Cloning and Expression of Wild-type and Mutant Forms of the Cardiotonic Polypeptide Anthopleurin B*  

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Venom of the sea anemone Anthopleura xanthogrammica contains a minimum of three polypeptide toxins capable of prolonging the repolarization phase of the action potential. A synthetic gene for the most toxic of the Anthopleura toxins, anthopleurin B (ApB), has been designed, synthesized, and expressed as a fusion protein with the gene 9 product of bacteriophage T7 in Escherichia coli. The fusion protein has been purified and its disulfide bonds reoxidized using glutathione redox couples. Upon cleavage with staphylococcal protease, this protocol yields approximately 1 mg of native ApB/liter of original culture. The recombinant protein has been shown to be identical to natural ApB with respect to amino acid composition, amino-terminal sequence, secondary structure, high pressure liquid chromatographic mobility, and biological activity. A second form of ApB containing additional residues of glycine and arginine at its amino terminus has also been characterized. This protein, termed GR-ApB, is identical in specific activity to the wild-type form. This work lays the groundwork for a detailed analysis of ApB structure and action by site-directed mutagenesis.

The venom of the Pacific coast sea anemone Anthopleura xanthogrammica is a rich source of vasoregulatory substances, cytolysins, and neurotoxins (1–3). Polypeptide neurotoxins produced by this anemone belong to a class of toxins which bind to fast voltage-dependent sodium channels of nerve and muscle (4–6). Toxin binding delays the repolarization phase of the action potential, causing the channels to persist in the open conformation (2, 7–9). The primary toxin of the scorpion Leiurus quinquestriatus also delays the inactivation of sodium channels (10–13), although this toxin lacks any sequence or structural homology to the anemone toxins. Catterall and Beress (11) have shown that toxin II from Anemonea sulcata, a close homologue to the anthopleurin toxins, can compete with Leiurus toxin for the same binding region on the sodium channel (11), which has been mapped for scorpion toxin to domain I of the α-subunit at the extracellular loop between transmembrane helices S5 and S6 (14). Since both scorpion and anemone toxins delay repolarization, this overlapping binding region is believed to be tightly coupled to the inactivation gate of the sodium channel (11).

A. xanthogrammica produces two major neurotoxins, anthopleurin A (ApA) and anthopleurin B (ApB) (1–3), which are 49-residue polypeptides with a molecular mass 5.2 kDa and are cross-linked by three disulfide bonds. Analysis of the structure of ApA by two-dimensional NMR is consistent with a protein containing a four-stranded antiparallel β-pleated sheet encompassing residues 2–4, 21–23, 34–36, and 45–49, with a type II β-turn formed by residues 30–33 (15). ApB is likely to have a generally similar structure because of 85% sequence conservation and retention of all three disulfide pairs.

Although the anthopleurins are clearly structurally related, ApB is 7–10-fold more active than ApA on mammalian cardiac and neuronal channels (3). ApB is also the most cationic of all the Anthopleura and Anemonea toxins. Of the seven amino acid differences between ApA and ApB, two involve replacement of basic residues: Arg-12 and Lys-49 of ApB are replaced by serine and glutamine, respectively, in ApA. It has been suggested that the increased basicity of ApB provides the basis for its increased specific toxicity (16). Indeed, among this family of polypeptide neurotoxins, ApB is the most potent homologue known (17).

The anthopleurin toxins have been the subject of a good deal of interest for their therapeutic potential. In whole animal studies, anthopleurin A has been shown to exert a powerful positive inotropic effect (18, 19) which is correlated to an increase in the amplitude of the action potential and is suppressed by tetrodotoxin (20). Studies involving intravenous administration of ApA have led to the suggestion that the polypeptide may have significant advantages over glycosides and other inotropic agents in treatment of heart failure (21, 22). In this report we describe the cloning and expression in Escherichia coli of a synthetic gene for ApB and the refolding and reoxidation of the recombinant toxin into a biologically active protein. We have also expressed an altered form of ApB having an amino-terminal extension, Gly-Arg. This toxin, designated GR-ApB, was found to be as active as natural ApB at enhancing the veratridine-dependent 22Na uptake by neuroblastoma cells in culture.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents—β-Cyanoethylphosphoramidites were obtained from ABN, Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and T4 polynucleotide kinase were obtained from either Bethesda Research Laboratories or New England Biolabs. Avian myeloblastosis virus reverse transcriptase is a product of Life Sciences. Staphylococcal protease (Staphylococcus aureus, strain V8) was obtained from ICN. Isopropyl β-D-thiogalactopyranoside was obtained from U. S. Biochemical Corp., and restriction protease factor
Xa (bovine) was from Boehringer Mannheim. Sea anemone toxin ApB was purified from 5 kg of A. saxostrommica by the method described previously (3), except with the addition of a final purification step by HPLC. Tetrodotoxin was obtained from Calbiochem. All other reagents were of the highest purity and purchased from standard biochemical sources.

Cell Cultures—Murine neuroblastoma cells (N18) used in the solid uptake experiments were generously provided by Dr. Richard Akeson (Children’s Hospital Research Foundation, Cincinnati, OH). Cells were maintained in 90% Dulbecco’s modified Eagle’s medium, 5-10% fetal calf serum under conditions described previously (25). E. coli strain JM109 was routinely used for bacterial transformations and propagation of M13 phage, whereas the strain UV5 (duf ung) was used for isolating uracil-containing templates for site-directed mutagenesis (26). The E. coli expression strain B1.21(DE3) was used for production of fusion protein.

DNA Methods—Bacterial transformations, colony hybridizations, and plasmid or phage DNA isolation were performed as described previously (26). Oligonucleotide-directed mutagenesis was carried out using the method of Kunkel et al (25). Oligonucleotides were synthesized on a Pharmacia Gene Assembler and purified by denaturing polyacrylamide gel electrophoresis prior to use. Sequencing of plasmid and phage DNA was by the dye methodology on either single- or double-stranded templates (27).

The nucleotide sequence of the ApB gene was based on reverse transcription of the known amino acid sequence (2); the gene was initially synthesized as eight strands ranging in size from 20 to 55 nucleotides (see Fig. 1). Equimolar amounts of complementary strands were annealed and phosphorylated using T4 polynucleotide kinase. These DNA duplexes were then ligated and cloned directly into pUC19. The coding sequence was verified by double-stranded dideoxy sequencing.

Expression and Fusion of Protein—For expression, the ApB coding sequence was inserted in-frame, 3′ to the sequence encoding the gene 9 polypeptide of bacteriophage T7 in the plasmid pRS9 (28, 29) (for details) and fused to alanine to construct transformed into the expression strain B1.21(DE3) (28). Cells were grown in Luria broth in the presence of 100 µg/ml ampicillin to mid-log phase and induced with 0.5 mM isopropyl β-D-thiogalactopyranoside for 2 h. Cells were harvested by centrifugation and resuspended in TNE buffer (10 mM Tris, pH 8.6, 50 mM NaCl, 1 mM EDTA). The protease inhibitors leupeptin, phenylmethylsulfonyl fluoride, and pepstatin were added to final concentrations of 0.5, 100, and 1 µM, respectively. After 30 min of lysozyme (5 mg/liter of culture) digestion on ice, β-mercaptoethanol was added to the cell suspension frozen. After three freeze-thaw cycles, the cells were passed through a French pressure cell and the resulting suspension clarified by centrifugation for 40 min at 27,000 × g. Nucleic acids were removed from the supernatant by gradually bringing the streptomycin sulfate concentration to 1% (w/v). After centrifugation, the ammonium sulfate concentration of the supernatant was gradually brought to 50% saturation (w/v) to preferentially precipitate the fusion protein. The precipitate was dissolved in column buffer (50 mM Tris, pH 7.2, 100 mM NaCl, 5 mM β-mercaptoethanol) and dialyzed against the same buffer to remove residual ammonium sulfate. The fusion protein was purified further by anion exchange chromatography on DE52 and eluted using a linear gradient from 100 to 400 mM NaCl in column buffer. Fractions were pooled based on their absorbance at 280 nm. Protein concentration was determined by the method of Bradford (29), with bovine serum albumin as standard.

Reduction, Cleavage, and Purification of Recombinant ApB—ApB can be reoxidized prior to cleavage by dialyzing the protein, at a concentration of 2 mg/ml, against 100 mM Tris, pH 8.0, 100 mM NaCl, and 1 mM β-mercaptoethanol. The β-mercaptoethanol is then replaced by dialysis against 1 mM reduced glutathione. Oxidized glutathione is then added to a final concentration of 0.2 mM and the solution incubated overnight at room temperature. The protein solution is then passed through a Sephadex G-25 column to remove β-mercaptoethanol. The fusion protein cleaved by overnight treatment with staphylococcal protease (3%) by weight at 37°C with constant stirring. Insoluble peptides are removed by centrifugation and the supernatant concentrated by ultrafiltration and lyophilized. Pure recombinant ApB is separated from residual gene 9-derived peptides on a preparative reverse phase C4 column.

Immunoblotting—Antibodies against ApB were raised in New Zealand White rabbits. The neurotoxicity of natural ApB was first demonstrated by reducing the toxicity of the kallikrein by incubating with Kunitz trypsin inhibitor for 30 min at 55°C for 6 h in the presence of 50 mM β-mercaptoethanol. Animals were injected with 100 µg of reduced ApB emulsified in complete Freund’s adjuvant; subsequent boosts used similar toxin amounts in incomplete Freund’s. Antibodies in the sera were capable of detecting 10–50 ng of ApB by dot-blot analysis. Western blots were carried out using the anti-peptide goat-anti-rabbit IgG as the secondary antibody and the chromogenic substrate diaminobenzidine (30).

Analytical Methods—Samples for amino acid analysis were prepared by acid hydrolysis of 5 nmol of protein in vacuo at 110°C for 22 h. In some cases samples were reduced and alkylated with iodoacetamide prior to hydrolysis. Hydrolysates were analyzed by HPLC on a PicoTag analyzer. Amino-terminal sequences of 1-nmol samples were determined by automated Edman degradation using a Porter Industries protein sequenator (model PI-2090E). Far-UV CD spectra were obtained on a Cary 61 instrument calibrated with camphorsulfonic acid. CD data were analyzed by the computer program described by Chen et al (31).

Functional Characterization of Recombinant ApB—The ability of ApB to enhance veratridine-dependent 22Na uptake by cultured cells was measured according to the procedures of Lazdunski and coworkers (3). N18 cells were grown to confluence (4–5 days) in 24-well plates. After removal of the medium by aspiration, cells were preincubated at 37°C with sodium-free binding solution containing 25 mM Hepes/Tris, pH 7.4, 140 mM choline chloride, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 5 mM glucose, 0.1 mg/ml bovine serum albumin, 20 µM veratridine, and varying concentrations of ApB. After 30 min, the rate of 22Na uptake was measured by incubation for 1 min in a solution containing 25 mM Hepes/Tris, pH 7.4, 130 mM choline chloride, 10 mM NaCl, 5.4 mM KCl, 1.3 mM MgSO4, 5 µM ouabain, 22NaCl (1 Ci/ml), and the same concentration of ApB as the binding solution. After uptake, cells were washed with 3 × 2.5 ml of ice-cold 25 mM Hepes/Tris, pH 7.4, 145.6 mM choline chloride, 1.8 mM CaCl2, 0.8 mM MgSO4. Cells were then solubilized in 0.5 M NaOH and the total protein as well determined by the method of Bradford (29). Each data point of the dose-response curve was assayed in quadruplicate. Control experiments with no effectors, and veratridine only were also carried out as described above. The rate of 22Na uptake with 20 µM veratridine only was subtracted from the toxin uptake data. The specificity of uptake was estimated by measuring its sensitivity to inhibition by tetrodotoxin (3). In the absence of ApB, 10 nM tetrodotoxin reduced the rate of 22Na uptake/min/mg of protein. Kinetic constants Vmax and Km were determined by the computer program of Cleland fitting the curve to a hyperbolic function (38).

RESULTS

Design of the Synthetic ApB Gene—The synthetic ApB gene was designed with unique PstI and EcoRI overhangs at the 5′ and 3′ ends, respectively, along with internal Apal, BamHI, and NcoI sites to facilitate subcloning and cassette mutagenesis (Fig. 1). The 162-base pair ApB gene was first ligated into the PstI/EcoRI sites of pUC19, yielding the plasmid pKB-1 whose nucleotide sequence was verified by double-stranded dideoxy sequencing. All constructs were initially cloned and maintained in E. coli strain JM109 because it lacks the T7 RNA polymerase necessary for induction of plasmid encoded protein (24).

Expression Constructs—The steps involved in plasmid construction for expression are summarized in Fig. 2. Initial experiments were carried out by transforming pKB-1, having the coding sequence of ApB under the control of the lac UV5 promoter, into E. coli JM109. Because induction with isopropyl β-D-thiogalactopyranoside afforded only very low levels of expression (data not shown), use of this system as a production strain was not pursued further. Small foreign peptides overexpressed in E. coli are frequently unstable or even cytotoxic (28, 32) so that this result was not entirely surprising.
The nucleotide sequence of the ApB gene was derived by reverse translation of the known protein sequence (2). The gene was synthesized in eight strands which were annealed to form four sets of complementary duplexes, containing the following overhangs: PstII/ApaI, ApaI/BanHI, BamHI/NcoI, and NcoI/EcoRI. The four duplexes were ligated to form the intact gene. The restriction sites are highlighted in boldface.

AATCATCGAAGGACGGTGCA
Xa linker
GTAGCTCCTGCC
Deletion of three codons (cys-arg-met)

Deletion of last two codons

It is often possible to express foreign peptides as fusion proteins. We have previously reported successful expression of small polypeptides as chimeras with the gene 9 protein of bacteriophage T7. The vector pSR9 (28) (Fig. 2) contains the coding sequence for the gene 9 protein under control of the T7 RNA polymerase promoter. One advantage of this system is that gene 9 fusions are soluble and highly acidic, facilitating both their isolation and purification by anion exchange chromatography. Another advantage is that the gene 9 protein has evolved to be stable in E. coli, permitting high levels of fusion protein to accumulate in the cytoplasm. We therefore used the gene 9 fusion system for production of ApB.

The ApB gene was fused to the 3' end of gene 9 of bacteriophage T7 in the following manner. To allow for efficient cleavage of ApB from the gene 9 protein, a linker encoding the factor Xa recognition sequence (Ile-Glu-Gly-Arg) was ligated to the 5' end of the PstI/EcoRI fragment encoding ApB in pKB-1 (Fig. 2). After hydrolysis with EcoRI and gel purification, this 174-base pair fragment was inserted into the EcoRI site of plasmid pSR9 to yield the plasmid pKB-2. This construct retained codons for 3 amino acid residues (Cys-Arg-Met) lying between the cleavage site for factor Xa and the amino-terminal glycine of natural ApB. Deletion of the three extra codons was accomplished by site-directed mutagenesis using the “loopout” primer 5'-GCCCATCGAAGGACGGGGGTACCGTGCCTGT-3' (see “Experimental Procedures”). Reinsertion of the shortened ApB gene into pSR9 yielded the plasmid pMG-1. Induction of BL21(DE3) cells harboring pMG-1 resulted in expression of a soluble protein with an apparent molecular mass of 43 kDa which was immunoreactive with antibodies raised against natural ApB (Fig. 3). Although we could readily prepare gram quantities of fusion protein using this system, all attempts to cleave ApB from the gene 9 protein using factor Xa were unsuccessful.

FIG. 1. Design of the synthetic gene for ApB. The nucleotide sequence of the ApB gene was derived by reverse translation of the known protein sequence (2). The gene was synthesized in eight strands which were annealed to form four sets of complementary duplexes, containing the following overhangs: PstII/ApaI, ApaI/BanHI, BamHI/NcoI, and NcoI/EcoRI. The four duplexes were ligated to form the intact gene. The restriction sites are highlighted in boldface.

FIG. 2. Expression constructs. Details of plasmid constructions are under “Experimental Procedures.” A linker encoding the factor Xa recognition sequence was ligated to the 5' end of the ApB gene, and this was ligated into the vector pSR9 at the 3' end of gene 9. Removal of the intervening codons between the Xa cleavage site and the start of the ApB gene yielded the plasmid pMG-1, which was used to express GR-ApB. Deletion of the last two codons of the Xa linker yielded the plasmid pMG-2 used in the staphylococcal protease digestion system. b.p., base pairs.

FIG. 3. Expression and purification of recombinant ApB. Panel A is a Coomassie-stained 12% Laemmli gel (39) showing the purification and cleavage of the gene 9-ApB fusion protein; panel B is a Western blot of an identical gel. From left, 1st and 2nd lanes 1, total cellular protein with or without induction by isopropyl-β-D-thiogalactopyranoside, respectively; 3rd lane, crude lysate; 4th lane, streptococcal sulfate supernatant; 5th lane, ammonium sulfate pellet; 6th and 7th lanes, pellet and supernatant after staphylococcal protease hydrolysis; 8th lane, HPLC-pure recombinant ApB; 9th lane, natural ApB; 10th lane, aprotinin; 11th lane, molecular weight markers: amylase, bovine serum albumin, cytochrome c, insulin.
Possible explanations for this insensitivity to factor Xa will be discussed later.

Since the expression system was so efficient and because the ApB protein lacks glutamic acid, the plasmid pMG-1 was modified such that cleavage of the fusion protein by staphylococcal protease could be used to release full-length ApB. To place a glutamate residue immediately amino-terminal to the ApB sequence, the codons for the last two amino acids of the Xa linker (Gly-Arg) were deleted by site-directed mutagenesis using the primer 5'-GCACGGTACCCCTTCGATGAA; subcloning yielded the plasmid pMG-2. This plasmid can be used to express fusion protein as described above, and cleavage of the fusion protein with staphylococcal protease has successfully yielded recombinant ApB in reasonable quantities. Because the gene 9 protein is highly acidic, containing 48 glutamic acid residues, a limit digest with staphylococcal protease yields for the most part peptides considerably smaller than ApB, greatly facilitating further purification (see below).

Purification, Reoxidation, and Cleavage of Fusion Protein—Upon induction of E. coli strain BL21(DE3) harboring the plasmids pMG-1 or pMG-2, large amounts of a soluble protein of a molecular mass of 43 kDa are produced (Fig. 3). Both proteins react with antibodies raised against natural ApB. In crude extracts of induced cells, the chimeric proteins typically represent 20-25% of the total cellular protein. After purification as described under "Experimental Procedures," the approximate yield of fusion protein obtained was 50-100 mg/liter of culture, consistent with previous characterization of this expression system (28).

Reoxidation of the three disulfide bonds in ApB is most readily accomplished prior to cleavage from the gene 9 protein. Reoxidation is achieved by subjecting the purified fusion protein to a redox couple of oxidized and reduced glutathione (see "Experimental Procedures"). The ratio of oxidized to reduced glutathione affording optimal yields of active ApB was empirically determined to be 1:5, with a total glutathione concentration of 1.2 mM. This ratio reoxidizes ApB into a conformation that is biologically active after cleavage and which has an HPLC elution profile indistinguishable from natural toxin. Other glutathione ratios yield multiple products by HPLC, most of which lack biological activity.

One of the characteristics of staphylococcal protease digestion of the ApB fusion protein is extensive precipitation of insoluble peptide products; therefore, optimization of digestion was critical for efficient release of ApB. It is apparent from a western blot (Fig. 3) that most of the ApB in the fusion remains in the insoluble fraction after hydrolysis as a mixture of complete and incomplete digestion products. The soluble fraction contains primarily completely digested ApB and smaller peptides derived from the gene 9 protein.

The biologically active form of ApB is found wholly in the soluble fraction. After removal of the insoluble peptides, ApB can be purified from the residual contaminating gene 9 peptides in a single step by HPLC; this material is homogeneous by the criteria described below. We report the yield of active ApB from 1 g of starting fusion protein as 3 mg. Although this yield is far from the theoretical amount of ApB obtainable, the relative ease of producing gram quantities of fusion protein makes this procedure reasonable for production of milligram quantities of ApB.

Protein expressed using the factor Xa construct pMG-1 was also cleaved with staphylococcal protease to yield ApB with the amino-terminal extension Gly-Arg (GR-ApB). Both forms of ApB have been purified to homogeneity from the soluble fraction by HPLC.

Characterization of Recombinant ApB—The amino acid compositions for recombinant ApB, natural ApB, and GR-ApB are shown in Table I. The amino acid compositions of the natural and recombinant forms of ApB were indistinguishable from each other, whereas GR-ApB contains an additional residue each of glycine and arginine. The compositions of both samples are also in excellent agreement with those predicted from the amino acid sequence (2).

Because multiple glutamate residues are present near the carboxyl terminus of the gene 9 protein, it was necessary to verify that the recombinant proteins lacked any amino-terminal extensions because of incomplete cleavage by staphylococcal protease. Ten cycles of automated Edman degradation were carried out on the ApB proteins expressed from pMG-1 and pMG-2 (Table II). Amino-terminal sequence analysis of both samples gave the expected sequence and compositions for recombinant ApB, natural ApB, and GR-ApB.
revealed no detectable contaminating sequences, indicating a high level of purity.

To ascertain whether the recombinant toxins share the same secondary structure as natural ApB, far-UV circular dichroism spectra were obtained for ApB, GR-ApB, and natural ApB and analyzed by the computer program of Chen et al (31). All three proteins were found to contain 5% α-helix and 27% β-pleated sheet.

**Functional Characterization**—ApB has been shown to be a potent enhancer of the veratridine-dependent uptake of 22Na by neuroblastoma cells in culture (3, 4). We have measured the 22Na uptake response of N18 cells pretreated with increasing concentrations of toxin (see "Experimental Procedures"). Dose-response curves for natural and recombinant toxins are shown in Fig. 4. The uptake curves for all forms of ApB are virtually superimposable. Table III summarizes the calculated $V_{\text{max}}$ and $K_{\text{m}}$ values obtained in these experiments. All forms of natural and recombinant ApB exhibit half-maximal stimulation at a concentration of 28 ± 12 nM, in close agreement with previously reported values (3, 17).

**DISCUSSION**

We report the successful cloning and expression of a synthetic gene encoding the neurotoxin anthopleurin B. The ApB gene was initially cloned in pUC19 under the control of the lacUV5 promoter and later transferred into the plasmid pSR9, where expression is regulated by the T7 RNA polymerase present in the host bacterium (E. coli strain BL21(DE3)). In this latter system, it was possible to obtain large amounts of recombinant ApB in the form of a chimeric protein. Expression levels for the ApB-gene 9 chimera are expressed from GR-ApB and analyzed by the computer program of Chen et al. (32) have recently reported

**TABLE III**

**Functional characterization of natural and recombinant ApB**

Activities of the various forms of ApB were assessed by measuring the effects of the toxins on veratridine-induced, tetrodotoxin-sensitive sodium uptake by N18 cells in culture as described under "Experimental Procedures." All results are corrected for basal uptakes obtained in the presence of 20 μM veratridine and no ApB. Kinetic constants were derived by fitting the data to a single hyperbolic function (38).

| Toxin form   | $V_{\text{max}}$ (nmol/min/mg) | $K_{\text{m}}$ (nM) |
|--------------|-------------------------------|---------------------|
| Recombinant ApB | 68 ± 5 | 22 ± 7 |
| Natural ApB   | 76 ± 8 | 32 ± 12 |
| GR-ApB        | 95 ± 6 | 30 ± 6 |

factor Xa inhibitor antistasin (37) in the active site region of the latter (ApB<sup>16</sup>-Gly-Pro-Arg-Pro-Arg-Gly; antistasin<sup>36</sup>-Gly-Val-Arg-Cys-Arg-Val), we tested the ability of ApB to inhibit the hydrolysis of the homocromatic substrate N-benzoyl-Ile-Glu-Gly-Arg-para-nitroanilide by factor Xa (data not shown). However, we were unable to demonstrate any concentration-dependent inhibition of the initial rates of hydrolysis, suggesting that the observed inability to cleave the fusion protein with factor Xa must be caused by an inherent property of this specific chimera. It is possible that the positive carboxyl terminus of ApB interacts tightly with highly acidic sequences of the gene 9 protein and thus occludes the factor Xa recognition sequence. Fiordalisi et al. (32) have recently reported the inability of a K-bungarotoxin fusion protein to cleave with factor Xa, demonstrating that occlusion of the Xa cleavage site is not a property unique to our system.

Attempts to oxidize recombinant ApB after cleavage from the fusion protein have yielded only very small amounts of biologically active toxin. However, consistent with previous studies in this and other laboratories (28, 33), refolding and reoxidation of ApB are efficiently accomplished prior to cleavage with staphylococcal protease. In addition, the extent of staphylococcal protease digestion increases significantly after refolding and reoxidation. Hydrolysis of the fusion protein is most efficient at high substrate concentrations: when the fusion protein is cleaved at 20 μg/ml, all products obtained are less than 14 kDa, with more than 75% being below 6 kDa. Precipitation of peptide by-products also increases with increasing substrate concentrations. We have been able to use this to our advantage since the insoluble peptides can be easily removed from the correctly folded ApB, which remains soluble. We have attempted to digest the fusion protein under conditions that prevent precipitation (data not shown), but without success. Although digestion in the presence of 1% sodium dodecyl sulfate proceeds to completion with the generation of minimal precipitate, the digest is more difficult to purify and the final yield of active toxin no greater than otherwise obtained.

We have fully characterized both recombinant ApB and the amino-terminal extension product GR-ApB. Both recombinant proteins have an apparent molecular mass of 5–5.5 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and are immunoreactive with antibodies raised against natural ApB. The recombinant form of ApB eluted on a reverse-phase HPLC column at the same retention time as natural toxin, unlike GR-ApB which elutes slightly earlier because of the extra arginyl residue. The recombinant forms of ApB were shown to be pure by amino acid and amino-terminal sequence analysis. Secondary structures, as measured by circular dichroism, of both recombinant proteins and the natural toxin were all shown to be identical.

The recombinant protein GR-ApB was shown to be as active as either natural or recombinant ApB in enhancing veratridine-dependent, tetrodotoxin-sensitive sodium uptake by N18 cells. The addition of 2 residues to the amino terminus of ApB appears to have no effect on either the folding or the biological activity of the protein. Thus, more subtle mutations of the ApB peptide sequence will likely retain structure and function, allowing for analysis of recombinant toxins bearing specific point mutations.

ApB has the highest activity on mammalian voltage-dependent sodium channels of all of the anemone toxins characterized thus far. Although ApB shares 85% peptide sequence homology with the primary toxin of *A. xanthogrammica*, ApA, its activity is from 7–10 fold higher (3, 17). We now have an ideal system for detailed analysis of structure-function rela-
tionships of ApB by site-specific mutagenesis. Coupled with high resolution analysis of the three-dimensional structures of wild-type and mutant forms, such studies should provide a clearer understanding of the molecular interactions important for altering channel gating.

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