Synthesis, Structure, and Properties of MeSer[1]-Tentoxin, a New Cyclic Tetrapeptide Which Interacts Specifically with Chloroplast F_1 H^+-ATPase Differentiation of Inhibitory and Stimulating Effects[†]

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ABSTRACT: A new tentoxin analogue, in which the L-methyl alanine residue is substituted by L-methylserine, has been prepared following the synthetic pathway recently described for the synthesis of tentoxin [Cavelier, F., & Verducci, J. (1995) *Tetrahedron Lett. 36*, 4425−4428]. Using two-dimensional homonuclear proton nuclear magnetic resonance and structural analysis, we observed that MeSer[1]-tentoxin, like tentoxin, adopts several conformations in aqueous solution and presents self-aggregative properties. This analogue was found to be conformationally similar to the natural toxin. It showed the same efficiency as tentoxin in inhibition of ATPase activity of the isolated chloroplast F_1 proton ATPase (CF_1) as well as in inhibition of the ATP synthase activity of the membrane-bound enzyme (CF_0CF_1) in thylakoids and proteoliposomes. At concentrations above 10 μM, MeSer[1]-tentoxin did not reactivate CF_1 to a high extent, contrary to tentoxin. It appeared, however, to bind in the same way, since the reactivating effect of tentoxin was inhibited by MeSer[1]-tentoxin. These results show that it is possible, using tentoxin analogues, to separate inhibitory and activating effects on the chloroplast ATPase, despite the limited chemical difference between the two toxins.

Tentoxin is a natural cyclic tetrapeptide [*cyclo*-(L-MeAla[1]-L-Leu[2]-MePhe[(*Z*)Δ][3]-Gly[4])] which is excreted by some phytopathogenic fungi from *Alternaria* species (Meyer et al., 1971; Liebermann & Oertel, 1983). This toxin causes chlorosis in many sensitive plants (Durbin & Uchytil, 1977), affects the development of chloroplasts (Schadler et al., 1976), and inhibits ATP synthesis in isolated chloroplasts (Steele et al., 1976). The chloroplast H^+-ATPase (CF_1)[1] is a species-specific receptor for tentoxin (Steele et al., 1976; Avni et al., 1992; Hu et al., 1993; Dahse et al., 1993), but very little is known about the mode of action of the toxin. For sensitive plants, tentoxin binding inhibits the CF_1-ATPase activity at a low concentration (10^{-8} M) of the toxin (Steele et al., 1976, 1978a,b; Dahse et al., 1994) and starts to stimulate it at a concentration of 10^{-5} M and higher (Dahse et al., 1994; Steele et al., 1978a). Reactivation also exists in the membrane-bound CF_0CF_1, although it is limited to a partial recovery of the activity (Sigalat et al., 1995). Interestingly, a tightly coupled proton transport is preserved in the CF_0CF_1 partially reactivated by a high concentration of tentoxin (Sigalat et al., 1995), although the interaction between the nucleotide binding sites, which is supposed to play a central role in the functioning of this complex, is severely altered (Hu et al., 1993; Fromme et al., 1992). In order to investigate the molecular basis of these phenomena and their mechanistic implications, it is advantageous to dissociate the inhibitory and the activatory effects. A possible approach is the design of tentoxin derivatives which are only slightly different in chemical structure but which exhibit large and well-characterized changes in the biological activity.

A crucial step in this kind of study is the determination of the structure in solution of the effector and its analogues. Since they have a limited number of conformations, small cyclic peptides are especially suitable for such investigations (Blout, 1981; Kessler, 1982; Kessler et al., 1982). In addition, such an approach can give some clues concerning the molecular pattern relevant to a given biological activity, on the basis of experimental structural differences and similarities.

Several analogues of tentoxin have been synthesized and characterized using various biological assays (Rich et al., 1978a,b, 1981; Rich & Bhatnagar, 1978a; Edwards et al., 1986, 1987). Among these analogues, only Pro[1]-tentoxin (Edwards et al., 1986), Sar[1]-tentoxin (Rich et al., 1981), and

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[1] Abbreviations: BR, bacteriorhodopsin; Boc, *N-tert*-butoxycarbonyl; CF_0CF_1, chloroplast F_0F_1 H^+-ATPase; CF_1, chloroplast F_1 H^+-ATPase; CF_1-ϵ, chloroplast F_1 H^+-ATPase with its ϵ-subunit removed; Chl, chlorophyll; COSY, correlated spectroscopy; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMF, dimethylformamide; DPPA, diphenylphosphoryl azide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FAB, fast atom bombardment ionization; HOBT, hydroxybenzoyltriazole; MeAla, L-alanine, N-methylated; MeΔ^ZPhe or ΔPhe, α,β-dehydrophenylalanine, N-methylated in *Z* configuration; MeSer, L-serine, N-methylated; NBA, nitrobenzylalcohol matrix; ROE, rotating frame nuclear Overhauser effect; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; T_1, spin−lattice relaxation time; T_2, spin−spin relaxation time; τ_c, correlation time; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography.

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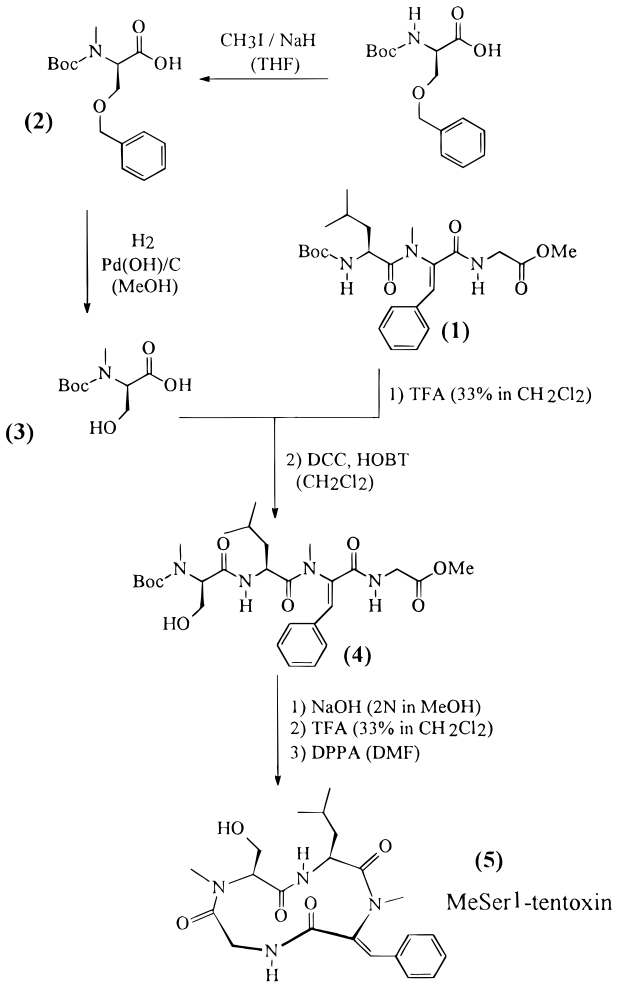
L-Ala[1]-tentoxin (Edwards et al., 1987) exhibited biological effects comparable—but not identical—to those of the natural phytotoxin. Nevertheless, the low solubility of these analogues made impossible at the time the NMR study of their conformational features in water. Another cyclic analogue modified in the position 1, D-MeAla[1]-tentoxin, was proven to present two interconverting conformations in chloroform (Rich & Bhatnagar, 1978b). The two conformers were separated at low temperature, and only one of them retained a significant ability to inhibit CF_1 (Rich et al., 1978b). Recently, we have shown that natural tentoxin also exists in multiple conformations in aqueous solution (Pinet et al., 1995), and the conformers were identified using two-dimensional proton NMR spectroscopy.

So far, a complete set of structural and biological data on a given tentoxin analogue was not available. We report here the synthesis and the characterization of a new synthetic tentoxin analogue, MeSer[1]-tentoxin, where L-MeAla[1] has been replaced by L-MeSer[1]. This compound is particularly relevant for the following reasons: first, like the aforementioned analogues, it presents a minor chemical modification on the residue 1, and is thus expected to retain significant activity; second, it offers an improved solubility in water by the presence of an hydroxylic group; third, the serine side chain is sterically not too different from the alanine side chain. Our investigations include (i) the detailed conformational analysis of MeSer[1]-tentoxin in aqueous solution by NMR spectroscopy and (ii) a functional study of the chloroplast H^+-ATPase, in its solubilized form (CF_1) and its membrane-bound form (CF_0CF_1), treated with MeSer[1]-tentoxin and assayed for ATP synthesis as well as for ATP hydrolysis. Our results show that, as compared to natural tentoxin, MeSer[1]-tentoxin exhibits similar structural features, and identical inhibitory action on isolated or membrane-bound CF_1. However, its ability to reactivate the ATPase is dramatically reduced. This lack of activatory effect is likely to be due to a non-productive binding of MeSer[1]-tentoxin.

EXPERIMENTAL PROCEDURES

Synthesis of MeSer[1]-Tentoxin. MeSer[1]-tentoxin was synthesized using the procedure summarized in Scheme 1, which will be described in detail elsewhere. This synthetic pathway is derived from our recently described procedure for the synthesis of tentoxin, where the protected tripeptide (**1**) was synthesized using the homogeneous phase synthetic pathway employing an azlactone as key intermediate (Cavelier & Verducci, 1995). In the subsequent steps, there were several modifications due to the introduction of MeSer amino acid: the N-methylation of the serine was carried out on commercial Boc-L-Ser(OBn)-OH using the procedure of Cheung and Benoiton (1977). The *O*-benzyl cleavage of Boc-L-MeSer(OBn)-OH (**2**) was then carried out by hydrogenation using 10% $Pd(OH)_2/C$ as catalyst. We then applied the strategy described in Cavelier and Verducci (1995), which has the advantage introducing the MeSer amino acid at the last step of the linear tetrapeptide synthesis (**4**). The formation of esters on the free alcohol function was limited using mild coupling conditions, even with unprotected MeSer. After having deprotected both extremities of the tetrapeptide (**4**) in two steps, the cyclization was carried out using diphenylphosphoryl azide (DPPA) under high dilution conditions. The cyclopeptide (**5**) was obtained after chromatography on silica gel (AcOEt/MeOH 90/10 as eluent), followed by semi-preparative HPLC (C_{18} Nucleosil column, 5 μm, 1 × 25 cm, flow 4 mL min^{-1}, elution by H_2O/CH_3CN 65/35 monitored at 214 nm, retention time 9.8 min). The compound (**5**) was isolated with 5% yield and characterized by TLC ($R_f = 0.60$, AcOEt/MeOH 90/10), analytical HPLC (C_{18} Nucleosil column, 5 μm, 250 × 4.6 mm, CH_3CN/H_2O 35/65, 1 mL min^{-1}, retention time 9.2 min), mass spectrometry FAB(+) (NBA) (453 [M + Na^+] 5%; 431 [M + H^+] 15%), and proton NMR (see Results). The presence of the hydroxylic side chain of MeSer is suspected to decrease the cyclization yield as compared to the 18% yield obtained for tentoxin (Cavelier & Verducci, 1995). This is due to additive effects of steric hindrance and possibly side reactions involving the alcohol function. But despite the low yield in purified compound (5%), the MeSer[1]-tentoxin obtained presented a very satisfactory homogeneity in analytical HPLC (not shown) and conclusive characterization by mass spectroscopy and proton NMR. Its degree of purity was finally higher than 95%.

Scheme 1: Synthetic Pathway of MeSer[1]-Tentoxin[a]



[a] Bold numbers refer to key intermediates (see text).

High-Resolution Proton NMR Spectroscopy for Structural Studies. All NMR samples contained 1.0 mg of HPLC-purified MeSer[1]-tentoxin solubilized in 0.5 mL of 10 mM phosphate buffer containing 200 mM NaCl and 0.1 mM EDTA. The MeSer[1]-tentoxin samples (4.7 mM) were freeze dried and then dissolved in either D_2O 99.95% (CEA-Eurisotop) with pD adjusted to 7.0 or in H_2O/D_2O 90/10 (v/v) with pH adjusted to 4.0 for observation of exchangeable

protons. Samples with concentration ranging from 20 μM up to 9 mM were also prepared in the last buffer in order to investigate concentration dependence of amide protons. Concentrations were controlled by UV absorption assuming $\epsilon_M = 17\,500\ M^{-1}\ cm^{-1}$ at 284 nm.

^{1}H NMR spectra were recorded using a Bruker AMX 500 spectrometer operating at 500.13 MHz. Signal processing was done on an Indy SGI workstation using the TRIAD-SYBYL program (Tripos Associates, St. Louis, MO, version 6.1). The acquisition of one- and two-dimensional spectra followed the procedures described in Pinet et al. (1995), except that for homonuclear two-dimensional experiments (COSY and ROESY) a total of 96 transients and four dummy transients were acquired at −5 °C. Mixing times of 100−200 ms were used for ROESY experiments, with a spin-locking field of 5 kHz applied in the rotating frame. ROESY spectra were apodized using Hanning functions and ROESY cross-peak integrals were corrected for offset effects (Griesinger & Ernst, 1987). For generating distance constraints, a further correction of peak integrals was made according to the species proportions.

Modeling and Structural Calculations. Model-building, energy minimization, and molecular dynamics simulations were performed *in vacuo*, using the SYBYL molecular modeling package (Tripos Associates, St. Louis, MO, version 6.1). Initial structures were generated using the published φ, ψ, ω angles for tentoxin (Pinet et al., 1995) as a starting point of a linear molecule (open peptide). The molecule was then closed and allowed to relax to its minimum of potential energy. All the calculations were then carried out as previously described (Pinet et al., 1995), with the following adjustments. For each molecular species, 320 structures were generated by the initial molecular dynamics simulation runs without the NMR constraints (total simulation time of 320 ps). These were then clustered by a least-squares procedure, where the non-hydrogen atoms are matched to those of the lowest energy structure of each cluster, with a root mean square (rms) smaller than 0.1 Å. A family of about 200 clusters was thus generated. All of the clusters were then subjected to extensive minimization and clustered again in the same manner, leading to about 20 unique structures. At this stage, NMR distance and torsion constraints were added to the resulting structures. These were energy-minimized and clustered once again. Six structures were selected, including the best and the worst of the minimized structures, and were used as starting structures for restrained molecular dynamics simulations. Final runs were performed as described in Pinet et al. (1995), with structures stored every 1 ps, and subjected to the same process as in the first dynamics run.

In addition, the positions of leucine and serine side chains were explored using a systematic conformational search on the best structure, by generating all the rotamers with a 10° step for χ_1 and a 30° step for χ_2, resulting in 432 rotamers of each lateral chain. All rotamers were then minimized under NMR constraints.

Preparation, Storage and Assay of Solubilized CF_1-ϵ. The soluble chloroplast ATPase (CF_1) was extracted and purified from spinach (*Spinacia oleracea* L.) leaves, in the active form with its inhibitory ϵ subunit removed (Berger et al., 1987). The enzyme was stored at 5 °C in ammonium sulfate (50% saturation) at a concentration of 15 mg mL^{-1}. Protein concentration was determined using the Bio-Rad protein assay (Bradford, 1976) with bovine serum albumin as a standard or by UV absorption spectroscopy assuming for CF1-ϵ an absorbance of 0.48 at 280 nm for 1 mg of protein mL^{-1} and an optical pathway of 1 cm (Berger et al., 1987).

In order to assay the ATPase activity, the enzyme (about 2 μg) was preincubated at room temperature for at least 45 min in 30 μL of 20 mM Tricine (pH 8.0), 3 mM DTT, in the presence of variable concentrations of toxin. ATP hydrolysis was started by adding a 5 μL aliquot of the enzymatic solution to 200 μL of a medium containing 60 mM Tris-SO_4, 40 mM $KHCO_3$ (pH 8.0), 3 mM DTT, 1.0 mM ATP, 0.18 mM $MgSO_4$ (Berger et al., 1994), and the same concentration of toxin as in the incubated solution. The reaction mixture was thermostated at 37 °C. From the beginning of ATP hydrolysis, aliquots of 10 μL of the reaction mixture were taken up every 3 min (up to 15 min) and injected into a TSK DEAE 2SW 5 μm analytical HPLC column (25 × 0.46 cm). The nucleotides were separated by isocratic elution with 0.1 M KH_2PO_4, pH 4.3, 0.25 M NaCl, at a rate of 1.0 mL/min. With this procedure, the reaction was immediately stopped after injection, by dilution and drop of the pH to 4.3. ADP concentration was measured by the height of the peak detected at 260 nm (elution time 4.5 min). This peak increased linearly with the time of reaction, which allowed the calculation of ATP hydrolysis rates, through calibration with known concentrations of ADP (Berger et al., 1990, 1994). Blanks without CF_1-ϵ were made before and after each kinetic run and were subtracted from the progression curves to eliminate the background ADP. No ATP hydrolysis was detected in the absence of enzyme. The activity in the presence of toxin was normalized to the control without toxin. We were then able to draw a unique plot of the ATPase activity *vs* toxin concentration from different experiments.

Thylakoids Preparation, Storage, and Assay. Envelope-free chloroplasts from lettuce (*Lactuca sativa* L.) leaves with thiol-reduced (DTT-treated) ATPase were prepared as previously described (Valerio et al., 1992), stored on ice at 1 mM [Chl], and assayed at 10 μM [Chl] in the previously described set-up (Sigalat et al., 1995). The assay medium contained 0.1 M sorbitol, 50 mM KCl, 5 mM $MgCl_2$, 2 mM K_2HPO_4, 2 mM Tricine, 50 μM pyocyanine, and 4 μM 9-aminoacridine (9-AA) to control the magnitude of the proton gradient (Schuldiner et al., 1972). Other additions are indicated below. The sample, adjusted to pH 8.0, was stirred and thermostated at 20 °C. Thylakoids were energized by red light (intensity, 1.5 kW m^{-2}). ATP synthesis or hydrolysis was monitored from scalar H^+ consumption or production (Nishimura et al., 1962), as previously described (Valerio et al., 1992). The following protocol was used for ATP synthesis: (1) incubation for either 2 or 30 min with toxin at the indicated concentration, in the dark; (2) switching on the light; (3) addition of 250 μM ADP 90 s later; (4) depending on the activity, addition of 1 μM venturicidin to stop ATP synthesis 2 or 3 min later; (5) switching off the light 1 min later. The rate of ATP synthesis was constant for at least 30 s. The protocol for measuring the ATP hydrolysis was previously described (Sigalat et al., 1995).

Preparation and Assay of the Co-Reconstituted System. Proteoliposomes containing bacteriorhodopsin (BR) and CF_0-CF_1 from spinach (*Spinacia oleracea* L.) were prepared as previously described (Sigalat et al., 1995; Rigaud & Pitard, 1994) and stored on ice before use at a lipid concentration

of 4 mg mL^{-1}, BR (0.2 mg mL^{-1}), CF_0CF_1 (0.03 mg mL^{-1}), in 50 mM Na_2SO_4, 50 mM K_2SO_4, 25 mM KH_2PO_4, pH 7.3. For the assays, proteoliposomes were diluted 5-fold in the same medium, stirred, and thermostated at 40 °C, and supplemented with 10 μM diadenosine-5′-pentaphosphate and with toxin at indicated concentrations. 15 minutes after the beginning of the illumination (yellow light, intensity 1.2 kW m^{-2}), once the steady state proton gradient was reached, ATP synthesis was initiated by adding 500 μM ADP and 2 mM $MgSO_4$. Aliquots were taken up, quenched by 2% trichloroacetic acid, and titrated for ATP using the luciferin−luciferase method (Lemaster & Hackenbrock, 1978).

RESULTS

NMR Determination of Interconverting Conformers of MeSer[1]-Tentoxin. In aqueous solution, tentoxin was reported to present multiple interconverting conformers in multisite chemical exchange which can be explored from slow (at −5 °C) to fast (at +82 °C) chemical exchange conditions (Pinet et al., 1995). MeSer[1]-tentoxin also presents multisite chemical exchange with at least three interconverting conformers, as revealed by temperature dependence of proton NMR spectra (not shown). The coalescence temperature, *i.e.*, the temperature at which the three NMR lines corresponding to each conformer coalesce into a single resonance, was determined from the line shape of the *N*-methyl protons resonances of MeSer residue. This temperature was about 45 °C, a value close to that found for tentoxin (47 °C).

The signal assignment was achieved using the step-by-step procedure described (Pinet et al., 1995). At −5 °C, where the chemical exchange is slow, we were able to assign all the proton resonances of the three detectable forms, which will be referred to as [A], [B], and [C] in the following sections. There is a direct correspondence between this notation and the one we used for tentoxin. Contrary to those recorded with tentoxin, the NMR spectra of MeSer[1]-tentoxin did not reveal the presence of a fourth conformer. According to the average surface ratio of the resonances, the relative proportions of the [A], [B], and [C] forms of MeSer[1]-tentoxin at −5 °C were 44%, 48%, and 8%, whereas those of the [A], [B], [C], and [D] forms of tentoxin were 51%, 37%, 8%, and 4%. Thus, [B] form becomes the predominant form of MeSer[1]-tentoxin in aqueous solution.

Self-Association Properties of MeSer[1]-Tentoxin in Aqueous Solution As Revealed by ^{1}H NMR Study. We had evidence that tentoxin, which is a hydrophobic molecule, presents in aqueous solution a micelle-like structure involving 10−15 monomers, in the concentration range of NMR samples (Pinet et al., 1995). The analogue MeSer[1]-tentoxin contains an alcohol group, and, as expected, its solubility in water (>10 mM) is higher than that of tentoxin (about 4 mM). Nevertheless, the following observations strongly support that MeSer[1]-tentoxin is also organized in small micelles in aqueous solution. The detailed interpretation of the NMR parameters can be found in detail in our NMR study of tentoxin (Pinet et al., 1995).

Transverse relaxation times (T_2) of protons not affected by chemical exchange at −5 °C (*e.g.*, [A] Hα_{Leu}, [A] Hβ_{Leu}, [A] Hβ'_{Ser}, [B] Hα_{Ser}, and [C] Hβ_{Phe}), corresponded to an average rotational correlation time τ_c of 1.3 ns at 500 MHz, consistent with the association of about eight molecules of MeSer[1]-tentoxin (for a monomer, τ_c would be about 0.2 ns). τ_c can also be derived from the ratio T_1/T_2, where T_1 is the spin−lattice relaxation time. For the above-mentioned protons, this ratio was found to be between 5 and 10, giving a τ_c of about 1−1.5 ns, also in favor of the presence of aggregates. Moreover, for the three forms [A], [B], and [C], there was an important shielding effect of 0.2−0.6 ppm for Leu Hγ proton, and of 0.3−0.4 ppm for Leu Hδ protons, when compared to chemical shifts found in random coil structures. For methyl protons of hydrophobic residues in peptides, such a shielding is associated with an unusual molecular environment. This noticeable shielding is even stronger than for tentoxin (Pinet et al., 1995). It is very likely to result from ring-current shift effects, both by intramolecular side chain/side chain stacking, where the leucine side chain is close to the hydrophobic MeΔ^ZPhe residue, and by intermolecular interactions. Thus, it is suggested that MeSer[1]-tentoxin, like tentoxin, is self-associated in aqueous solution, in a micelle-like organization gathering the two hydrophobic residues (Leu and MeΔ^ZPhe) in the center of the aggregate and exposing Gly and MeSer toward the solvent.

Finally, one-dimensional ^{1}H NMR spectra of MeSer[1]-tentoxin were recorded at −5 °C in H_2O at concentrations ranging from 20 μM to 9 mM. The position and the linewidths of all resonances, including amide protons resonances (known to be very sensitive to aggregation phenomena), did not vary significantly. Thus, aggregation is likely to occur at lower concentrations.

In conclusion, according to concentration-dependence NMR experiments and relaxation data analysis, it was found that MeSer[1]-tentoxin tends to be organized above 10 μM in aqueous solution into small aggregates of roughly 5−10 molecules.

Tridimensional Structure of the Conformers. The conformation of the cyclic backbone was determined using chemical shifts, *J* coupling constant information, and distance constraints derived from ROESY negative cross-peaks, as described in Pinet et al. (1995). All the NMR data that allowed the structural determination of MeSer[1]-tentoxin will be available in detail elsewhere, and only the main structural results are outlined below. In addition, chemical shifts, *J* couplings, and observed ROEs of the three conformers [A], [B], and [C] of MeSer[1]-tentoxin are strikingly similar to those of the conformers [A], [B], and [C] of tentoxin, respectively. This is strongly in favor of close conformational features, that can be summarized as follows [for nomenclature, see Pinet et al. (1995), and the semideveloped formula of MeSer[1]-tentoxin in Scheme 1]: (i) *cis-trans-cis-trans* conformation of the amide bond sequence for all the conformers; (ii) *S-cis* $C_\beta H{=}C_\alpha{-}C{=}O$ configuration for the MeΔPhe residue in [A] and [B] forms, and *S-trans* $C_\beta H{=}C_\alpha{-}C{=}O$ configuration in the [C] form; (iii) "boat-like" conformation of the peptide ring predominant in all conformers; (iv) [B] form derived from [A] form by applying a peptidic bond inversion of about 180° around φ_{Leu} and ψ_{Ser}; (v) [C] form derived from [B] form with a ring flip of 180° around φ_{Gly} and $\psi_{\Delta Phe}$.

This initial conformational assignment could be directly inferred from a careful proton chemical shift analysis. It was fully confirmed by molecular modeling and structural calculations, using the distance constraints derived from homonuclear two-dimensional ROESY spectra. In MeSer[1]-tentoxin, most of the observed ROEs corresponded to close

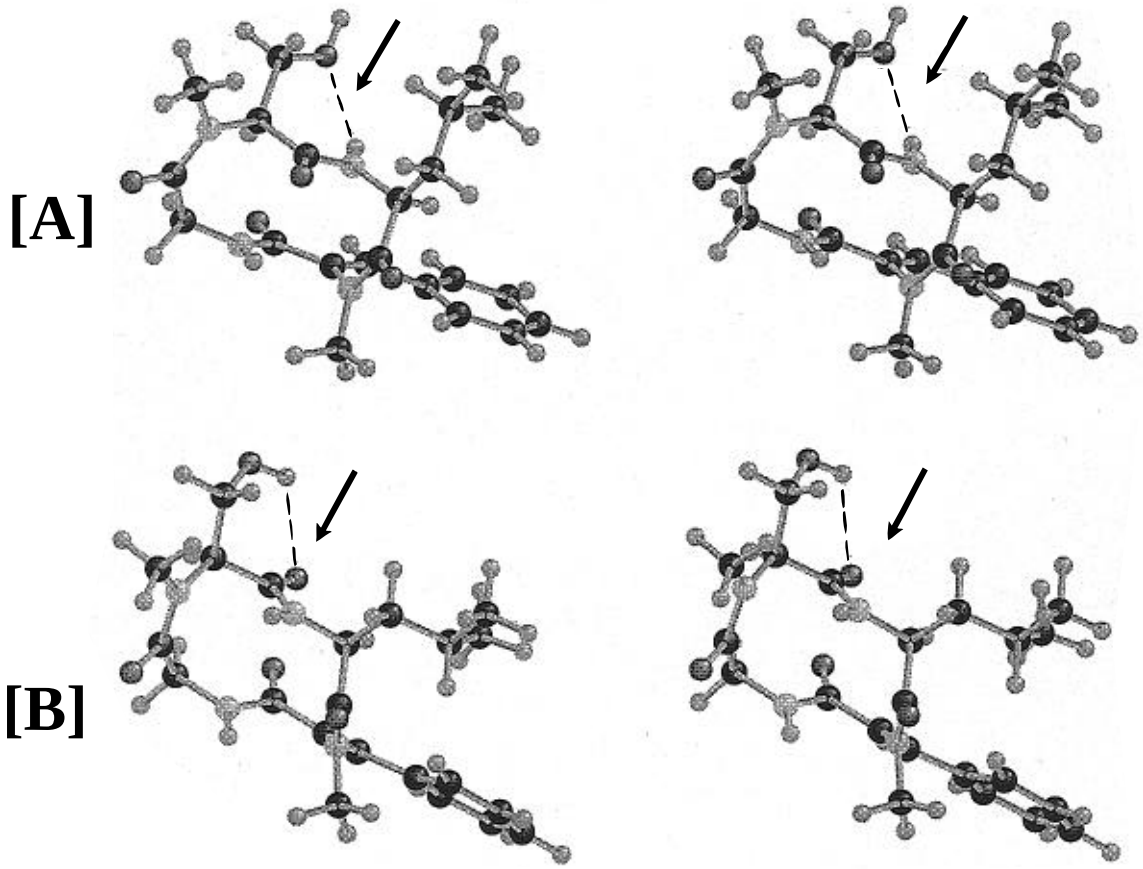


FIGURE 1: Stereoviews of the models proposed for the two majors forms of MeSer[1]-tentoxin in aqueous solution and derived from restrained molecular dynamics simulations. Top view, [A] form; bottom view, [B] form. The arrow points the peptidic bond inversion when switching from one form to the other. The dashed lines indicates *intramolecular* hydrogen bonding, which does not exist in natural tentoxin.

or sequential contacts and arose from *intramolecular* contacts. But, contrary to tentoxin, some very weak cross-peaks detected in 100 ms ROESY spectra could be eventually assigned to *intermolecular* contacts, thus accounting for the existence of a micellar-like organization of MeSer[1]-tentoxin in solution. However, the very limited number of these ROEs, as well as the poor accuracy of their intensity measurement, did not allow the building of a model of the supramolecular organization. The conformational analysis of the three conformers of MeSer[1]-tentoxin resulted from the interpretation of all *intramolecular* contacts. The structural models described below were derived from constrained molecular dynamics calculations, *i.e.*, using the experimental interproton distance constraints derived from the observed ROEs analysis. These models therefore accounted for the actual structure of each conformer in aqueous solution.

Initial structural models were built using appropriate φ, ψ, ω angles published for tentoxin. The complete dynamics simulations were only performed for [A] and [B], for which a reasonable set of NMR constraints was available. Respectively, 26 and 22 distance constraints were collected (data not shown), as well as two dihedral constraints (the synthetic cyclic tetrapeptide has only two non-methylated peptide bonds). The addition of NMR constraints during the dynamics simulations fixed the backbone ring into the conformation presented in the final models (Figure 1 and Table 1). The different structures derived from restrained dynamics differed essentially by the conformation of the side chains. Figure 1 shows the lowest energy structure of [A] and [B] forms, generated by the constrained dynamics runs.

Table 1: Torsion Angles of [A] and [B] Forms of MeSer[1]-Tentoxin[a]

residue	angle	[A] form	[B] form
MeSer[1]	φ	−83	−66
	ψ	165	−32
	ω	180	176
	χ_1	175	−169
	χ_2	180	−21
Leu[2]	φ	59	−121
	ψ	53	102
	ω	−8	−19
	χ_1	−53	−179
	χ_{21}	173	65
	χ_{22}	−64	−174
MeΔ^ZPhe[3]	φ	−94	−53
	ψ	0	−34
	ω	178	176
Gly[4]	φ	−121	−122
	ψ	51	91
	ω	0	0

[a] The peptidic backbone (φ, ψ, ω) and side chain (χ) torsion angles of the lowest energy structures derived from restrained molecular dynamics simulations for the two major forms [A] and [B] (for the minor form [C], the set of NMR constraints was not sufficient to allow restrained molecular simulations).

In forms [A] and [B], the atoms of the peptidic bond between MeSer and Leu were found to be involved in an *intramolecular* H-bond with the serine alcohol function as indicated in Figure 1. The presence of this H-bond in the structure should stabilize the two conformers and make their interconversion more difficult, since flipping the peptidic bond implies the transient rupture of the H-bond. As a result, the coalescence temperature should be higher for the

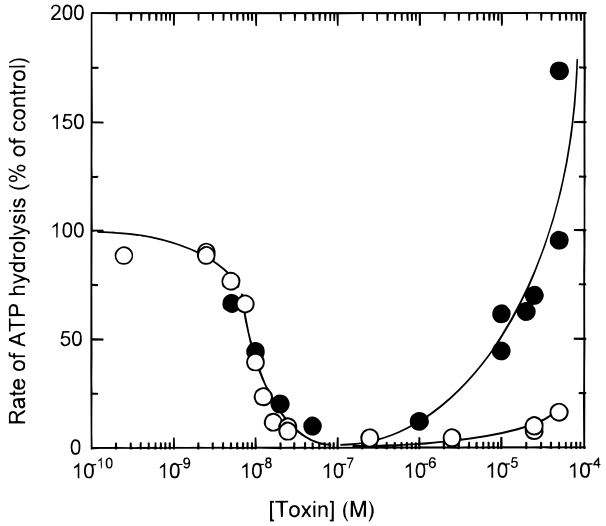


FIGURE 2: Rate of ATP hydrolysis by CF1-ϵ as a function of tentoxin and MeSer[1]-tentoxin concentrations. Conditions as described under Experimental Procedures. (●) Tentoxin; (○) MeSer[1]-tentoxin. Absolute activities without toxin ranged from 2.5 to 6.0 μmol (mg of protein)$^{-1}$ min^{-1}.

synthetic toxin than for the natural one. However, the opposite situation occurs. This indicates that *intermolecular* interactions in the aggregate are predominant in the case of MeSer[1]-tentoxin.

Compared Effects of MeSer[1]-Tentoxin and Tentoxin on Isolated CF$_1$-ϵ. As mentioned above, the effect of changing the MeAla to MeSer residue induces only minor changes in the structural characteristics of the molecule. These strong similarities suggest that biological activity of the analogue should match that of the natural product for sensitive plant species. Figure 2 reports the effect of increasing concentrations of tentoxin and of MeSer[1]-tentoxin on the ATPase activity of isolated CF$_1$-ϵ. It appears that both toxins inhibit the enzyme with the same efficiency as tentoxin, with almost complete inhibition around 2.10^{-8} M. At higher concentrations (10^{-5}—10^{-4} M), a very low release of inhibition was observed with MeSer[1]-tentoxin, whereas the natural tentoxin, as previously observed (Steele et al., 1978a; Dahse et al., 1994), stimulated the ATPase activity to a great extent. This experiment clearly demonstrates that inhibition and activation of the enzyme by the toxin can be separated by manipulating the structure of the effector.

Competition between Synthetic and Natural Toxins. We wondered whether the very limited reactivation of ATP hydrolysis by MeSer[1]-tentoxin at high concentration is due to a weak affinity for a putative activation site. To answer this question, we have investigated the effect of different concentrations of MeSer[1]-tentoxin on the ATPase activity in the presence of 10 μM tentoxin, a concentration which induces a significant reactivation of the enzyme (Figure 2). Figure 3 shows that MeSer[1]-tentoxin, added together with tentoxin, prevents reactivation by the natural toxin. It also confirms that MeSer[1]-tentoxin, added alone, reactivated the ATPase to a moderate extent. At 50 μM MeSer[1]-tentoxin, the activity with and without 10 μM tentoxin was practically the same, showing that MeSer[1]-tentoxin competes efficiently with tentoxin. So the weakness of its activating effect, as compared to tentoxin, is not due to a lower affinity for the enzyme.

Inhibition of Photophosphorylation in Thylakoids by MeSer[1]-Tentoxin and Tentoxin. Since the study of isolated

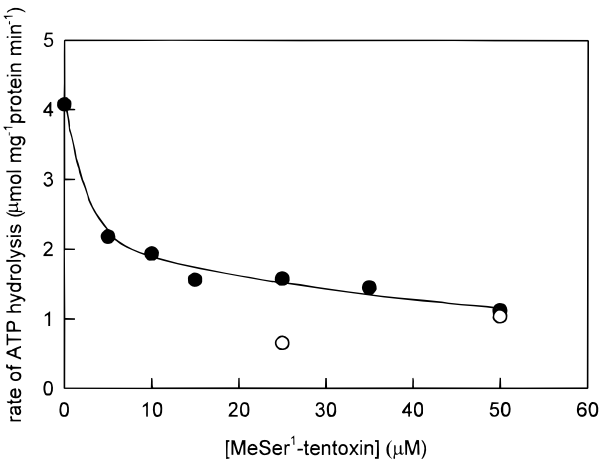


FIGURE 3: Effect of MeSer[1]-tentoxin on the rate of ATP hydrolysis by CF1-ϵ in the presence of 10 μM tentoxin (●). Conditions as described under Experimental Procedures (see also Fig. 2). Protein concentrations: incubation medium, 8.64 mg of protein mL^{-1}; reaction medium, 1.44 mg of protein mL^{-1}. Tentoxin and MeSer[1]-tentoxin were simultaneously present in the incubation and reaction media. (○) Control experiments without tentoxin. The activity with 10 μM tentoxin and without MeSer[1]-tentoxin represented 65% of the activity of the control [6.0 μmol (mg of protein)$^{-1}$ min^{-1} without inhibitor].

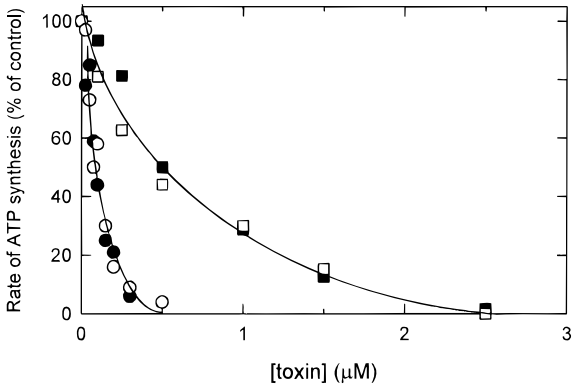


FIGURE 4: Inhibition of photophosphorylation by tentoxin and MeSer[1]-tentoxin in DTT-treated thylakoids. Conditions as described under Experimental Procedures. (■, ●) Tentoxin; (□, ○) MeSer[1]-tentoxin. Thylakoids were incubated for either 2 min (■, □) or 30 min (●, ○) with toxin. Two different chloroplast preparations. Activity of the controls: 72 mmol of ATP (mol of Chl)$^{-1}$ s^{-1} (■, □) and 74 mmol of ATP (mol of Chl)$^{-1}$ s^{-1} (●, ○).

CF$_1$ does not bring information on the ability of the enzyme to function as a coupled proton pump, we have studied the effect of toxins on CF$_0$CF$_1$ bound to natural membranes (thylakoids). Figure 4 shows the effect of MeSer[1]-tentoxin and tentoxin, in the micromolar range, on the light-induced ATP synthesis in DTT-treated thylakoids at 20 °C. The effect depended on the time of incubation: with 2 min of incubation in the dark, half-inhibition was reached at about 0.5 μM of MeSer[1]-tentoxin or tentoxin, and full inhibition practically occurred at about 2.5 μM. After 30 min of incubation, the concentration of half-inhibition shifted from 0.5 to approximately 0.1 μM, the inhibition being almost complete at 0.5 μM. In all cases, both substances inhibited the reaction with the same apparent efficiency, although the binding of toxins to CF$_1$ was obviously not complete in 2 min So MeSer[1]-tentoxin and tentoxin bind with the same rate and the same affinity to the site(s) responsible for the inhibitory effect.

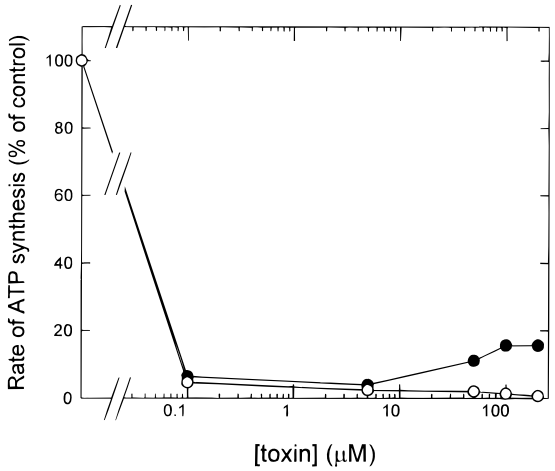


FIGURE 5: Effect of tentoxin and MeSer[1]-tentoxin on the light-driven ATP synthesis by proteoliposomes co-reconstituted with bacteriorhodopsin and CF_0CF_1. Conditions as described under Experimental Procedures. CF_0CF_1 concentration in the assay medium: 6 μg mL^{-1}. Activity of the control: 0.3 μmol (mg of protein)$^{-1}$ min^{-1}. (●) Tentoxin; (○) MeSer[1]-tentoxin.

Recovery of the Activity of Membrane-Bound CF_0CF_1 at High Toxin Concentrations. The effects of high concentrations of tentoxin and MeSer[1]-tentoxin on ATP synthesis were studied. Since reactivation of ATP synthesis by tentoxin was difficult to reveal in thylakoids (Sigalat et al., 1995), these experiments were carried out on proteoliposomes containing CF_0CF_1 and a light-dependent proton pump, bacteriorhodopsin. The temperature was 40 °C, and the time of incubation with the toxins was 15 min. The results are displayed in Figure 5. In the sub-micromolar range, MeSer[1]-tentoxin and tentoxin inhibited around 95% of the activity. At much higher concentrations, tentoxin induced a significant recovery of ATP synthesis (about 15% of the control), whereas MeSer[1]-tentoxin was ineffective. We verified, by the pH-dependent fluorescence of pyranine entrapped into liposomes (Seigneuret & Rigaud, 1986), that this lack of ATP synthesis was not due to some trivial effect of MeSer[1]-tentoxin on the proton gradient. So, although the release of the inhibition of ATP synthesis by tentoxin at high concentrations was smaller than that observed on ATP hydrolysis by the isolated enzyme, the difference between tentoxin and MeSer[1]-tentoxin was preserved.

We also investigated the reactivation of membrane-bound ATPase activity by toxins at high concentrations in DTT-treated thylakoids. In this case, instead of light-driven ATP synthesis which is poorly reactivated by tentoxin [see above and Sigalat et al. (1995)], we measured the rate of the ATP hydrolysis triggered by simultaneous addition of an uncoupler and switching off the light, as in Sigalat et al. (1995). This activity was in all cases fully sensitive to venturicidin, then tightly coupled to proton pumping. It was 100% inhibited by 5 μM tentoxin or 5 μM MeSer[1]-tentoxin. 100 μM tentoxin reactivated 20% of the control activity, while MeSer[1]-tentoxin at the same concentration did not reactivate the enzyme at all (not shown). These data confirm the pattern of reactivation obtained with isolated CF_1-ϵ or with CF_0CF_1 bound to liposomes.

DISCUSSION

In this work, we have examined the effect of a small chemical modification of the tentoxin molecule on its tridimensional structure and its biological activity. The substitution of an alanine by a serine was shown to result in limited changes in the conformation of the tetrapeptide. The three different conformers of MeSer[1]-tentoxin which were characterized by NMR resemble the three major conformers previously reported for native toxin (Pinet et al., 1995), and their proportions were not very different. In the inhibitory range of concentrations (10^{-8}−10^{-6} M), both molecules were also found to inhibit the enzyme with the same efficiency (same I_{50}), and the time-dependence of the inhibitory effect is also unchanged. These results demonstrate that the thermodynamic constant of association of the toxin to the inhibitory site(s), as well as the kinetic constants of binding and release are not modified. At concentrations where tentoxin reactivates the enzyme (10^{-5}−10^{-4} M), MeSer[1]-tentoxin seems to bind to the enzyme in the same way as tentoxin, since it was shown to compete with tentoxin and to prevent its activating effect. It is thus very interesting to note that, despite these similar binding properties, MeSer[1]-tentoxin is far from reactivating the enzyme to the same level as does tentoxin.

Our data on the ATPase reactivation have to be compared to those previously reported with other tentoxin analogues modified in position 1, Sar[1]-tentoxin (Rich et al., 1981) and Pro[1]-tentoxin (Edwards et al., 1986). The most simple interpretation of such kinetic data is to propose that CF_1 has two classes of sites for binding tentoxin and analogues, one of high affinity responsible for inhibition and one of low affinity responsible for activation. This is consistent with the existence of two binding sites of different affinities recently characterized by dialysis experiments (Pinet et al., 1996). Sar[1]-tentoxin inhibits ATPase activity of CF_1 grossly with the same efficiency as tentoxin and reactivates it at lower concentration (Rich et al., 1981). Although direct evidence is lacking, one can speculate that Sar[1]-tentoxin has a higher affinity than tentoxin for the activation site. Pro[1]-tentoxin was mentioned to inhibit the ATPase at low concentration without reactivating it at high concentrations (Edwards et al., 1986). At first sight, this behavior resembles that of MeSer[1]-tentoxin reported here, but no quantitative data were given and no competition experiments were carried out, so it is not possible to know whether Pro[1]-tentoxin fails to bind to the activator site or if it binds to it in a non-productive way. In the case of MeSer[1]-tentoxin, the lack of significant reactivation clearly resides in its behavior once it is bound. Due to the presence of an alcohol function, the synthetic toxin can interact with the enzyme by supplementary hydrogen bonds and (or) can introduce a steric hindrance sufficient to prevent the activating process. So a limited change in the structure of the ligand (toxin) results in a highly significant modification of the receptor (CF_1).

On the basis of the similarities and differences in binding and inhibitory properties of the available tentoxin analogues, one can notice that, whereas the alanine position appears to be crucial for the putative activating site(s), the conservation of the two hydrophobic residues (Leu[2] and MeΔ^ZPhe[3]) pattern seems to be important for recognition in the inhibiting site(s) of CF_1. Indeed, the L-Ala[1]-tentoxin analogue has the same activity as tentoxin has on lettuce seedling chlorosis assay, whereas the Δ^ZPhe[3]-tentoxin analogue is much less efficient (Edwards et al., 1987). These ideas have naturally to be confirmed by the development of other new analogues

associated with *in vitro* studies in order to better understand the structure−function relationships of the natural phytotoxin.

Among the several effects of tentoxin on CF_1 ATPase previously reported in the literature, some deserve a special attention: the reactivation of ATPase at high toxin concentrations (Steele et al., 1978a; Dahse et al., 1994), the parallel restoration of coupled proton transport in CF_0CF_1 (Sigalat et al., 1995), and the disruption of the interactions between the nucleotide binding sites (Fromme et al., 1992; Hu et al., 1993). Taken together, these data could lead to the idea that proton pumping and cooperativity between the catalytic sites are not necessarily linked. In the actually prevailing model of rotational catalysis by F_0F_1 ATPase (Abrahams et al., 1994; Vik & Antonio, 1994; Aggeler et al., 1995), proton-driven rotation of the γ subunit is responsible for energy conversion by sequential changes of affinity of the catalytic sites for ADP, phosphate, and ATP. So the proton pumping and the transmission of information (cooperativity) between the catalytic nucleotidic sites are expected to be strictly linked to the same process, namely, γ movements, whereas they appear to be disconnected in CF_0CF_1 reactivated by high concentrations of tentoxin. This would question the validity of the model of rotational catalysis, and then makes tentoxin and derivatives a powerful tool to study the ATPase mechanism. In this context, the possibility to dissociate the inhibitory and the activating effects is especially attractive.

As regards the binding sites of tentoxin and derivatives, numerous questions should be addressed. The number and location of these sites are to be precisely determined. Moreover, we do not know whether the inhibitory and activatory sites are different in nature, or if they are potentially equivalent, their different affinities being then due to a negative cooperativity of the toxin binding. It also remains to check, when reactivatory properties of tentoxin and MeSer[1]-tentoxin are compared, whether the reactivation is determined by the presence of the natural toxin in the low-affinity site, in the high-affinity site, or in both sites. In parallel to the characterization of these sites, new derivatives are being prepared and studied in structural and functional aspects. These various approaches are expected to bring a new insight into the effect of the phytotoxin as well as in the very mechanism of the proton ATPase.

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