complexes bearing the same molecule on both sites (Table II). It is not possible, at the present time, to understand the molecular rules that would determine the activity of all of these ternary complexes, but there are two main points. First, the activity depends on the toxin bound on the loose site; compare, for example, the CF\(_1\)-TTX-lys\(^2\)-TTX and CF\(_1\)-TTX-Lys\(^2\)-TTX complexes (negligible activities). Second, the activity also seems to depend on the toxin bound on the tight site; compare the CF\(_1\)-TTX-Lys(Z)\(^2\)-TTX complex (negligible activity) with the CF\(_1\)-Lys(Z)\(^2\)-TTX-Lys(Z)\(^2\)-TTX complex (30% activity) and see also the competition between TTX and MeSer\(^1\)TTX. So, although the CF\(_1\)-ATPase enzymes complexed with a single molecule of TTX or with a single molecule of an analogue cannot be discriminated on the basis of their activity, they are potentially different. This difference is revealed only when a second molecule of the analogue is bound on the loose site. All of our data are consistent with an interaction between the two TTX binding sites.

---

\(^2\) F. André, unpublished results.
**Tentoxin Binding Sites in Chloroplast F$_1$-ATPase**

Cooperative Binding, an Alternative Hypothesis—To fit our data, we have considered only a simple model with two preexisting and absolute $K_D$ values, which assumes that binding of a first toxin molecule to the complex has no influence on the affinity of the complex for a second molecule. The activities of ternary complexes are then the only way to detect interactions between the two sites. However, it would be also possible to fit them with a model of cooperative binding, and in this case the $K_{D2}$ of a given toxin would depend on the toxin bound on the tight site. For example, data of Fig. 6 could be fitted satisfactorily with a model assuming that all of the ternary complexes have a negligible activity, provided Lys(Z)$_2$-TTX binds to the loose site with a $K_{D2}$ equal to 100 μM instead of 11 μM, the value drawn from Fig. 4. This means that replacing Lys(Z)$_2$-TTX by TTX at the tight site would dramatically decrease the affinity of Lys(Z)$_2$-TTX for the loose site. Even though the mechanism differs from that assumed in our first model, this would demonstrate even more directly the interaction between the two binding sites of the toxin. Understanding this interaction should be an important element in the knowledge of the catalytic mechanism of CF$_0$CF$_1$-ATP synthase.

Acknowledgments—We thank Véronique Mary for extraction of the spinach chloroplast F$_1$-ATPase. We are indebted to Drs. Florine Cavelier and Jean Verducci for the chemical synthesis of tentoxin and all of its analogues. Dr. Catherine Berthomieu performed the FTIR analysis of tentoxin solutions.

**APPENDIX**

The Single-site Model—The binding equilibrium is governed by $K_{DT1}$, the dissociation constant of the enzyme-toxin complex,

$$K_{DT1} = \frac{[ET]}{[E]T}$$

(Eq. 1)

where $[E]$ is the concentration of the free form of the enzyme, $[ET]$ the concentration of the complex, and $[T]$ the concentration of free toxin. If $[E]_c$ refers to the total concentration of the enzyme, the relative concentrations of the two forms of the enzyme are

$$\frac{[E]}{[E]_c} = 1$$

(Eq. 2)

$$\frac{[ET]}{[E]_c} = \frac{1}{1 + \frac{[T]}{K_{DT1}}}$$

(Eq. 3)

If $v_o$ and $v_i$ are the rates of the reaction catalyzed by the forms $E$ and $ET$, respectively, the total reaction rate is

$$V = \frac{v_o}{1 + \frac{[T]}{K_{DT1}}} + \frac{v_i}{1}$$

(Eq. 4)

The rate $V$ normalized to the control reaction rate $v_o$ is

$$\frac{V}{v_o} = \frac{1}{1 + \frac{[T]}{K_{DT1}}} + \frac{V_i}{v_o}$$

(Eq. 5)

with $V_i = v_i/v_o$.

In the experimental plots, the free concentration of toxin, $[T]$, will be identified to the total concentration of toxin $[T] + [ET]$, since the toxin is always in large excess under our conditions. This approximation also applies to the two-sites model.

The Two-sites Model—Let us consider the binding equilibria where two molecules of toxin are successively bound to sites called 1 and 2 (Scheme 1, where $ET_1$ refers to the complex with one molecule of toxin bound to the high affinity site ($K_{D1}$), $ET_2$ to the complex with one molecule of toxin bound to the low affinity site ($K_{D2}$), and $ET_1T_2$ to the complex with the toxin bound to the two sites. One has

$$K_{D1} = \frac{[ET_1T]}{[ET_1][T_2]} = \frac{[ET_1][T]}{[ET_1][T_2]}$$

(Eq. 6)

$$K_{D2} = \frac{[ET_2]}{[E][T]} = \frac{[ET_2]}{[E][T]}$$

(Eq. 7)

and

$$[ET] = [E] + [ET_1] + [ET_2] + [ET_1T_2].$$

(Eq. 8)

Because experimentally $K_{D1} \ll K_{D2}$, one can consider that $[ET_2]$ is negligible. The relative concentrations of the different states are therefore

$$\frac{[ET]}{[ET]} = \frac{1}{1 + \frac{[T]}{K_{D1}}} + \frac{[ET]}{[ET]}$$

(Eq. 9)

$$\frac{[ET_1T_2]}{[ET]} = \frac{1}{1 + \frac{[T]}{K_{D2}}}$$

(Eq. 10)

and

$$\frac{[ET_1T_2]}{[ET]} = \frac{1}{1 + \frac{K_{D2}}{K_{D1}}}$$

(Eq. 11)

To simplify, one assumes that binding of a single toxin molecule fully inhibits the enzyme, i.e. the activity of the state $ET_1$ is 0. The reaction rate $V$ (with toxin) normalized to that of the control (without toxin) becomes

$$\frac{V}{V_o} = \frac{1}{1 + \frac{[T]}{K_{D1}}} + \frac{V_A}{1 + \frac{[T]}{K_{D2}}}$$

(Eq. 12)

where $V_A$ is the ratio between the catalytic activities of the states $ET_1T_2$ and $E$.

**Competition between Two Different Toxins at the Two Binding Sites—**Let us consider the same equilibria where two different toxins (T and X) are bound. One has

$$K_{D1} = \frac{[ET_{1X}][T][X]}{[ET_1][X]} = \frac{[ET_{1X}][T]}{[ET_1][X]}$$

(Eq. 13)

$$K_{D2} = \frac{[ET_{2X}]}{[E][X]} = \frac{[ET_{2X}]}{[E][X]}$$

(Eq. 14)

$$K_{D1} \ll K_{D2}$$

(Eq. 15)

$$K_{D2} \ll K_{D2}$$

(Eq. 16)

By convention, T refers to TTX and X to an analogue. Indexes 1 and 2 still refer to the high and low affinity sites, respectively. $K_{D1}$ and $K_{D2}$ are the dissociation constants corresponding to toxin X. The concentrations of all of the possible complexes are given by

$$[ET] = \frac{[E]}{1 + \frac{[T]}{K_{D1}} + \frac{[X]}{K_{D2}}}$$

(Eq. 17)

$$[ET_1T_2] = [E] \frac{[T]}{K_{D1}}$$

(Eq. 18)
This equation was used to fit the data of Fig. 3. \( K_{d1}, K_{d2}, V_{TT} \) and \( V_{XX} \) were determined previously from the data of Fig. 2. \( V_{TX} \) and \( V_{XX} \) cannot be discriminated in Equation 27; only their sum (or their average) can be derived from the fit. If the activity of the ternary complex depended only on the nature of the toxin bound on the second site, thus \( V_{XX} + V_{TX} = V_{TT} \) and \( V_{TT} \) becomes then \( V_{TX} + V_{XX} = V_{TT} \). The sum equality should be found if the activity of the ternary complex depended only on the nature of the toxin bound on the first site, i.e. \( V_{XX} \) and \( V_{TX} = V_{TT} \). This equality can be checked easily with the results derived from the fit.

REFERENCES

1. Boekema, E. J., Berden, J. A., and van Heel, M. G. (1986) Biochim. Biophys. Acta 851, 353–360

2. Yoshimura, H., Matsumoto, M., Endo, S., and Nagayama, K. (1990) Ultramicroscopy 32, 265–274

3. Boekema, E. J., Xiao, J., and McCarty, R. E. (1990) Biochim. Biophys. Acta 1020, 49–56

4. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621–628

5. Collinson, I. R., Runswick, M. J., Buchanan, S. K., Fearney, I. M., Skehel, J. M., van Raaij, M. J., Griffiths, D. E., and Walker, J. E. (1994) Biochemistry 33, 7971–7978

6. Arselin, G., Vaillier, J., Graves, P. V., and Velours, J. (1996) J. Biol. Chem. 271, 20284–20290

7. Vick, S. B., and Antonio, J. B. (1994) J. Biol. Chem. 269, 30384–30389

8. Singh, S., Turina, P., Bastamante, C. J., Keller, D. J., and Capaldi, R. (1996) FEBS Lett. 397, 30–34

9. Durban, T. M., Bubynin, V. V., Zhou, Y., Hutcheon, M. L., and Cross, R. L. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 10964–10968

10. Boyer, P. D. (1993) Biochim. Biophys. Acta 1140, 215–250

11. Wang, J. H., Cesana, J., and Wu, J. C. (1987) Biochemistry 26, 5527–5533

12. Musier, K. M., and Hammes, G. G. (1987) Biochemistry 26, 5982–5988

13. Aggeler, R., Haughton, M. A., and Capaldi, R. A. (1995) J. Biol. Chem. 270, 9185–9191

14. Schäffer, D., Engelbrecht, S., and Junge, W. (1996) Nature 381, 623–628

15. Noji, H., Yasuda, R., Yoshida, K., and Kinosa, K. (1997) Nature 386, 299–302

16. Meyer, W. L., Templeton, E. G., Grable, C. T., Sigel, C. W., Jones, R., Woodhead, S. H., and Sauer, C. (1971) Tetrahedron Lett. 25, 2357–2360

17. Liebermann, B., and Oertel, B. (1983) Z. Allg. Mikrobiol. 23, 503–511

18. Durbin, R. D., and Uchytíl, T. F. (1987) Phytopathology 67, 602–603

19. Steele, J. A., Uchytíl, T. F., Durbin, R. D., Bhatnagar, P. K., and Rich, D. H. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2245–2248

20. Steele, J. A., Durbin, R. D., Uchytíl, T. F., and Rich, D. H. (1978) Biochim. Biophys. Acta 501, 72–82

21. Steele, J. A., Uchytíl, T. F., and Durbin, R. D. (1978) Biochim. Biophys. Acta 504, 136–141

22. Steele, J. A., Uchytíl, T. F., Durbin, R. D., Bhatnagar, P. K., and Rich, D. H. (1978) Biochim. Biophys. Acta 501, 72–82

23. Dahse, I., Pezenec, S., Girault, D., Berger, G., André, F., and Liebermann, B. (1994) J. Plant Physiol. 143, 615–620

24. Sigalat, C., Pitard, B., and Haraux, F. (1995) FEBS Lett. 368, 253–256

25. Fromme, P., Dahse, I., and Graber, P. (1992) Z. Naturforsch. 47c, 239–244

26. Hu, N., Mills, D. A., Huchzermeier, B., and Richter, M. L. (1993) J. Biol. Chem. 268, 8536–8540

27. Pinet, E., Gomis, J. M., Girault, G., Cavelier, F., Verducci, J., Noé, J.-P., and André, F. (1996) FEBS Lett. 395, 217–220

28. Arni, A., Anderson, J. D., Holland, N., Rochaix, J.-D., Gromet-Elhanan, Z., and Edelman, M. (1992) Science 257, 1245–1247

29. Sokolov, M., and Gromet-Elhanan, Z. (1996) Biochemistry 35, 1242–1248

30. Pinet, E., Cavelier, F., Verducci, J., Girault, G., Dubart, L., Haraux, F., Sigalat, C., and André, F. (1996) Biochemistry 35, 12504–12511

31. Berger, G., Girault, G., André, F., and Galmiche, J.-M. (1987) J. Liquid Chromatogr. 10, 1507–1517

32. Pinet, E., Neumann, J.-M., Dahse, I., Girault, G., and André, F. (1995) Biopolymers 36, 155–152

33. van Raaij, M. J., Abrahams, J. P., Leslie, A. G. W., and Walker, J. E. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 6913–6917
Interrelation between High and Low Affinity Tentoxin Binding Sites in Chloroplast F$_1$-ATPase Revealed by Synthetic Analogues

Jérôme Santolini, Francis Haraux, Claude Sigalat, Laurence Munier and François André

J. Biol. Chem. 1998, 273:3343-3350.
doi: 10.1074/jbc.273.6.3343

Access the most updated version of this article at http://www.jbc.org/content/273/6/3343

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 33 references, 8 of which can be accessed free at http://www.jbc.org/content/273/6/3343.full.html#ref-list-1