Interactions of the Neurotoxin Vipoxin in Solution Studied by Dynamic Light Scattering

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ABSTRACT   The neurotoxin vipoxin is the lethal component of the venom of *Vipera ammodytes meridionalis*. It is a heterodimer of a basic toxic His-48 phospholipase A_2 (PLA_2) and an acidic nontoxic Gln-48 PLA_2. The shape of the neurotoxin and its separated components in solution as well as their interactions with calcium, the brain phospholipid phosphatidylcholine, and two inhibitors, elaidoylamide and vitamin E, were investigated by dynamic light scattering. Calcium binding is connected with a conformational change in vipoxin observed as a change of the hydrodynamic shape from oblate ellipsoid to a shape closer to a sphere. The Ca^{2+}-bound form of vipoxin, which is catalytically active, is more compact and symmetric than the calcium-free heterodimer. Similar changes were observed as a result of the Ca^{2+}-binding to the two separated subunits. In the presence of aggregated phosphatidylcholine, the neurotoxic complex dissociates to subunits. It is supposed that only the toxic component binds to the substrate, and the other subunit, which plays a chaperone function, remains in solution. The inhibition of vipoxin with the synthetic inhibitor elaidoylamide and the natural compound vitamin E changes the shape of the toxin from oblate to prolate ellipsoid. The inhibited toxin is more asymmetric in comparison to the native one. Similar, but not so pronounced, effects were observed after the inhibition of the monomeric and homodimeric forms of the toxic His-48 PLA_2. Circular dichroism measurements in the presence of urea, methylurea, and ethylurea indicate a strong hydrophobic stabilization of the neurotoxin. Hydrophobic interactions stabilize not only the folded regions but also the regions of intersubunit contacts.

INTRODUCTION

Neurotoxins are the most lethal components of snake venoms. They are monomeric or multichain phospholipase A_2s (phosphatide *sn*-2 acylhydrolase, PLA_2, EC 3.1.1.4) which specifically hydrolyze the 2-acyl ester bond of 1,2-diacyl-3-*sn*-phosphoglycerides releasing fatty acids and lysophospholipids. Snake venom PLA_2s possess a wide variety of pharmacological activities: neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant, convulsant, antiplatelet, hemorrhagic, hemolytic, and edema-inducing effects (Huang et al., 1997). They exert neurotoxicity through binding to the pre- or postsynaptic membranes of the neuromuscular junctions. The presynaptic PLA_2s block the transmission across the neuromuscular junctions of the breathing muscles and cause a rapid death (Westerlund et al., 1992). Postsynaptic neurotoxins prevent the binding of acetylcholine to its receptor (Changeux et al., 1970). Two types of PLA_2 receptors have been identified. The N (neuronal) receptors are located in brain membranes and recognize toxic secretory PLA_2s from snake and bee venoms (Lambeau et al., 1989). The M receptors have a binding profile distinct from that of the N-type proteins and have a high affinity for nontoxic secreted PLA_2s (Valentin and Lambeau, 2000). The hydrolysis of cell membrane phospholipids by PLA_2s is connected with a liberation of arachidonic acid, which is a precursor of eicosanoid

mediators of inflammation: prostaglandins, leukotrienes, and thromboxanes. In this way PLA_2s are involved in human diseases like rheumatoid arthritis, asthma, and septic shock (Scott et al., 1990, 1991).

Vipoxin is a heterodimeric postsynaptic neurotoxin isolated from the venom of *Vipera ammodytes meridionalis*, the most toxic snake in Europe (Aleksiev and Shipolini, 1971). It is composed of two oppositely charged 13.5 kDa subunits with the same length of the polypeptide chain: a basic, strongly toxic His-48 PLA_2 with a pI of 10.4 and an acidic, nontoxic Gln-48 PLA_2 with a pI of 4.6 (Tchorbanov et al., 1978). In the complex, the acidic subunit reduces considerably the toxicity and phospholipase A_2 activity of His-48 PLA_2 (Aleksiev and Tchorbanov, 1976). The two components of the neurotoxic dimer are closely related proteins with 62% sequence identity (Mancheva et al., 1987). Most probably, Gln-48 PLA_2 is a product of evolution of the toxic subunit. Vipoxin is a unique example of modulation of the toxic function generated by molecular evolution and demonstrates transformation of catalytic and toxic function into an inhibitory and nontoxic one.

Here, we describe interactions between vipoxin and Ca^{2+}, the brain phospholipid phosphatidylcholine, a natural inhibitor (*d*-*α*-tocopherol, vitamin E), a synthetic inhibitor (the amide of *trans*-9-octadecenoic acid, elaidoylamide), and ureas with increasing hydrophobicity. Calcium is necessary for the enzymatic activity of the toxin. The inhibition of PLA_2 is of medical importance, because this enzyme is involved in human inflammatory diseases (Yedgar et al., 2000). We have investigated hydrodynamic properties of the native proteins and their complexes with effectors using

dynamic light scattering—a method very sensitive for detection of changes in the shape of protein molecules in solution and their aggregation state. The effects of denaturants, studied by circular dichroism measurements, revealed the nature of the forces stabilizing the 3-D structure.

MATERIALS AND METHODS

Neurotoxins and chemicals

Vipoxin was isolated from the venom of *Vipera ammodytes meridionalis* as described previously (Tchorbanov and Aleksiev, 1981). The two components of the neurotoxin, His-48 PLA_2 and Gln-48 PLA_2, were separated after dissociation of the complex and purified by the procedure given in Mancheva et al. (1986). Phosphatidylcholine, vitamin E, urea, methyl- and ethylurea were purchased from Fluka Chemie GmbH (Taufkirchen, Germany). Elaidoylamide was a generous gift from Prof. M. Jain (University of Delaware).

Dynamic light scattering

Dynamic light scattering (DLS) measurements were made using a RiNA GmbH system (Berlin, Germany) with a He-Ne laser providing a 690-nm light and an output power in the range of 10–50 MW. An autopiloted run with 20 measurements at every 30 s, with a wait time of 1 s, was used. Measurements were performed with protein solutions in 10 mM Tris/HCl buffer, pH = 7.2, at a constant temperature of 20°C. A total of 50 mM $CaCl_2$ was used for the experiments in the presence of added calcium and a twofold molar excess of elaidoylamide or vitamin E for the inhibition studies. The samples to be analyzed were filtered directly to the cell.

Hydrodynamic parameters of the heterodimeric neurotoxin vipoxin, separated toxic and nontoxic components, and respective complexes were determined as follows: the measured translational diffusion coefficient D_T is related to the frictional coefficient f by the Einstein-Sutherland equation:

$$D_T = k_B T / f (\text{cm}^2/\text{s}),$$

where k_B is the Boltzmann constant and T is the temperature Kelvin. The frictional coefficient of a spherical particle, f_{sph}, is a function of the fluid viscosity, η, and the radius of the particle, r_{sph}. It is defined by the Stokes law:

$$f_{sph} = 6\pi\eta r_{sph}.$$

The shape of vipoxin, separated His-48 PLA_2, and Gln-48 PLA_2 in solution as well as those of their complexes with effectors were characterized using the so-called Perrin or shape factor F which is informative for the shape of the molecule. This factor represents a ratio of the measured frictional coefficient f to the frictional coefficient f^{Theo} of a hypothetical sphere for which a hypothetical radius is calculated using the molecular mass:

$$F = f / f^{Theo}.$$

It can be shown that

$$f / f^{Theo} = R_H / R_H^{Theo},$$

where R_H is the measured hydrodynamic radius, and R_H^{Theo} is the radius of the hypothetical sphere, calculated from the molecular mass. The theoretical hydrodynamic radius was calculated from the formula:

$$R_H^{Theo} = [(3M(V_s + h))/(4\pi N_A)]^{1/3},$$

where M is the molecular mass, V_s is the particle specific volume, h is the hydration, and N_A is the Avogadro constant. The molecular masses

of vipoxin and the homodimers are 27 kDa, and those of the separated components, His-48 PLA_2 and Gln-48 PLA_2 in monomeric state, are 13.5 kDa. V_s has values between 0.69 and 0.75 $\text{cm}^3\,\text{g}^{-1}$ for proteins containing only amino acid residues (Cantor and Schimmel, 1980). A value of 0.73 $\text{cm}^3\,\text{g}^{-1}$ was used for the investigated proteins. According to the authors mentioned above, hydrations between 0.3 and 0.4 g H_2O (g-protein)$^{-1}$ are needed to account for the hydrodynamic behavior of globular proteins. We have used a value of 0.35 g H_2O (g-protein)$^{-1}$.

Circular dichroism measurements

Circular dichroism (CD) spectra were recorded by a Jobin Yvon (France) dichrograph. Protein solutions with a concentration of 0.1 mg ml^{-1} in quartz cuvettes with a path length of 0.2 cm were placed in a cell holder, which was thermostatically controlled. The samples were kept for 10 min to ensure the attainment of thermal equilibrium, confirmed by the constancy of the ellipticity. Each spectrum represents an average of three measurements.

Computer graphic studies

Computer graphic studies of the vipoxin three-dimensional structure were carried out using our own coordinates at 1.4 Å resolution (Banumathi et al., 2001; Protein Data Bank; code 1 jlt). The program TURBO Frodo (Roussel and Cambilau, 1991) was applied.

RESULTS AND DISCUSSION

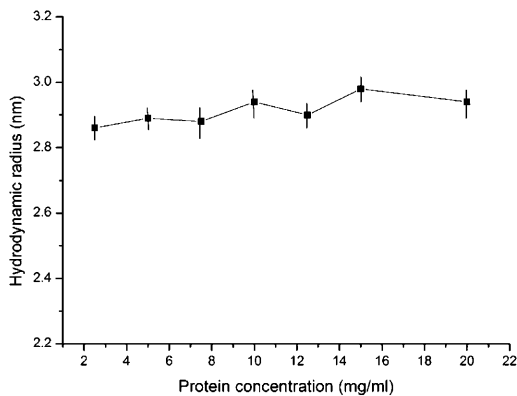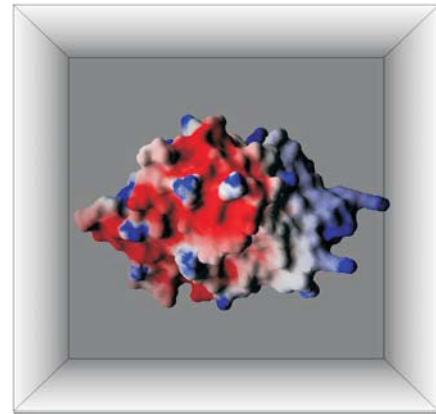### Interaction of the neurotoxin vipoxin and its components with calcium

Calcium is essential for the binding of substrate and catalysis by secreted PLA_2s. The metal ion forms the positively charged oxyanion hole that stabilizes the negatively charged transition state of the hydrolytic reaction, which lowers the activation barrier. Ca^{2+} is heptacoordinated in a pentagonal bipyramidal cage by the two carboxylate oxygens of Asp-49; the backbone carbonyl oxygens of residues 28, 30, and 32; and two water molecules (Scott et al., 1990; Six and Dennis, 2000). The region responsible for the metal ion binding is called the ''calcium binding loop'' and consists of residues 25–33. Protein loops are often involved in biological activities, and, in the case of PLA_2, the calcium-binding loop directly participates in catalysis. Both subunits of vipoxin contain this loop. The neurotoxic complex and the toxic His-48 PLA_2 require Ca^{2+} to exhibit their catalytic activity (Aleksiev and Tchorbanov, 1976). In a previous paper (Banumathi et al., 2001) we have described the high resolution (1.4 Å) x-ray structure of the Ca^{2+}-free vipoxin. However, we could not obtain crystals of the neurotoxin with bound calcium suitable for high-resolution x-ray studies. DLS measurements proved to be a suitable tool for investigating the interaction of the toxin with Ca^{2+} and other effectors.

Hydrodynamic parameters of vipoxin and its separated components, the toxic His-48 PLA_2 and nontoxic Gln-48 PLA_2, in the absence and presence of Ca^{2+}, are present in Table 1. The hydrodynamic radius of the calcium-free heterodimeric neurotoxin is 2.91 ± 0.07 nm at protein concentrations in the range 2.5–20 mg/ml (Fig. 1). Similar

**TABLE 1 Parameters calculated from dynamic light scattering measurements**

Species	R_H (nm)	R_H^{Theo} (nm)	f/f^{Theo}
Vipoxin	2.90	2.26	1.28
Vipoxin-Ca^{2+}	2.60	2.26	1.15
Vipoxin-elaidoylamide	3.70	2.27	1.63
Vipoxin-vitamin E	3.84	2.27	1.69
His-48 PLA_2-dimer	2.70	2.26	1.19
His-48 PLA_2-dimer-Ca^{2+}	2.42	2.26	1.07
His-48 PLA_2-dimer-elaidoylamide	2.86	2.27	1.26
His-48 PLA_2-dimer-vitamin E	2.89	2.27	1.27
His-48 PLA_2-monomer	2.18	1.80	1.21
His-48 PLA_2-monomer-Ca^{2+}	1.93	1.80	1.07
His-48 PLA_2-monomer-elaidoylamide	2.43	1.81	1.34
His-48 PLA_2-monomer-vitamin E	2.38	1.81	1.32
Gln-48 PLA_2-dimer	2.85	2.26	1.26
Gln-48 PLA_2-dimer-Ca^{2+}	2.53	2.26	1.12

FIGURE 2 Calcium-free conformation of vipoxin (Protein Data Bank, code 1jlt). The surface potentials are shown, the positive in blue and the negative in red.

curves were obtained for the separated subunits. In practice, the globular macromolecules in solution are nonspherical and hydrated. The measured radius, R_H, is influenced by the asymmetric shape and hydration of the protein molecule. The theoretical hydrodynamic radius R_H^{Theo} of the toxin represents the radius of a hypothetical hard sphere which diffuses with the same speed and was calculated to be 2.26 nm for a molecular mass of 27 kDa. From the high resolution x-ray structure at 1.4 Å (Banumathi et al., 2001), vipoxin can be described as an oblate ellipsoid (Fig. 2). For equal volumes the surface area of the ellipsoid is greater than that of the sphere, and the respective frictional coefficient is larger than f^{Theo}. As the ratio f/f^{Theo} is equal to R_H/R_H^{Theo}, the DLS-measured hydrodynamic radius is larger than that of the hypothetical sphere. In the presence of calcium, R_H decreases to 2.60 ± 0.05 nm, which suggests a metal ion binding-induced conformational change. The respective frictional coefficient and the Perrin ratio also decrease. For constant mass this means less deviation of the molecule from the spherical shape. After the binding of Ca^{2+} the neurotoxin becomes more compact and more symmetric. This is the catalytically active form of vipoxin. Computer graphic

studies using our own coordinates at 1.4 Å resolution showed that both components of the toxin from the venom of *V. a. meridionalis* contain conformationally flexible calcium-binding loops. In the absence of bound Ca^{2+}, the local conformation is stabilized by a salt bridge between Lys-69 of one subunit and Asp-49 of the other; i.e., the ε-NH_2 group of Lys-69 plays the role of the metal ion (Fig. 3 *a*). Comparison of the Ca^{2+}-free structure of the vipoxin PLA_2 (Protein Data Bank; code 1 jlt) and that of the *Naja naja atra* calcium-bound PLA_2 (Protein Data Bank; code 1 pob) (Fig. 3 *b*), taken as a representative of a calcium-dependent PLA2 with bound metal ion, shows that the conformation of the loop (residues 25–33) is changed. The DLS measurements revealed that the binding of calcium changes not only the conformation of the calcium binding loop but also the structure of the whole toxin which we have observed as a change of the shape of the neurotoxic complex.

The hydrodynamic studies show that the separated toxic His-48 PLA_2 exists as a monomer with R_H of 2.18 ± 0.07 nm up to a protein concentration of 5 mg/ml (Table 1). The theoretical hydrodynamic radius was calculated to be 1.80 nm; i.e., the nonspherical shape of this protein leads to an

FIGURE 1 Hydrodynamic radii of vipoxin obtained at pH 7.2 and 20°C as a function of the protein concentration.
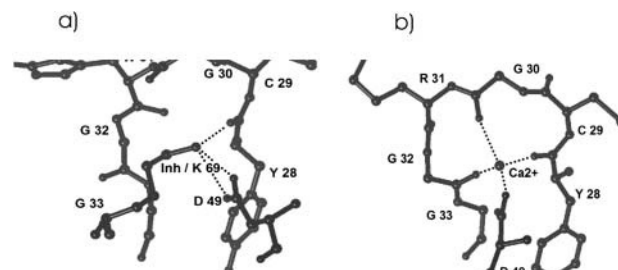
FIGURE 3 Comparison of the calcium binding loop region of two PLA_2 structures: (*a*) calcium-free conformation of the vipoxin PLA_2 (Protein Data Bank, code 1jlt) and (*b*) calcium-bounded conformation of the *Naja naja atra* PLA_2 (Protein Data Bank, code 1 pob).

increase in the radius. R_H decreases to 1.93 ± 0.05 nm upon binding of Ca^{2+}, and the Perrin factor becomes 1.07, which means a shape near to that of a sphere for which the ratio f/f^{Theo} is 1.00. At concentrations higher than 5 mg/ml, the vipoxin toxic PLA_2 exists in a dimeric form (Table 1). In the presence of calcium, the hydrodynamic radius decreases from 2.70 to 2.42 nm and the Perrin factor from 1.19 to 1.07. The Ca^{2+}-bound forms of either the monomer or the dimer of the vipoxin His-48 PLA_2 have a close to spherical shape and are more compact and symmetric in comparison to the Ca^{2+}-free protein.

The DLS results show that the separated nontoxic Gln-48 PLA_2 exists in solution as a dimer, even at low protein concentrations. It possesses a Stokes radius equal to 2.85 ± 0.06 nm, which decreases to 2.53 ± 0.08 nm upon binding of calcium (Table 1). The Perrin ratio changes from 1.26 to 1.12 suggesting that the Ca^{2+}-bound protein has a hydrodynamic shape closer to a sphere. The metal ion-bound form of the chaperone subunit is more compact, as it was observed also for vipoxin and the toxic PLA_2.

Interaction of the neurotoxin vipoxin with the brain phospholipid phosphatidylcholine and the substrate analog 1-palmitoyl-*sn*-glycero-3-phosphocholine; probable mechanism of action in the presence of aggregated substrates

Phospholipids are natural substrates of PLA_2s. The interaction of vipoxin with phosphatidylcholine (PCh), the major structural phospholipid of the brain, was investigated by DLS measurements at pH 7.2. The activity of secreted PLA_2s toward aggregated/micellar substrates is several times higher than that on monomolecular dispersed substrates, which is known as "interfacial activation" (Warwicker, 1997). The neurotoxin was added to a solution of PCh for which DLS measurements showed the presence of aggregated/micellar particles. Immediately after that a new peak corresponding to particles with R_H of 2.16 ± 0.07 nm, a hydrodynamic radius typical for the separated monomeric subunits of vipoxin, was observed. Several minutes later only aggregates of these particles with $R_H = 5.98 ± 0.09$ nm were registered. Similar dissociation of the subunits was observed also when vipoxin was added to a solution containing aggregates of 1-palmitoyl-*sn*-glycero-3-phosphocholine. Five minutes after adding the neurotoxin to the aggregated substrate analog, a new peak was observed and existed for a period of 15 min with $R_H = 2.19 ± 0.07$ nm. After that, this peak was transformed into a new one with $R_H = 5.92 ± 0.08$ nm. Most probably, the neurotoxic complex dissociates in the presence of micellar substrates. It can be supposed that only the toxic His-48 PLA_2 binds to the substrate. The second component, the nontoxic and enzymatically inactive Gln-48 PLA_2, acts as a chaperone subunit to avoid nonspecific binding of the toxic enzyme and remains in solution. This hypothesis explains the absence of

enzymatic and pharmacological activities of the nontoxic subunit because there is not a biological necessity for them. The separated Gln-48 PLA_2 easily aggregates, as shown by DLS experiments.

Interaction of neurotoxin vipoxin and its components with synthetic and natural inhibitors

Secreted PLA_2s hydrolyze phospholipids of the cell membranes producing mediators of inflammatory diseases (Yedgar et al., 2000). For this reason the protection of cell membranes from the phospholipolytic action of PLA_2s is of medical importance and can be used for treatment of inflammatory processes. The results from the inhibition of a Viperidae snake venom PLA_2 can be used also for the human lipolytic enzymes because they have similar/identical active sites. Human synovial fluid PLA_2s and the related enzymes from viper venom belong to the same group, II A, of secreted phospholipase A_2s (Six and Dennis, 2000).

We have used DLS measurements to study the interaction of vipoxin and its components with a synthetic (elaidoylamide) and a natural (vitamin E) inhibitor. Elaidoylamide (the amide of *trans*-9-octadecenoic acid) inhibits the phospholipase A_2 activity of both vipoxin and its separated toxic subunit. The effective Stokes radius of the neurotoxin complexed to the synthetic inhibitor increases by 8 Å from the one determined for the free vipoxin (Table 1). The Perrin ratio also increases from 1.29 to 1.63, which indicates different asymmetry for the inhibited toxin. The shape of the complex can be assigned as that of prolate ellipsoid because a disk shape with a Perrin factor greater than 1.5 would have an unrealistically minor axis (Cantor and Schimmel, 1980).

Naturally occurring compounds are prospective as drugs because they are more tolerant to the living organisms. Recently, we have described the inhibitory effect of vitamin E (*d*-α-tocopherol) toward vipoxin and its isolated toxic His-48 PLA_2 (Nötzel et al., 2002). This compound is a physiological membrane lipid antioxidant. The binding of vitamin E to vipoxin results in considerable change in the native toxin structure and the hydrodynamic radius increases by 9–10 Å (Table 1). The ratio of frictional coefficients also increases from 1.29 to 1.69, which indicates considerable change in the asymmetry of the molecule. This ratio suggests that the enzyme-inhibitor complex can be described as prolate ellipsoid, for reasons discussed before.

The same tendency of increase of the hydrodynamic radius and the Perrin factor was observed also for the interaction of either monomeric or dimeric His-48 PLA_2 with both inhibitors, but the effect on the shape of the protein molecules was not so pronounced (Table 1). In all cases the inhibition of monomeric, heterodimeric, or homodimeric neurotoxins was connected with conformational changes observed as changes in the shape of the macromolecules, which becomes more asymmetrical after the complex formation. Recently we have crystallized the homodimer of

the vipoxin PLA$_2$ in the presence of elaidoylamide, and the preliminary x-ray data show that the toxin is complexed to the inhibitor (Georgieva et al., 2003).

Gln-48 PLA$_2$ exists in solution as a dimer even at low protein concentration. The hydrodynamic radius of the dimer is similar to that of vipoxin (Table 1). Again, in the presence of calcium the R_H value and the Perrin ratio decreases, which suggests more spherical shape of the Ca^{2+}-bound form of the chaperone subunit. We found no evidence for a complex formation between the dimeric Gln-48 PLA$_2$ and the two inhibitors. Most probably this is due to steric hindrances for the inhibitors to bind the substrate-binding channel, imposed by the dimeric structure of the chaperone subunit.

Hydrophobic stabilization of the neurotoxic complex

The role of hydrophobic interactions for the structural integrity of vipoxin was investigated by CD measurements using a series of ureas with increasing hydrophobicity. At pH 7.0 the neurotoxin exhibits a great resistance to unfolding in the presence of urea. A period of 24 h incubation at 7 M concentration of this reagent did not change significantly the CD spectrum (Fig. 4). The increase of incubation time to 42 h had no additional effect. The influence of urea series of hydrophobic reagents on the secondary structure of the toxin was followed by the changes in ellipticity at 221 nm, which is connected mainly with the α-helical structure. The high resolution x-ray structure of vipoxin (Banumathi et al., 2001) shows that ~50% of the polypeptide chain of each subunit is folded into α-helices. The results of the CD measurements are presented in Fig. 5. It is evident that urea has no
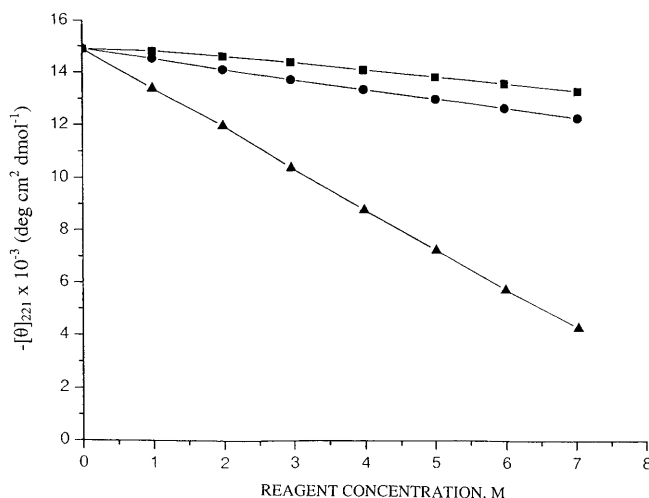
FIGURE 5 Dependence of the ellipticity at 221 nm on the reagent concentration after 24 h incubation of vipoxin in the presence of urea (■), methylurea (●) and ethylurea (▲).

significant effect on the protein structure. An increasing effectiveness of the more hydrophobic members of the series in the order ethylurea > methylurea > urea was observed. In the presence of methylurea slight changes in the ellipticity at 221 nm suggest that the folded regions of vipoxin remain largely unaltered. Considerable unfolding was observed with the more hydrophobic and more effective ethylurea. However, even at 7 M concentration of this reagent the changes were not complete after 24 h (Fig. 5). The trend of effectiveness of the ureas as unfolding reagents suggests a strong hydrophobic stabilization of the neurotoxin.

Vipoxin is a complex of two oppositely charged polypeptide chains, a basic and toxic subunit with a pI of 10.4 and an acidic and nontoxic component with a pI of 4.6 (Tchorbanov et al., 1978). For this reason it was assumed that electrostatic forces are mainly responsible for the association of the subunits. The results presented here demonstrate that aside from the electrostatic interactions, hydrophobic forces are very important for the structural integrity of the neurotoxin. The dissociation of the vipoxin heterodimer requires the simultaneous influence of low pH and a high urea concentration for disruption of the intersubunit contacts. Only at pH close to 4.0 and in the presence of 7 M urea was a dissociation of the complex to subunits observed. It is important to note that at these conditions both components of the neurotoxin can be separated and isolated with a preserved secondary structure and pharmacological activity. In conclusion, hydrophobic interactions stabilize not only the folded regions in vipoxin but also the intersubunit region.
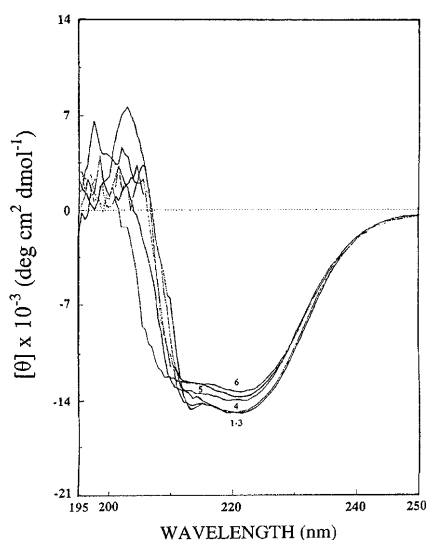

FIGURE 4 Circular dichroism spectra at pH 7.0 of vipoxin after 24 h incubation in the presence of 0–2 M urea (1–3), 5 M urea (4), 6 M urea (5), and 7 M urea (6).

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