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Thiol exchange catalysed refolding of small proteins utilizing solid-phase supports

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The study of isolated snake toxin refolding has been a valuable tool in the understanding of protein folding dynamics. We report here differences in the refolding characteristics of three toxin classes and introduce a novel method for overcoming disulphide mismatching and oligomer formation by utilizing solid-phase thiol exchange gels.

1. Introduction

Snake toxins form a large group of homologous proteins that can be subdivided into three main classes: short neurotoxins, long neurotoxins and cytotoxins [1]. These are basic molecules (molecular weight approx. 7000) with iso-electric points above pH 9.0. They are also highly cross-linked, containing either four or five disulphide bonds. Primary sequence analysis has shown a high degree of homology due to conserved residues within and between toxin classes, especially in the positioning of disulphide bonds [2].

These characteristics render them ideal for refolding studies. Microheterogeneity within 'hypervariable' regions in the molecule and the effect of an extra disulphide bond in the long neurotoxins can be studied and some early findings have been reported [3].

In contrast to many of the short neurotoxins, refolding of both α-cobratoxin (long neurotoxin) and cytotoxin CM6 does not lead to a high proportion of native toxin. Slow refolding rates are observed and oligomer formation, due to intermolecular disulphides, predominates over a wide range of conditions [4].

In order to overcome these problems, a novel method of refolding reduced toxin molecules was sought which would both favour formation of the correctly folded species and permit refolding of the proteins at concentrations above 5×10^{-5} M, the normal limit for bulk phase conditions [5].

The method reported here uses a column of modified agarose gel to which are attached exchangeable disulphide groups. These are capable of forming intermolecular disulphide bonds with reduced toxin molecules, permitting a dynamic exchange to take place. Mixed disulphides and intramolecular disulphides form and break as the toxin molecule passes down the column. Assuming that the native toxin conformer is the most stable, a high proportion of the native species should be eluted from the column.

In this paper, we describe some studies on the refolding of members of each of the three toxin groups monitored by iso-electric focussing, SDS-polyacrylamide gel electrophoresis, reverse-phase HPLC and circular dichroism.

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2. Materials and methods

2.1. Materials

All toxin samples of *Naja naja siamensis* were obtained by fractionation of the crude venom as described previously [6]. Toxin α from *Naja nigricollis* was kindly supplied by Dr. A. Menez. All toxins were identified by amino acid analysis and monitored for phospholipase contamination as described in ref. 6. All toxin concentrations were determined spectrophotometrically on a Perkin Elmer Lambda 5 spectrophotometer.

Activated thiol-Sepharose in the form of thiol-Sepharose 4B (agarose glutathione-2-pyridyl disulphide) was obtained from Pharmacia (Uppsala). Oxidized glutathione and dithiothreitol were purchased from Sigma. Guanidine was purchased from Aldrich and was twice recrystallized from hot ethanol. All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Iso-electric focussing

Purified short neurotoxin (α-toxin *N. nigricollis*), long neurotoxin (α-cobratoxin from *N. naja siamensis*) and cytotoxin (CM6 from *N. naja siamensis*) were reduced, refolded with glutathione (final concentration 1 mM) and trapped using iodoacetic acid to form the carboxymethylated species. Desalted samples were then applied to iso-electric focussing gels (Serva precotes pH 3.5–10) and separated according to iso-electric point as described previously [3].

2.2.2. SDS-polyacrylamide gels

Samples of refolded toxin were applied to a discontinuous 10–20% polyacrylamide gel system and separated according to molecular weight under non-reducing conditions. Gels were made according to Laemmli [7], the running time being 5–7 h and subsequent fixing and staining being performed with Kenacid blue/acetic acid solution. Gels were destained in a 1 : 2 : 8 methanol/acetic acid/water solution.

2.2.3. Reverse-phase HPLC

Samples of native and refolded toxin were run on a Waters HPLC system using a C8 reverse-phase radial compression cartridge in conjunction with an RCM 100 radial compression module. The solvent system used was a sodium phosphate (0.01 M, pH 2.5), sodium sulphate (0.1 M) aqueous phase run against an increasing gradient of acetonitrile. All materials were analytical grade. Buffers were filtered through a 2 μm Millipore solvent filter, samples being filtered through an Acro LC3A sample filter prior to injection.

2.2.4. Circular dichroism

CD studies on toxin samples were performed on a Jasco J40 spectrophotometer using a 0.02 cm path-length cell. All samples were run in distilled water and were measured using units of ΔE.

2.2.5. Binding of toxin samples to thiol-agarose followed by elution

Before attempting immobilization of the toxin on activated thiol-agarose, the reaction of the reduced protein with 2,2-dipyridyl disulphide was monitored in the coupling/refolding medium to be used. The reaction was followed in a spectrophotometer by monitoring the release of 2-thiopyridone as previously described [8]. This permitted assessment of buffer suitability for the thiol exchange reaction. When the reaction was complete, the amount of reacted thiol groups was determined using the molar absorbance of 2-thiopyridone at 343 nm ($8.08 \times 10^3\ cm^{-1}\ M^{-1}$). For long neurotoxins, the coupling/refolding buffer used was sodium phosphate (50 mM, pH 8.7), KCl (100 mM), for cytotoxins, guanidine (1 M) was added. All buffers were flushed with N_2 to minimize reoxidation, the reaction being carried out in sealed cuvettes.

Activated thiol-Sepharose was washed with sodium phosphate buffer (pH 7.0) using 250 ml/g dry gel. The washed gel was packed in a plastic column (total volume 20 ml) and equilibrated with the coupling/refolding medium which had been thoroughly saturated with N_2 prior to use (fig. 1). The toxin was reduced in guanidine buffer (6 M guanidine, 0.1 M Tris-HCl; pH 8.0) using dithiothreitol (40 mM) for 75 min at room temperature. The reduced toxin was desalted on a Pharmacia PD10 (G25) column equilibrated with the coupling/refolding buffer and passed directly onto

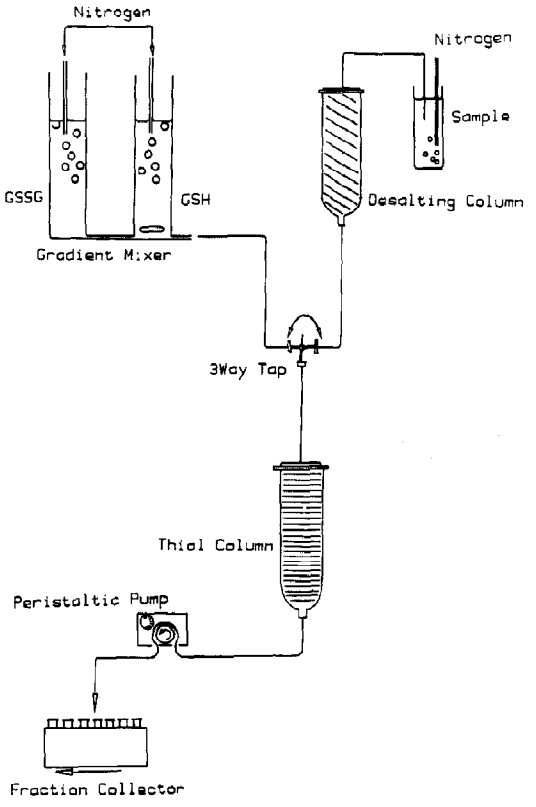


Fig. 1. Schematic representation of column refolding apparatus used in cytotoxin and neurotoxin refolding. All buffers were saturated with N_2 prior to use. Column switching was performed using a three-way tap.

the activated thiol-agarose gel. This mixture was incubated for 18 h at room temperature. The column was washed with buffer in order to monitor the amount of toxin bound and the eluted fraction was desalted, lyophilized and monitored at 280 nm. A gradient of reduced to oxidized glutathione was applied to the column at a flow rate of 0.25 ml min^{-1} and the eluent collected. This gradient produces a continually changing redox potential on the gel which influences the disulphide content of the bound toxin molecules. The fractions collected were desalted, lyophilized and monitored by HPLC and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions.

2.2.6. Immediate elution of the toxin from the thiol gel

This procedure is identical to that described above, except that no incubation period was used. Thus, the redox gradient of glutathione was applied immediately after the reduced toxin was passed to the gel. The eluent was collected, and the fractions combined, desalted, lyophilized and monitored by HPLC and SDS-PAGE as detailed previously. In order to optimize the production of native material, the flow rate, redox conditions (i.e., the gradient of oxidized and reduced glutathione) and column length were systematically varied. Refolding of 1.5 and 3.0 μmol of toxin was attempted for long neurotoxins and cytotoxins.

3. Results

3.1. Initial refolding studies of toxins

Initial refolding studies of α-cobratoxin, toxin α and cytotoxin CM6 using iso-electric focussing to separate intermediates are presented in figs. 2–4. Both toxin α and cytotoxin CM6 refold back to a species which has the same iso-electric point as the native toxin when oxidized glutathione is used. In contrast, the long neurotoxin failed to refold fully to the native material even after 72 h. Although this technique indicated the fully oxidized cytotoxin only to be present after 24 h of

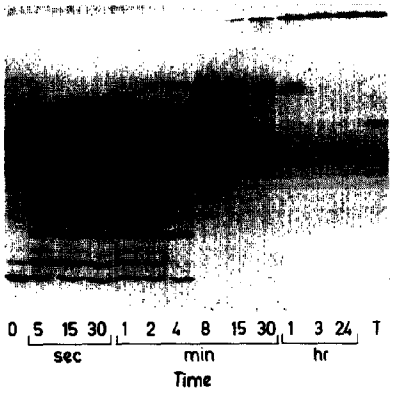


Fig. 2. Iso-electric focussing on polyacrylamide gel of species trapped during the refolding of reduced, short neurotoxin (toxin α, *Naja nigricollis*); T, native toxin.

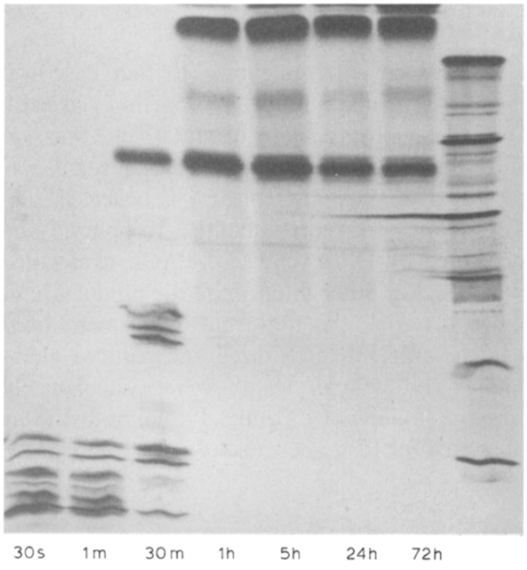


Fig. 3. Iso-electric focussing on polyacrylamide gel of species trapped during the refolding of reduced, long neurotoxin (α-cobratoxin, *N. naja siamensis*).

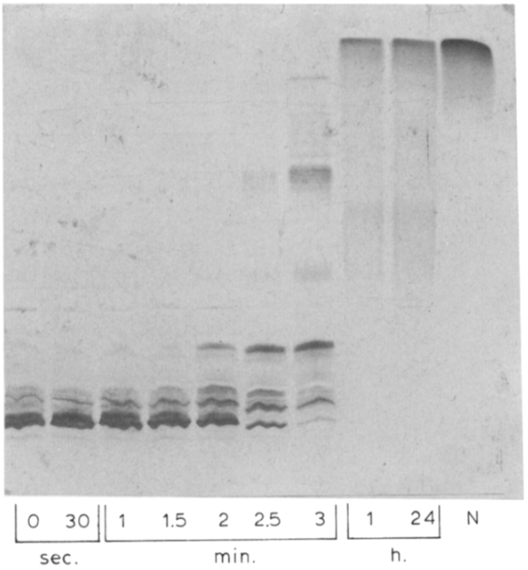
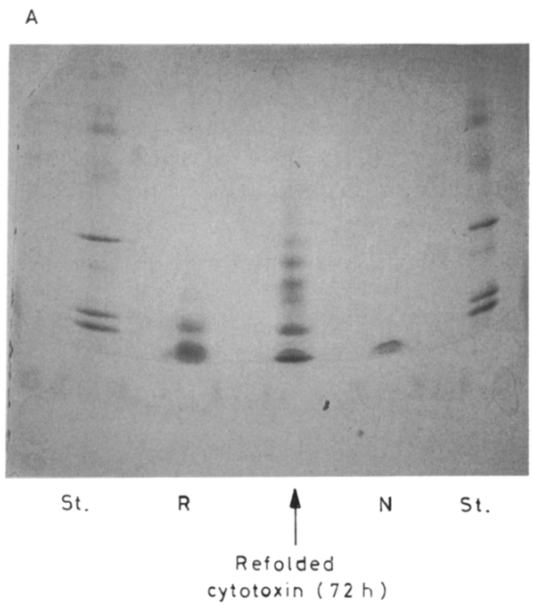


Fig. 4. Iso-electric focussing on polyacrylamide gel of species trapped during the refolding of reduced, cytotoxin CM6 (*N. naja siamensis*); N, native toxin.



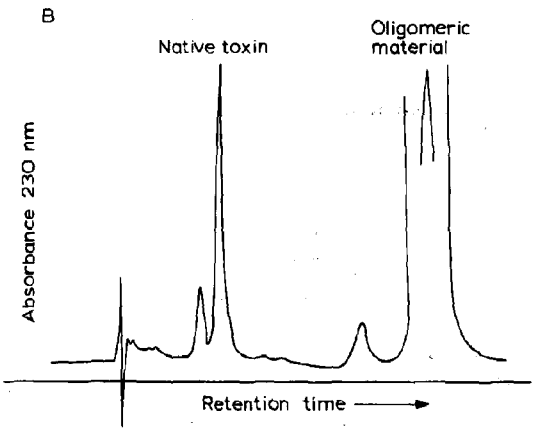


Fig. 5. (A) SDS-polyacrylamide gel (non-reducing 10–20%) of reduced, 72 h refolded and native cytotoxin. The presence of oligomeric material dominates the refolded cytotoxin fraction. R, fully reduced toxin; N, native toxin; St., molecular weight standards (M_r 14000–78000) (B). Reverse-phase HPLC profile of oligomeric and native toxin formed in bulk phase refolding of cytotoxin CM6. Small amounts of native toxin are formed. Samples were run on a C8 reverse-phase RCM cartridge.

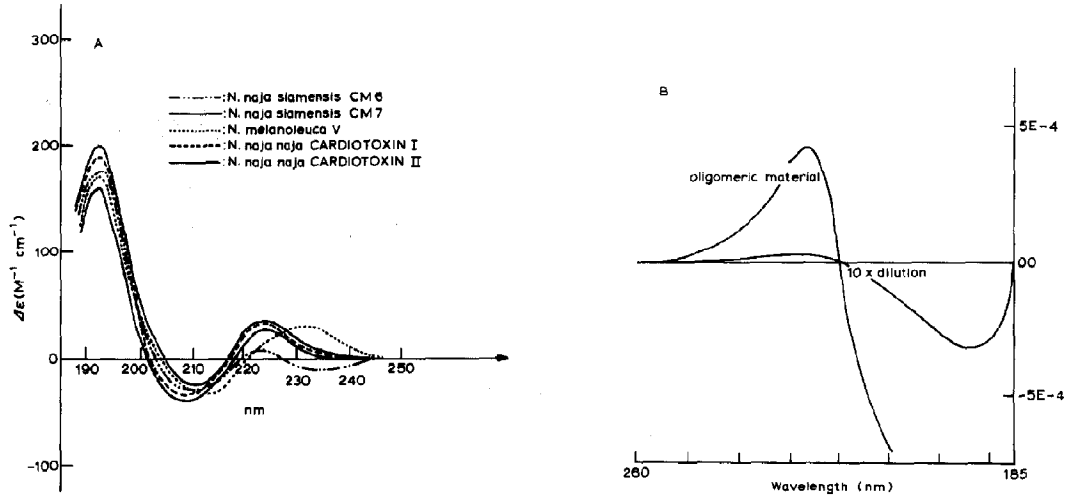


Fig. 6. (A) CD spectra of cytotoxins. The positive 192 nm band is indicative of extensive β-sheet structure. The samples were at 1 mg ml^{-1} in water, using a 0.02 cm path length cell. (B) CD spectrum of a typical sample of refolded cytotoxin (1 mg ml^{-1}) containing oligomeric material. The spectrum possesses a negative band centred on 195 nm which is indicative of a random coil conformation.

refolding, when the samples were analysed by reverse-phase HPLC, heterogeneous material was observed even after 72 h (fig. 5). Thus, a number of species each containing four disulphides were produced. Indeed, SDS-PAGE gels (10–20% non-reducing conditions) detected the presence of appreciable quantities of oligomeric material. This was not found to be the case with the short neurotoxin, toxin α (D.C. Smith, unpublished data). The CD spectra of the refolded samples possess a broad trough centred around 195 nm, typical of a random coil conformation and quite different from that of the native toxin (fig. 6A and B). A small band in the 228 nm region is also seen which tentatively can be assigned to disulphides.

The tendency to form oligomers was minimized by the inclusion of guanidine (1 M) in the refolding medium. The refolding buffer finally chosen for cytotoxins was sodium phosphate (50 mM). KCl (100 mM) and guanidine (1 M), pH 8.7. However, non-native protein still dominated the refolded product as demonstrated by HPLC, even after 72 h (fig. 7). Repeated attempts to refold cytotoxin CM6 under a variety of conditions failed to increase the yield of native refolded material to more than 35%, again in contrast to the short neurotoxins [3].

3.2. Column refolding studies of toxins

The use of columns for refolding toxins was introduced in an attempt to improve the yield of native refolded toxin which is desirous particularly in the case of cytotoxins and long neurotoxins. Furthermore, bulk phase refolding of toxins is severely limited by toxin concentration. The introduction of a column into the refolding procedure could in principle remove this limitation. 'Activated thiol-Sepharose 4B' was chosen as the matrix because it contains a readily exchangeable disulphide group and the hydrophilic glutathione residue acts as a spacer group. Thereby, steric interference with exchange reactions at the terminal thiol group is decreased [9].

Initially, cytotoxin refolding was monitored. High levels of cytotoxin conjugation were possible, the typical range being 80–90% bound. However, when eluted by the glutathione gradient after 18 h incubation the protein emerged in two distinct fractions. A small amount (approx. 4%) was rapidly eluted from the column and analysis showed this to be native toxin. The remainder of the protein eluted over a wide range of fractions and was found to contain a number of components when analysed by HPLC. Although this

fraction contained some native cytotoxin, a large proportion was oligomeric. Similar results were obtained using different column flow rates (i.e., slower flow rates to allow greater thiol exchange), redox gradients and column lengths. In contrast, when freshly reduced cytotoxin was eluted from the column with no 18 h incubation period a much lower proportion of oligomeric material was produced.

The bulk of the material was centred around a narrow range of fractions and although oligomeric material was evident, it was much reduced. With this system, again only 35–40% of toxin was in the native conformation. When oxidized glutathione concentration and column length were increased (fig. 8), the amount of oligomeric material was greatly reduced, being confined to a central fraction. Over 50% of toxin was seen in the native conformation. The refolding of 3 μmol toxin did not reduce this yield. In comparison with bulk phase refolding this is a vast improvement, although refolding conditions have yet to be optimized.

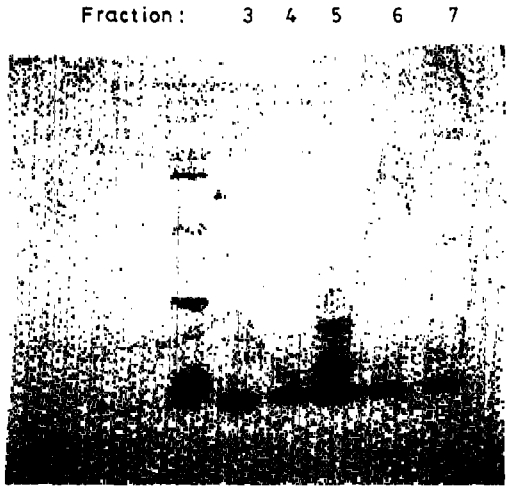


Fig. 8. SDS-polyacrylamide gel (non-reducing 10–20%) and HPLC profiles of fractions of refolded cytotoxin CM6 eluted from an activated thiol-Sepharose 4B (40 ml) column. St., molecular weight standards (M_r 14000–78000).

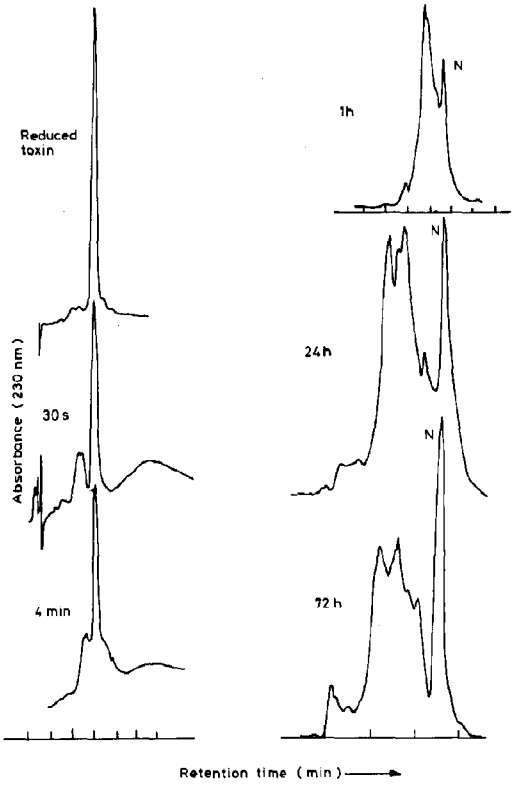


Fig. 7. Reverse-phase HPLC profiles of cytotoxin CM6 after bulk phase refolding in guanidine buffer. Samples were run on a C8 reverse-phase RCM cartridge. Traces show progressive loss of the reduced toxin peak and emergence of the native peak (N), with a simultaneous build up of non-native material.

When α-cobratoxin was refolded on a column matrix, no oligomeric material was observed and the refolding HPLC profiles were dominated by the native refolded toxin (fig. 9). Only extremely small amounts of oligomeric material were seen in the later fractions. In this experiment, 3.0 μmol (21 mg) were successfully refolded. This is in contrast to bulk phase refolding where no native toxin was formed after 24 h refolding and the material was largely oligomeric.

4. Discussion

The above results show that (i) cytotoxins and short neurotoxins refold at a faster rate than the long neurotoxins (figs. 2–4) and (ii) ostensibly native, fully oxidized cytotoxin is seen only after 24 h reoxidation with oxidized glutathione. However, this material has been found to consist

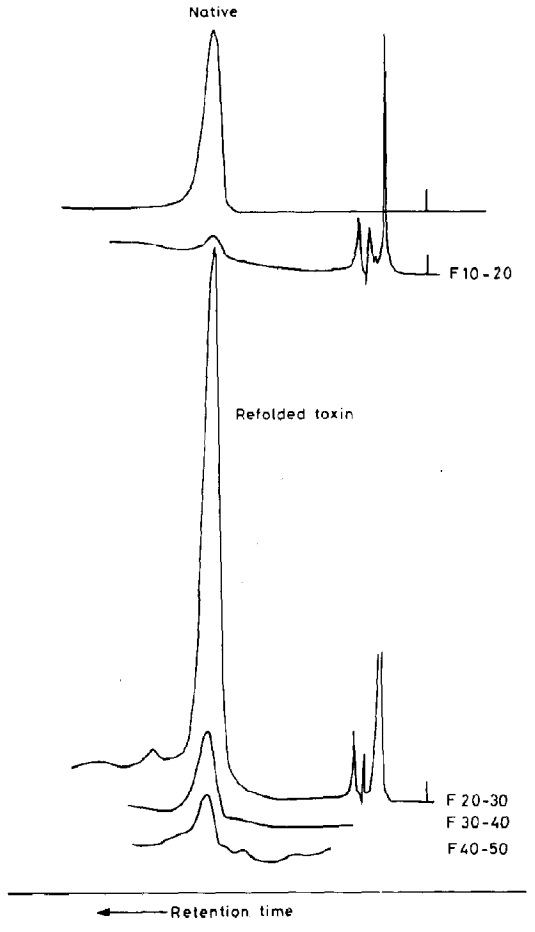


Fig. 9. HPLC column refolding profiles of refolded long neurotoxin (α-cobratoxin) displaying the production of almost pure native toxin subsequent to elution from an activated thiol-Sepharose 4B column.

of oligomeric and other non-native material, in contrast to short neurotoxins, which have been shown to refold to native toxin in the bulk phase [3].

Both long neurotoxins and cytotoxins have hydrophobicities greater than that of short neurotoxins [1]. This is particularly evident with the cytotoxins where sequencing, backed up by X-ray crystallographic data, shows an extended hydrophobic surface [10]. In addition, the extra disulphide bond and C-terminal section of long neurotoxins could play an important role in determining the refolding characteristics.

In view of this exended hydrophobicity, in vitro bulk phase refolding of cytotoxins might well be expected to be complicated by problems due to aggregation. This is confirmed somewhat by the finding that refolding in trifluoroethanol/water mixtures slows formation of the native toxin, as judged by iso-electric focussing [4]. Intermolecular disulphide formation at this stage would make aggregation irreversible. Guanidine overcomes these aggregation problems but is reported to randomize disulphide formation [11], thus leading to greater probability of mismatching disulphide bonds. In vivo intermolecular interactions would be minimized as each toxin molecule is independently synthesized and folded.

For a disulphide bond to be formed between cysteine residues, the peptide backbone conformation must restrict them to a proximity of 4–9 Å. Protein disulphide formation would therefore be expected to depend on the conformation and environment of the polypeptide chain and, by implication, would be dependent on the folding pathway [11]. It has been shown that reduced trapped cytotoxin CM6 has a strong helical character as determined by CD analysis (fig. 10) and that this

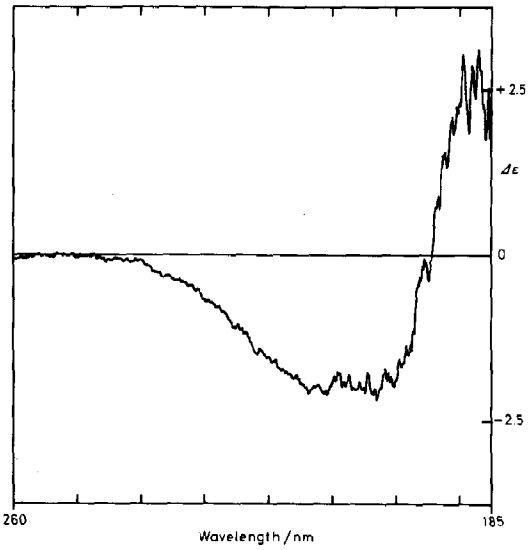


Fig. 10. CD spectrum of reduced, trapped (iodoacetamide) cytotoxin CM6 in water, which is indicative of the presence of extensive α-helical character. Toxin concentration, 1 mg ml^{-1}; 0.02 cm path length cell.

is consistent with structural predictions which show α-helical tendency to be high for cytotoxins [12,13]. This is not the case for short neurotoxins and long neurotoxins [12,13]. The helical structure is stable in 2 M guanidine and at temperatures up to approx. 50 °C (D.C. Smith, unpublished data). This property may contribute to major differences in the folding pathways of the three toxin classes. Some cysteine residues may be located in an ordered conformation in the reduced cytotoxins and therefore be in a different environment from those in other reduced toxins. This may have a profound effect on disulphide bond formation and the refolding pathway.

The column refolding system detailed in the text was designed to overcome some of the problems encountered with the refolding of both long neurotoxins and cytotoxins. It was hoped that column refolding would influence the folding pathway catalytically and lead to a greater yield of the native toxin. The high sulphydryl content of the column matrix is an attempt to mimic protein disulphide isomerase [14].

The reduced toxin was found to bind to the column, probably forming a mixture of bound species. Once the redox gradient is initiated, the toxin will begin to refold. The redox potential of the system will be continually changing as the toxin folds, the gradient of glutathione species becoming more oxidizing. Under these conditions, toxin-toxin interaction will be minimized and therefore intramolecular disulphides will generally be favoured over intermolecular bonds. Eventually, fully oxidized material will pass from the column. Partially oxidized material with incorrectly paired disulphides will normally be less stable than the native toxin and will therefore be more likely to undergo dynamic thiol exchange. Thus, native toxin formation will be favoured.

To summarize the result, it is evident that although yields of refolded cytotoxin of greater than 50%, have not been achieved, greatly increased amounts of cytotoxin have been successfully refolded (i.e., the refolding of 3.0 μmol, 21 mg cytotoxin with no loss of yield). In the case of long neurotoxins, very high yields of correctly refolded material have been obtained. This difference in yields may well reflect the greater hydrophobicity of the cytotoxins in comparison to the long neurotoxins. This problem must be overcome before successful refolding is achieved.

This column-based technique, when perfected, may prove useful in the refolding of both cysteine-containing, genetically engineered proteins and synthetic polypeptides, on a preparative scale.

Acknowledgement

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