Separation of intermediates in the refolding of reduced erabutoxin b by analytical isoelectric focusing in layers of polyacrylamide gel

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Isoelectric focusing in a thin layer of polyacrylamide gel is shown to be a suitable method for the resolution of intermediates trapped during the refolding process of reduced cystine-containing proteins. The method has been applied to the well-characterized snake neurotoxin erabutoxin b. An explanation is offered for the relatively low rate of refolding of this polypeptide.

The sequences of more than 35 'short' snake neurotoxins have been elucidated. These polypeptide chains contain 60, 61 or 62 amino acids and share 27 common residues, including the eight thiol groups, which are paired in an identical manner (Yang *et al.*, 1970; Endo *et al.*, 1971). As a result, short neurotoxins are thought to possess an homologous structure (Dufton & Hider, 1977; Ménez *et al.*, 1978), and all possess a similar curare-like activity (Ishikawa *et al.*, 1977). Despite these analogies, it has been shown that reduced 'short' neurotoxins refold, *in vitro*, with significantly different rates (Ménez *et al.*, 1980). The variation in length of the hairpin segment situated between the two cysteine residues 17 and 24 was tentatively proposed as being responsible for the observed kinetic differences. Since erabutoxin b, isolated from *Laticauda semifasciata* (Sato & Tamiya, 1971), is one of the toxins that refolds relatively slowly, it was decided to study its folding process in more detail. This has been achieved by trapping the intermediates that form during the renaturation process and by fractionating these species by using polyacrylamide-gel isoelectric focusing. The results obtained are compared with the corresponding data on the refolding processes of bovine pancreatic trypsin inhibitor and ribonuclease (Creighton, 1978).

Materials and methods

Materials

Erabutoxin b was given by Professor N. Tamiya (Sendai, Japan). ³H-labelled toxin α was prepared as previously described (Ménez *et al.*, 1971). Oxidized

and reduced glutathione and dithiothreitol were purchased from Sigma Chemical Co., ¹⁴C-labelled glutathione (1 or 3 mCi/mmol) was purchased from New England Nuclear Corp., and ¹⁴C-labelled iodoacetic acid was purchased from C.E.A. (Saclay, France). Erabutoxin b concentrations were determined as described previously (Ménez *et al.*, 1980). Absorption spectra were recorded with a Cary 210 spectrophotometer. Densitometer tracings were obtained with a Vernon densitometer. The receptor-protein-rich membrane (20 μ m of [³H]toxin-binding sites) was prepared from *Torpedo marmorata* electric organ and generously given by Professor J. P. Changeux (Institut Pasteur, Paris, France).

Protein-refolding experiments

Reduction of erabutoxin b disulphide bonds (0.8 mM) was performed as a routine with 40 mM-dithiothreitol in 6 M-guanidinium chloride in 0.1 M-Tris/HCl buffer, pH 8, at room temperature for 75 min. Reduced toxin was desalted by filtration through a Bio-Gel P-2 column (15 cm $\times$ 1 cm) equilibrated in 10% (v/v) acetic acid, freeze-dried and kept under vacuum before use (within 1 week). The reduced toxin was then diluted to 0.05 mM in 50 mM-sodium phosphate buffer, pH 7.0, containing 0.1 M-NaCl. Renaturation was initiated by the addition of oxidized glutathione (1 mM final concentration) at 37°C. The reaction was stopped at different times by the method reported by Creighton (1974). In brief, 0.25 ml of 0.2 M-iodo[¹⁴C]acetic acid (0.3 mCi/mmol) in 0.2 M-NaOH containing 50 mM-phosphate buffer and 0.1 M-NaCl was added to 0.7 ml of toxin solution. The mixture was then desalted on a Bio-Gel P-2 column (10 cm $\times$ 1 cm) equilibrated in 10% acetic acid and freeze-dried.

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Separation of protein-refolding intermediates

The protein was dissolved in pure water to a final concentration of approx. 0.3 mM, and 10 μ l was applied to an 11 cm $\times$ 20 cm polyacrylamide gel. The gel (2 mm thick) contained 8% acrylamide, 0.2% *NN'*-methylenebisacrylamide, 12% (w/v) sucrose, 2% (v/v) pH 3.5–10 Ampholine (LKB Produkter) and 0.15% ammonium persulphate. Electrode strips were soaked in 1 M-NaOH (cathode) or 1 M-H₃PO₄ (anode). Electrophoresis was run at 30 W at 4°C for 75 min. The resulting gels were immersed in 20% (w/v) trichloroacetic acid overnight and stained for 3 h in 0.1% Coomassie Blue in 20% trichloroacetic acid. Gels were subsequently destained by washing in 20% trichloroacetic acid for 3 h followed by 10% acetic acid. Densitometer tracings were recorded on wet gels. Gels were dried before submission to radioautography or sectioning for scintillation counting of radioactivity.

Assessment of biological activity

The concentration of biologically active material present in the renaturation solution was estimated from the ratios of the IC₅₀ values characteristic for the binding of [³H]toxin α (8 nM) with the nicotinic acetylcholine receptor (7 nM) (Ishikawa *et al.*, 1977) in the presence of various amounts of (i) native erabutoxin b as a standard (IC₅₀ = 45 nM) and (ii) protein in the renaturation process.

Results

The free thiol groups of fully reduced erabutoxin b (8 $\pm$ 0.1 SH groups/molecule) were blocked with carboxymethyl groups in a manner similar to that described by Creighton (1974), thus introducing eight additional negative charges ($pK \approx 3$), which obviously will diminish the original value of the isoelectric point of the reduced molecule. This procedure leads to a good separation of carboxymethylated reduced erabutoxin b from native erabutoxin b by polyacrylamide-gel isoelectric focusing; the former migrates with an apparent pI 3.8 $\pm$ 0.1 (mean of four experiments) whereas the native toxin shows an apparent pI 9.4 $\pm$ 0.1 (Fig. 1). A similar method was used to trap and to fractionate the intermediates occurring during the renaturation process of reduced erabutoxin b. In principle any monomeric population of erabutoxin b molecules having incorporated a number of acidic groups between none and eight should display an intermediate pI , i.e. between pI 9.4 and 3.8. The isoelectric-focusing patterns obtained at various times of renaturation (Figs. 2 and 3) demonstrate that this is actually the case. At least 26 resolved bands, excluding those migrating like native (N) and fully blocked reduced (R) erabutoxin b, have been detected, with apparent pI values between those of the N and R proteins.

Fig. 1. Migrations of native (N) and reduced carboxymethylated (R) erabutoxin b on polyacrylamide gel after submission to isoelectrofocusing over the pH range 3–10. For experimental details see the text.

They have been arbitrarily divided into nine groups, which have been designated as A–I.

The number of free thiol groups in each species was estimated by measuring the number of carboxymethyl groups incorporated per molecule of protein, by using iodo[¹⁴C]acetate. The ratio of the area of the densitometer tracing of the stained gel (Fig. 3) over the area of the corresponding radioautogram (not shown) was calculated for each of the nine species. Since the number of carboxymethyl groups (and thus the number of SH groups) incorporated per molecule of protein is 8 for species R, the number of SH groups found was 6.3 $\pm$ 0.2 for species A, B, C and D, 4.0 $\pm$ 0.3 for species E, 2.0 $\pm$ 0.4 for species F and 2.0 $\pm$ 0.2 for species G. This number was not determined for the radioactive band H. No radioactivity was detected at the level of bands I and N. This indicates that these two bands correspond to species with four disulphide bonds. Despite the fact they have the same net charge (they bear no additional carboxymethyl groups), these species migrate differently, however. This may result from appreciable differences in their configuration. It should be stressed that renaturation of erabutoxin b was performed in the presence of 1 mM-oxidized glutathione. This reagent, which is known to accelerate the renaturation process of proteins, including erabutoxin b (Ménez *et al.*, 1980), may lead to the formation of mixed disulphides. This was checked by using ¹⁴C-labelled oxidized glutathione during the renaturation process of the toxin. A radioautogram (not shown) of the pattern obtained indicates that some radioactivity was associated with bands R, A, B, C, D, E and G, but the number of mixed disulphides per molecule of protein was found for populations R, A, B, C, D and E to be less than 3% of the total free thiol groups (estimated as described above) and less than 12% for population G. Therefore, since the fraction of mixed disulphides is small,

Fig. 2. *Isoelectric focusing on polyacrylamide gel of species trapped at different times during the refolding of reduced erabutoxin b*
For experimental details see the text. Species R and N are blocked fully reduced and regenerated erabutoxin b respectively. Species A–I are groups of observable trapped intermediates, and species T is native erabutoxin b.

Fig. 3. *Densitometer tracings of the isoelectricfocusing pattern represented in Fig. 2*
To clarify the Figure, broken lines have been artificially added between the closest bands.

it may be concluded that the number of carboxymethyl groups incorporated per molecule of protein correctly reflects the number of thiol groups not involved in a disulphide bond. Thus the number of internal disulphide bonds per molecule of protein is 0 for species R, 1 for species A, B, C and D, 2 for E, 3 for F and G, and 4 for I and N.

The formation of the native-like species (N) approximates to a first-order reaction with a half-time of more than 3 h, which is consistent with the previous observations made in solution without blocking of the free thiol groups (Ménez *et al.*, 1980). There is a good correlation between the proportion of native-like species (N) present in a mixture trapped during the renaturation and its capacity to compete with native [^3H]toxin, which is specifically bound to the cholinergic receptor (Fig. 4). This indicates that species other than N have little, if any, biological activity, and that this species, which migrates like native erabutoxin b, corresponds to the fully renatured toxin.

In order to ensure that the experimental conditions used in the present work did not interfere with protein folding, renaturation was repeated at pH 8 with 10 mM-oxidized dithiothreitol. The reaction was much slower, in agreement with previous observations (Ménez *et al.*, 1980), but the spectrum of trapped species remained the same.

Fig. 4. Percentage of species N in trapped samples (densitometer tracing) as a function of neurotoxin activity (inhibition of [^3H]toxin α binding to the acetylcholine receptor) of samples containing species N (renaturation longer than 8 min)

For experimental details see the text. ○ and ● correspond to different experiments. The horizontal and vertical error bars indicate the limits of the experimental variation.

Discussion

The present study has shown that isoelectric focusing in a thin layer of polyacrylamide gel is a suitable method to separate the numerous intermediates that were trapped during the folding process of erabutoxin b. In all 28 different species have been resolved and, in principle, this method should be appropriate for the refolding study of any cysteine-containing protein.

The number of different species with a single disulphide bond is rather large. There are at least ten such bands in populations A, B, C and D (Fig. 3). Such a spectrum suggests that the formation of the first intramolecular disulphide bond is essentially random, as with other proteins (Creighton, 1978), and also that this step is not followed by a rapid intramolecular rearrangement favouring the accumulation of a preferred population, as in the case of bovine trypsin inhibitor.

Fully reduced erabutoxin b disappears with a apparent half-time of about 17 s, which is consistent with the values reported for the reaction of model thiol compounds and oxidized glutathione (Creighton, 1975). This suggests that the rate-limiting step in the formation of most of the first intramolecular disulphide bonds is the reaction of toxin thiol groups with the disulphide reagent. This conclusion is further supported by the failure of mixed disulphides to accumulate in the reduced toxin. A similar situation holds for the formation of the second and third bonds, but not for some of the fourth bonds, as accumulation of mixed disulphides occurs to a small extent at the level of band G (molecules with three disulphide bonds).

Clearly the refolding process of erabutoxin b is consistent with those of bovine trypsin inhibitor (Creighton, 1974) and ribonuclease (Creighton, 1977): (i) the formation of intramolecular disulphide bonds is successive; (ii) the rate of the process decreases as the number of disulphide bonds increases; (iii) the rate-limiting step is the formation of the native-like species. Since reduced erabutoxin b and ribonuclease have 8 SH groups/molecule whereas reduced inhibitor has only 6, one could expect a smaller number of single- and/or double-intramolecular-disulphide species for the latter protein. This is indeed observed: 28 single-disulphide intermediates were estimated during the refolding of ribonuclease (Creighton, 1978), and at least 10 single-disulphide intermediates are observable during the refolding of erabutoxin b (Figs. 2 and 3), whereas only two single-disulphide intermediates predominate during the folding of bovine trypsin inhibitor (Creighton, 1978). A further similarity between erabutoxin b and ribonuclease is that the refolding of both proteins occurs only after extensive incubation (several hours), whereas it is almost complete

within 30 min for bovine trypsin inhibitor (Creighton, 1974). Thus it would appear that the refolding of both ribonuclease and erabutoxin b is less well directed than that of the bovine trypsin inhibitor. We suggest that the low rate of folding of the two former proteins is due to the observed accumulation of intermediates, and in particular of those possessing one and two disulphide bonds.

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