Functional and structural characterization of a novel member of the natriuretic family of peptides from the venom of *Pseudocerastes persicus*[1]

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Abstract A novel peptide, PNP (*P*seudocerastes *p*ersicus *n*atriuretic *p*eptide), was isolated from the venom of the Iranian viper *P. persicus*. Amino acid sequencing revealed that the 37-residue peptide belongs to the family of natriuretic peptides. The physiological effects of intra-venously PNP infused into anesthetized rats on urine flow, sodium excretion and blood pressure were comparable to those of atrial natriuretic peptide (ANP). In PC12 cells that were treated with either PNP, ANP, or C-type natriuretic peptide, PNP induced a similar cGMP response as ANP. Since PC12 cells only express the natriuretic peptide receptor (NPR)-A receptor we conclude that PNP binds to the NPR-A receptor. The solution conformation of PNP was characterized using ^{1}H nuclear magnetic resonance spectroscopy and indicates a high degree of conformational flexibility.

Key words: Natriuretic peptide; Snake venom; Nuclear magnetic resonance characterization

1. Introduction

The natriuretic peptide family includes many structurally related peptides that are involved in physiological processes controlling natriuresis, diuresis, blood pressure, homeostasis, and inhibition of aldosterone secretion (for review see [1–3]). Natriuretic peptides are characterized by a homologous sequence of 17 amino acids that form a ring closed by a cysteine disulfide bridge. atrial natriuretic peptide (ANP), the first factor identified, is primarily synthesized in the heart [4] whereas brain natriuretic peptide (BNP), which was originally isolated from porcine brain, was later found in heart as well [5]. C-type natriuretic peptide (CNP), a natriuretic peptide that lacks the C-terminal extension [6] is expressed predominantly in the central nervous system and vascular endothelial cells and is a more potent vasoactive agent than ANP and BNP while its natriuretic activity is weaker [7].

Natriuretic peptides mediate their function through binding to high affinity receptors that are found at high concentrations in the target organs [6,8]. These receptors belong to the family of membrane-bound guanylate cyclase receptors that catalyze the production of the second messenger cyclic GMP upon stimulation. Three types of receptors of natriuretic peptides have been identified, natriuretic peptide receptor A, B, and C (NPR-A, NPR-B, and NPR-C). NPR-A is activated by ANP, but it also binds BNP, albeit with a lower affinity. NPR-B is preferentially activated by CNP, while NPR-C, that has only a small intra-cellular segment and lacks guanylate cyclase activity, binds all three types of peptides ith equal affinity. It primarily controls the local concentration of natriuretic peptides available to NPR-A and NPR-B by internalizing them as a clearance receptor [9].

Natriuretic peptides have also been isolated from a range of other vertebrates. Notably, some were found in snake venoms: *Dendroaspis auguticeps* natriuretic peptide was detected in the venom of *Dendroaspis auguticeps* (the green mamba) and has properties similar to both ANP and CNP [10]. CNP analogs were also cloned from the venom glands of snakes of the Crotalinae subfamily [11]. Here we present a functional study of a novel member of the natriuretic peptide family, *Pseudocerastes persicus* natriuretic peptide (PNP), isolated from the venom of the Iranian snake *Pseudocerastes persicus*.

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Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; NPR-A, natriuretic peptide receptor A; NPR-B, natriuretic peptide receptor B; NPR-C, natriuretic peptide receptor C; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE correlation spectroscopy; PNP, *Pseudocerastes persicus* natriuretic peptide; TOCSY, total correlation spectroscopy

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2. Materials and methods

2.1. Purification

P. persicus snakes were milked and the pooled venom was lyophilized and stored at −20°C. The dried crude venom (1 g) was dissolved into 25 ml of mobile phase (0.05 M ammonium acetate, pH 6.8) and was applied on a Sephadex G-50 column (210×3.5 cm; flow rate: 60 ml/h; fraction: 5 ml) (Pharmacia, Uppsala, Sweden). Lyophilized fractions were further purified by a semi-preparative reversed phase (RP)-HPLC C8 column (250×10 mm, 5 μm) (Merck (Darmstadt, Germany) or Vydac (Hesperia, CA, USA)) equilibrated in 0.1% w/v trifluoroacetic acid. Elution was performed using a linear gradient (10–30%) of acetonitrile containing 0.1% w/v trifluoroacetic acid in 40 min (2 ml/min) and absorbance measured at 215 nm. The fraction corresponding to PNP was lyophilized and further analyzed by an analytical RP-HPLC C8 column (250×4.6 mm, 5 μm).

2.2. Reduction and alkylation

PNP (1.8 nmol) was reduced for 1 h at 37°C (N_2 atmosphere) with a 10-fold excess of dithiothreitol (with respect to disulfide) in 100 mM Tris–HCl buffer, pH 8.0, containing 6 M guanidium–HCl. The generated thiol groups were alkylated in dark with a five-fold excess of iodoacetamide.

2.3. Enzymatic cleavage

PNP (3 nmol) was cleaved with trypsin (Promega, Madison, WI, USA) at an enzyme/substrate ratio of 1:50 (w/w) for 18 h at room temperature in 100 mM Tris–HCl buffer, pH 8.0, in the presence of 20 mM iodoacetamide to prevent disulfide exchange. Reduced and alkylated PNP (500 pmol) was cleaved with endoproteinase Asp-N (Boehringer Mannheim, Germany) at an enzyme/substrate ratio of 1:50 (w/w) for 18 h at 37°C in 50 mM Tris–HCl buffer, pH 8.0.

2.4. RP-HPLC

The peptide mixtures were separated by RP-HPLC on an Aquapore Butyl column (2.1×100 mm, wide-pore, 30 nm, 7 μm; Applied Biosystems) with a Hewlett Packard 1090 liquid chromatograph (Hewlett Packard) using acetonitrile gradients.

2.5. Amino acid analysis

Samples were hydrolyzed in the gas phase with 6 M hydrochloric acid containing 0.1% (by volume) phenol for 24 h at 115°C under N_2 vacuum [12]. The liberated amino acids were reacted with phenylisothiocyanate and the resulting phenylthiocarbamoyl amino acids analyzed by RP-HPLC on a Nova Pak ODS column (3.9×150 mm, 4 μm; Waters) in a Hewlett Packard liquid chromatograph 1090 with an automatic injection system [13].

2.6. Amino acid sequence analysis

The N-terminal sequence analysis was carried out either in a Procise cLC 492 protein sequencer or in a pulsed-liquid phase sequencer 477A, both from Applied Biosystems. The released amino acids were analyzed on-line by RP-HPLC. In the case of the disulfide-linked peptide the di-phenylthiohydantoin-cystine was detected as a double peak in the vicinity of phenylthiohydantoin-Tyr [14,15]).

2.7. Mass spectrometry

Masses of the peptides were determined by electrospray ionization mass spectrometry with a single-stage quadrupole instrument (VG Platform, Micromass).

2.8. Sequence comparison

The sequences of the natriuretic peptides were obtained by a Blast search using the PNP sequence as query. A multiple sequence alignment was obtained with ClustalW [16] and manually adjusted.

2.9. Physiological assay

Experiments were performed on Wistar rats (200–250 g) in accordance with local guidelines of animal care. The animals were deprived of food the night before the experimentation but were allowed free access to water. After anesthesia with thiobutabarbital (Inactin, 100 mg/kg i.p., Byk Gulden, Konstanz, Germany), they were placed on a thermostat table to maintain body temperature at 36–37°C. The trachea was cannulated for free breathing. Cannulas were inserted into the left femoral vein for infusion of saline (1.5 ml/h), ANP (kindly provided by Dr. Forssmann, Lower Saxony Institute for Peptide Research, Hannover, Germany), or PNP and into the left femoral artery for monitoring arterial blood pressure. After opening the abdomen by a sub-umbilical incision both ureters were cannulated. Urine from both ureters was collected into pre-weighed Eppendorf tubes to determine diuresis gravimetrically. Sodium excretion was determined by measuring its concentration in urine (Electrolyte Analyzer, AVL, Graz, Austria). Experiments commenced after a 1 h stabilization period. The experimental protocol consisted of six periods. In the first two control periods (C1 and C2) urine samples were collected for 10 min each. Thereafter, ANP or PNP was infused intra-venously for 25 min at a rate of 3×10^{-11} mol/min (3×10^{-6} stock solution, 10 μl/min). For the two experimental periods (E1 and E2, 5 min each) urine was collected 10 and 20 min past the beginning of the infusion. For the recovery periods (R1 and R2, 10 min each) urine collection was started at the 10th and 20th min after stopping the infusion.

2.10. Determination of receptor specificity

PC12 cells and differentiated conditionally immortalized mouse podocytes were each cultured and seeded at confluent density in multiwell plates. After 2–3 days, cells were washed with RPMI 1640 medium and incubated for 20 min with agonists (PNP, ANP, CNP) in RPMI 1640 at various concentrations at 37°C. Thereafter cells were lysed. Total cGMP (cell+medium) concentration was measured with a colorimetric enzyme immuno-assay (Amersham Biosciences) according to the manufacturer's protocol including an acetylation reaction. Optical densities were read with a microplate reader (Bio-Rad) at 450 nm. CNP was purchased from Bachem.

2.11. Nuclear magnetic resonance (NMR) spectroscopy

The peptide was dissolved in 600 μl H_2O:D_2O 9:1 either at pH 4.0 or pH 6.0 to a final concentration of 1 mM. NMR spectra of PNP in water, pH 4.0, were acquired at 4°C on either a Bruker DR×500 or DR×600 spectrometer equipped with a cryoprobe. Additional spectra were recorded at 14°C and 24°C to resolve signal overlap. Spectra were also recorded at pH 6.0 to probe the influence of pH on the conformation.

Total correlation spectroscopy (TOCSY) spectra were measured using the DIPSI-2rc sequence [17]. Water suppression was achieved by applying WATERGATE [18]. A series of mixing times between 40 and 65 ms was used for the TOCSY spectra. Two-dimensional nuclear Overhauser effect correlation spectroscopy (NOESY) spectra were acquired with 150 and 300 ms mixing time to identify long range NOEs and to facilitate sequential assignments. $^3J_{HN-H\alpha}$ couplings were derived from COSY spectra [19].

Data were processed using the Bruker XWIN-NMR software (version 2.6) and analyzed with XEASY [20].

2.12. Restraints and structure calculation

Structure calculation was performed with a simulated annealing protocol using CNS (version 1.1) [21] and the standard protocol of ARIA (version 1.2) [22]. Three different ensembles each comprising 50 structures were generated, of which the 10 best structures were further refined in a shell of water [23]. The first set contained information of three additional NOEs that were used as ambiguous distance restraints. In a second calculation these NOEs were assigned to medium range distance restraints between His17/Ile19 and His25/Leu28, and in a third calculation they were assigned to long range distance restraints between His17/Leu28 and His25/Ile19.

The structures were superimposed onto the backbone heavy atoms of the lowest energy structure of residues 14–30. The structural models were visualized with the program MOLMOL [24].

3. Results

3.1. Purification of PNP

Fig. 1A shows the chromatogram of the crude venom applied to a Sephadex G-50 gel filtration column. PNP was eluted in fractions between the third and the fourth peak. These fractions were applied on a semi-preparative RP-HPLC C8 column, Fig. 1B. The peak corresponding to 23.15% (26.29 min) acetonitrile contained PNP. To verify its

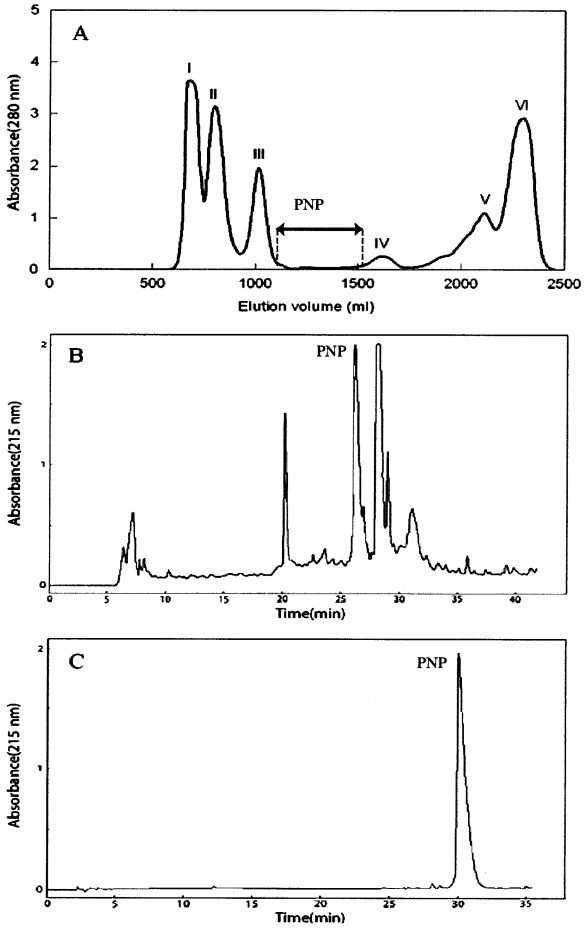

Fig. 1. Purification of PNP from the crude venom of *P. persicus*. A: Elution chromatogram of the crude venom on a Sephadex G-50 gel filtration column. B: The fractions between the third and the fourth peak of (A) were applied to a semi-preparative RP-HPLC C8 column. PNP elutes at 23.15% (26.29 min) of acetonitrile. C: Further analysis of PNP by analytical RP-HPLC C8 column.

purity, this fraction was further analyzed on an analytical RP-HPLC C8 column, Fig. 1C.

3.2. Protein analysis of PNP

PNP exhibits an experimental molecular mass of 3941.50 ± 0.17 Da when analyzed by mass spectrometry (calculated value 3942.45 Da). After reduction and alkylation the mass of PNP increased to a value of 4057.25 ± 0.23 Da (calculated value 4058.59 Da) indicative of two Cys residues. The reduced–alkylated peptide was sequenced by Edman degradation. Comparison of the sequence data with those of the amino acid composition and the molecular mass indicated that PNP contains two additional residues at the C-terminal end. Therefore, reduced and alkylated PNP was cleaved with endoproteinase Asp-N and the peptide mixture was separated by RP-HPLC and analyzed by mass spectrometry. Peak 6 contained the C-terminal peptide (Asp20–His37), which was confirmed by Edman degradation. The entire amino acid sequence of PNP (Swiss-Prot # P82972) is GENEPPKKKAPDGCFGHKIDRIGSHSGLGCNKFKPGH.

Cleavage of the native PNP with trypsin did not affect the region between the two Cys residues (14 and 30) which form a disulfide bridge. Analysis of the fragments by RP-HPLC,

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PNP       GENEPPKKKAPDGCFGHKIDRIGSHSGLGCNKFKPGH
lebetin   GDNKPPKKGPPNGCFGHKIDRIGSHSGLGCNKVDDNKG
hANP            SLRRSSCFGGRMDRIGAQSGLGCNSFR
mANP            SLRRSSCFGGRIDRIGAQSGLGCNSFR
hBNP          SPKMVQGSGCFGRKMDRISSSGLGCKVLRRH
mBNP          NSKVTHISSCFGHKIDRIGSVSRLGCNALKLL
pBNP           PKTMRDSGCFGRRLDRIGSLSGLGCNVLRRY
hCNP             GLSKGCFGLKLDRIGSMSGLGC
mCNP             GLSKGCFGLKLDRIGSMSGLGC
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Fig. 2. Multiple sequence alignment of natriuretic peptides. Conserved residues are printed in bold letters and highlighted. The disulfide bridge that is characteristic for these peptides is bridged. h, p and m are human, porcine and murine peptides.

mass spectrometry and sequence analysis yielded the expected double sequence with the release of di-phenylthiohydantoin-cystine in position 9.

3.3. Multiple sequence alignment

The family of natriuretic peptides is characterized by a conserved loop of 17 residues formed by an intra-molecular disulfide linkage. The N- and C-terminal ends vary considerably both in length and amino acid composition among the family (Fig. 2). Based on its primary sequence, PNP cannot be easily grouped together with either ANP or BNP. Its closest homologs found through a BLAST search were lebetins, peptides isolated from the venom of *Macrovipera lebetina* [25].

3.4. Physiological assay

Since PNP is highly homologous to the natriuretic peptides we tested its influence on the renal and blood system. For this purpose, purified PNP was infused intra-venously into rats. A control group was treated with ANP. At period C2 which served as baseline the values for blood pressure (mm Hg), diuresis (μl/min), and Na^+ excretion (μmol/min) were 111 ± 2, 20.1 ± 2.8, and 2.4 ± 0.3 for the group injected with

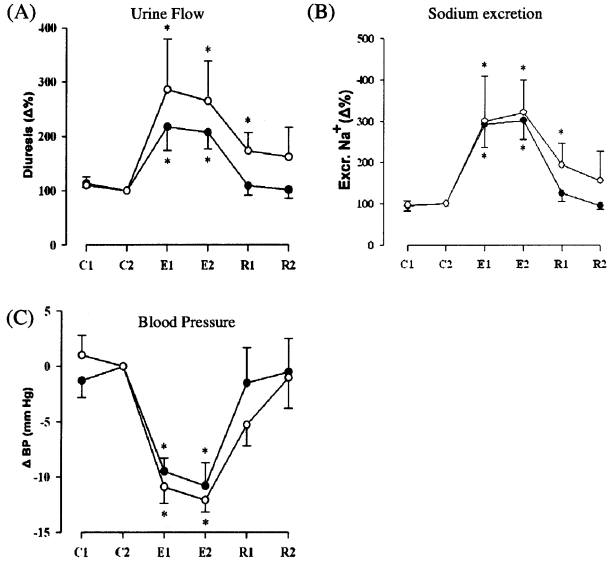

Fig. 3. Effects of PNP after intra-venous injection into rats. A: Urine flow, B: urinary sodium excretion, C: systemic blood pressure. Open circles, PNP; closed circles, ANP. C1 and C2 are control periods, E1 and E2 are experimental periods during infusion of the peptides and R1 and R2 are the recovery periods after stopping the infusion.

ANP ($n = 6$) and 110 ± 4, 12.7 ± 3.1 and 2.3 ± 0.7 for the group injected with PNP ($n = 7$), respectively. Fig. 3A shows the effect of ANP and PNP on diuresis. Both peptides increase urine flow significantly and to a comparable degree during the experimental periods E1 and E2. In contrast to the ANP group, the rise in urine flow did not recover completely in the PNP group during the recovery period R1. Equipotent effects of ANP and PNP on natriures are clearly seen in Fig. 3B although the natriuretic effect of PNP did not recover completely in the period R1, either. As shown in Fig. 3C, both peptides reduced systemic blood pressure by about 10 mm Hg, and the pressure change recovered during periods R1 and R2. The slightly prolonged effects of PNP versus ANP may be related to the slower rate of removal of PNP in vivo, i.e. removal by neutral endopeptidase and the clearance receptor (NPR-C).

3.5. Interaction with NPR-A receptor

The results obtained from the physiological experiments in rats suggested that PNP binds in a similar way to the natriuretic peptide receptors as ANP. Thus, we tested its ability to induce the formation of cGMP in two previously characterized cell lines. PC12 cell lines have been reported to express only the NPR-A receptor [26] which we confirmed by RT-PCR. CNP did not increase the basal cGMP levels, consistent with the fact that CNP specifically binds the NPR-B receptor (Fig. 4A). In contrast, the cGMP concentration after treatment of the cells with either ANP or PNP was comparable.

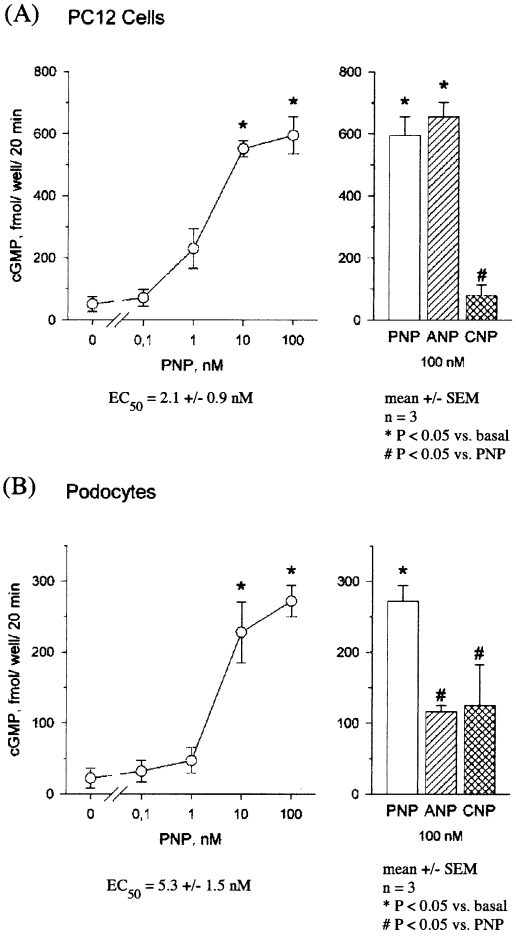

Fig. 4. Effects of PNP, ANP, and CNP on the formation of cGMP in (A) PC12 cells, (B) podocytes. The formation of cGMP depending on the amount of PNP is shown on the left. The EC_{50} value represents the effective concentration of PNP that is needed to induce 50% of the maximum effect. The effects of PNP, ANP and CNP are compared at equal concentrations on the right. SEM, standard error of the mean; $P < 0.05$ significant difference (Student's t-test).

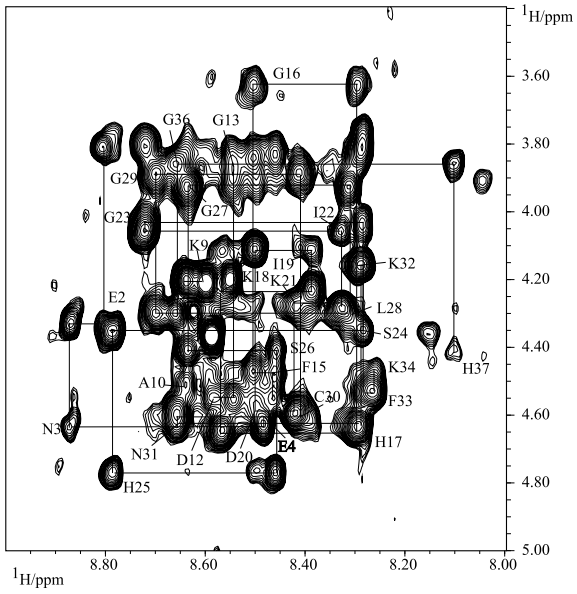

Fig. 5. Part of the NOESY fingerprint region of PNP in water, pH 4.0, 4°C with a mixing time of 150 ms. Sequential connectivities are indicated.

ANP and CNP induce a similar response in podocytes. These cells express both NPR-A and NPR-B (data not shown) and were used as control for the specificity of the interaction (Fig. 4B). Surprisingly, the amount of cGMP induced by PNP was higher than that induced by either ANP or CNP. Mass spectrometry analysis revealed that the PNP was partially degraded, such that the C-terminal part beyond residue 32 was lost. Since such a truncated PNP resembles more closely CNP (which lacks any C-terminal residues beyond the second cysteine) it is possible that the increased cGMP response in podocytes resulted from the loss of the C-terminal sequence of PNP and thus enabled it to bind both the NPR-A and NPR-B receptors.

3.6. NMR analysis

Using standard methods for sequential assignments based on homonuclear TOCSY and NOESY spectra, essentially all of the backbone and side chain protons could be assigned (Fig. 5). Side chain resonances were unaffected by temperature (4 vs. 24°C) or pH (4 vs. 6) indicating that no major structural rearrangements occur. The low deviation of the H_α chemical shifts from random coil values together with most values for the vicinal $J_{HNH\alpha}$ couplings at 6–8 Hz (see supporting information) indicates a high degree of conformational averaging. Nevertheless, we found sequential H_N–H_N NOEs throughout the sequence (Fig. 6). Likewise, a few weak NOEs were observed involving the aromatic side chains of the histidines. Due to signal overlap we could not unambiguously assign these peaks nor find others that either support or contradict them. They could be either assigned to the interactions His17/

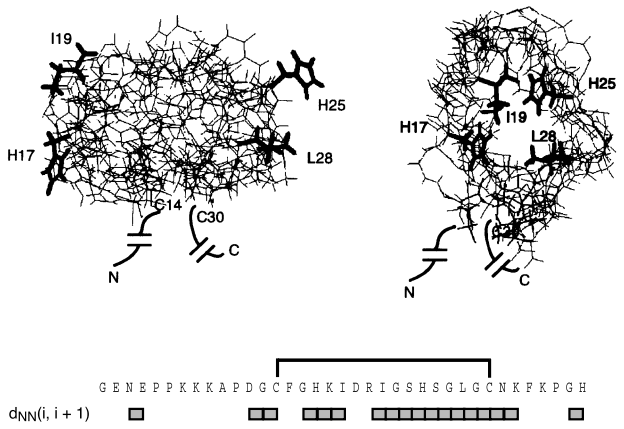

Fig. 6. Structural bundles of the 'open' (left) and 'closed' (right) conformation of PNP superimposed on the backbone atoms of residues 14–30. A summary of the observed H_N–H_N connectivities is given below. Empty fields represent missing connectivities due to signal overlap.

Ile19, His25/Leu28 or His17/Leu28, His25/Ile19, residues within the central 17-membered ring of the peptide (Fig. 6). To test these assignment possibilities we performed a series of structure calculations. When the ambiguous NOEs were either not assigned, or assigned to interactions between neighboring residues, PNP forms a loose structural bundle without any defined interactions ('open' conformation, Fig. 6). If the ambiguous restraints were assigned to the long range distance restraints His17/Leu28 and His25/Ile19 a more compact 'closed' structure was formed (Fig. 6). All structural bundles equally well fitted the experimental restraints, reflecting the low number of distance restraints.

4. Discussion

Based on the results of the physiological assay in Wistar rats, the receptor binding assay, and sequence homology we conclude that the newly isolated peptide PNP is a new member of the natriuretic peptide family with features similar to ANPs.

Our structural analysis is consistent with previous studies on natriuretic peptides that could only find signs of local interactions but no defined secondary structures [27–31]. The 'closed' conformation could resemble a transiently sampled conformation which might facilitate the transition to the receptor-bound conformation.

The high sequence conservation of the comparably long N-terminal sequence of PNP with lebetin 2α is intriguing. The N-terminal sequence in both PNP and lebetin 2α exhibits several conserved charged residues. ANP, which appears to be the natriuretic peptide that is closest in function, only contains two positively charged residues within a six residue short tail. Notably, initial tests to see whether PNP likewise is capable to prevent platelet aggregation induced by thrombin were positive. Thus, lebetin peptides and PNP may be prototypes of a new class of natriuretic peptides that also exhibit platelet aggregation properties.

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