Formation of a native-like β-hairpin finger structure of a peptide from the extended PDZ domain of neuronal nitric oxide synthase in aqueous solution

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Neuronal nitric oxide synthase (nNOS) is targeted to the cell membrane via interactions of its extended PDZ domain with PDZ domains of membrane-associated proteins including PSD-95 and α1-syntrophin. The formation of heterodimers between the nNOS PDZ domain and the PDZ domains of nNOS-binding proteins requires a stretch of continuous amino-acid residues C-terminal to the canonical nNOS PDZ domain. In this work, we show that a 27-residue peptide comprising the C-terminal extension of the extended nNOS PDZ domain is capable of binding to PSD-95. The structure of the 27-residue peptide in aqueous solution was determined using multidimensional NMR-spectroscopic techniques. The free peptide adopts a native-like β-hairpin finger structure in aqueous solution. The results indicate that the C-terminal extension peptide of the nNOS PDZ domain may represent a relatively independent structural unit in the mediation of the interaction between nNOS and PDZ domain-containing proteins including PSD-95 and α1-syntrophin.

Keywords: nitric oxide synthase (nNOS); NMR structure; PDZ; peptide conformation; PSD-95.

Synthesis of NO in neurons is largely regulated by the neuronal isoform of nitric oxide synthase (nNOS). nNOS differs from the endothelial and inducible isoforms of the enzyme in having an approximately 250-residue N-terminal extension which contains an extended PDZ domain and an 8-kDa dynein light chain/protein inhibitor-binding domain [1–4]. Many of the unique functional properties of nNOS, with respect to the other two isoforms, can be attributed to this unique 250-residue N-terminal extension. For example, the PDZ domain of the enzyme is responsible for coupling nNOS to the N-methyl-d-aspartic acid receptor via a versatile synaptic organization protein PSD-95 [2]. The association of nNOS with the N-methyl-d-aspartic acid receptor ensures direct coupling of Ca²⁺ influx to the activation of the enzyme via a Ca²⁺/calmodulin-mediated pathway. The functional significance of the interaction between dynein light chain/protein inhibitor and nNOS is still not known.

Subcellular localization experiments showed that the majority of nNOS is associated with the cell membrane even though the enzyme itself does not contain transmembrane domains [5]. Membrane localization of nNOS in neuronal cells is mediated via the specific interaction of the extended PDZ domain of the enzyme with the second PDZ domain of PSD-95 [2]. In skeletal muscle, nNOS was found to be localized to the sarclemma, and membrane association of nNOS in skeletal muscle is also mediated by a PDZ–PDZ interaction between nNOS and α1-syntrophin [6]. In α1-syntrophin ‘knock-out’ mice, nNOS was found to be localized to cytosol instead of the normal sarclemma location [7]. In Duchenne muscular dystrophy and its experimental mouse model, mdx, which lacks dystrophin expression, nNOS is absent from the sarclemma [6].

Canonical PDZ domains contain ≈90–100 amino-acid residues [8]. A typical domain consists of a six-stranded antiparallel β-barrel flanked by two α-helices forming a compact globular structure [9,10]. A common mode for interactions of PDZ domains involves associations of short peptide fragments located at the extreme C-termini of interacting proteins [9,11]. The carboxy peptides bind to a groove formed by the αB helix and the βB strand of the PDZ domains [9,12,13]. Formation of the PDZ/PDZ dimers of nNOS/PSD-95 and nNOS/α1-syntrophin represents a distinct class of interaction mode of PDZ domains, as the interactions do not involve carboxy peptides. To form such PDZ/PDZ heterodimers, an ≈25-residue peptide fragment C-terminal to the canonical nNOS PDZ domain is absolutely required [2]. Both NMR and X-ray studies showed that this 25-residue extension of the nNOS PDZ domain adopts a two-stranded antiparallel β-sheet structure (hence named β-finger) [13,14]. The crystal structure of the nNOS PDZ–α1-syntrophin PDZ complex further showed that the β-finger of nNOS PDZ binds to the carboxy peptide binding groove of α1-syntrophin PDZ via β-invasion [14]. Before the formation of complexes with PSD-95 and α1-syntrophin, the β-finger loosely packs with the preceding canonical PDZ domain [13]. Formation of the PDZ/PDZ dimer with α1-syntrophin considerably reduces the conformational flexibility of the β-finger, and further contacts between the β-finger and the canonical PDZ domain were observed ([14]; H. Tochio & M. Zhang, unpublished results). In addition, a number of tertiary interactions between the nNOS PDZ and the α1-syntrophin PDZ domain are observed in the complex, and these interactions have been suggested to be important to explain the binding specificity between the nNOS PDZ domain and the PDZ domains from α1-syntrophin and PSD-95 [14].

In this work, we studied the interaction of a 27-residue biosynthetically prepared peptide comprising the β-finger of the nNOS PDZ (the β-finger peptide) with the second PDZ

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Abbreviations: nNOS, neuronal NO synthase; PSD-95, postsynaptic density-95; PDZ, PSD-95, Disc-large, and ZO-1; HSQC, heteronuclear single-quantum correlation spectroscopy.

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domain of PSD-95. The solution structure of the $\beta$-finger peptide was determined using NMR spectroscopy. The data obtained from this work suggest that the $\beta$-finger peptide contains most of the structural properties for its interaction with the PDZ domains from PSD-95 and $\alpha$-1-syntrophin.

**MATERIALS AND METHODS**

**Preparation of the $\beta$-finger peptide**

The $\beta$-finger peptide used in this study corresponds to the amino-acid residues from Glu101 to Gly127 of rat nNOS. The gene coding for the 27-residue $\beta$-finger peptide was amplified from the rat nNOS PDZ cDNA using a pair of primers with sequences: 5'-CAGGATCCCATGGAGGGCTTCACTAC-3' (coding strand); and 5'-GGGATCCTAACCAGGGGCTGGGT-3' (non-coding strand). The resulting PCR fragment was inserted into the NotI sites of a modified version of pET32a (Novagen), which was designed for large-scale biosynthetic production of linear peptides (Q. Zhang & M. Zhang, unpublished results). The resulting bacterial culture was grown in standard M9 medium using $\text{NH}_4\text{Cl}$ as the sole nitrogen source.

The resulting $\beta$-fingers were expressed as a thioredoxin–His$_6$-fused form in Escherichia coli (non-coding strand). The fusion protein was purified using a Ni$_2^+$-nitrilotriacetate affinity column, and the $\beta$-finger peptide alone was further purified by passing the thrombin digestion mixture through a C$_{18}$ RP-HPLC column. The $\beta$-finger peptide was prepared in an identical manner except that the $\beta$-finger peptide was further purified by passing the thrombin digestion mixture through a C$_{18}$ RP-HPLC column. The details of the preparation and purification of the $\beta$-finger peptide will be described elsewhere (Q. Zhang & M. Zhang, unpublished results). The resulting $\beta$-finger peptide contains four extra amino acids (GlySerAlaMet) from cloning artefacts at its N-terminus. Uniformly $^{15}$N-labeled $\beta$-finger peptide was prepared in an identical manner except that the bacterial culture was grown in standard M9 medium using $^{15}$NH$_4\text{Cl}$ as the sole nitrogen source.

**NMR spectroscopy**

Three samples were prepared for structural determination of the $\beta$-finger peptide in aqueous solution by NMR spectroscopy (unlabeled $\beta$-finger peptide in 90% H$_2$O/10% D$_2$O and 99.99% D$_2$O, respectively; and a $^{15}$N-labeled peptide in 90% H$_2$O/10% D$_2$O). The concentrations of the NMR samples were $\approx 3.0$ mM, and pH values of the samples were adjusted to 5.0 using diluted KOH or KOD (direct meter reading).

All NMR spectra were recorded on a Varian Inova 750-MHz spectrometer equipped with an actively z-gradient shielded triple-resonance probe at 278, 283 and 303 K. 2D $^1$H DQF-COSY, TOCSY, and NOESY were obtained using standard pulse sequences [15]. A 3D sensitivity-enhanced, $^{15}$N-separated NOESY spectrum of the $^{15}$N-labeled $\beta$-finger peptide was recorded using a pulse sequence described previously [16]. Mixing times of 100 and/or 200 ms were used in the NOE experiments. A typical data matrix for $^1$H 2D spectra was 2048 x 512 complex data points ($f_2 \times f_1$) with 8096 Hz spectral width in both dimensions. The data matrix for the 3D $^{15}$N-NOESY experiment was 128 x 24 x 1024 ($f_3 \times f_2 \times f_1$) complex data points. NMR data were processed using the NMRPipe software package [17], and analysed using PIPP [18].

**Structural calculation**

Approximate interproton distances were obtained from the volume integration of NOE peaks from the 2D $^1$H-NOESY and 3D $^{15}$N-NOESY experiments. The NOEs were classified into three categories as strong (1.8–2.9 Å), medium (1.8–3.5 Å), and weak (1.8–5.0 Å). The NMR structures were calculated using a distance geometry/simulated annealing protocol using the program XPLOR [19].

**RESULTS**

**The $\beta$-finger peptide alone is capable of binding to PSD-95**

The $\beta$-finger peptide is capable of binding to PSD-95. The nNOS PDZ domain contains a loosely packed two-stranded $\beta$-hairpin finger comprising $\approx 25$ amino-acid residues C-terminal to the canonical PDZ domain [13], and this part of the protein is directly involved in the formation of the nNOS–$\alpha$-1-syntrophin complex [14]. To investigate whether this $\beta$-finger peptide alone is capable of binding to the partners of the nNOS PDZ domain, we titrated the $^{15}$N-labeled second PDZ domain of PSD-95 (PSD-95 PDZ2) with the unlabeled $\beta$-finger peptide. In the course of the titration, a new set of resonances appeared (the peaks drawn in red in Fig. 1A) in the $^1$H-$^1$N heteronuclear single-quantum correlation (HSQC) spectra of the PSD-95 PDZ2 on addition of a substoichiometric amount of the $\beta$-finger peptide, and the intensities of the new set of resonances increased with increasing amounts of the $\beta$-finger peptide. The intensities of the free PSD-95 PDZ2 peaks continued to decrease on addition of increasing amounts of the $\beta$-finger peptide. Figure 1A shows the overlay plot of the $^1$H-$^1$N HSQC spectra of the free PSD-95 PDZ2 (black) and the $\beta$-finger peptide-saturated form of the protein (red). The slow exchange between the free and $\beta$-finger peptide-saturated forms of PSD-95 PDZ2 at the NMR time scale indicated that the $\beta$-finger peptide binds to PSD-95 PDZ2 with a reasonably high affinity.

Figure 1B shows a chemical-shift perturbation plot of the PSD-95 PDZ2 resulting from binding of the $\beta$-finger peptide. It is clear that the $\beta$-finger peptide binds to the groove formed by the $\alpha$B helix and the $\beta$B strand of the protein, as residues in both the $\alpha$B helix and the $\beta$B strand undergo large chemical-shift changes. In addition, the chemical-shift data also indicate that the ‘GLGF’ motif immediately preceding the $\beta$B strand and the extended $\beta$B/$\beta$C loop are also involved in the $\beta$-finger peptide binding. Taken together, we conclude that the $\beta$-finger peptide and the carboxy peptides such as CAPON and the NR2 subunit of N-methyl-D-aspartic acid receptor share the same binding site on PSD-95 PDZ2 [20].

**Chemical-shift assignment of the $\beta$-finger peptide**

Initially, the sequence-specific assignment of the $\beta$-finger peptide was tentatively obtained using standard $^1$H 2D NOE and TOCSY experiments with an unlabeled peptide sample [15]. However, because of the chemical-shift degeneracy (for example, eight Thr out of a total of 27 residues of the peptide), the assignment of the peptide contains some ambiguity. To obtain an unambiguous chemical-shift assignment of the $\beta$-finger peptide, we prepared a $^{15}$N-uniformly labelled peptide sample. The 3D $^{15}$N-TOCSY and NOESY spectra of the $\beta$-finger peptide were valuable in making unambiguous assignments of the $\beta$-finger peptide in aqueous solution. In addition, the 3D $^{15}$N-NOESY spectrum was vital in identifying a large number of medium-range and long-range NOEs necessary for defining the conformation of the peptide (an example is shown in Fig. 2). The backbone amide proton NOEs derived from the 3D $^{15}$N-NOESY spectrum of the peptide were cross-checked with $^1$H 2D NOESY spectra of the...
unlabeled peptide at various temperatures (5, 10, and 20 °C) with two mixing times (100 and 200 ms). The rest of the NOEs were obtained from 1H 2D NOESY spectra of the β-finger peptide dissolved in 99.99% D2O at 5 and 10 °C with a mixing time of 100 ms.

**Secondary structure of the β-finger peptide**

For a linear peptide with a random coil structure in solution, one expects to observe medium to weak sequential dαN(i, i+1) and medium to strong intraresidue dαN NOE connectivities. However, initial analysis of the backbone NOE pattern of the β-finger peptide showed continuous medium to strong dαN(i, i+1) NOE connectivities for residues from Phe103 to Thr110 and from Pro117 to Gln124, indicating that these two stretches of the β-finger peptide may adopt a β-strand like structure (data not shown). Significant amounts of long-range NOEs between the backbones of the two stretches further indicated that the two β-strands of the β-finger peptide form an antiparallel sheet structure (Figs 2 and 3). A number of long-range NOEs involving the side chains of the two opposite strands of the peptide also substantiate the formation of the antiparallel β-sheet structure. Figure 4 representatively shows NOEs between the side chain of Val122 and the imidazole ring of His106, and those between the aromatic ring of Phe117 and the side chain of Pro117. The possibility of peptide aggregation-induced long-range NOEs can be ruled out, as both the chemical shifts and the line shape of the peptide did not change on sample dilution (data not shown).

NOE analysis also showed that the six-residue fragment (Thr112–Thr116) linking the two strands of the β-finger peptide adopts a turn-like structure. Many characteristic medium-range and long-range NOEs (e.g. dαNThr112/Thr116, dαNThr112/Gly115, dαNAsp114/Thr116, and dαNAsp114/Gly115; see Fig. 3) characteristic of a turn-like structure were observed in this six-residue stretch. Taken together, we conclude that the β-finger peptide forms a two-stranded antiparallel β-hairpin-like secondary structure in aqueous solution.

**Three-dimensional structure of the β-finger peptide**

The 3D structure of the β-finger peptide was calculated using a total of 201 interproton distance restraints (102 sequential, 49 medium, and 50 long-range) derived from NOE cross-peaks. It
is well known that a linear peptide like the β-finger peptide is likely to have multiple conformations in aqueous solution, and these conformers are under rapid equilibrium. Therefore, one cannot use the interproton distances directly converted from the volumes of the NOE cross-peaks for structural calculation, as the intensities of the NOE cross-peaks represent weighted averages of all conformations. Here, we assume that the β-finger peptide exists as simple two-state conformers (random coil and the β-hairpin). The intraresidue and sequential NOE connectivities derived from the random-coil structure should be filtered for the calculation of the β-hairpin structure. However, it is difficult to distinguish/deconvolute the intraresidue and sequential NOEs contributed by the two conformers without a systematic analysis of the conformational status of the peptide. To circumvent such a multiconformation equilibrium problem, we simply omitted all intraresidue NOEs for the β-hairpin structure calculation, on the grounds that the inclusion of intraresidue NOEs does not significantly contribute to the quality of the calculated structures. In addition, we have adjusted some of the $d_{NN(i,i+1)}$ connectivities in the two β-strand regions from medium (3.5 Å) to weak (5 Å) on the basis that these NOEs are largely contributed by the random-coil conformer of the peptide. We note that such NOE intensity adjustments are rather approximate. Fortunately, the distance restraints used for the structure calculation by NMR are also approximate in nature. The interproton distance ranges used in the structure calculation are likely to accommodate the approximation used in NOE adjustments. Figure 5 shows the 10 calculated β-finger peptide structures with the lowest energies. No NOE violations greater than 0.3 Å were observed in the final structures. Superposition of the 10 structures using the two opposite strands (Phe103–Leu107 and Arg121–Pro125) and the turn region (Thr110–Lys118) gives rise to rmsd values of 0.84 and 0.45 Å, respectively. There seems to be
some sort of hinge motion between the turn and the mini β-sheet region as superimposition of the calculated structures using the entire peptide resulted in a much higher rmsd value.

DISCUSSION

Other than binding to carboxy peptides, PDZ domains have also been shown to form homodimers and heterodimers [21–24]. Formation of homodimers and heterodimers of PDZ domains often leads to homomultimerization and/or heteromultimerization of multi-PDZ-domain-containing proteins. Such PDZ-domain-mediated multimerization is an important step in assembling various functional signal-transduction units [25,26]. Extensive biochemical and structural studies have uncovered the mechanism of the interaction between PDZ domains and short carboxy peptides [9,11–13]. However, the molecular basis of the formation of PDZ/PDZ homo/heterodimers is just beginning to be understood. A prototype model of PDZ/PDZ interactions was constructed from the 3D structures of the extended nNOS PDZ domain and its complex with the α1-syntrophin PDZ [13,14]. Formation of the nNOS and α1-syntrophin dimer is primarily mediated by docking of the β-finger peptide of the nNOS PDZ domain to the carboxy peptide binding groove of the α1-syntrophin PDZ domain. The nNOS–α1-syntrophin complex structure also indicated that the tertiary interactions may play some roles in the formation of the PDZ dimer. However, it is not known whether the β-finger peptide alone is capable of binding to the PDZ domains of PSD-95 and α1-syntrophin.

In the present study, we aimed to investigate the structural and functional properties of the β-finger peptide without it being attached to the canonical nNOS PDZ domain. Both automatic and manual syntheses of the β-finger peptide using standard solid-phase-coupling methods failed, presumably because of intrinsic sequence properties of the peptide. Therefore, we opted to prepare the β-finger peptide using a biosynthetic approach. The β-finger peptide was expressed in a thioredoxin-fused form, and the peptide was cleaved from the fusion protein and purified to homogeneity in large amounts necessary for NMR structural studies. Owing to a high degree of 1H chemical-shift degeneracy, it was difficult to obtain a complete chemical-shift assignment of the β-finger peptide using conventional 1H-NMR spectroscopy. Spectral overlap also resulted in only a limited amount of unambiguous NOEs (particularly medium-range and long range NOEs).

![Fig. 4. Two regions of the 1H 2D NOESY spectrum (100 ms mixing time) of the unlabeled β-finger peptide showing the NOEs between the side chains of the aromatic residues (His106, Phe103 and Phe111) and aliphatic residues in the two opposite strands.](image)

![Fig. 5. Best-fit superposition of the backbone atoms (N, Cα, and C') of the final 10 calculated structures of the β-finger peptide derived from the NMR experimental restraints. (A) Superposition using the backbone atoms of the amino-acid residues in the two β-strand regions (Phe103–Leu107 and Arg121–Pro125) (see also [13]). (B) Superposition using the backbone atoms of the amino-acid residues in the turn region (Thr110–Lys118).](image)
that could be assigned from the 2D $^1$H NOESY spectra. To improve spectral resolution, we prepared a $^{15}$N-uniformly labeled β-finger peptide using the same biosynthetic approach. The 3D $^{15}$N-separated NOESY experiment allowed us to identify many medium-range and long-range NOEs crucial for determining the solution conformation of the peptide (see Fig. 2 for example).

The solution structure of the β-finger peptide in aqueous solution closely approximates the structure of the same peptide attached to the canonical PDZ domain [13,14]. However, the population of the β-hairpin-like finger structure of the free peptide in aqueous solution is much lower than that in the same peptide attached to the protein. In fact, the majority of the interstrand long-range NOEs characteristic of an antiparallel β-sheet structure as shown in Figs 3 and 4 are predicted to be strong, whereas these NOEs were experimentally detected as weak, indicating that the β-hairpin-like finger structure may represent a minor population of the peptide conformers in solution. It is likely that the weak tertiary interactions between the β-finger peptide and the canonical PDZ domain serve to promote and partially stabilize the β-hairpin finger structure of the peptide [13]. Docking of the β-finger peptide to the peptide-binding groove of another PDZ domain further stabilizes the β-hairpin finger structure ([13]; H. Tochio & M. Zhang, unpublished results).

The observation that the β-finger peptide alone in solution can form a transient native-like β-hairpin finger structure suggests that the peptide contains a majority of the structural properties necessary for its interaction with target PDZ domains such as PSD-95 PDZ2 and α1-syntrophin PDZ. The robust interaction observed between the β-finger peptide and PSD-95 PDZ2 further indicates that the peptide may be able to function as a PDZ domain-binding motif when embedded within the polypeptide chain of a protein. Similar PDZ domain–internal peptide fragment interaction has been observed in other proteins. For example, the third PDZ domain of the *Drosophila* multi-PDZ protein INAD can form a PDZ homodimer, and the dimerization requires an additional $\approx 30$ amino-acid residues C-terminal to the third canonical PDZ domain. Disruption of the canonical PDZ structure does not seem to affect the binding of the mutated PDZ3 to the wild-type PDZ3 in INAD [23]. Therefore, it is possible that tertiary interaction between the β-finger peptide-like peptide segment with a PDZ domain may not be a prerequisite for the interaction between the peptide and another PDZ domain. Further studies are in progress dissecting the contribution of the canonical PDZ domain of nNOS to the enzyme’s binding to PSD-95. The interactions observed between PDZ domains and internal peptide of target proteins without PDZ domains may also use the interaction mode between the β-finger peptide and the PDZ domain from PSD-95 and α1-syntrophin shown in this study.

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**REFERENCES**


