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Refolding of Reduced Short Neurotoxins: Circular Dichroism Analysis†

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ABSTRACT: The four disulfide bonds of nine homologous short curare-like polypeptides are cleaved by reduced dithiothreitol. Air oxidation renaturations of the reduced compounds are followed by far-ultraviolet circular dichroism analysis, and the kinetics of refolding thus determined are compared. They indicate that three toxins refold 4–10 times more slowly than the six others. It is shown that a significant difference between the refolding kinetics still subsists when renaturations are made in the presence of various concentrations of thiol–disulfide exchange reagents or at various pH values. From an examination of the toxin sequences, it is proposed that a single additional amino acid insertion is responsible for the difference in the observed kinetics. This proposal is supported by temperature studies of renaturation kinetics.

The refolding of reduced polypeptides is known to occur spontaneously as originally demonstrated by Anfinsen and co-workers (Sela et al., 1957; Haber & Anfinsen, 1962). Such a spontaneity strongly suggests that the required information for refolding is contained in the amino acid sequence of the polypeptide chain. Although there is no definitive explanation of the mechanism by which a reduced molecule refolds to its native structure, evidence is now accumulating which indicates that interactions between nearest-neighbor side chains play an important role in directing the refolding of a polypeptide chain (Lewis et al., 1971; Zimmerman & Scheraga, 1977; Go & Taketomi, 1978; Matheson & Scheraga, 1978). Such interactions are thought to trigger the formation of nucleation sites, as summarized by Anfinsen & Scheraga (1975). Obviously, longer range interactions are also involved in the refolding process but probably to a lesser extent than the short-range interactions (Anfinsen & Scheraga, 1975).

In order to gain a clear understanding of the refolding process, it is important to establish which amino acids define the nucleation sites. Selective modifications and the preparation of synthetic analogues of the polypeptide chain should, in principle, allow an experimental examination of the influence of the constituent amino acids upon the refolding process. For instance, in the particular case of ribonuclease, it has been shown that specific iodination of Tyr-115 prevents the refolding of the reduced molecule (Friedman et al., 1966). Unfortunately, the synthesis of protein analogues is a prohibitively difficult task. However, groups of natural polypeptides possessing homologous sequences do exist. Such protein families have the potential of being useful tools for the study of the relationships between amino acid sequence and the ability of a chain to refold.

The short neurotoxins isolated from snake venoms constitute a large group of homologous polypeptides of 60, 61, or 62 residues, cross-linked by four disulfide bonds (Lee, 1972; Yang, 1974). These molecules possess a curare-like activity, specifically blocking the nicotinic acetylcholine receptor. Extensive data are now available which highlight the close structural (Dufton & Hider, 1977; Ménez et al., 1978) and biological (Ishikawa et al., 1977) homologies of these polypeptides. In addition, the X-ray structure of one of the toxins has been elucidated in two different laboratories (Tsernoglou & Petsko, 1976; Low et al., 1976). Thus, this polypeptide group would appear to be particularly well suited for a comparative refolding study.

In the present work, nine reduced short neurotoxins are subjected to air oxidation at 37 °C and pH 7, and the kinetics of refolding, measured by the variations of optical activity, are determined. Under these conditions, it appears that the neurotoxins do not all refold at the same rate. When renaturation experiments are carried out at different pH values or in the presence of thiol–disulfide exchange reagents, a significant difference of behavior still subsists. In order to establish the origin of this phenomenon, the kinetics of the air

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oxidation refolding process are measured over a wide temperature range.

Materials and Methods

Toxin α[1] from *Naja nigricollis* was purified from venom (Institut Pasteur, Paris) according to the method described by Fryklund & Eaker (1975). Erabutoxins a and b were prepared from dried venom glands of *Laticauda semifasciata* (kindly supplied by Professor N. Tamiya), according to the method of Tamiya & Abe (1972). In some cases, erabutoxins were prepared and donated to us by Professor N. Tamiya. Cobrotoxin from *Naja naja atra* was a kind gift of Dr. K. Hayashi (Kyoto). Toxin d from *Naja melanoleuca* was kindly provided by Dr. E. Barnard (London). Toxin IV from *Hemachatus haemachatus* was a generous gift from Dr. D. Eaker (Uppsala). Toxin α from *Naja naja philippinensis* was kindly provided by Dr. J. P. Bargetzi (Genève), and toxin a from *Astrotia stokesii* and toxin b from *Aipysurus laevis* were generously donated by Professor N. Tamiya. Reduced and oxidized dithiothreitol and oxidized glutathione are from Sigma Chemical Co. Concentrations of toxins were determined on the basis of their molecular absorbance at 278 nm, as previously reported (Ménez et al., 1978). Absorption spectra were recorded with a DRM10 Zeiss spectrophotometer. Circular dichroism measurements were made at 22 °C with a Jouan CD II dichrograph. Dichroic ellipticity [Θ] is expressed in deg cm^2 dmol^{-1}, in moles of protein. The mean residue ellipticity has not been used because throughout the wavelength range used the toxin CD spectra display significant and variable contributions due to aromatic chromophores, making unsuitable, in this particular case, the concept of mean residue ellipticity (e.g., in moles of peptides).

Reduction of Toxins. Reduction of the toxin (2×10^{-4} M) disulfide bonds was achieved by the addition of an excess of reduced dithiothreitol (4×10^{-2} M) in 8 M urea and 0.1 M Tris-HCl, pH 8.5, at room temperature, in the dark and under an N_2 atmosphere. Although disulfide bonds were disrupted within a few seconds, the mixtures were left for $\sim$1 h in order to ensure completion of the reaction. After removal of the reagent by gel filtration (Bio-Gel P-2 equilibrated in 10% acetic acid), the peptide was lyophilized to dryness and stored at 4 °C. The sulfhydryl content was measured by the Ellman method (Ellman, 1959).

Toxin Renaturation. The process of air oxidation of the reduced toxins was performed at 37 °C in the dark, at pH 7, in the presence of 0.1 M phosphate and 0.1 M NaCl. The protein concentration was always 50 μM. At different times during the renaturation process, an aliquot was taken for circular dichroic measurements. The cell path length was 0.2 cm. The CD spectra were routinely recorded between 250 and 220 nm, for 12 min. A base line was recorded before and after each experiment. Variations of ellipticity were expressed as a percentage by

$$(\Theta_t - \Theta_\infty)/(\Theta_r - \Theta_\infty) \times 100$$

where Θ_t, Θ_∞, and Θ_r are ellipticities at time t, at infinite time, and in the reduced state, respectively. Ellipticity at infinite time was estimated from ellipticity of an equivalent concentration of native toxin. Actually, when kinetics of refolding were rapid enough to be entirely analyzed, it was observed that the experimental values obtained at infinite time were equal to 90–95% of native ellipticity but, when kinetics of refolding were slow, the incubation was followed until 65–70% of native ellipticity was recovered and then infinite time renaturation ellipticity was checked by extrapolation. When changing the pH of the solutions, 0.1 M phosphate buffer was used for pH comprised between 6.5 and 7.5 whereas 0.1 M Tris-HCl buffer was used for pH above 7.5. In some instances, oxidized dithiothreitol or glutathione was added to the renaturation buffer. For experiments made with the former reagent, CD spectra were directly recorded in the presence of the reagent whereas with glutathione, the renaturation mixture was passed rapidly ($\sim$10 min) through a Bio-Gel P-2 column (10 × 1 cm) equilibrated in a 0.1 M phosphate buffer, pH 6.5, in order to remove the reagent prior to recording the spectra. It must be noticed that at this pH and in the absence of oxidized mercaptan reagents, the rate of CD recovery becomes very small for all proteins (see Results). All comparative experiments were run simultaneously.

Results

Choice of Neurotoxins. Toxins isolated from eight snake species belonging to both *Elapinae* and *Hydrophiinae* subfamilies are used in this study (Table I). The polypeptide chain length varies from 60 to 62 amino acids, and residue changes can greatly differ from one toxin to another; for instance, with cobrotoxin and erabutoxin b the residue difference reaches 45% whereas with erabutoxin a and erabutoxin b it is less than 2%.

Circular Dichroism Spectra of Native and Reduced Neurotoxins. CD spectra were recorded between 270 and 210 nm for both native and reduced toxins in aqueous solution (Figure 1). In their native state, the toxins display a broad positive dichroic band at 227–229 nm and a negative one at 216 nm. It was previously reported that such a shape is a common characteristic of the CD spectra of all neurotoxins so far studied (Ménez et al., 1978), indicating that these polypeptides possess a close overall secondary structure. The negative band at 216 nm, together with a very large positive peak (not shown here) observable at 197 nm (Ménez et al., 1976; Chen et al., 1977; Ménez et al., 1978), indicates that the toxin structure is largely composed of β sheet (Greenfield & Fasman, 1969) together with β turns (Chen et al., 1977). The positive band at 227–229 nm contains a contribution from both the invariant chromophores Tyr-25 and Trp-29 (Ménez et al., 1976, 1978). It should be noted that in the presence of additional phenolic or indolic groups in the toxin sequences, the ellipticity of this band is further increased. This is readily observed both in the case of cobrotoxin (one supplementary tyrosine at the 35th position) and in that of toxin α from *N. naja philippinensis* (one additional tryptophan at the 28th position). It was therefore concluded that this positive band reflects the molecular structure localized in the vicinity of tryptophan and tyrosine chromophores of the toxins (Ménez et al., 1978).

Once the four disulfide bonds are reduced, the CD spectra of the toxins become markedly different from those observed for native toxins (Figure 1). The strong negative ellipticity ($[\Theta]_{227-229}$ is comprised of between -11×10^4 and -13×10^4 deg cm^2 dmol^{-1}), together with a minimal intensity value around 200 nm (inset of Figure 1), is indicative of a random polypeptide backbone (Tiffany & Krimm, 1969). However, a slight negative shoulder is observable at 220–223 nm which might correspond to the presence of some residual structure in the reduced polypeptides. Indeed, the CD spectra of a set of disulfide-free polypeptides in 6 M guanidine hydrochloride [shaded area of Figure 1, from Cortijo et al. (1973)] do not actually exhibit such a negative shoulder. However, this difference may be also a manifestation of the solvation phenomena (Privalov, 1979).

[1] Abbreviations used: erabutoxin b or Eb, erabutoxin b from *Laticauda semifasciata*; toxin α, toxin α from *Naja nigricollis*.

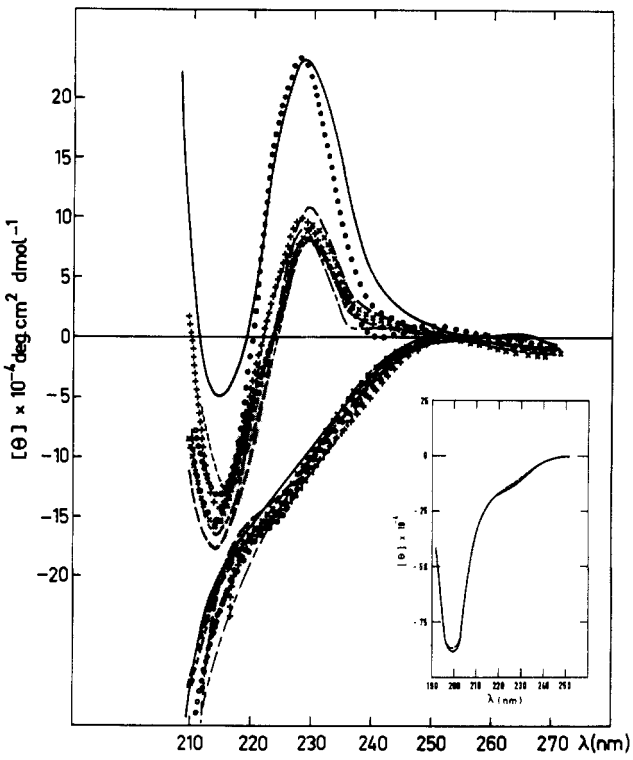

FIGURE 1: Circular dichroism spectra of nine native and reduced short neurotoxins in the far-ultraviolet region. The native toxins were dissolved in pure water whereas reduced polypeptides were in a 10^{-2} M phosphate buffer, pH 7. For native toxins the concentrations were calculated from their molar absorbance at 278 nm as described (Ménez et al., 1978). For reduced toxins, the molar absorbance was experimentally determined for two toxins (erabutoxin b and toxin α from *N. nigricollis*) and found to be 6900. Since this value corresponded nicely to the actual sum of the molar absorbance of individual aromatic chromophores (Beaven & Holiday, 1952), it was assumed that a similar situation held for other reduced polypeptides. In all cases the protein concentration was close to 5×10^{-5} M and the cell path length was 0.2 cm. Shaded area corresponds to the range of ellipticities observed for nine disulfide-free proteins in 6 M guanidine hydrochloride (Cortijo et al., 1973). (—) Cobrotoxin; (···) toxin α from *N. naja philippinensis*; (– – –) *A. stokesii* toxin a; (×) *A. laevis* toxin b; (+) erabutoxin a; (- - -) *H. haemachatus* toxin IV; (○) *N. melanoleuca* toxin d; (– · –) erabutoxin b and toxin α from *N. nigricollis*. Inset shows the CD spectra of reduced toxin α from *N. nigricollis* (- - -) and reduced erabutoxin b (—).

Rates of Ellipticity Regain during Reoxidation of Reduced Toxins. Since the neurotoxins of this study display similar configurations in their native and reduced states, kinetics of structural renaturation were compared by measuring the variations of ellipticity at a constant wavelength between these two states. The 227–229-nm range was chosen for most experiments because of the large ellipticity change associated with this spectral region. Accurate measurements (±5%) have thus been routinely performed with different toxin batches and with no sign of aggregation provided the pH is kept below 8.5. The data obtained at pH 7.0 for three representative toxins are shown as semilogarithmic plots (Figure 2). Similar results have been obtained at other wavelengths between 270 and 210 nm and for the other toxins. The reactions followed biphasic first-order kinetics, the second phase only becoming apparent for erabutoxin b after 40 h. A short lag period is observable for most toxins, but its significance is not elucidated.

Clearly, erabutoxin b renatures more slowly than both toxin α from *N. nigricollis* and toxin a from *A. stokesii*; for instance, after 10 h, 80–90% of the total ellipticity is recovered for the two latter toxins while it is only 20% for the former. As stated above, some residual structure might subsist in reduced po-

Table 1: Chart Alignment of Nine Snake Toxin Amino Acid Sequences[a]

neurotoxins	sequences	no. of aa	snake subfamily	ref
L. semifasciata erabutoxin a	RICFNQHSSQPQTTKTCPGESSCYNKQWSDFRGTIIERGCGCPTVKPGIKLSCCESEVCNN	62	(H)	Sato & Tamiya (1971)
L. semifasciata erabutoxin b	RICFNQHSSQPQTTKTCPSGESSCYHKQWSDFRGTIIERGCGCPTVKPGIKLSCCESEVCNN	62	(H)	Sato & Tamiya (1971)
N. naja atra cobrotoxin	LECHNQQSSQTPTTTGCSGGETNCYKKRWRDHRGYRTERGCGCPSVKNGIEINCCTTDRCNN	62	(E)	Yang et al. (1969)
N. nigricollis toxin α	LECHNQQSSQPPTTKTCP●GETNCYKKVWRDHRGTIIERGCGCPTVKPGIKLNCCTTDKCNN	61	(E)	Eaker & Porath (1967)
N. melanoleuca toxin d	MECHNQQSSQPTTTKTCP●GETNCYKKQWSDHRGTIIERGCGCPSVKKGVKINCCTTDRCNN	61	(E)	Botes (1972)
H. haemachatus toxin IV	LECHNQQSSQTPTTQTCP●GETNCYKKQWSDHRGSRTERGCGCPTVKPGIKLKCCTTDRCNK	61	(E)	Botes & Strydom (1969)
N. naja philippinensis toxin α	LECHNQQSSQAPTTKTCS●GETNCYKKWWSDHRGTIIERGCGCPKVKPGVKLNCCKTDRCNN	61	(E)	Hauert et al. (1974)
A. stokesii toxin a	MTCCNQQSSQPKTTTNCA●C●NSCYKKTWSDHRGTIIERGCGCPQVKSGIKLECCHTNECNN	60	(H)	Maeda & Tamiya (1978)
A. laevis toxin b	LTCCNQQSSQPKTTTDCA●●DNSCYKMTWRDHRGTRIERGCGCPQVKPGIKLECCKTNECNN	60	(H)	Maeda & Tamiya (1976)

[a] The one-letter notation is used [IUPAC–IUB Commission on Biological Nomenclature (1968)]. (E) and (H) indicate that the snake belongs to the *Elapinae* and *Hydrophiinae* subfamilies, respectively. (●) indicates a deletion in the toxin sequence.

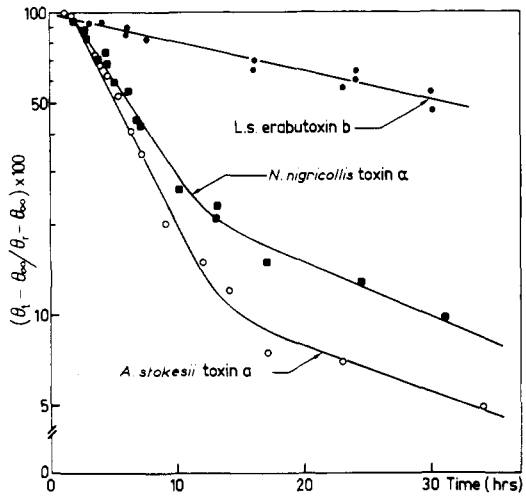

FIGURE 2: Kinetics of ellipticity recovery for three reduced toxins in the presence of just air. Reduced toxins (8 ± 0.1 sulfhydryl groups of *L. semifasciata* erabutoxin b and *N. nigricollis* toxin α and 8.6 SH groups for *A. stokesii* toxin a) were dissolved in 0.1 M phosphate buffer, pH 7, and 0.1 M NaCl at 37 °C at a final concentration of 5×10^{-5} M. At the indicated times, aliquots were taken and submitted to circular dichroism analysis. Θ_t is ellipticity at 228 nm at the indicated time (see the text); Θ_∞ and Θ_r are ellipticities at infinite time and in the reduced state, respectively.

Table II: Comparison of the Initial Rate Constants of Structural Recovery for Nine Different Short Neurotoxins

neurotoxins	$k_i \times 10^5$ (s^{-1})
L. semifasciata erabutoxin a	0.8
L. semifasciata erabutoxin b	0.7
N. naja atra cobrotoxin	0.6
N. nigricollis toxin α	4.0
N. melanoleuca toxin b	2.9
H. haemachatus toxin IV	6.4
N. naja philippinensis toxin α	6.4
A. stokesii toxin a	5.6
A. laevis toxin b	5.5

lypeptide chains in aqueous solution. For this reason, renaturation experiments have been repeated for both erabutoxin b and toxin α by incubating a sample of concentrated reduced toxin in 8 M urea for 2 h prior to initiating the refolding process by diluting the sample with the renaturation buffer. The data obtained under these conditions are similar to those obtained by directly solubilizing the reduced toxin in the renaturation buffer excluding the influence of any residual structure on the rate of renaturation. It should be noticed also that incubation of toxin α with 1 mM EDTA failed to induce any appreciable changes in the observed kinetics, indicating that the possible presence of metal ions in the solution does not alter significantly the renaturation rate.

The rate constants for the initial renaturation phase of nine neurotoxins (mean value obtained from four to six experiments) are represented in Table II. Two groups of values are readily distinguished. Those obtained for erabutoxin a, erabutoxin b, and cobrotoxin possess k_i values around 0.7×10^{-5} s^{-1} whereas those obtained for the six other toxins have k_i values ranging between 2.9×10^{-5} and 6.4×10^{-5} s^{-1}. The slope of the second phase which corresponds to a limiting step of the renaturation process of all toxins could not be consistently determined and was not considered further.

pH Dependence of Refolding Kinetics. The pH dependence of the initial rate constants of short neurotoxin renaturations was analyzed for the three following polypeptides: erabutoxin b, toxin α from *N. nigricollis*, and toxin a from *A. stokesii*. The data obtained between pH 6.4 and pH 8.2 are shown in

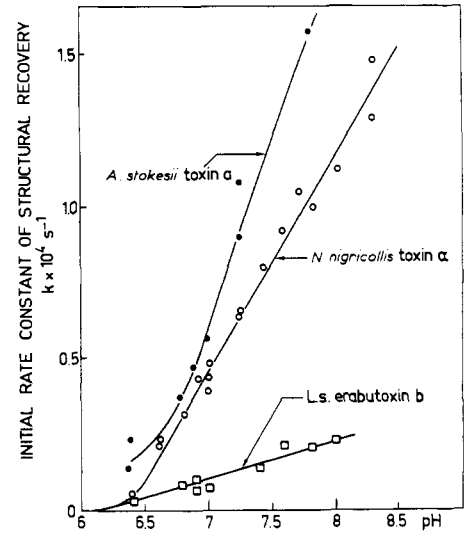

FIGURE 3: pH dependence of initial rates of structural refolding for three toxins. The experimental conditions are similar to those described in the legend of Figure 2 except that the pH of the renaturation buffer is varied.

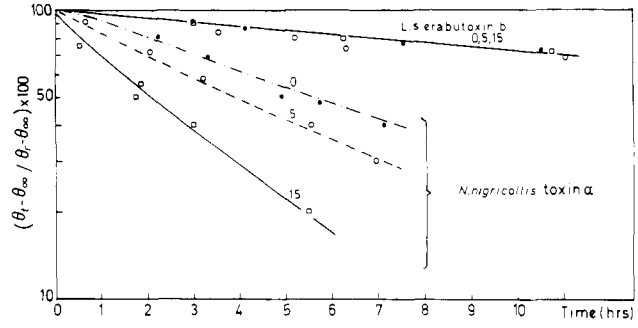

FIGURE 4: Kinetics of renaturation of two reduced toxins with oxidized dithiothreitol as disulfide reagent. The experimental conditions are similar to those reported in the legend of Figure 2 except that the renaturation buffer contains indicated mM concentrations of oxidized dithiothreitol.

Figure 3. Above pH 8.5 some turbidity sometimes occurs in the solution. Throughout the pH range studied, the rate of renaturation varies linearly for each toxin. However, the variation of the rate of erabutoxin b always remains slower than that observed for the two other toxins. It is therefore concluded that the difference of kinetics previously observed between "slow" toxins on one hand and "rapid" toxins on the other hand is independent of pH changes. That the rate of renaturation increases with pH is probably related to the increased degree of ionization of sulfhydryl groups which consecutively favored the basic thiol–disulfide exchange reaction (Jocelyn, 1972).

Effect of Oxidized Mercaptan Reagents. It is well established now that oxidized mercaptan reagents accelerate the formation of protein disulfide bonds (Saxena & Wetlaufer, 1970). At slightly alkaline pH, cysteine residues and the reagent participate in a thiol–disulfide exchange reaction to reach a thermodynamic equilibrium (Lumper & Zahn, 1965). The effect of the addition of oxidized dithiothreitol and glutathione upon the kinetics of refolding has been analyzed with both erabutoxin b and *N. nigricollis* toxin α. Provided the concentration of oxidized dithiothreitol is kept below 15 mM, circular dichroism spectra can be directly recorded in the presence of this reagent. The variations of ellipticity observed at 228 nm under these conditions are shown in Figure 4. Surprisingly, the presence of dithiothreitol accelerates the rate of refolding for toxin α while it is virtually without effect on

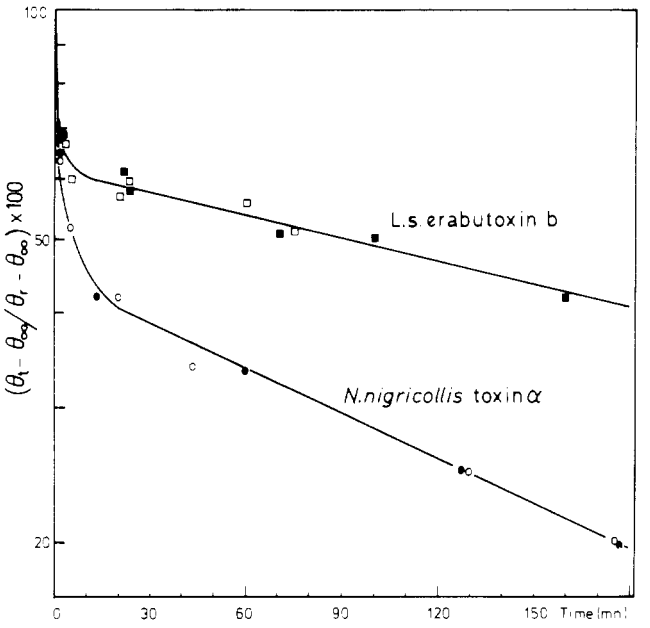

FIGURE 5: Kinetics of renaturation of two reduced toxins with oxidized glutathione as disulfide reagent. The experimental conditions are similar to those reported in the legend of Figure 2 except that the renaturation buffer contains 1 mM concentration of glutathione. For both toxins, ellipticity at infinite time was observed to be more than 85% of native ellipticity. Filled and open symbols indicate two different sets of experiments: (□ and ■) erabutoxin b; (○ and ●) toxin α from *N. nigricollis*.

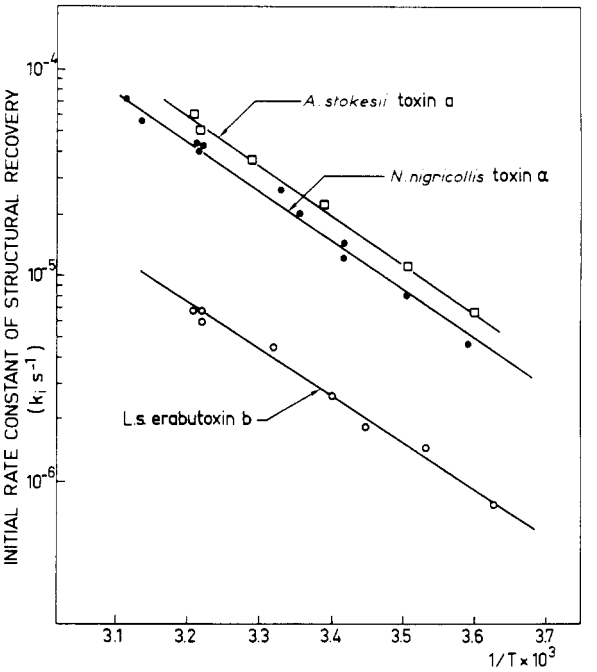

FIGURE 6: Arrhenius plot of the refolding kinetics of three short neurotoxins. All experiments were at pH 7.0 in 0.1 M phosphate buffer containing 0.1 M NaCl. The straight lines were calculated by the method of least squares.

the refolding of erabutoxin b. Thus, the difference in refolding kinetics observed above between the two toxins appears to be even more pronounced in the presence of the reagent. Oxidized glutathione displays a perturbating ellipticity throughout the useful wavelength range. As a result, the reagent must be removed from the toxin solution before CD spectra can be recorded. In the presence of 1 mM glutathione, the refolding process proceeds much more rapidly (Figure 5). This is consistent with oxidized glutathione having a greater redox potential than oxidized dithiothreitol. In both cases the reaction is distinctly biphasic. The first phase is very rapid (half-time of 2–3 min) and is followed by a second one which indicates a first-order reaction. In the latter case, the rate of appearance of ellipticity indicates a rate constant for refolding of 3.8×10^{-5} and 7.6×10^{-5} s^{-1} for erabutoxin b and toxin α, respectively. Then the difference between the refolding velocities of the two toxins is much less pronounced in the presence of glutathione, but still, it appears that the renaturation process of toxin α proceeds more rapidly.

Temperature Dependence. Renaturation of three reduced toxins (one slow toxin and two rapid toxins) has been undertaken over the temperature range 4–37 °C. Arrhenius plots of the data obtained are shown in Figure 6. The resulting slopes are, within the limits of experimental error, identical, indicating that a constant enthalpy of activation exists for the refolding process of the three toxins. $\Delta H^{\ddagger}$ was found to be 10 ± 0.5 kcal mol^{-1} (Table III). Thus, differences between the free energies of activation of the folding process of the slow and the rapid toxins seem to be derived from an entropic origin.

Table III: Activation Enthalpy, Entropy, and Free Energy for the Unfolded to Refolded Transition of Three Short Neurotoxins

neurotoxins	$\Delta H^{\ddagger}$ (kcal mol^{-1})	$\Delta S^{\ddagger}$ (eu)	$\Delta G^{\ddagger}$ (kcal mol^{-1})
L. semifasciata erabutoxin b	10.4	−48.9	25.6
N. nigricollis toxin α	10.2	−43.3	23.6
A. stokesii toxin a	10.3	−44.3	24.1

Discussion

That short neurotoxins possess a similar native conformation is well documented (Dufton & Hider, 1977; Ménez et al., 1978; Visser & Louw, 1978). The structure is largely constituted of β sheet and β turn as judged from crystallographic and spectroscopic data (Tsernoglou & Petsko, 1976; Low et al., 1976; Ménez et al., 1976; Chen et al., 1977). After reduction of the four disulfide bonds, this structure collapses and the polypeptide chain adopts what is essentially an unfolded state. However, it was previously shown that under appropriate conditions, the reduced neurotoxins spontaneously regain part of their native biological potency as, for instance, in the case of cobrotoxin (Yang, 1967) and toxin α from *N. nigricollis* (Bouet et al., 1977).

From an analysis of structural recovery expressed in terms of variations of dichroic ellipticity, it has been demonstrated that reduced short neurotoxins refold spontaneously at different rates (Table II). Two sea snake toxins, erabutoxin a and erabutoxin b, together with one land snake toxin, cobrotoxin, renature 4–10 times more slowly than six other homologous toxins. This phenomenon persists under various experimental conditions. Thus, the renaturation process can be accelerated via an increase of the rate of disulfide bond formation either by increasing the degree of ionization of thiol groups or by the addition of thiol–disulfide exchange reagents in the medium. In both cases, a significant difference between the refolding kinetics of the slow and the rapid toxins is observed. Even, in the case of the addition of oxidized dithiothreitol, the difference is further increased. This suggests that the appropriate cysteine residues are poised in a more favorable orientation for disulfide bond formation in the case of the rapid toxins than in the corresponding situation for that of the slow toxins. Therefore, it is likely that these two toxin groups are distinguishable by some peculiarity which differentiates the folding tendency of the polypeptide chains. Clearly, it is important to establish the origin of this difference.

It is generally accepted that for single-chain proteins, all the information necessary for folding is contained in the sequence (Anfinsen & Scheraga, 1975; Nemethy & Scheraga, 1977; Creighton, 1978). However, none of the amino acid substitutions occurring between the toxin sequences (Table I) appears to account for the observed phenomenon as large differences occur within both the slow and rapid toxin groups. Thus, comparison of the rapid toxin sequences reveals that the number of substitutions varies between 8 (*N. nigricollis* toxin α and *N. melanoleuca* toxin d) and 18 (*N. nigricollis* toxin α and *A. stokesii* a). Similarly, the number of substitutions occurring between the sequences of the slow toxins varies between 1 residue (erabutoxin a and erabutoxin b) and 28 residues (erabutoxin b and cobrotoxin). There is no obvious trend in the site or nature of these substitutions. Recently, Brandts and co-workers (Brandts et al., 1975, 1977; Lin & Brandts, 1978) and others (Schmid & Baldwin, 1978) have highlighted the importance of cis–trans isomerism of proline residues in generating a slow phase of protein refolding. However, in the present case, the three slow toxins do not possess any additional proline residues over and above those of the rapid toxins which might account for such a phenomenon (Table I). Furthermore, the activation enthalpy reported by Brandts for proline cis–trans isomerism (18–20 kcal mol^{-1}) is much higher than that reported in this study ($\sim$10 kcal mol^{-1}). Additionally, the phenomenon described by Brandts and co-workers deals with refolding of proteins devoid of disulfide bonds (Lin & Brandts, 1978) or with proteins containing intact disulfide bonds (Brandts et al., 1975, 1977). In our case, it has been observed that when the disulfide bonds are intact, refolding of the toxin is extremely rapid, occurring on a time scale of seconds (unpublished data). Thus, cis–trans isomerism of a proline residue cannot explain the observed phenomenon reported in this work. It was recently shown that the secondary structure of homologous short neurotoxins can be accurately predicted on the basis of an averaged scheme procedure (Ménez et al., 1978) derived from the basic predictive method developed by Chou & Fasman (1974) for individual chains. As a consequence, it is unlikely that the secondary structure of the native protein appreciably varies from one toxin to another. This is confirmed from an examination of individual toxin secondary structure predictions (Dufton & Hider, 1977). Therefore, no correlation between individual secondary structure and the observed refolding kinetics is anticipated.

The toxin sequences listed in Table I have been aligned essentially with respect to their cysteine residues since these are undoubtly paired in an invariant manner (Yang et al., 1970; Endo et al., 1971). It is clear that erabutoxin a, erabutoxin b, and cobrotoxin all possess one additional amino acid inserted at the 19th position of their sequence. Apparently, this is the single peculiarity which may account for the kinetic difference in toxin refolding. Helinski & Yanovski (1963) have demonstrated that a single amino acid replacement observed in an abnormal form of tryptophan synthetase prevented the enzyme from refolding to its native conformation. In the case of neurotoxins, however, we propose that the change which alters the rate of refolding consists of an insertion of a critical additional residue rather than a simple substitution. In agreement with this proposal is the increase of entropy change observed for the folding process of these toxins (see Table III). The additional degree of freedom brought to the unfolded chain by the presence of the supplementary 19th residue may account for this variation. It is worth noting that the four toxins with 61 residues possess one more amino acid in position

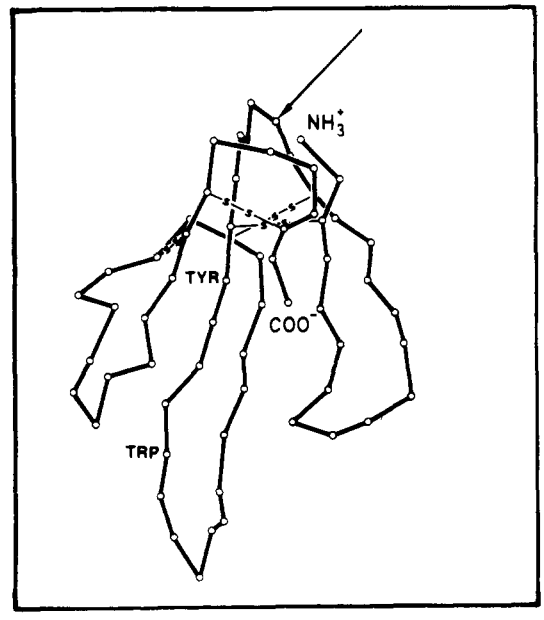

FIGURE 7: X-ray structure of erabutoxin b from *L. semifasciata* according to Tsernoglou & Petsko (1976) [reprinted with permission from Tsernoglou & Petsko (1976); copyright 1976 Federation of European Biochemical Societies]. The arrow indicates the location of the 19th residue.

18 than the toxins of *A. stokesii* and of *A. laevis*. Nevertheless, additional insertion has no effect since the six toxins renature at the same rate. Only with the addition of amino acids at both positions 18 and 19 is an effect seen. Thus, it seems that the amino acids 18 and 19 are present within a region whose size is critical for the rate of renaturation of the toxins. Furthermore, it is interesting that if this insertion of an additional amino acid at position 19 is responsible for the slowing down of the renaturation rate, a change in the nature of this residue (either glycine or serine depending on the case) is without effect since erabutoxin b and cobrotoxin renature at the same rate.

The X-ray structure of erabutoxin b which is a slow toxin has been resolved (Tsernoglou & Petsko, 1976; Low et al., 1976). As indicated in Figure 7, the additional residue is located in a hairpin region situated between the invariant cysteine residues 17 and 24. This section which includes a type I β turn 18–21 (Kimball et al., 1979) connects the two β strands 11–15 and 24–31 (low, 1979). Therefore, it is likely that the formation of the two disulfide bonds could depend upon the configuration adopted by the 17–24 segment during the refolding of the chain. Also, it is probable that the formation of these two disulfide bonds will have a crucial influence on the formation of the connecting β-sheet strands 3–8, 11–15, 24–31, and 34–40 (Low, 1979). Due to its variable length the 17–24 segment demands conformational changes. Obviously, elucidation of the 17–24 segment conformation of different short toxins will be extremely useful to understand the mechanism which induces a retardation of the refolding process.

Another interesting feature of this study is the apparent lack of sensitivity that the refolding kinetics show toward extensive amino acid substitutions. For example, although the erabutoxin b and cobrotoxin sequences differ by 28 residues, the two chains refold approximately at the same rate. This might indicate that the amino acids which are responsible for directing the folding of the toxins are invariant residues or alternatively that mutations have been properly compensated. Many reports dealing with renaturation of reduced polypeptides typically concern a single protein which is considered

to be representative of other homologous proteins. However, from this study it is clear that the data obtained for a particular protein cannot be automatically applied to others in the same homologous series.

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