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Two Conformations of the Cyclic Tetrapeptide, [D MeAla[1]]-Tentoxin Have Different Biological Activities[1,2,3]

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The cyclic tetrapeptide [D-MeAla[1]]-tentoxin (**2**) has been found to exist in multiple conformations in solution. Two of these (**2U** and **2L**) have been isolated. Circular dichroism spectroscopy was used to show that the conformations of **2U** and **2L** differed from each other in water. The potencies of **2U** and **2L** as inhibitors of coupled electron transport in isolated chloroplasts and as inhibitors of chloroplast coupling factor one ATPase (CF_1-ATPase) were determined. Conformer **2L** is the more potent inhibitor of coupled electron transport. In the CF_1-ATPase assay, conformer **2L** is a more potent inhibitor than **2U** but the potency of each is much less than that of tentoxin. The reported results provide the first demonstration that separate conformers of a single molecule can have differing biological activities.

INTRODUCTION

A knowledge of solution conformation is thought to be essential for understanding the binding of biologically active peptides to their receptors and ultimately for determining the molecular mechanism of action of the peptide (*1*). To interpret conformation activity data, it is assumed that the target receptor (or enzyme) recognizes a biologically active conformation of the peptide, where the "active" conformation may be either the entire peptide or a subunit (*2*), and that peptide analogs which do not adopt the active conformation have less biological activity or are inactive. This concept also is invoked in structure–activity studies of other biologically active substances that have some degree of conformational flexibility. Although studies of conformationally restricted or "rigid" analogs of neurotransmitters, e.g., acetylcholine (*3*), histamine (*4*), dopamine (*5*), and enkephalin (*6*) provide data consistent with the concept of an active conformation, the "rigid" analogs employed have been structural isomers or diastereomers of each other. No system has been reported in which individual *conformations* (conformers) of a single active substance have been bioassayed (*7*).

During our studies of the relationships between the structure and activity of the

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[2] Abstracted in part from the Ph.D. thesis of Pradip K. Bhatnagar, June 1977. Financial support from the National Institutes of Health (GM 19311) is gratefully acknowledged.

[3] All amino acids except glycine are of the L-configuration unless noted. Standard abbreviations for amino acids as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [*J. Biol. Chem.* **247**, 977 (1972)] are used. Additional abbreviations used: MePhe[(*Z*)Δ], *N*-methyl-(*Z*)-dehydrophenylalanine; MeAla, *N*-methylalanine; CF_1, Chloroplast coupling factor one.

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phytotoxin, tentoxin, cyclo(-L-MeAla-L-Leu-MePhe[(*Z*)Δ]-Gly-) (**1**) (*8–10*), a species-specific inhibitor of photophosphorylation and chloroplast coupling factor one (CF_1) (*11*), we synthesized [D-MeAla^1]-tentoxin (**2**) (*12*) and found that this analog existed in multiple conformations in solution. These were sufficiently stable that they could be separated by thin-layer chromatography (tlc) on silica gel at 4°C into two fractions designated **2U** and **2L** (*13*, *14*) on the basis of their faster (**2U**) and slower (**2L**) mobilities on tlc. Further study established that the rate of conformational interconversion is sufficiently slow that the biological activity of each fraction could be measured. We report here the effects of **2U** and **2L** as inhibitors of coupled electron transport and CF_1-ATPase. Our results provide the first demonstration that separate conformers of a single molecule can have differing biological activities.

MATERIALS AND METHODS

[D-MeAla^1]-tentoxin was synthesized by the reported procedure (*12*). Samples of **2U** used in these studies were prepared by equilibrating pure **2L** at room temperature and then rechromatographing the mixture at 4°C (*13*).

Circular dichroism spectra were measured on a Cary 16 spectrophotometer equipped with a model 6001 CD attachment using, generally, a 1.0-cm path length and 0.04° full range and peptide concentrations near 10^{-5} *M*. A blank run of solvent and cell was subtracted from the measured spectrum.

Inhibition of coupled electron transport was obtained from polarographic measurements of oxygen uptake following the reported procedures (*17*). The data presented in Fig. 3 represent an average of three determinations.

CF_1-ATPase was prepared from CF_1 by gentle trypsin activation following the method of Lien and Racker (*15*). ATPase activities were determined by measuring phosphate release (*17*).

RESULTS

Before describing the chemical and biological results, it is useful to clarify what is meant by the term individual conformations. The conformations of the molecular species contained in fractions **2U** and **2L** in chloroform have been determined by nmr spectrometry (Fig. 1) (*13*). The substance designated **2L** is a mixture of two non-separable conformers designated $\mathbf{2L}_1$ and $\mathbf{2L}_2$, which interconvert rapidly at 27°C (*13*). In addition to the three ring–system conformations shown (Fig. 1), other conformations are present. Rotation about the ψ_{MeAla}, ϕ_{Leu} torsion angle generates a second set of ring conformations in which the carbonyl groups of MeAla and MePhe[(*Z*)Δ] are aligned in **2U** and $\mathbf{2L}_1$ and opposed in $\mathbf{2L}_2$. Rotation of the leucine side chain (χ_1) is also possible. The nmr data indicate that both rotational processes occur rapidly with respect to the nmr time scale (*13*). Consequently, the substances designated **2U** and **2L** are, in fact, mutually exclusive populations of several conformations and are not limited to the molecular species depicted in Fig. 1. For convenience these will be referred to as conformers **2U** and **2L**.

The conformational properties of **2U** and **2L** previously were studied by circular dichroism (CD) spectroscopy in methanol (Fig. 2A) in conjunction with the nmr analysis (*13*).[4] Circular dichroism data of aqueous solutions were obtained (Fig. 2B) to confirm that these conformers also differed in water, a biologically relevant solvent.[5] The CD spectra of **2U** obtained in water or methanol are very different from those of tentoxin or **2L**, especially between 260 and 300 nm. Therefore, **2U** does not resemble the conformations of either tentoxin or **2L** in either solvent. The general similarity between the CD curves for **2U** in the two solvents suggests the corresponding conformations of **2U** are related.

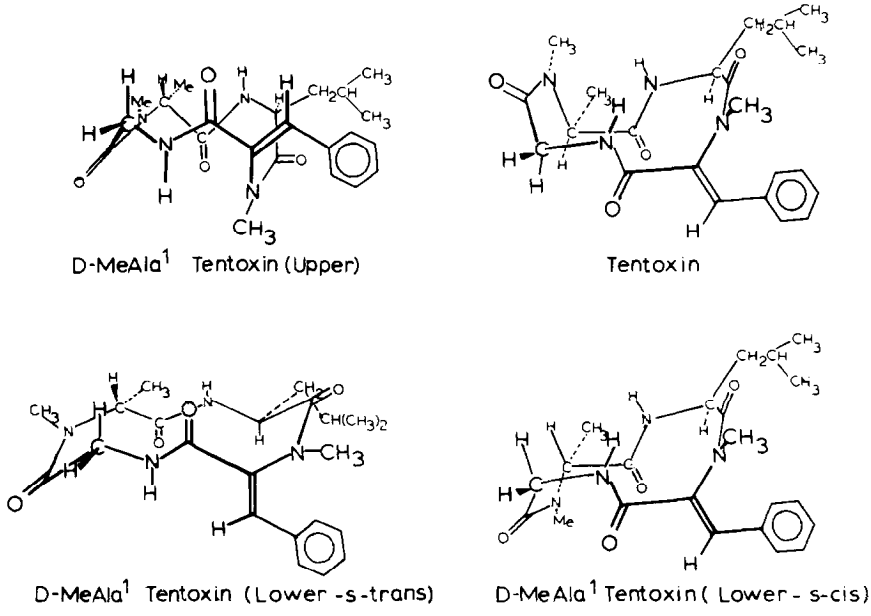

FIG. 1. Solution conformations of tentoxin and [D-MeAla[1]]-tentoxin conformers at 27°C in chloroform. Rotation about ψ_{MeAla}, ϕ_{Leu} in all structures is rapid on the nmr time scale and other rotomers are present. Upper right, tentoxin (**1**); upper left, **2U**. The substance designated **2L** in the text is a mixture of the *S–trans* conformer ($\mathbf{2L_1}$) and the *S–cis* conformer ($\mathbf{2L_2}$). At equilibrium **2L** contains $\mathbf{2L_1}$ and $\mathbf{2L_2}$ in a ratio of 1 : 1 while in water the ratio is 0.20 : 0.80.

The shift in the maximum ellipticity for **2L** from 260 nm in methanol to 280 nm in water is consistent with a ratio of $\mathbf{2L_2}$: $\mathbf{2L_1}$ in water greater than the 1 : 1 ratio found in methanol (*13*). This assumes that $\mathbf{2L_2}$ has a CD maximum at 280 nm as does tentoxin. However, the ellipticity at 280 nm is greater for **2L** than for tentoxin so that the respective conformational distributions about the dehydrophenylalanine chromophore cannot be identical. Although the CD spectrum in water for each substance is clearly related to the corresponding spectrum in methanol, the spectra are not identical. Consequently, the precise conformations of **2U** and **2L** in water cannot be determined

[4] Nuclear magnetic resonance studies in methanol-d_4 have shown that the conformation of **2U** and **2L** are the same in methanol as in chloroform.

[5] The low solubility of tentoxin and its analogs in water has prevented us from obtaining conformation data by nmr spectrometry.

by comparative CD data alone. The solvent-dependent spectral changes could easily be caused by several factors. Different rotomer populations about ψ_{MeAla}, ϕ_{Leu}, or χ_{Leu} torsion angles caused by lowering of dipole–dipole repulsions upon shifting from organic to aqueous media are especially likely.

Conformers **2U** and **2L** were allowed to equilibrate at 25°C for 24 hr in water and the CD spectrum of the equilibrium mixture was obtained (Fig. 2B). The composition of the equilibrium mixture that would give the observed spectrum is 65 ± 2% **2L** and

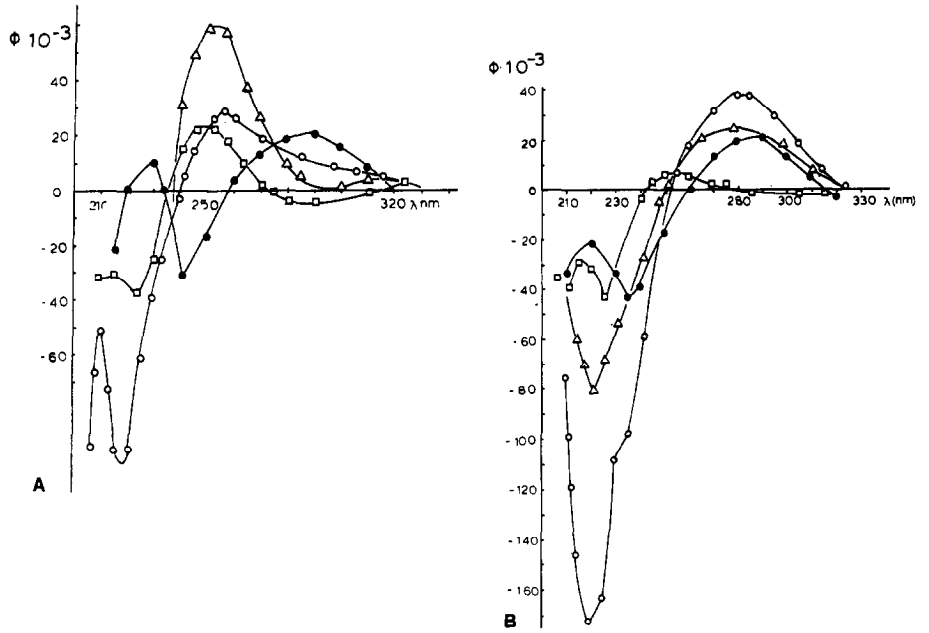

FIG. 2. Circular dichroism spectra of tentoxin analogs at 25°C. A. Spectra taken in methanol: tentoxin (**1**), ●—●; higher R_f conformer of [D-MeAla$_1$]-tentoxin, (**2U**), □—□; lower R_f conformer of [D-MeAla^1]-tentoxin, (**2L**), which is a mixture of $\mathbf{2L}_1$ and $\mathbf{2L}_2$, ○—○; calculated spectrum for *S–trans* conformer $\mathbf{2L}_1$, △—△. Spectrum was calculated by asssuming the elipticity for the *S–cis* conformer, $\mathbf{2L}_2$, at 280 nm would equal that of tentoxin. B. Spectra taken in water: tentoxin (**1**), ●—●; higher R_f conformer of [D-MeAla^1]-tentoxin, (**2U**), □—□; lower R_f conformer of [D-MeAla^1]-tentoxin, (**2L**), ○—○; equilibrium mixture of **2U** (35%) and **2L** (65%), △—△.

35 ± 2% **2U**. The rate of interconversion was measured at 27°C starting with either conformer by following the change in the CD spectrum with time. The half-life in water is about 200 min ($k_{obs} = 3.4 \times 10^{-3}$ min^{-1}). Thus, the rate of interconversion in water is much slower than that occurring in chloroform ($T_{\frac{1}{2}} = 20$ min; $k_{obs} = 4 \times 10^{-2}$ min^{-1}) (*13*).

The biochemical properties of **2U** and **2L** were measured in two systems, coupled electron transport and CF_1-ATPase. Inhibition of coupled electron transport in lettuce chloroplasts by tentoxin and its analogs is shown in Fig. 3. The compounds were incubated with the chloroplasts for 1 min and then electron transport rates were measured polarigraphically for 1 min (*11*). Under these conditions less than 10% interconversion of **2L** and **2U** would occur in each sample if interconversion occurs at a rate

approaching that found in chloroform. At the slower rate found in water, less than 1% interconversion would occur in each sample. The chloroform rate is included since interconversion might occur in the hydrophobic chloroplast membranes.

Inhibition of CF_1-ATPase by **2U** and **2L** was also measured (Fig. 4). The conformers were incubated with CF_1-ATPase for 10 min, substrate was added, and inorganic phosphate was measured after 10 min (*15*, *17*). Under these conditions the rate of conformational interconversion should be close to that obtained in water so that each conformer would be contaminated with less than 3% of the other. The relatively low

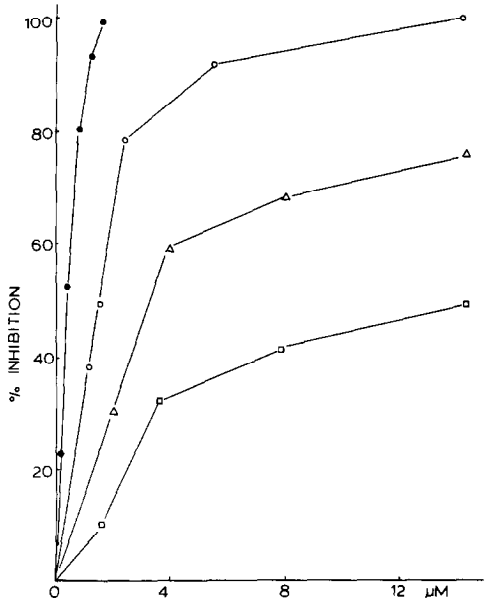

FIG. 3. Inhibition of coupled electron transport in lettuce chloroplasts by tentoxin analogs: tentoxin (**1**), ●—●; [D-MeAla^1]-tentoxin, lower R_f conformer, (**2L**), O—O; [D-MeAla^1]-tentoxin, higher R_f conformer, (**2U**), □—□; equilibrium mixture of **2L** and **2U** (0.65 : 0.35), △—△.

potency of **2U** in this assay is consistent with the aqueous rate of interconversion. Since tentoxin must be incubated with CF_1-ATPase for about 30 min to produce maximal inhibition (*17*), it is possible that the binding of **2U** and **2L** had not reached equilibrium during the 20-min incubation and assay period.

DISCUSSION

The data for inhibition of coupled electron transport in isolated chloroplasts (Fig. 3) establish that the two isolable conformational populations of [D-MeAla^1]-tentoxin differ in their biochemical properties and that **2L** is a substantially more potent inhibitor than **2U**. The rate of conversion of **2U** to **2L** is too slow to cause the inhibition produced by **2U**. Thus, the inhibition observed with **2U** is real.

The inhibition curve for **2U** given in Fig. 3 is unusual in that maximum inhibition

does not approach 100% at high inhibitor concentrations. Nevertheless, the hyperbolic shape of the curves indicates that some process is being saturated. Furthermore, **2U** and **2L** compete with each other since the effects of **2U** and **2L** are not additive. For example, an 8 μM solution of **2** at equilibrium is 5.2 μM in **2L**. This concentration alone will produce 90% inhibition of coupled electron transport, whereas the equilibrium mixture produces only about 65% inhibition. In fact, the inhibition curve found for equilibrated [D-MeAla^1]-tentoxin is exactly an average of those produced by each conformer.

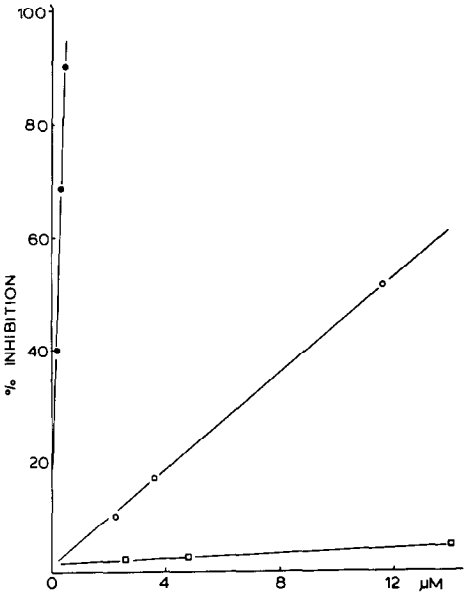

FIG. 4. Inhibition of CF_1-ATPase by tentoxin analogs: tentoxin (**1**), ●—●; [D-MeAla^1]-tentoxin, lower R_f conformer, (**2L**), O—O; [D-MeAla^1]-tentoxin, higher R_f conformer, (**2U**), □—□.

These results indicate that **2U** and **2L** are mutually antagonistic. However, because tentoxin must be equilibrated with chloroplasts for about 1 hr to achieve maximum inhibition (*11*), the conditions used to assay **2U** and **2L** are not at equilibrium. Consequently, the relative potencies at **2U** and **2L** in this assay cannot be used to estimate relative affinities for tentoxin receptors.

The relative apparent partition coefficients of the analogs (Table 1) do not appear to explain the different potencies. Since determination of partition coefficients by the usual methods (*16*) is not possible for **2U** and **2L** because of conformational interconversion, R_f values were used to estimate relative polarity. Tentoxin (**1**) and [L-Pro^1]-tentoxin (**3**) are equally effective inhibitors of CF_1 and CF_1-ATPase yet have very different polarities (*12*, *13*). The R_f values of conformers **2U** and **2L** fall between those of **1** and **3**. The least potent inhibitor, **2U**, has an R_f virtually identical with that of tentoxin in several solvent systems. Thus, there is no apparent linear or parabolic pattern that indicates that polarity itself explains the different potencies of **1** through **3**.

To compare the biochemical properties of **2U** and **2L** without the complex effects

that occur in the chloroplast assay, we measured inhibition of CF_1-ATPase by tentoxin analogs (Fig. 4). CF_1 has been identified as a receptor for tentoxin (*11*). This isolable protein can be converted by gentle trypsin treatment into CF_1-ATPase (*15*). Tentoxin binds tightly to and inhibits CF_1-ATPase uncompetitively with respect to ATP (*17*). This pattern of inhibition is consistent with inhibition of the enzyme after the first irreversible reaction takes place (*17*).

Conformer **2L** is a much better inhibitor of CF_1-ATPase than is **2U**, which is nearly inactive over the concentration range tested. However, the potency of both **2U** and **2L**

TABLE 1

COMPARISON OF ED_{50} WITH POLARITY OF TENTOXIN ANALOGS

Compound	R_f[a]	ED_{50}[b] (μM)
Tentoxin (**1**)	0.45	0.4
[D-MeAla[1]]-Tentoxin (**2U**)	0.45	6[c](14)
[D-MeAla[1]]-Tentoxin (**2L**)	0.30	1.4
[L-Pro[1]]-Tentoxin (**3**)	0.15	0.8

[a] r_f value taken from tlc of compound on Brinkman silica gel G plates developed with 5% ethanol in ethyl acetate (*12*).

[b] Values determined from the inhibition curves shown in Fig. 3 and from Ref. (*14*).

[c] The 6 μM value for the ED_{50} was estimated by a linear extrapolation of the data from 0 to 4 μM out to about 12 μM. This procedure provides an estimate of the ED_{50} in the absence of saturation effects and may be an artifically low value.

relative to tentoxin is very low in this assay. Under the conditions of the experiment reported here, tentoxin has an ED_{50} of about 0.1 versus 12 μM for **2L**. The cause of the large decrease in potency of **2L** relative to tentoxin is not known. However, because of the potential for conformational interconversion between **2U** and **2L**, the inhibitors could not be incubated with the enzyme long enough to ensure equilibrium, and the difference in potency between **2L** and **1** might vary with incubation time.

Our results establish that different conformational populations of [D-MeAla[1]]-tentoxin have different biochemical activities in both isolated chloroplasts and CF_1-ATPase. In both systems **2L** is the more potent inhibitor. In chloroform this fraction contains conformers $\mathbf{2L}_1$ and $\mathbf{2L}_2$ in equal amounts (*13*). The latter conformer is closely related to tentoxin except for the projection of the –Gly–MeAla– amide bond (*13*, *14*). The CD data presented here suggest that a species related to $\mathbf{2L}_2$ also exists in water and is a major contributor to the aqueous conformation of **2L**. If this is correct, it would be consistent with the more potent inhibition produced by **2L**, particularly in the CF_1-ATPase assay.

Both conformations **2U** and **2L** inhibit coupled electron transport in isolated chloroplasts but it is not known if both inhibit by the same mechanism. Recently it was found that tentoxin affects CF_1-ATPase in two ways. At nanomolar concentrations tentoxin inhibits CF_1-ATPase (*17*) but at micromolar concentrations tentoxin

stimulates CF_1-ATPase. Furthermore, native CF_1 is converted to a Ca^{2+}-dependent ATPase at micromolar concentrations of tentoxin (J. A. Steel, T. F. Uchytil, and R. D. Durbin, unpublished results). Either action of tentoxin on CF_1 in chloroplasts inhibits coupled electron transport. It is possible that **2U** and **2L** might act at different sites to inhibit coupled electron transport. Experiments are in progress to test this possibility.

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