Multiple 6-Bromotryptophan Residues in a Sleep-Inducing Peptide†

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ABSTRACT: We have characterized a novel sleep-inducing peptide comprising 33 amino acids with three residues of the unusual posttranslationally modified amino acid, 6-bromotryptophan. The peptide, termed "light sleeper" or the r7a conotoxin, was purified from the venom of the fish-hunting *Conus radiatus*. The light sleeper peptide has additional notable biochemical properties; it equilibrates slowly between two distinct conformers, and has four γ-carboxyglutamate residues. The pattern of posttranslational bromination in the light sleeper peptide suggests that tryptophan residues at N- and C-termini may be preferential sites for posttranslational bromination.

We describe the isolation and characterization of a peptidic gene product with the highest number of posttranslationally modified tryptophan residues yet characterized. The peptide has other unusual biological and biophysical features: it is sleep-inducing, and it equilibrates slowly between two different conformations.

The bromination of tryptophan (to 6-bromotryptophan) was only recently established to be a posttranslational modification (*1*, *2*) (for a review, see ref *3*). Natural products that seem to be derived from 6Br-Trp are not uncommon in the marine environment. However, it was the characterization of *Conus* peptides with 6Br-Trp and the accompanying demonstration that these were *bona fide* gene products directly translated from mRNA that firmly established that bromination is a true posttranslational modification. Initially, this might have been regarded as an esoteric adaptation in a highly specialized biological system. More recently however, an effort to characterize ligands for orphan G-protein-coupled receptors led two different laboratories to demonstrate that this posttranslational modification occurs in a neuropeptide from mammalian brain (*4*, *5*).

Unusual posttranslational modifications have often been initially characterized in highly specialized biological systems, and then subsequently shown to be much more widely distributed. One classic example is the γ-carboxylation of glutamate to γ-carboxyglutamate (Gla),[1] which for many years was thought to be a specialization of the vertebrate blood clotting cascade. Some years later, this posttranslational modification was shown to occur in *Conus* peptides. Furthermore, it appears from more recent biochemical and molecular work that the γ-carboxylation enzyme machinery is expressed in a variety of mammalian tissues (suggesting diverse physiological roles), and that the relevant modification enzyme, γ-glutamyl carboxylase, is found in *Drosophila* and *Anopheles*, as well as in vertebrate systems and *Conus* (*6*, *7*).

Thus, the demonstration that bromination occurs in both the *Conus* peptide system and in mammalian brain raises the strong possibility that this posttranslational bromination is similarly likely to be much more widely distributed in biology. For such posttranslational modifications, it would be of use to predict when they may occur. With genomic technology being ever more widely used, the possibility becomes ever greater that posttranslationally modified gene products will be missed when using conventional molecular analyses. The ability to pinpoint when posttranslational modification may occur has been key to the rapid development of such fields as protein phosphorylation and N-glycosylation. To begin to recognize potential sites for posttranslational modification, it is first necessary to identify actually modified amino acids from native modified gene products in as many different sequence contexts as possible.

The peptide characterized in this report is highly unusual in that it contains three different Br-Trp residues, more than in any other gene product characterized so far. Thus, it could provide one important guidepost for predicting when tryptophan residues may be brominated. Indeed, the pattern of bromination we have found suggests that closer scrutiny for potential posttranslational bromination in certain mammalian neuropeptides is justified.

Additionally, the peptide described here is noteworthy in that it induces a sleep-like state in mice. Pharmacological agents that induce sleep are potential tools for understanding central nervous system function. This peptide was isolated from the venom of a cone snail, *Conus radiatus*, that has three unrelated peptides that all induce sleep (*2*, *8*). Each

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[1] Abbreviations: 6Br-Trp, 6-bromotryptophan; Gla, γ-carboxyglutamate; ACN, acetonitrile; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; MALDI, matrix assisted laser desorption ionization; DTT, dithiothreitol; PTH, phenylthiohydantoin; Tris, tris-hydroxymethylaminomethane; PCR, polymerase chain reaction; UTR, untranslated region; EST, expressed sequence tag; NMDA, *N*-methyl-D-aspartate.

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causes a sleep-like state with different properties. Upon injection of the peptide characterized in this report, animals enter into a sleep from which they are very easily arousable, symptomatology notably different from the other two sleeper activities isolated from the same venom. As a consequence, we refer to this activity as the "light sleeper" peptide. Thus, the unique symptomatology elicited by this peptide, as well as the unprecedented number of 6Br-Trp residues present make it of potential multidisciplinary interest, in biochemistry, genomics, and neuroscience.

EXPERIMENTAL PROCEDURES

Purification of the Peptide. Crude venom extract was prepared as described previously (*9*). The extract was applied into a Vydac C_{18} semipreparative HPLC column (10 mm × 250 mm, 5 μm particle size, Rainin Instruments) and eluted at 5 mL/min using a gradient of solvent A (0.1% TFA) and solvent B_{90} (0.085% TFA in 90% ACN). Further purification by HPLC was done on a Vydac C_{18} analytical column (4.6 mm × 250 mm, 5 μm particle size, Rainin). The effluents were monitored at 220 nm and aliquots of fractions were assayed for biological activity.

Bioassay. The peptide was lyophilized and dissolved in normal saline solution before injection using a 29-gauge insulin syringe. Swiss Webster mice (14 days old) were injected intracranially with 20 μL of the peptide solution as described earlier (*10*). Control animals were similarly injected with normal saline solution.

Mass Spectrometry. MALDI mass spectra were obtained using a Voyager DE STR mass spectrometer through the Mass Spectrometry and Proteomic Core Facility of the University of Utah.

Peptide Sequencing. The purified peptide was reduced with DTT and alkylated with 4-vinylpyridine as described previously (*9*). The alkylated peptide was sequenced by automated Edman degradation (*11*) on an Applied Biosystem model 492 sequenator, courtesy of Dr. Robert Schackmann of the DNA/Peptide Facility, University of Utah. The PTH-amino acid derivatives were purified by HPLC.

Peptide Conformation Analysis. Five nanomoles of lyophilized peptide was dissolved in a 500 μL solution containing 50% solvent A and 50% solvent B_{90}. The pH of the solution was adjusted to 7 with 0.5 M Tris. The mixture was incubated at 25 °C for 30 min, and then diluted with 500 μL of solvent A before applying it on an analytical HPLC C_{18} column. An aliquot of each of two peak fractions was taken, and the pH was adjusted to 7 with 0.5 M Tris. The sample was allowed to sit at 25 °C for 3 h, and then quenched in ice and diluted with an equal volume of solvent A before loading on an analytical C_{18} HPLC column.

Identification and Sequencing of cDNA Clones Encoding the Peptide. The cDNA was prepared by reverse transcription of RNA isolated from *C. radiatus* venom duct as previously described (*12*). The resulting cDNA was used as a template for PCR using oligonucleotides corresponding to the conserved signal sequence and 3′ UTR sequence of prepropeptides from *C. gloriamaris* and *C. textile*, which were generated by the EST cloning technique (C. Walker and B. M. Olivera, unpublished results). The resulting PCR products were purified using the High Pure PCR Product Purification kit (Roche Diagnostics, Indianapolis, IN) following the manufacturer's suggested protocol. The eluted DNA fragments were annealed to pAMP1 vector and the resulting products were transformed into competent DH5α cells, using the CloneAmp pAMP system for Rapid Cloning of Amplification Products (Life Technologies/Gibco BRL, Grand Island, NY). The nucleic acid sequences of the resulting peptide-encoding clones were determined according to the standard protocol for automated sequencing.

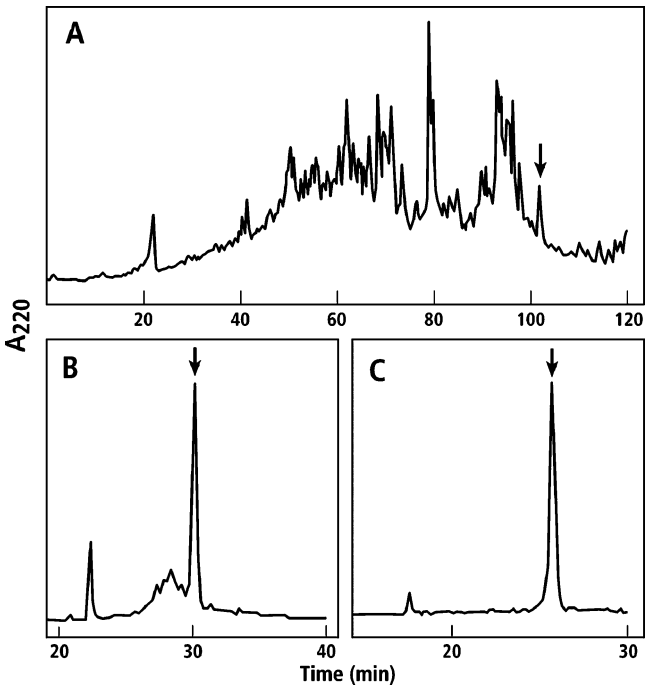

FIGURE 1: Purification of the peptide. (A) Fractionation of crude extract from *C. radiatus* venom using a C_{18} semipreparative column eluted with a gradient of 0−60% solvent B_{90} (0.085% TFA in 90% acetonitrile) over 120 min at a flow rate of 5 mL/min. The solid arrow indicates the fraction containing conotoxin r7a. (B) The fraction marked by arrow in A was eluted using a C_{18} analytical column at 30−70% solvent B_{90} over 40 min at a flow rate of 1 mL/min. (C) The fraction marked by arrow in B was further purified using a gradient of 35−65% B_{90} over 30 min at 1 mL/min.

RESULTS

Purification and Characterization of the Peptide. Using a C_{18} semipreparative reverse-phase column, a crude *C. radiatus* venom extract gave an elution profile typical of *Conus* venoms (see Figure 1A). A hydrophobic peak fraction of the venom elicited light sleep in young mice. Further fractionation was carried out by monitoring this behavioral symptomatology elicited upon i.c. injection in mice. The biologically active component from the major hydrophobic peak was purified to homogeneity as shown in Figure 1B,C, and described under Experimental Procedures.

A standard Edman analysis was carried out on the purified venom component identified by the arrow in Figure 1C. The following sequence assignment indicated that the biologically active toxin was a peptide with 33 amino acid residues: XFGHXXCTYXLGPCXVDDTCCSASCXSKFCGLX. Seven positions (marked by X) could not be immediately assigned. For positions 1, 10, and 33, a late-eluting peak was detected in the chromatogram after Edman degradation; in previous sequencing work with conotoxins, this late-eluting material indicated the presence of 6Br-Trp. Positions 5, 6, 15, and

Table 1: cDNA Clone Sequence Encoding Conotoxin r7a[a]

```
-5                  -1 ↓ +1
 A   P   A   K   R   W   F   G   H   E   E   C   T   Y
GCA CCT GCT AAG CGT TGG TTT GGA CAC GAA GAA TGC ACT TAT

+10                                             +20
 W   L   G   P   C   E   V   D   D   T   C   C   S   A
TGG TTG GGG CCT TGT GAG GTG GAC GAC ACG TGT TGT TCT GCC

                            +30
 S   C   E   S   K   F   C   G   L   W   *
AGT TGT GAG TCC AAG TTC TGC GGG TTG TGG TGA
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[a] ↓ indicates cleavage site of the mature peptide; *, stop codon. The cDNA clone was obtained and sequenced as described under Experimental Procedures. The Trp residues at positions 1, 10, and 33 are posttranslationally modified to 6Br-Trp, and the Glu residues at positions 5, 6, 15, and 26 are posttranslationally modified to Gla in the mature peptide.

26 showed low levels of glutamate, which previously had suggested the presence of Gla residues.

A cDNA clone from *C. radiatus* venom duct mRNA was identified. A sequence analysis of this clone, shown translated in Table 1, revealed the presence of tryptophan residues at positions 1, 10, and 33, glutamate residues at positions 5, 6, 15, and 26, and a stop codon after the 33rd amino acid residue. MALDI mass spectrometric analysis gave a mass *M* of 4203.61.

The results above are consistent with the peptide having 6Br-Trp residues at positions 1, 10, and 33, Gla residues at positions 5, 6, 15, and 26, and a free acid C-terminus. The MALDI mass spectra also showed M of 4159.26, 4114.81, and 4069.74 consistent with decarboxylation of one, two, and three Gla residues, respectively, a routine occurrence during MALDI mass spectrometry. Thus, the complete sequence assignment for the light sleeper peptide is $\underline{W}$FGH$\gamma\gamma$CTY$\underline{W}$LGPCγVDDTCCSASCγSKFCGL$\underline{W}$, where $\underline{W}$ = 6Br-Trp and γ = Gla. Consistent with previous suggestions for nomenclature, we will refer to this peptide as the r7a conotoxin since it has the Type 6/7 Cys pattern. Although there is a great diversity of *Conus* peptides (>50 000), there are only a few characteristic patterns of Cys residues within the primary sequence (*13*). For *Conus* peptides with six Cys residues, the Type 3 (- -CC- -C- -C- -CC- -) and the Type 6/7 (- -C- -C- -CC- -C- -C- -) Cys patterns are the most common.

Biological Effects of Purified Peptide. Bioassay of the purified r7a conotoxin was carried out as described under Experimental Procedures (see Table 2). Injection of the purified peptide in 14-day-old mice caused a light sleep syndrome. The mice were easily aroused when touched, but would revert to the light sleep state after arousal. The duration of the light sleep state was dose-dependent, but even at the highest dose (1 nmole), the mice recovered. Recovery was scored when the injected mice had the same degree of activity as the control mice.

Analysis of Interconversion of Conformers. The native r7a conotoxin did not produce a symmetrical elution peak when analyzed by reverse-phase HPLC under a variety of experimental conditions. If the purified peptide were allowed to incubate at room temperature, an HPLC profile consisting of two well-resolved peaks was observed (Figure 2A). To confirm that these peaks were the same peptide, the two fractions separated by HPLC were each incubated at 25 °C for 3 h after adjusting the pH to 7. Upon application to the

Table 2: Biological Activity of the Peptide[a]

dose (nmol)	symptomatology
1.0	Light sleep within 3−5 min after injection. Recovery after ~120 min.
0.50	Light sleep within 3−4 min after injection. Recovery after ~25 min.
0.25	Light sleep within 5−7 min after injection. Recovery after ~12 min.

[a] Swiss Webster mice (14 days old, 7.1−7.8 g) were injected intracranially with 20 μL of conotoxin r7a dissolved in normal saline solution (NSS) as described under Experimental Procedures. Control animals were similarly injected with NSS. Two to four mice were injected per dose. Light sleep was indicated when mice easily awakened when touched.

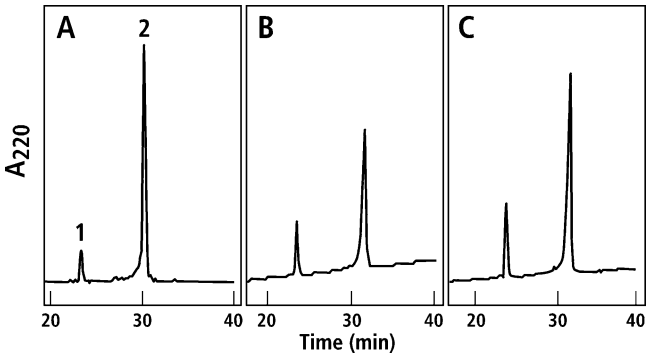

FIGURE 2: Analysis of conformational states. Reverse-phase HPLC analyses were done using a gradient of 30−70% solvent B over 40 min at a flow rate of 1 mL/min. (A) Lyophilized conotoxin r7a was dissolved in a mixture of solvents A and B_{90}. The pH was adjusted to 7 with 0.5 M Tris, and the mixture was allowed to sit at 25 °C for 30 min before analysis was done with HPLC. (B,C) Peak 1 and 2 fractions, respectively, in panel A were separately incubated at 25 °C for 3 h.

Table 3[a]

A. Sleeper peptides from *C. radiatus* venom

Name	Sequence	Ref
Bromosleeper	WATIDγCγγTCNVTFKTCCGOOGDWQCVγACPV^	(*2*)
Conantokin-R	GEγγVAKMAAγLARγNIAKGCKVNCYP^	(*8*)
Light sleeper	WFGHγγCTYWLGPCγVDDTCCSASCγSKFCGLW^	This work

B. Bromotryptophan-containing peptides from *C. radiatus* venom and mammalian brain

Name	Sequence	Ref
Bromocontryphan	GCO*W*EPWC*	(*1*)
Bromosleeper	WATIDγCγγTCNVTFKTCCGOOGDWQCVγACPV^	(*2*)
Light sleeper	WFGHγγCTYWLGPCγVDDTCCSASCγSKFCGLW^	This work
Bovine NPB	WYKPTAGQGYYSVGRAAGLLSGFHRSPYA^	(*4, 5*)
Human NPB	WYKPAAGHSSYSVGRAAGLLSGLRRSPYA^	(*5, 18*)
	? ?	
Human NPW	WYKHVASPRYHTVGRAAGLLMGLRRSPYLW^	(*5, 17, 18*)

[a] W, 6-bromotryptophan; γ, gamma-carboxyglutamate; O, 4-*trans*-hydroxyproline; *W*, D-tryptophan; ^, C-terminal free acid; *, C-terminal amidation. The amino acid sequences of human neuropeptides NPB and NPW were deduced from cDNA sequences. The human form of NPB is presumed to have been posttranslationally modified to 6Br-Trp at the N-terminus, as is found in bovine NPB. The question marks over the N- and C-terminal residues of human NPW denote the possibility that these are sites for potential bromination under certain physiological conditions (see Discussion).

same reverse-phase HPLC column, each gave an elution profile with two peaks in the HPLC chromatogram (Figure 2B,C). These results are consistent with a conformational interconversion of conotoxin r7a between two major states. Although the two conformational states of the peptide are stable enough to separate on an HPLC elution column, they are clearly interconvertible.

DISCUSSION

We have described the purification and characterization of the "light sleeper peptide" from *C. radiatus* venom. This is the third peptide from *C. radiatus* venom that induces a sleep-like state. The present peptide is unusual in that the sleeper symptomatology elicited is distinctly different from sleeper peptides characterized previously from this venom (i.e., bromosleeper and conantokin-R) (*2*, *8*). Although i.c. injection of conotoxin r7a induced a sleep-like state, the mice were much more easily arousable. Thus, at a gross physiological level, the light sleeper peptide appears to be acting through a different mechanism from other sleeper peptides characterized from *C. radiatus* venom, bromosleeper and the NMDA receptor antagonist conantokin-R, which cause a much less arousable sleep state. This also suggests that the light sleeper peptide has a different molecular target in the mammalian central nervous system.

Another unusual characteristic of the peptide is the equilibration between alternative conformational states. The nature of these conformational interconversions needs to be elucidated. It is worth noting that two previous cases of slow conformational interconversion have been reported among *Conus* peptides: the contryphans (*12*, *14*, *15*) and α-conotoxin MI (*16*).

Like the two other sleeper peptides, the light sleeper peptide has posttranslationally modified amino acids, including multiple Gla residues. A similar number is found in both the bromosleeper peptide and conantokin-R with no obvious sequence homology between the peptides in the regions containing Gla residues (Table 3A). The light sleeper peptide has three 6Br-Trp residues, at both the N-terminus and C-terminus, as well as one internal 6Br-Trp residue within the disulfide loops. This differs from bromocontryphan and bromosleeper, described earlier from the same venom, both of which have a single 6Br-Trp residue (Table 3B). In both the bromosleeper and light sleeper peptides, the N-terminal amino acid is 6Br-Trp. In bromocontryphan, only one of the two Trp residues is posttranslationally brominated; furthermore, both brominated and nonbrominated forms of the peptide were found in venom (*1*, *9*). All three Trp residues of the light sleeper peptide are modified, and so far, no undermodified forms have been found in *C. radiatus* venom.

The identification of three 6-bromotryptophan residues in the light sleeper peptide is based on three results: (1) the characteristic late-eluting hydrophobic peak obtained after chromatography using standard Edman methods, (2) the mass

spectrometry data, and (3) the presence of Trp codons at the relevant positions in the cDNA clones. The rigorous evidence that modified Trp residues were 6Br-Trp was presented for two different *Conus* peptides (see refs *1* and *2*). However, the possibility that another brominated tryptophan residue coelutes at the same position as 6Br-Trp in the Edman analysis is not absolutely eliminated (for example, the D-6-bromotryptophan would probably have the same elution properties). Unequivocal proof for the sequence assignment presented is the successful synthesis of the biologically active peptide, and a demonstration of its chemical identity to the native material.

The identification of multiple bromination sites in the light sleeper peptide suggests some potential general features of this newly characterized posttranslational modification. Particularly notable is the high frequency of modified N-terminal tryptophan residues in the small set of known 6Br-Trp-containing polypeptides; there is no apparent sequence similarity in the different peptides. As is shown in Table 3B, not only are the N-termini of the light sleeper and bromosleeper peptides from *C. radiatus* venom modified, but 6Br-Trp is also found at the N-terminus of NPB, the first mammalian (bovine) peptide characterized with this posttranslational modification (*4*, *5*). Since all of these peptides are processed from propeptide precursors, this raises the possibility that the modification enzyme may be associated with propeptide (or prohormone) convertase complexes, which proteolytically cleave propeptide precursors to the mature gene product. It should also be noted that in at least one case, bromocontryphan, both modified and unmodified forms of the peptide were found in *C. radiatus* venom (*1*, *9*). Thus, there may be a regulation of tryptophan bromination, and potentially modifiable gene products may exist in either brominated or nonbrominated states, depending on physiological conditions.

The characterization of the light sleeper peptide provides additional signposts for the potential occurrence of posttranslationally brominated tryptophan residues: the presence of tryptophan codons in the N-terminal and/or C-terminal positions of a processed mature polypeptide. It was previously suggested that there may be recognition signal sequence motifs for posttranslational bromination enzymes (*1*); the further definition of such recognition signals, combined with the presence of tryptophan residues in favored positions for modification should lead to the reliable prediction of when posttranslationally modified tryptophan residues will be present, using DNA sequence alone. However, to rigorously test such predictions, a characterization of the activity of the posttranslational modification enzyme on a set of model substrates would be required; at this time, there is no concrete information regarding the tryptophan-brominating enzyme system.

The findings with bromocontryphan and the light sleeper peptide raise the question of whether some gene products characterized without bromination may be potential substrates for modification under a given set of physiological conditions. In particular, in the mammalian brain, the neuropeptide NPW, which like NPB is a ligand for GPCR7 and GPCR8, has Trp residues at both the N- and C-termini, as determined from cDNA sequences (*5*, *17*, *18*). These are the positions of two of the three brominated Trp residues found in the light sleeper peptide. It is notable that neither for bromocontryphan, nor mammalian peptide NPB, does there seem to be a great difference in activity between brominated and nonbrominated forms of the peptides (*1*, *4*). In these cases at least, bromination is clearly not absolutely required for biological activity. Thus, while the biochemistry of this posttranslational modification is becoming better defined, insights into the larger physiological significance of bromination have yet to be obtained.

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