Analysis of folding intermediates from the main toxin of *Naja naja samarensis* containing two blocked cysteines

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The formation of folding intermediates blocked by iodoacetate from the main toxin of *Naja naja samarensis* was monitored by FPLC and the populations were identified by amino acid analyses. We have determined conditions allowing for the highest yield in the populations with two blocked cysteines. The free cysteines remaining under these conditions were labeled with iodo[^{14}C]acetate and localized by peptide mapping in one of the products isolated by ion-exchange chromatography (NT^3III). We have also investigated the effects on the native-like characteristics of such molecules of an incubation at equilibrium with a mixture of cysteine and cystine. We find that many different molecular populations are present during the folding process and that disulfide exchanges allow for the reconstitution of native-like products having open disulfides even under strongly denaturing conditions.

Folding intermediate *Disulfide bond* *Renaturation* *Fast protein liquid chromatography* *(Snake venom)*

1. INTRODUCTION

It is now well recognized that several specific biological properties of proteins depend on their three-dimensional conformation, which in turn is a function of their amino acid sequence. Anfinsen [1] has clearly demonstrated that generation of the folded conformation occurs spontaneously. The process of folding and the stabilisation of the tertiary conformation are nevertheless comprised of a multitude of events which have been reviewed partially in recent literature [2–7].

There is much evidence that proteins do not simply flip from the denatured state to the native one, but that many intermediary states are involved in this process [8–11]. The debated question is, however, whether there are a few obligatory intermediates along the folding pathway which the protein forms sequentially, or if many different and parallel pathways lead to the same native structure.

To address this question several authors have studied the folding of small proteins containing disulfide bonds, since the chemical modification of cysteines is an elegant way to trap partially oxidized forms of polypeptides. The folding of BPTI [12], lysozyme [13] and RNase [14] has been studied recently in this manner.

The native conformation of a protein can be reached after (i) the formation of wrong disulfide bridges, followed by (ii) the rearrangement of such bonds by thiol/disulfide exchange [15], a process that can be efficiently catalysed by the addition of low molecular mass thiol/disulfide reagents [16].

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Abbreviations: NT, *Naja naja samarensis* main toxin; NT^1, NT^2, NH^3, NT^4, NT with respectively 1, 2, 3 or 4 reformed disulfide bonds; CM-NT, NT with 8 CM-Cys; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; Gu-HCl, guanidine hydrochloride; CM-Cys, carboxymethylcysteine; BPTI, bovine pancreatic trypsin inhibitor; RNase, ribonuclease A

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The main toxin of *Naja naja samarensis* is a short postsynaptic toxin from elapid venoms and contains 4 disulfide bonds. Its amino acid sequence [17] and the three-dimensional structure of a very similar toxin [18] are known. The physiological activity of this molecule consists in a specific block of the nicotinic acetylcholine receptor [19].

We analyze in this report the folding of the fully reduced toxin. Renaturation and oxidation kinetics were measured and experimental conditions were set to lead to the highest amount of molecules having an average number of two blocked cysteines. We address the questions of the uniqueness of the folding pathway and of the integrity of all disulfide bonds in the native structure.

2. MATERIALS AND METHODS

N. naja samarensis venom was purchased at the Miami Serpentarium, Miami. Iodo[^{14}C]acetate was from the Radiochemical Center, Amersham. Trypsin and trypsin inhibitor were purchased from Sigma. The Filter-solv scintillation cocktail was obtained from Beckman and Aquassure from New England Nuclear. All other reagents were of an analytical grade.

Amino acid analyses were performed on a Beckman 119CL amino acid analyzer. Samples were hydrolyzed in 1 ml of constant boiling HCl at 110°C for 24 h in evacuated, sealed tubes [20]. High-performance ion-exchange chromatography was performed on a Pharmacia FPLC system equipped with a Mono S HR 5/5 column, using a linear gradient of ammonium acetate (from 50 to 335 mM in 20 ml) in 7 M urea at pH 5.0. The flow rate was 1.5 ml/min. 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% triethylamine and 0.05% trifluoroacetic acid, with a flow rate of 1 ml/min. The reduced toxin was prepared according to [21].

2.1. *Analysis of disulfide formation*

2.5 mg of previously reduced toxin were oxidized at a concentration of 5×10^{-5} M in 50 mM Na phosphate, 100 mM NaCl, 1 mM EDTA, 1 mM GSSG and 1 mM GSH, pH 7.0 at 37°C. Aliquots of 750 μl were withdrawn after 1, 2, 3, 4, 5, 6, 8, 10 and 20 min of oxidation and carboxymethylated with 200 μl of a solution containing 0.2 M iodoacetate, 0.2 M NaOH, 50 mM Na phosphate, 100 mM NaCl, pH 7.0, in the absence of light [22]. After 15 min at room temperature, the reaction was stopped by the addition of 100 μl glacial acetic acid. The samples were desalted on a Sephadex G-10 column (100 × 1 cm) in 0.1 M acetic acid, lyophilized and dissolved in 100 μl of 50 mM ammonium acetate, pH 5.0 in 7 M urea and chromatographed by FPLC. Each of the major peaks was collected, analyzed by HPLC and the content in CM-Cys determined by an amino acid analysis.

The samples containing three disulfide bonds (NT^3I, NT^3II and NT^3III) were incubated separately for 72 h at 5×10^{-5} M in 50 mM Na phosphate buffer, 100 mM NaCl, 1 mM EDTA, 1 mM cysteine and 1 mM cystine, pH 7.0, at room temperature, then desalted, lyophilized and chromatographed by FPLC. The peaks that had the same elution volume as the native toxin were analyzed by HPLC.

2.2. *^{14}C labeling of cysteines*

2 mg of reduced toxin were oxidized as described above. After 255 s of oxidation at 37°C, free cysteines were labeled with a 60-fold molar excess of iodo[^{14}C]acetate (1.72 mCi/mmol). Before the fractionation of the labeled incubation mixture by ion-exchange chromatography, two aliquots were withdrawn. The first one was immediately analyzed by FPLC; 250 μl fractions were collected and their radioactivity was determined. The second sample was incubated for 72 h at room temperature at 5×10^{-5} M in the presence of 1 mM cysteine and 1 mM cystine and analyzed as described above.

A fully [^{14}C]carboxymethylated toxin was obtained by reacting 0.65 mg of entirely reduced toxin with a 60-fold molar excess of iodo[^{14}C]acetate (1.72 mCi/mmol) in 100 mM Tris-HCl, pH 8.0, 6 M Gu-HCl and 1 mM EDTA for 15 min at 37°C. The protein fraction was desalted and lyophilized.

2.3. *Peptide mapping*

Before any peptide mapping could be performed, it was mandatory to carboxymethylate completely the remaining cysteines. The fraction

NT^3III, isolated by FPLC and containing an average of 2 [^{14}C]CM-Cys/mol toxin, was reduced again and carboxymethylated with non-radioactive iodoacetate. 50 nmol of the completely carboxymethylated toxin or NT^3III were dissolved in 300 μl of 50 mM ammonium acetate, pH 8.3, 10 μl of 160 mM $CaCl_2$, 5 μl of 1% trypsin in 1 mM HCl and digested for 24 h at room temperature. The reaction was stopped by the addition of 5 μl of 2% trypsin inhibitor and the solution was lyophilized. The tryptic peptides were dissolved in a minimal amount of water. A 10 μl spot was applied on top of a Whatman 3MM paper (40 cm long) and developed in a chamber saturated with butanol/acetic acid/water (4:1:5) (organic phase) for 36 h. As a control, a completely non-radioactive carboxymethylated and digested toxin was simultaneously analyzed; the peptides were detected with ninhydrin.

The chromatogram was dried in a warm air stream and cut into 2 mm wide bands. The radioactivity of every other band was determined by dissolving them for 1 h in 3 ml of Filter-solv scintillation cocktail. The samples were counted for 10 min. The remaining bands corresponding to radioactivity peaks were pooled and the spots hydrolyzed to determine their amino acid composition and specific activity.

3. RESULTS

From the kinetics of disulfide bond formation, as illustrated in fig.1, it can be concluded that the major populations that accumulate contain 3 disulfide bridges. The fully carboxymethylated species, containing no disulfides, were not taken into account for the calculation of the percentage of the different populations since they were not retained by ion-exchange chromatography. Because the fully carboxymethylated toxin represents only a significant percentage of the total population at very early times of refolding [11], it can be ignored without significantly affecting the experimental values. The largest amount of folding intermediates with three reconstituted disulfide bridges occurred after an incubation of 255 s in the oxidation mixture.

At least three different sets of molecules containing three closed disulfides can be isolated by

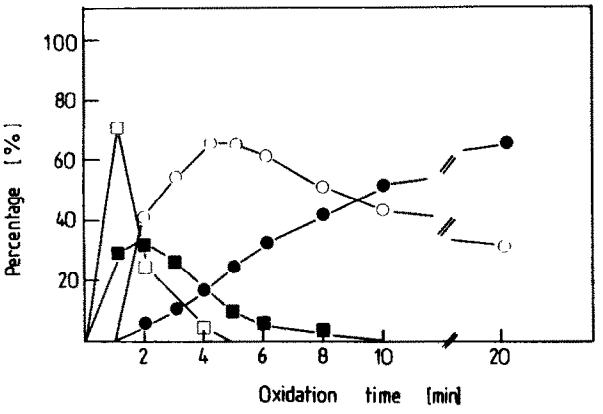

Fig.1. Kinetics of the folding intermediates with 1–4 reconstituted disulfide bonds. Each point corresponds to the percentage of each population compared to the total optical density area determined by FPLC. The intermediates were trapped after the oxidation time indicated. (□) NT^1, (■) NT^2, (○) NT^3, (●) NT^4.

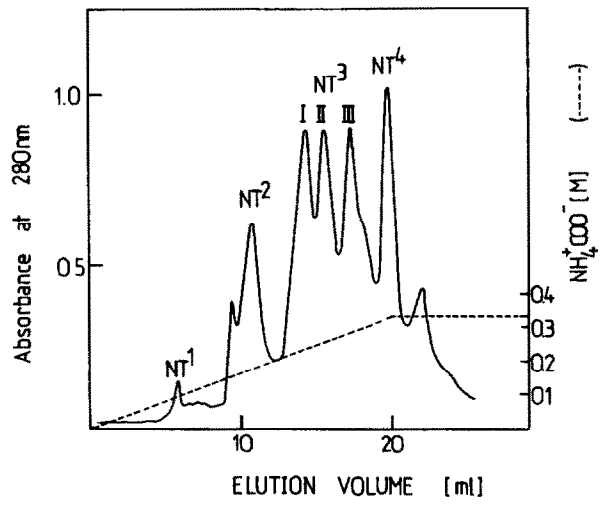

Fig.2. FPLC elution profile of an NT reoxidation mixture blocked by carboxymethylation after 255 s incubation.

FPLC (fig.2) and the content in CM-Cys of each is indicated in table 1.

When the oxidation mixture, quenched with iodoacetate after 255 s, is incubated for 72 h with reduced and oxidized glutathione, its FPLC pattern changes markedly: 7.0% of the radioactivity co-elutes with the native toxin (fig.3) in 7 M urea at pH 5.0.

Table 1

CM-Cys present in FPLC peaks

Peak	CM-Cys
NT^1	6.3
NT^2	4.8
NT^3I	2.2
NT^3II	2.1
NT^3III	2.4
NT^4	0.7

CM-Cys was determined by a standard amino acid analysis including carboxymethylcysteine in the standard calibration. Samples were prepared as described in section 2

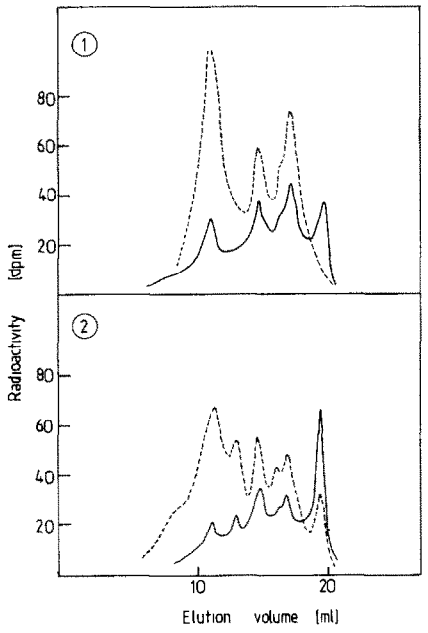

Fig.3. FPLC elution profile of an iodo[^{14}C]acetate labeled reoxidation mixture (panel 1) prior to and (panel 2) after incubation at equilibrium in presence of 1 mM cysteine and cystine. (——) A_{280}, (– – –) radioactivity.

Which are the free cysteines and which cysteines are involved in disulfide bonds? Digestion with trypsin gave at least a partial answer to this question, as seen in fig.4. For this purpose the partially [^{14}C]carboxymethylated toxin was reduced again and the remaining cysteines blocked with non-radioactive iodoacetate. Digestion with trypsin of the now chemically homogeneous toxin and subsequent peptide mapping showed radioactivity in all cysteine peptides (fig.5). The specific activity of NT^3III and NT fragments are compared in table 2. Peptides 1–15, 16–25 and 16–26 of NT^3III are strongly labeled, when compared to other peptides of the same population and to those of CM-NT.

Analyses of samples NT^3I, NT^3II and NT^3III by reverse-phase HPLC are presented in fig.6. The elution patterns of the three populations are markedly different and at least 14 peaks are detected. We have isolated by FPLC, after incubation at the equilibrium in the presence of cysteine and cystine to generate the thermodynamically more stable populations, one peak for each NT^3 product that had the same retention time as the native toxin. The HPLC elution profiles of these species are shown in fig.6, where a minimum of 17 peaks can be observed.

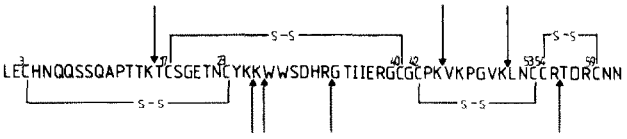

Fig.4. Primary structure of NT. The positions of disulfide bonds are indicated by a solid line and tryptic cleavage sites by arrows [17].

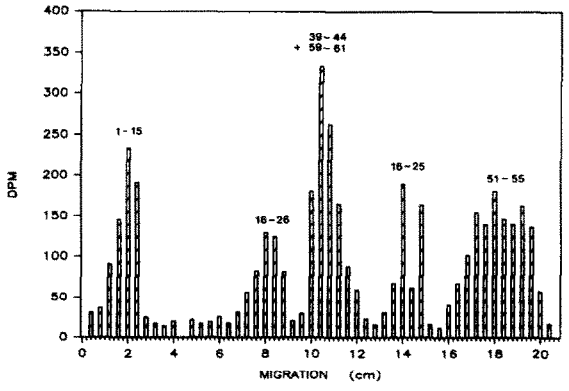

Fig.5. Distribution of radioactivity after descending chromatography of the tryptic peptides from NT^3III. The sequence numbers of amino acids in the peptides are shown. Experimental conditions are described in section 2.

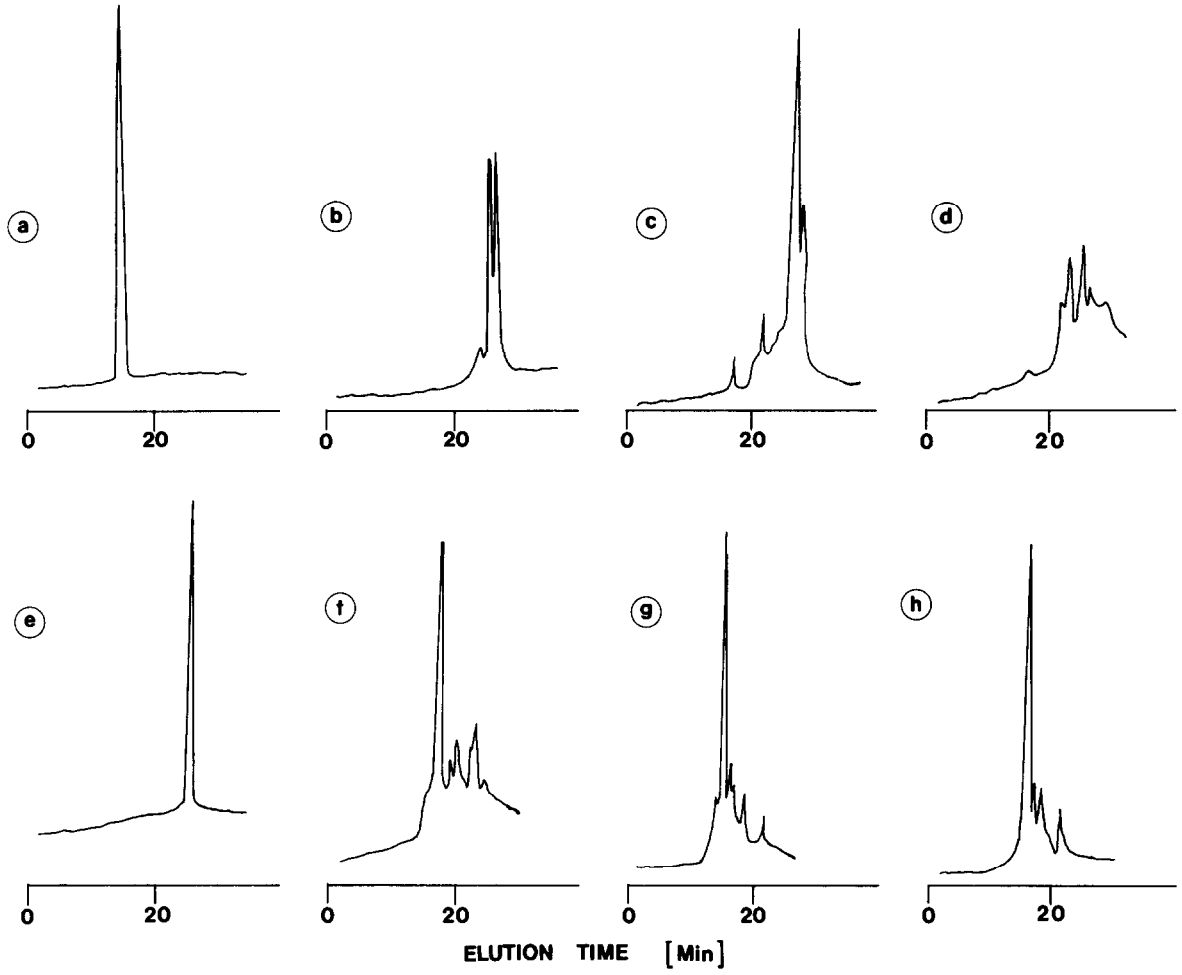

Fig.6. HPLC elution profiles of (a) NT, (b) NT^3I, (c) NT^3II, (d) NT^3III, (e) CM-NT, (f) native-like NT^3I, (g) native-like NT^3II and (h) native-like NT^3III. Samples were analyzed on a Waters μBondapak C-18 column in a gradient of acetonitrile (10% to 35% in 40 min) in 0.025% triethylamine + 0.05% trifluoroacetic acid. The flow rate was 1 ml/min and the absorbance was determined at 210 nm.

4. DISCUSSION

The renaturation of the main toxin from *N. naja samarensis* is efficiently catalyzed by the addition of a mixture of low molecular mass thiols and disulfides.

The half-life for the formation of the toxin having 4 reconstituted disulfide bonds, which elutes with the same retention time as the native toxin on FPLC, is approx. 10 min. When glutathione is omitted, the half-time increases to 160 min [21]. In the given experimental conditions, molecules with 3 disulfides are predominant between 3 and 8 min reoxidation time. If we consider only the disappearance of NT^1, NT^2 or NT^3, the overall half-times are 45 s, 2 min and 16 min, respectively. The last data are approximate since none of the mentioned populations (NT^1, NT^2 or NT^3) is homogeneous [11].

When the oxidation is quenched after 4 min 15 s and the reaction mixture is incubated with cysteine and cystine, the FPLC elution profile is considerably modified (fig.3). It appears that an appreciable percentage of the radioactivity is eluted

Table 2

Specific activities and CM-Cys content of NT and NT^3III tryptic peptides

Peptide	cpm/nmol		CM-Cys/peptide		
	CM-NT	NT^3III	CM-NT	NT^3III	NT
1–15	138	36	0.9	0.34	1
16–25/16–26	308	68	2.1	0.64	2
39–44 + 59–61	435	63	3.0	0.60	3
51–55	276	45	1.9	0.42	2

The left column indicates the specific activity of the cysteine-containing peptides. The radioactivity was determined for each peptide hydrolysate on an aliquot withdrawn immediately before the hydrolysis step. The specific activity was calculated from the peptide concentration derived from amino acid analysis. The right column indicates the average of cysteines per peptide. For this purpose, we have summed up the specific activities and divided by a total of 8 cysteines for the completely labeled toxin and by 2 for NT^3III

with the same retention time as the native toxin, indicating that even partially oxidized molecules can display the same elution characteristics as the parent toxin. The presence of the 4 disulfide bonds is thus not mandatory for the reconstitution of native-like properties of the toxin, even in 7 M urea. These findings are in good agreement with the results of earlier publications [21] in which the hydrodynamic volume of folding intermediates was analyzed.

Renaturation and disulfide exchange lead to very heterogeneous populations of folding intermediates. A large number of conformers showing 1–4 disulfide bonds was observed by FPLC (fig.2). At least three peaks with one open disulfide bond: NT^3I, NT^3II and NT^3III could be distinguished. The partial surface charges of such peaks are sufficiently different to display a dissimilar behavior on ion-exchange matrix, which suggests a different conformation and hence a different disulfide pairing.

The only covalent reaction that occurs during renaturation is the formation of disulfide bonds, which are physico-chemically equivalent. The various forms of disulfides have similar enthalpies of formation [22]. For example, if species with 3 disulfide bridges show a higher percentage of native-like populations, it is likely that the disulfide bonds of NT^3 must have a higher energy barrier for the disulfide interchange reaction than those of NT^1 and NT^2.

The distribution of radioactivity observed in peak NT^3III, in which every cysteine-containing peptide is labeled, demonstrates that this product is a mixture of different pairings of cysteines in the molecules. The distribution of radioactivity stated in table 2 suggests that molecules having a blocked disulfide between Cys 3 and Cys 23 are present in NT^3III. It is very unlikely that peaks NT^3I and NT^3II are constituted by a single type of disulfide pairing, since reverse-phase HPLC profiles (fig.6b–d) show very different patterns. Furthermore, the analysis of products that have the same elution volume as the native toxin by FPLC (not shown) yielded different HPLC elution profiles (fig.6f–h). A total of at least 17 peaks could be observed.

We conclude therefore that, during the folding process of the snake toxin, no dominant disulfide assemblies are generated in the renaturation/reoxidation process, in contrast to what was found for BPTI [23].

Many sterically favorable populations of intermediates, leading to the final conformation, are formed and apparently reshuffled in parallel folding pathways, rather than by following a single pathway, in which the native cysteine pairings are reconstituted sequentially.

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