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High-Level Production and Isotope Labeling of Snake Neurotoxins, Disulfide-Rich Proteins

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The aim of this work was to produce and to label snake neurotoxins, disulfide-rich proteins. A mutant of a snake toxin, erabutoxin a, was used as a model. Its N-terminal part was fused to ZZ, a synthetic IgG-binding domain of protein A (B. Nilsson *et al.*, 1987, *Protein Eng.* 1, 107–113), thus preventing degradation in the bacterial cytoplasm and providing a simple affinity-purification method on IgG Sepharose. A soluble fusion protein was obtained with a yield of 60 mg/L, corresponding to 20 mg/L toxin. The toxin moiety was folded on the column while the hybrid was still bound. The oxidoreducing conditions for the refolding were optimized and were found to be oxidative but with a need for reducing molecules. The concentration of the hybrid bound to the column could be increased up to 3.3 mg/ml without significantly altering the folding process. CNBr cleavage of the fusion protein followed by a purification step yielded about 2 mg of biologically active toxin mutant per gram of dry cell weight. This procedure was applied to produce 55 mg of a toxin uniformly labeled with ^{15}N. © 1997 Academic Press

Short-chain snake curaremimetic neurotoxins represent a familly of small proteins of 60 to 62 amino acids. They adopt a typical three-finger-shape structure containing three adjacent loops rich in β-pleated sheets emerge from a globular core in which four invariant disulfides are located (2–4). They bind tightly to the nicotinic acetylcholine receptor (AcChR), thus altering nerve–muscle transmission. The resulting paralysis of skeletal muscles, including the diaphragm, induces death as a result of respiratory failure (5). Recently, a mutational analysis of erabutoxin a (Ea), a curaremimetic toxin from sea snake venom, was undertaken, allowing identification of the determinant by which this toxin blocks AcChR (6,7).

A monoclonal antibody, named Mα1, was raised against the α toxin of *Naja nigricollis*, a 61-residue neurotoxin, and was found to possess some striking properties. It recognizes only 61-residue toxins, but a deletion mutant, EaΔS18, which we constructed from the previously unrecognized erabutoxin a, a 62-residue neurotoxin from the sea snake *Laticauda semifasciata*, was able to recover full Mα1-binding activity (8). The structure of this mutant may help us to understand how the deletion modified the structure of the toxin and allowed it to fit with a previously absent determinant. Also, the neutralization mechanism of this antibody seems results from a steric hindrance since it is capable of destabilizing an already formed toxin–AcChR complex (9), and its epitope is distant from the toxic site (10). It was suggested that the binding of Mα1 induced perturbations in the structure of the toxin which could dramatically reduce its affinity for the AcChR (9). This hypothesis could be tested by NMR experiments of a ^{15}N-labeled α toxin bound to Mα1.

As it was planned to solve the above questions using NMR techniques, milligram amounts of EaΔS18 mutant and ^{15}N-labeled α toxin were needed. Unfortunately the expression system we have used to this point led to a low yield of about 0.2 mg per liter of culture or per 8 g of biomass (11). This low yield made the production of quantities needed for NMR time consuming and prohibited the labeling of the protein with stable isotopes. In this paper we describe a method developed to produce the mutant and the labeled protein in amounts suitable for NMR studies.

The formation of disulfide bridges in these molecules was the main difficulty to overcome. In the previous

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system the synthesized toxin was directed to the periplasm of the bacteria, where conditions are compatible with the formation of disulfide bounds. This strategy gave a molecule with its native fold but at low yield (11–13). Possibly, this inadequate production yield is associated with difficulties met by bacteria in forming disulfides from eukaryotic proteins using their prokaryotic-specific disulfide-forming machinery (14). To overcome this difficulty, proteins rich in disulfides can be produced as inclusion bodies in bacteria (15,16). However, the recombinant proteins that are thus produced are usually required to undergo complex treatments including denaturation, refolding, and several purification steps which may lower the final yield of production. We wished to increase this yield and also to avoid systems which are based on the formation of inclusion bodies. Therefore, we tentatively produced in the cytoplasm, using T7 promoter, a soluble toxin fused to a synthetic double domain of the staphyloccocal protein A, ZZ (1). The subsequent refolding step was based on the exploitation of the IgG-binding activity of the fused ZZ protein to refold the toxin moiety directly on an IgG Sepharose column. Thus, we purified and refolded the fused toxin in a single step. The yield of this system, after the release of the mutant toxin by CNBr, was approximately 2 mg of fully active protein per gram of dry weight of cell culture, allowing us to produce enough ^{15}N-labeled toxin to perform NMR studies.

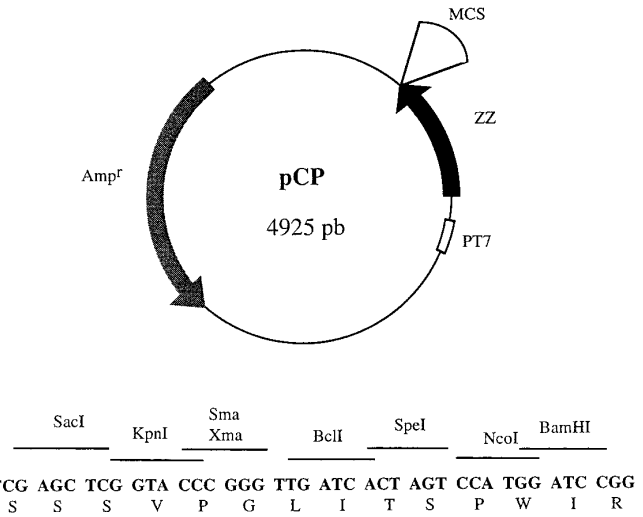

FIG. 1. Schematic representation of pCP1 vector showing the multicloning sites with the associated frame.

MATERIALS AND METHODS

Strains and plasmids. The pET3a derivative plasmid was used as described by Studier *et al.* (17) using the *Escherichia coli* strain BL21(DE3) LysS as host.

DNA manipulation. All DNA manipulations were carried out according to Sambrook *et al.* (18). Oligonucleotides used in PCR were synthesized in an Applied Biosystems DNA synthesizer. The restriction sites were created at each end of the ZZEaΔS18 coding region using pEZZEaΔS18 (8) as a template and the following single-strand oligonucleotides as primers: (I) 5′ *Nde*I site, 5′GGAGGA**CATATG**GCGCAACACGATGAGCCGTAGAC3′, and (II) 3′ *Bam*HI site, 5′GGGGGA**GGATCC**CTAATTGTTGCAGACCTCTGA3′. Restriction sites are indicated in boldface, stop codon is underlined.

A *Nde*I site was created after the codon corresponding to the last amino acid of the signal sequence of ZZ and a *Bam*HI site at the 3′ end of the EaΔS18. The amplified fragment was subcloned in M13mp18 to check its sequence and then inserted into pET3a. The resulting pETZZΔS18 vector was used to transform *E. coli* strain BL21(DE3) LysS.

To create a general purpose vector two oligonucleotides were synthesized: (A) 5′CCGGGTTGATCACTAGTCCATG3′ and (B) 5′GGATCCATGGACTAGTGATCAACCCGGGTAC3′. Once hybridized these nucleotides made a polylinker with the *Sma*I, *Xma*I, *Bcl*I, *Spe*I, and *Nco*I sites and the *Kpn*I and *Bam*HI 1/2 sites. This polylinker was ligated to the pEZZEaΔS18 plasmid in which the EaΔS18 coding sequence was excised by *Kpn*I/*Bam*HI digestion. The resulting plasmid was named pCP1 (Fig. 1).

Synthetic gene assembly. A 195-bp DNA fragment encoding the published α toxin sequence (19) was synthesized as follows. Ten oligomers ranging in lengh from 26 to 61 nucleotides were synthesized and purified. Five duplexes resulting from annealing of complementary nucleotides (100 pmol of each) were ligated to generate two segments having 82 and 113 bp, respectively. These two segments were finally ligated to yield the complete synthetic gene flanked by *Kpn*I and *Bam*HI restriction sites. It was purified, excised, and then inserted into M13mp18 to check its nucleotide sequence. The *Kpn*I/*Bam*HI fragment was then subcloned in the pCP1 vector, resulting in the vector pCPTx.

Protein expression. An overnight culture of BL21(DE3) LysS transformed cells was diluted 100 times in TSB medium (DIFCO) supplemented with 200 mg/L ampicillin and 5 g/L glucose and incubated at 37°C until OD_{600} nm reached 0.5. Then 0.5 mM IPTG was added and after 3 h of culture (OD_{600} = 1.7 to 3), cells were harvested by centrifugation and disrupted by sonication.

For large-scale production of the labeled protein BL21(DE3) LysS transformed cells were precultured in LB medium containing 200 mg/L ampicillin and 30 mg/L chloramphenicol for 6 h. Ten milliliters of this preculture was used to innoculate 1 L of M9 medium using

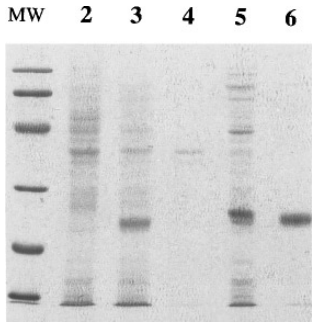

FIG. 2. Coomassie stained 15% SDS–PAGE. After 3 h of induction pEZZEaΔS18-containing cells were harvested then resuspended in Laemmli buffer (lane 3) or submitted to sonication. The sonicated cells were centrifuged, then the pellet (lane 4) and the supernatant (lane 5) were diluted in Laemmli buffer prior to boiling for 5 min. Culture of BL21(DE3) LysS transformed by pET3a was applied in lane 2 as a control. The equivalent of 5 μl of culture was applied. Lane 6 shows ZZEaΔS18 purified on IgG sepharose. Molecular weight markers were 97, 66, 45, 31, 21.5, and 14.4 kDa.

$(^{15}NH_4)_2SO_4$ as the nitrogen source and supplemented with ampicillin 200 mg/L, chloramphenicol 30 mg/L, and 1 ml of ($MnCl_2$ 5 g/L, $FeCl_3$ 6 g/L, $ZnCl_2$ 5 g/L, $CoCl_2$ 0.1 g/L, $CaCl_2$ 1 g/L, $CuSO_4$ 0.4 g/L, H_3BO_3 0.1 g/L). This second preculture was continued overnight at 37°C and used to innoculate 25 L of the same medium in a 30-L fermenter (Biolaffite, St. Germain en Laye, France). Induction was carried out as described above and the cells were harvested by centrifugation after 3 h of culture. The cell pellet was resuspended in 500 ml of lysis buffer [Tris 30 mM, pH 8, EDTA 5 mM, sucrose 20%, lysozyme (Sigma) 0.1 mg/ml, DNase I (Boerhinger) 0.1 mg/ml] and submitted to three cycles of freezing and thawing. Cell debris was discarded by centrifugation for 30 min at 12,000*g*, then 0.5 mM PMSF and 0.3 M Na_2SO_3 were added to the supernatant before it was subjected to purification/renaturation as described later.

Production of proteins in the periplasm of *E. coli* was carried as previously described (11). Concentration of total proteins was estimated by using the Bio-Rad Protein Assay (Bio-Rad).

Protein analysis. Level of protein expression was analyzed by SDS–PAGE using a PhastSystem (Pharmacia). At the end of the induction time 200 μl of culture was pelleted, resuspended in 50 μl of sample buffer, and boiled for 5 min; 1 μl of sample was applied.

Cytoplasmic solubility of hybrid protein was determined as follows: 20 ml of induced culture was centrifuged and the pellet was resuspended in 2 ml of (Tris 50 mM, pH 7.5; EDTA 1 mM) before sonication. After centrifugation of 50 μl of lysate, the pellet was resuspended in 100 μl sample buffer and the supernatant was diluted twofold in the same buffer.

Purification of recombinant protein. IgG Sepharose (Pharmacia) equilibrated in PBT [0.1 M phosphate buffer (pH8), 0.1% Tween 20] was incubated with extracellular extract for 3 h at 4°C, poured onto a column, washed with PBT until OD_{280} reached a baseline, then washed with 5 mM ammonium acetate, pH 5. The hybrid protein was eluted with 0.5 M acetate, pH 3.4. The eluted fraction was immediately frozen and lyophilized. Protein concentration was estimated by UV absorption, assuming a molar extinction coefficient at 280 nm of 11,460 for the hybrid protein.

In vitro refolding. The refolding step was introduced into the purification protocol after washing the column with PBT. The IgG Sepharose gel with the adsorbed hybrid protein was washed with two column volumes of renaturation buffer (0.1 M phosphate buffer, pH 8, 5 mM EDTA, appropriate ratio of GSH/GSSG). After incubation for 15 h at room temperature the purification was resumed at the ammonium acetate step.

Cyanogen bromide cleavage and purification of the recombinant toxin. The lyophilized hybrid was dissolved in 0.1 N HCl in the presence of a 500-fold excess CNBr for 24 h. The resulting recombinant toxin was separated from the ZZ moiety and other side products by chromatography on a reverse-phase HPLC column (Vydac; C_4, 5 μm 10 × 250 mm). The column was equilibrated in 0.1% trifluoroacetic acid, and elution was performed using a trifluoroacetic acid/CH_3CN/H_2O gradi-

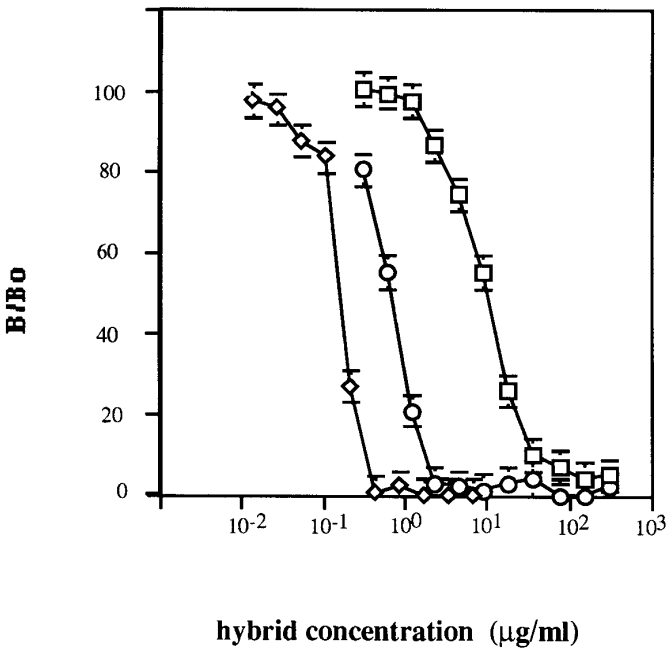

FIG. 3. Inhibition of binding of ^{3}H-α-toxin to monoclonal antibody Mα2,3 using increasing concentration of periplasmic-produced ZZEaΔS18 (◇) or cytoplasmic-produced ZZEaΔS18 with (○) or without (□) folding procedure.

ent system. The recombinant toxin was further purified by chromatography on a Mono S (Pharmacia) HPLC column equilibrated in 0.01 M ammonium acetate, pH 4.5, and eluted by a gradient of 0.01 to 1.5 M amonium acetate at the same pH.

Radioimmunoassay. The radioimmunoassay was carried out as previously described (9) using the monoclonal antibody Mα2,3 (20) as a probe for the native toxin and ^{3}H-labeled α toxin as a competitor. When biological activity was probed the acetylcholine receptor was used as a ligand as described by Faure *et al.* (21)

NMR spectroscopy. An NMR sample containing 10 mg of ^{15}N-labeled α toxin diluted in 400 μl of H_2O/D_2O (95/5%) was prepared. The pH of the solution was adjusted to 3.5 by adding microliter amounts of HCl. An HSQC experiment (22) was recorded at 308 K on 500 MHz Bruker spectrometer, using a 2.75-ns value for the 1/25 delay. The water signal was reduced by classical presaturation of 800 ms. The TPPI waste was used during acquisition (23). The final matrix is formed of 1024 $\times$ 256 ral points. The spectral width used was 6094 Hz for proton dimension and 2000 Hz for nitrogen dimension.

RESULTS

Synthesis of Recombinant Toxins

Taking advantage of the available pEZZΔS18 periplasmic expression vector (7), we introduced a restriction site after the codon of the last amino acid of the signal sequence of ZZ, and the fragment corresponding to the fusion protein was inserted into the pET3a vector to form the pETZZΔS18 vector. In this hybrid protein the EaΔS18 mutant was linked to the ZZ double domain of protein A by an amino acid sequence with a methionine in position -1 before the first residue of the mutant. This allowed the subsequent release of the toxin moiety by CNBr cleavage. The mutant (MW 6700) represented a third of the molecular weight of the hybrid protein.

Expression was carried out in the BL21(DE3) LysS strain. After 3 h of induction a major band at about 22 kDa appeared on SDS–PAGE (Fig. 2, lane 3). This size nicely matches the theoretical value calculated from the amino acid sequence of the ZZEaΔS18 hybrid protein. The overproduced protein was entirely soluble in the cytoplasm (Fig. 2, lane 5), since no accumulation of insoluble hybrid protein was observed. Since the hybrid was soluble in the cytoplasm, the extracellular extract could be purified without any need for additional denaturant. Purification on an IgG Sepharose column showed that the ZZ domain of the recombinant protein retained its ability to bind to IgG. The recombinant protein was stable in the cytoplasm, since the overproduced protein was found solely as hybrid (Fig. 2). In rich medium, the production yield of fusion protein determined by UV absorption after purification on IgG Sepharose (Fig. 2, lane 6) ranged between 60 and 90 mg/L of culture, corresponding to 18 to 27 mg/L toxin.

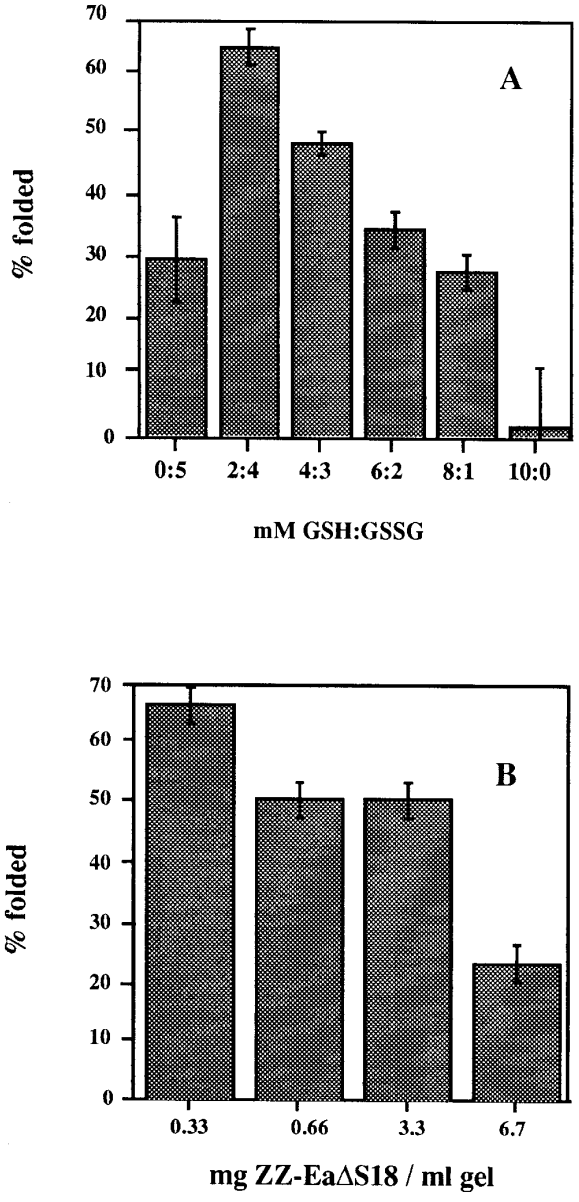

FIG. 4. Optimization of the ZZEaΔS18 folding conditions. An RIA experiment was done for each assay. The IC_{50} obtained was compared to the IC_{50} of the fully folded periplasmic-produced hybrid to give the amount of folded fraction. (A) One milligram of hybrid was incubated overnight with various GSH:GSSG ratios. (B) Different amounts of hybrid were bound to 1 ml of IgG Sepharose and incubated overnight in 2:4 GSH:GSSG.

Refolding of EaΔS18

The folding state of the toxin moiety in the recombinant protein was examined by radioimmunoassay us-

TABLE 1

Purification of Recombinant EaΔS18 from 1 Liter of Cell Culture Showing the Range of Production Obtained

	Total proteins (mg)	ZZEaΔS18 fusion protein (mg)	EaΔS18 equivalent (mg)
Crude extract	240 to 420	60 to 90	18 to 28
IgG Sepharose purification	60 to 90	60 to 90	18 to 28
CNBr treatment	ND	—	ND
RP-HPLC	ND	—	3.6 to 5.6
MonoS	2 to 3	—	2 to 3

ing toxin-specific monoclonal antibodies that recognize the toxin only when it is folded in a native-like structure. In particular, we used Mα2,3, which recognizes a topographical epitope that is similar to the determinant that is recognized by AcChR, the target of the toxin. ^{3}H-labeled α toxin of *N. nigricollis* was used as competitor. A reference curve was made with hybrid produced in the periplasm (Fig. 3) which was assumed to be fully folded (11); the IC_{50} deduced from this curve was about 0.4 ± 0.08 μg/ml. With the recombinant hybrid purified from the cytoplasm but without folding treatment (Fig. 3), an IC_{50} of 10 ± 2 μg/ml was obtained. This indicates that about 96% of the hybrid synthesized in the cytoplasm remains unfolded.

We then took advantage of the binding activity of the ZZ domain by oxidizing the hybrid protein while it was immobilized on an IgG Sepharose column. Thus, purification and renaturation were achieved in a single step. Various GSH upon GSSG ratios were tested for the refolding of the mutant toxin, and the resulting molecules were tested in the RIA experiments to give us an IC_{50}. Comparison with the IC_{50} of the fully folded periplasmic-produced hybrid protein gave an estimation of the amount of folded molecules in the assay, and the results are presented in Fig. 4. Optimal folding conditions were found to be identical to those of the native Ea (F. Bouet, personal communication), i.e., 2:4 mM in GSH:GSSG (Fig. 4A), and the refolding yield decreased if this ratio was changed.

In order to test the capacity of the solid-phase folding method, we increased the concentration of hybrid molecules per milliliter of IgG Sepharose gel. As can be seen in Fig. 4B, the concentration of hybrid could be increased up to 3.3 mg/ml with minimal loss of folding efficiency, but the yield decreased dramatically at 6.7 mg/ml.

The refolded hybrid protein was then cleaved by cyanogen bromide and the mutant toxin was purified according to the method previously described (13) and detailed above. Only the fraction that coeluted, as a single peak, with the native toxin in ion-exchange chromatography was collected. Table 1 summarizes the scheme of purification and the range of production we obtained. Total proteins were determined by colorimetric method. The amount of hybrid protein in the crude extract was estimated after IgG purification since this method was found to have a yield near 100%. As has been seen in previous work (13), the CNBr cleavage and the subsequent purification step were responsible for the principal losses of this method and yielded 10 to 20% of the starting equivalent toxin. The purified toxin had the expected amino acid composition and N-terminal sequence up to 5 residues (data not shown).

The biological activity of the purified recombinant mutant was probed by a competition binding assay, using *Torpedo* AcChR-enriched membranes and ^{3}H-labeled α toxin. The IC_{50} values obtained with the refolded protein and the natural erabutoxin were identical (Fig. 5A). The antigenic properties of the toxin were probed by RIA with Mα1 and Mα2,3 and IC_{50} of 10 ± 2 and 15 ± 3 nM, respectively, were found, which was identical to those expected for native toxin. The overall structure of the refolded mutant toxin was investigated by far-UV circular dichroism: recombinant protein and native toxin have the same secondary structure content, as indicated by CD analysis (Fig. 6A).

Labeling of the α Toxin of N. nigricollis with Stable Isotope

This production system was used to synthesize the homologous toxin α from *N. nigricollis* and to label it with stable isotopes. Since the cDNA for α toxin was not available we constructed a synthetic gene encoding the protein on the basis of its published sequence (19) using usable codons of *E. coli* (24). A methionine was inserted at the position −1 of the toxin to allow us to ultimately treat the hybrid protein with CNBr. The synthetic gene was inserted in a pCP1 vector (see Materials and Methods) using the *Kpn*I and *Bam*HI restriction sites.

Yield of production of ZZ–α-toxin hybrid protein in rich medium was comparable to that obtained with the mutant and was around 60 mg/L of culture. However, the simplest way to uniformly label a protein with ^{15}N is to use a minimal medium with $(^{15}NH_4)_2SO_4$ as the sole source of nitrogen, thus all the production assays of ZZ–α-toxin were made in this medium (see Materi-

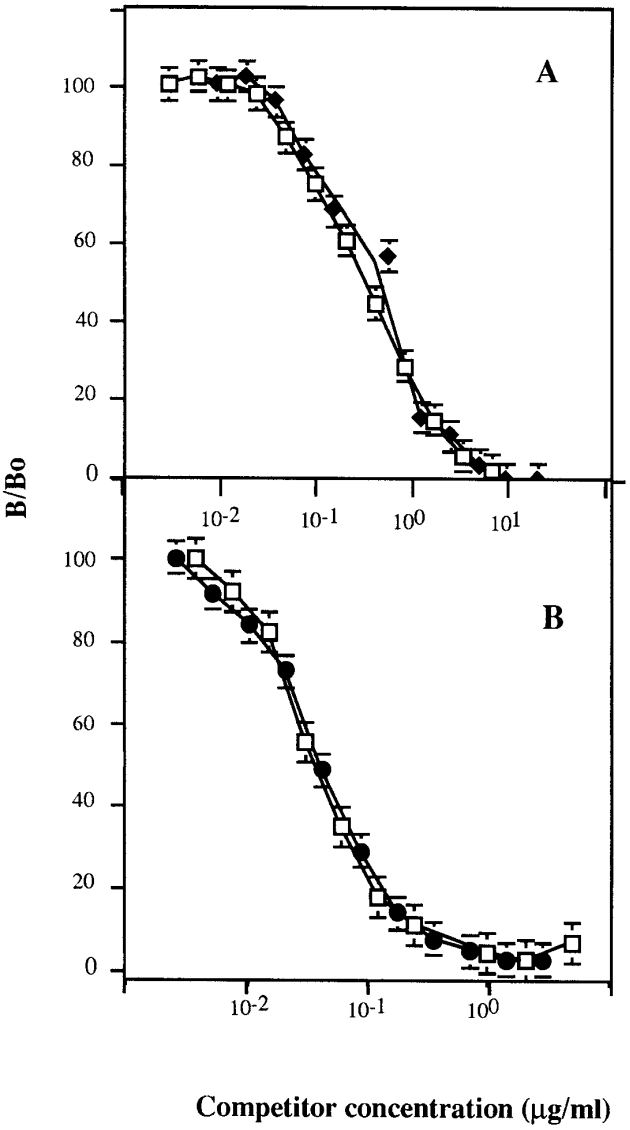

FIG. 5. Inhibition of binding of ^{3}H-α-toxin to acetylcholine receptor using increasing concentrations of: (A) natural erabutoxin a (□) or purified recombinant EaΔS18 (♦); (B) natural α toxin (□) or ^{15}N-α-toxin (●). Results were expressed as in Fig. 3.

als and Methods). A final yield of 44 mg of ZZ–α-toxin per liter, equivalent to 14 mg of toxin, was obtained in large-scale culture.

After the refolding procedure the IC_{50} obtained in RIA with this hybrid was about 0.48 ± 0.09 μg/ml. With the IC_{50} of the periplasmic-produced hybrid (see above) as a reference, the yield of refolding of the toxin moiety was estimated to be about 80%. After CNBr cleavage and purification we obtained 2.2 mg of toxin per liter, which corresponds to a final yield of 16%. Thus 55 mg of pure ^{15}N-α-toxin was isolated from 25 L of culture.

Biological and biophysical characteristics of the recombinant labeled toxin were tested, as for EaΔS18, and showed total identity with the native toxin (Figs. 5B and 6B). Mass spectrometry was used to determine the yield of labeling; comparison of the molecular weights of the native and isotope-labeled toxin indicates a yield of 92% ^{15}N incorporation. Heteronuclear NMR spectroscopy also allows quantification of the extent of labeling of the protein. By recording a 1D spectra with and without ^{15}N decoupling during acquisition, the amplitude of coupling could be measured for a number of residues. The amplitude measured was consistent with a protein labeled at 95 ± 5%. A HSQC experiment was recorded in order to check if all the ^{15}N of the protein was correctly labeled. Figure 7 shows the "fingerprint" of the labeled α toxin. The expected ^{15}N–^{15}NH correlation number is present on this spectrum.

DISCUSSION

The quantities of materials required for the study of proteins by NMR methods have often hampered this technique. When a protein must be modified, either by mutation or by labeling, recombinant methods are most appropriate. However, snake neurotoxins possess multiple disulfide bridges and, although expression in the periplasm provides a molecule with all the features of the native toxin (13,12), the yield of production is too low to satisfy NMR requirements. We have therefore tried to increase the production level by using the very

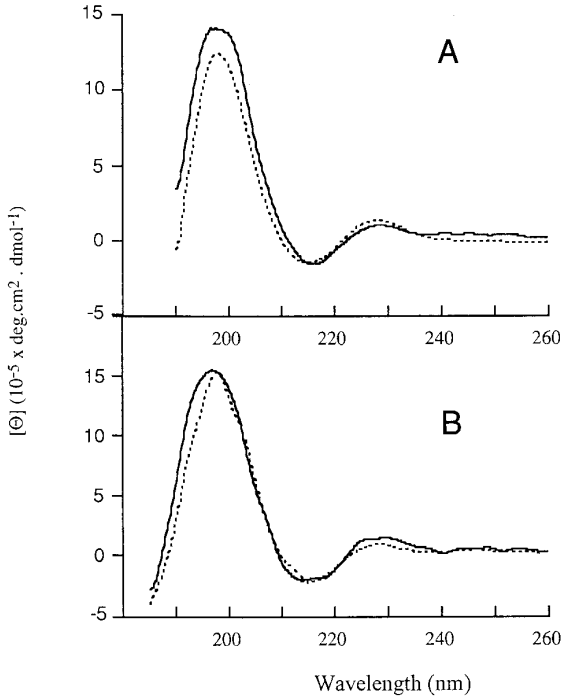

FIG. 6. CD spectra of: (A) natural Ea (——) and refolded EaΔS18 produced in the cytoplasm (· · ·); (B) natural α-toxin (——) and ^{15}N-labeled α toxin (· · ·).

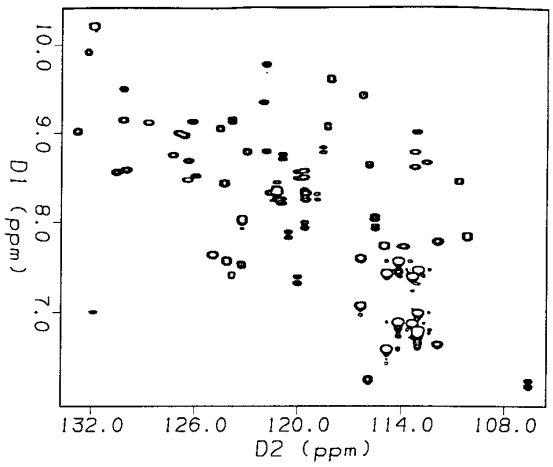

FIG. 7. Fingerprint of the ^{15}N-labeled α toxin showing all the backbone ^{15}N–^{15}NH correlations observed in the protein.

potent T7 promoter system and by directing the synthesis of the toxin to the cytoplasm of the bacteria. The ΔS18 mutant of erabutoxin a was used as a model protein because of our need for this molecule in other studies, and because of the difficulty in refolding the wild-type toxin (25), which presented a challenge for this method. As the mutant toxin was not stable alone in the cytoplasm (not shown) we stabilized it with a fusion partner. The choice of the partner was led by the need for a simple and efficient method of purification and refolding. The protein A of *Staphylococcus aureus* seemed to be a good candidate since its IgG-binding activity provided a simple one-step affinity purification scheme; furthermore, it has been produced in the cytoplasm of *E. coli* in which it keeps its IgG binding activity (26). In fact ZZ, a double synthetic domain of protein A (1), was preferred because of its smaller size and its absence of methionine, which facilitate the CNBr cleavage of the hybrid protein.

The resulting fusion protein was produced at a satisfying level but we observed variations in the production level which were highly dependent of the OD on the culture at the time of induction. The recombinant hybrid protein remains soluble and could be utilized without additional denaturation treatment. As expected, the ZZ part retained its binding activity, allowing the hybrid protein to be purified on IgG Sepharose. This was in accordance with recent work using the same fusion partner (27). We then took advantage of this activity to fold the toxin still bound to the Sepharose. This method offered two advantages. First, the purification and the folding step could be linked, reducing handling and materials. Second, as the hybrid molecule was immobilized during the folding process, molecular movements and thus aggregation were minimized; this allowed us to increase the concentration of the hybrid in the folding reaction to 3.3 mg/ml, which is more than three times that usually used in solution (28–30). However, a dramatic drop in the folding yield of the toxin was observed above this value. As this concentration corresponds to the maximum capacity of the gel, the hybrid molecules were probably close together and the benefit of the binding on a solid phase was lost.

RIA experiments were used to estimate the rate of folding of the toxin moiety in the hybrid protein and to compare different oxidoreducing conditions. Optimal conditions were found to be near that of the native toxin (25), i.e., oxidative but with a need for the presence of reduced molecules. This probably allows the reshuffling of incorrect bridges. The ΔS18 mutant of Ea was folded at a lower rate than the α neurotoxin, as it was also observed with the native Ea (25). Thus, the mutant and native toxin Ea seem share identical folding behavior. It should be noticed that under optimal conditions the ZZ hybrid form of the α neurotoxin was folded at good efficiency as was observed with the native toxin (25). Thus, it seems that the toxin part of the hybrid was folded independently, the ZZ exerting no influence in the folding process.

Finally, after cleavage and purification, a mutant toxin was produced with biological and structural characteristics identical to the parent molecule. The yield was about 10 times that observed with the periplasmic expression system (11) and, to our knowledge, it was the best yield yet reported for similar proteins. The main loss occurs during the cleavage process, but at this time the CNBr reaction remains the best method we have tried. Five liters of culture was sufficient to give the amount of mutant toxin needed.

The yield by biomass unit was 2 mg toxin per 0.5 g of biomass; this was 160 times higher than that obtained in the periplasmic production. With such a yield the labeling of the α neurotoxin of *N. nigricollis* could be considered. The labeling was done in minimal medium which caused an expected drop in production. Nevertheless, this loss was compensated for by the good folding efficiency of the α neurotoxin. Finally, this method is supported to by the fact that no detectable differences in structure or function are found between the labeled protein and the native toxin and that the ^{15}N-α-toxin was fully usable in NMR experiments.

The methods presented here describe a high-level expression system linked to a simple purification/renaturation procedure. This method allowed us to reach our goal, i.e., production of the desired amount of a mutant and a ^{15}N-labeled protein. It could also be of use in the production of small, disulfide-rich proteins. Furthermore, other kinds of labeling may now be considered such as ^{13}C/^{15}N or labeling of specific residues. The main limit of production lies in the separation of the protein of interest from the fusion partner. Engi-

neering a specific proteolytic digestion site may improve the yield at this point.

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