Reoxidation and renaturation studies on the main toxin of *Naja naja samarensis*

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The main toxin of *Naja naja samarensis* is a very small and rigid protein (M_r 6850, 8 cysteines). When fully reduced, it regains its native conformation by a mechanism involving a rapid cysteine oxidation and a slower, less temperature-dependent disulfide exchange. In a native-like form of the protein we observed a population whose cysteines were incompletely reoxidized. When intermediates with blocked cysteines were incubated with oxidized and reduced glutathione, the percentage of native-like molecules increased. These findings suggest a multiple folding pathway.

Folding *Renaturation* *Intermediate* *Disulfide exchange* *Toxin* *Snake venom*

1. INTRODUCTION

It is known that the renaturation of a reduced protein occurs in two observable steps. First by formation of an incorrect set of disulfide bonds, next followed by a disulfide exchange leading to the native structure [1]. The information necessary to obtain a native conformation is present in the amino acid sequence of the protein [2].

Such a folding mechanism has been analyzed for small proteins containing disulfide bonds like ribonuclease A [3–5], bovine pancreatic trypsin inhibitor [6,7], hen egg white lysozyme [2,8,9]. The main toxin of *Naja naja samarensis* belongs to the class of short postsynaptic toxins from elapid venoms, which specifically block the nicotinic acetylcholine receptor [10]. The polypeptide has 61 residues and 4 disulfide bridges. Its amino acid sequence and its binding constant to the nicotinic acetylcholine receptor have been determined [11]. The X-ray structure of one homologous toxin has been determined in two different laboratories [12,13], but little information is available concerning their renaturation mechanism [14–16].

Here we analyze the effect of temperature on the process of reoxidation and renaturation and the influence of oxidized and reduced glutathione on the stability of the folding intermediates. We find a partially oxidized refolding intermediate with a native-like conformation.

Abbreviations: NT, *Naja naja samarensis*, main toxin; n-NT, native-like toxin; d-NT, denatured toxin; HPLC, high-performance liquid chromatography; TEA, triethylamine; TFA, trifluoroacetic acid; Gu-HCl, guanidine hydrochloride; GSH, reduced glutathione; GSSG, oxidized glutathione; DTT, dithiothreitol; CM-Cys, carboxymethylcysteine

2. MATERIALS AND METHODS

N. naja samarensis venom was purchased at the Miami Serpentarium, Miami. Acetonitrile was of HPLC grade (Fisons). All other reagents were of analytical grade. Amino acid analysis was performed on a Beckman model 119 CL amino acid analyser [17]. Samples were hydrolyzed in 1 ml of constant boiling HCl at 110°C for 24 h in evacuated and sealed tubes. Reverse-phase HPLC was performed on a Spectra-Physics 8100 liquid chromatograph equipped with a Waters μBondapak C-18 column, using a gradient of acetonitrile (from 10 to 35% in 40 min) in 0.025 TEA, 0.05%

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TFA, with a flow rate of 1 ml/min. The main toxin was prepared as in [11].

2.1. *Reduction and oxidation*

NT (32 mg) was treated for 75 min at room temperature in 5 ml of 100 mM Tris–HCl; 6 M Gu–HCl; 1 mM EDTA; 100 mM DTT (pH 8.0), flushed with N_2. Then 1 ml of glacial acetic acid was added. The reduced NT was loaded on a column (25 × 3 cm) of Biogel P-2 in 10% acetic acid and lyophilized. The completely reduced toxin was oxidized at a final concentration of 3×10^{-5} M in 100 mM Na-phosphate; 100 mM NaCl (pH 7.1), flushed with O_2 at 21, 27, 35 and 40°C. The decrease in free sulfhydryl groups was monitored by the Ellman reaction [18]. When 80% reoxidation was attained, the samples were carboxymethylated with a 60-fold molar excess of iodoacetamide [19]. The protein was desalted on a column of Sephadex G-10 and lyophilized. The content of CM-Cys was determined by an amino acid analysis. Twenty μg of each sample were chromatographed by HPLC to determine the percentage of molecules with a native-like conformation. A second experiment was run at 21°C. All free cysteines were blocked as described above when 40% oxidation was attained. The sample was then desalted, lyophilized and chromatographed on a column (200 × 0.7 cm) of Sephadex G-50 superfine equilibrated in 50 mM ammonium acetate (pH 6.85) at 4°C. The products were analyzed by HPLC and amino acid analysis.

2.2. *Incubation in the presence of glutathione*

Twelve mg reduced toxin were dissolved in 30 ml of 100 mM Na-phosphate, 100 mM NaCl, 1 mM EDTA (pH 7.1) at 40°C and the reaction was followed by the Ellman reaction. After 1, 2, 3, 4, 5, 7 and 24 h, 3-ml aliquots were removed and treated with iodoacetamide as described. After desalting, the samples were divided into 2 equal parts. The first one was analyzed by HPLC. The second was incubated for 24 h in 1 mM GSSG, 1 mM GSH, 50 mM Na-phosphate, 1 mM EDTA (pH 7.1) at room temperature and then analyzed by HPLC.

3. RESULTS

The results of the oxidation experiments at different temperatures are summarized in table 1. When 80% of all cysteines are oxidized at different temperatures, the percentage of native-like protein determined by HPLC shows that the kinetics of renaturation does not follow the kinetics of oxidation in our experimental conditions. The native-like fraction of the toxin increases from 15.5 to 37.5% when temperature decreases from 40 to 21°C. An Arrhenius plot gives us an estimate of $\Delta H^* = 9.0$ kcal/mol, $\Delta G^* = 23.2$ kcal/mol and $\Delta S^* = -49.2$ e.u. at 21°C (fig.1) for cysteine oxidation.

Table 1

Reoxidation parameters at different temperatures

T (°C)	k (s^{-1})	$t_{1/2}$ (min)	% n-NT (80% reox)	% CM-Cys
21	3.37×10^{-5}	323	37.5	78.0
27	4.68×10^{-5}	246	33.6	82.5
35	7.16×10^{-5}	161	25.2	78.8
40	8.86×10^{-5}	130	15.5	77.4

Reoxidation was measured as described in section 2. The percentage of n-NT was measured by reverse-phase HPLC and the concentration in CM-Cys determined by an amino acid analysis. k is the reoxidation rate determined by plotting the log of the percentage of free cysteines *vs* time. $t_{1/2}$ represents the half-time of reoxidation

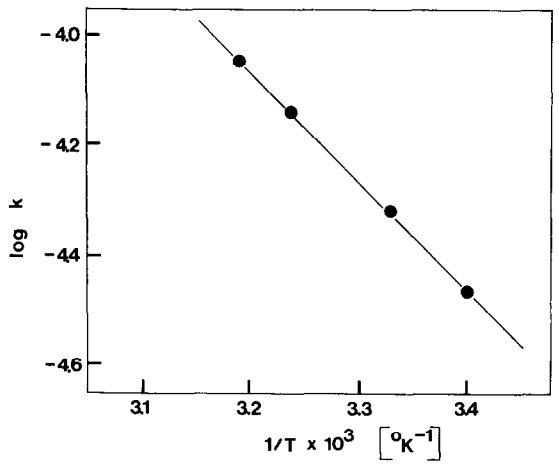

Fig.1. Arrhenius plot of the cysteine oxidation in NT. k is the reoxidation rate of NT. See section 2 for the experimental conditions.

The toxin with free cysteines blocked by carboxymethylation after 40% oxidation yields two peaks when analyzed by gel filtration (fig.2). The first peak (high hydrodynamic volume) corresponds to the elution volume of a completely reduced and carboxymethylated toxin (d-NT) and the second (low hydrodynamic volume) to the native toxin (n-NT). When the same sample is analyzed by HPLC it is also possible to differentiate a native-like and a denatured product but the relative amounts are very different: 33% n-NT by gel filtration and 11% by HPLC. Amino acid analysis of the products (table 2) indicates that 17.1% of the cysteines in the low hydrodynamic volume form are carboxymethylated (1.4 free Cys/molecule) whereas 90% of this material has the retention time of the native NT when analyzed by HPLC. In the high hydrodynamic volume form, 71.2% of the cysteines are carboxymethylated (1.2 disulfide bond/molecule). The apparent discrepancy between the percentage of n-NT measured in the total fraction and in the fraction showing a low hydrodynamic volume on Sephadex G-50 can be explained by the fact that the protein can undergo reshuffling and renaturation during the chromatography (40 h). A mixture of GSH and GSSG catalyzes the disulfide interchange and enhances the renaturation even in a protein whose free cysteines are blocked by carboxymethylation (fig.3). The observable renaturation starts after a

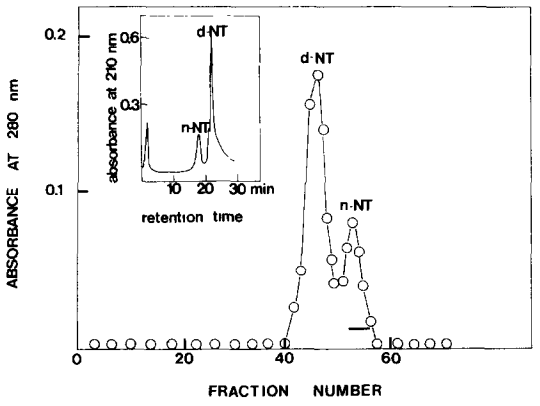

Fig.2. Gel filtration chromatography of NT after 40% oxidation on a column (200 × 0.7 cm) of Sephadex G-50 superfine; 720 1 fractions were collected and the absorbance was monitored at 280 nm. The fractions comprising the native-like peak were pooled as indicated. The same sample was loaded on a reverse-phase HPLC column and eluted with a gradient from 10–35% acetonitrile as shown in the inset.

Table 2
Amino acid composition of NT and n-NT

	NT	n-NT	theor
CM-Cys	–	1.4	–
Asx	7.1	6.8	7
Thr	5.7	5.6	6
Ser	4.3	3.5	4
Glx	6.1	6.2	6
Pro	3.9	3.7	3
Gly	5.2	5.2	5
Ala	1.2	1.1	1
Cys	7.8	6.8	8
Val	2.0	2.1	2
Leu	2.0	2.3	2
Ile	1.9	2.1	2
Tyr	0.8	0.8	1
His	2.0	2.0	2
Lys	5.9	5.8	6
Arg	3.9	4.0	4

Samples were hydrolyzed for 24 h at 110°C in constant boiling HCl

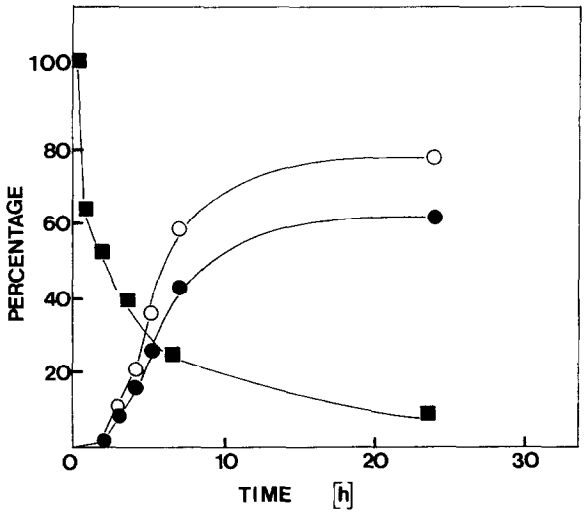

Fig.3. Kinetics of reoxidation and renaturation of a reduced toxin at 21°C and effect of oxidized glutathione on the renaturation of the protein. See section 2 for experimental conditions. (●—●) Percentage of n-NT upon oxidation in the presence of O_2 determined by reverse-phase HPLC. (○—○) Percentage of n-NT upon oxidation and followed by incubation for 24 h in the presence of 1 mM GSSG + 1 mM GSH. (■—■) Cysteine oxidation detected by Ellmann's reaction.

2-h lag period (50% reoxidation) and the subsequent increase in native form is very sharp.

4. DISCUSSION

The activation parameters and the kinetics of reoxidation for the main toxin of NT are very close to the parameters for renaturation obtained with the toxin of *Naja naja philippinensis* determined by CD. We have found a rate of oxidation of 7.2×10^{-5} s^{-1} at 35°C whereas it is 6.4×10^{-5} s^{-1} at 37°C for renaturation [14]. The amino acid sequence of both toxins [10] and the experimental conditions are identical. The number of CM-Cys detected in the n-NT peak isolated by gel filtration after 40% reoxidation indicates that part of this material is composed by toxin with an incomplete number of disulfide bonds and the observed low hydrodynamic volume is due to a compact structure very close to the native one. When we measured the renaturation of the toxin by chromatography on a reverse-phase HPLC, we observed that renaturation is much slower than reoxidation (table 1). However, the conditions used (10–35% acetonitrile, pH 2) could denature intermediates with incomplete or incorrect disulfide sets. The yield in the high hydrodynamic volume form (non-native species) increases upon reoxidation with higher temperatures. Similar findings were observed during the renaturation of lysozyme with an increase of Cu^{2+} in solution [8]. The higher rate of random motion of the polypeptide due to the rise in temperature reduces the average time in which cysteines are close enough for a disulfide interchange to occur and hence for the renaturation of the protein. A mixture of GSH and GSSG, as well as other small molecular mass thioldisulfide reagents, is known to catalyze the reshuffling of mismatched disulfide bonds and accelerate the renaturation of proteins by sulfhydryl–disulfide interchange [20]. We have determined that the increase in native-like conformation starts after incubation with GSSG and GSH when 20–25% of cysteines are previously carboxymethylated (1.6–2.0 CM-Cys/molecule) so that an average of 3 reconstituted disulfide bonds allows the reshuffling of disulfide bonds to obtain a correct and stable configuration. Statistically, 420 isomers are possible when 3 out of 4 S–S bonds are closed, from which only 4 are the native pairings [8]. Since it is very unlikely that the first disulfides formed are immediately the correct ones, we can assume from the kinetics of reoxidation and from the published data of renaturation [14] that in neutral, aqueous solution even a toxin with an incorrect pairing of cysteines acquires a sufficient stability to fold in a conformation similar to the native one. The CD analysis performed to follow the renaturation [14] shows a maximum at 228 nm which corresponds to the contribution of aromatic residues. What is detected is a local native-like environment for tyrosine and tryptophan, and not the native conformation of the protein as a whole. This could explain the similarities observed between renaturation and reoxidation. In fact, the four disulfide bridges of these toxins are very close to each other in space [12] and the pairing of cysteines, although not necessarily the correct one, may be sufficient to create a local environment around aromatic amino acids very similar to the one observed in the native protein. The CM-Cys present in the n-NT peak isolated by chromatography on Sephadex G-50, indicates that not all 4 disulfide bonds are mandatory to induce a native-like conformation. Nevertheless, the different yield observed in n-NT for the same sample, when analyzed by gel filtration or HPLC, demonstrates that a structure which is correct or native-like by one criterion, namely the hydrodynamic volume, does not have a sufficient stability to show the same behavior under stronger denaturing conditions. Earlier work showed that a synthetic peptide containing 33 residues, encompassing the amino acid sequence of the presumed active center, binds with a high affinity to the acetylcholine receptor [22]. This observation strengthens our conclusion that the integrity of the toxin is not necessary to obtain a compact structure displaying the characteristics of the native protein. It can be concluded that if not all four disulfides need to be intact or correctly paired and if the reshuffling with blocked cysteines can lead to a native-like structure, many parallel pathways are possible for the folding mechanism of snake neurotoxins.

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