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Structure of a novel PTH-related peptide hPTH′ and its interaction with the PTH receptor

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We have previously shown that a recombinant human PTH fragment, Pro-Pro-[Arg11] hPTH (1–34)-Pro-Pro-Asp (hPTH′), could be a potentially better and more cost-effective therapeutic agent than PTH (1–34) on osteoporosis. In this report, we characterized the solution conformations of hPTH′ by NMR spectroscopy and modeled the interactions between the hPTH′ and the PTH receptor. By comparing it with PTH (1–34) structures and their respective interactions with the PTH receptor, we identified two segments of helix extending from Ile5 to Met8 and from Glu22 to Gln29 with a divided kink between the two helixes around Arg20. Mutated arginine makes hPTH′ fill the receptor cavity better as well as forms hydrogen bonds with Val193. Understanding the ligand receptor interactions will help us design small molecules to better mimic the activities of PTH.

Keywords: parathyroid hormone; activity; structure

Introduction

Parathyroid hormone (PTH), an 84-amino acid polypeptide, is a key regulator of blood calcium levels, particularly in bone and kidney cells [1]. The effect of PTH is mediated primarily through the binding and activation of the PTH receptor (PTH1R), a class B G-protein-coupled receptor. Activation of the PTH1R results in the production and secretion of osteoclast-stimulating cytokines such as receptor activator of nuclear factor kappa B ligand, transforming growth factor beta, and interleukin 1. These cytokines are both indispensable for bone turnover and important in the pathology of osteoporosis and rheumatoid arthritis [2]. It has been shown that the first 34-amino-acid fragment of PTH is sufficient for its *in vivo* bioactivity, with the ability to reproduce biological response characteristic of the native intact PTH [3].

Recombinant human PTH 1–34 (teriparatide), approved by the Food and Drug Administration in 2002, is the first drug in a promising new class of osteoporosis medications. PTH has both anabolic and catabolic effects on the skeleton. The persistent elevation of PTH causes increased bone resorption, whereas the intermittently administered PTH results in enhanced bone formation. However, high costs of daily injections (day's treatment at US$624.18) would result in economic burdens for patients, let alone non-injection administrations with poor bioavailability [4]. To overcome these disadvantages, we have constructed a novel PTH (1–34) analog Pro-Pro-[Arg11] PTH (1–34)-Pro-Pro-Asp (hPTH′) by utilizing peptide tandem expression systems from inclusion bodies in *Escherichia coli* [5]. This expression system introduced an extra acid-labile Asp-Pro between two peptide fragments, and the peptide hPTH′ monomers with a Pro extension (the double prolines may be excised with dipeptidic peptidase IV *in vivo*) were generated by hydrochloric acid hydrolyzation. The hPTH′ monomer has been proven to have better activity than PTH (1–34) in increasing bone material density and trabecular width and improving other bone formation markers. More importantly, there is a 5.66-fold increase in yield between hPTH′ and PTH, which will reduce the cost significantly.

To understand the structural activity relationships between the hPTH′ and the PTH receptor and to facilitate the effort in identifying small-molecule candidates, we initiated a project to determine the structure of hPTH′ via NMR. By comparing it with PTH (1–34) structures and their respective interactions with the PTH receptor, we hope to shed light on the mechanism of action for the PTH peptide.

Experimental Procedures

Preparation of Pro-Pro-[Arg11] PTH (1–34)-Pro-Pro-Asp

The octa-repeat peptides were prepared and purified as described previously [5].

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Abbreviation used: *PTH, parathyroid hormone; hPTH, human parathyroid hormone; PTHrP, parathyroid-hormone-related peptide; NOESY 2D, NOE spectroscopy.*

NMR Spectroscopy

The NMR measurements of the hPTH′ peptide (2.5 mM in 50 mM sodium phosphate buffer) were conducted in 90% H_2O/10% D_2O mixture. NMR spectra were recorded at 298 K on a Unity Inova-600 spectrometer (Varian, Palo Alto, CA, USA) equipped with a triple-resonance probe and a *z*-axis pulse-field gradient. The distance restraints for structure generation were derived from NOESY spectra recorded in 90% H_2O/10% D_2O. The cross-peak intensities on the NOESY spectra were integrated and assigned using Sparky. A total of 457 NOE restraints were used for structure calculations. The Watergate approach was employed for suppression of the solvent peak. Quadrature detections in the F1 dimension were achieved with the approach of the states-TPPI. Data were collected with 256 t1 increments and 1024 complex data points, and signals were averaged over 32 transients. Proton chemical shifts were referenced to 3-(trimethyl-dilyl) propane-1,1,2,2,3,3-d6-sulfonicacid, sodium salt.

All spectra were processed by NMRPipe and NMRDraw (Biosym/Molecular Simulations, Inc., San Diego, CA, USA) software [6] and analyzed in SPARKY version 3.60 [7] . The NOE data were used as an input to ARIA 1.2 implemented in CNS 1.1 [8]. ^{1}H resonance assignments for both peptides were performed using NOESY spectra for identification of the scalar-coupled spin systems and the sequential connectivity. Four hundred fifty-seven distance constraints were obtained, including 301 intraresidue constraints, 83 sequential constraints, 72 medium constraints, and one long-range constraint. The structural calculations in combination with iterative NOE peak assignments were performed in nine cycles, and a total of 200 structures were finally obtained. The mean structure (referred to as the final structure) was obtained by averaging the coordinates of the ten lowest-energy structures. All calculations were carried out on an IBM workstation in accordance with the standard parameters of ARIA.

Molecular Modeling of PTH1 Receptor

The molecular modeling was performed with the application of DISCOVERY STUDIO 2.5 (Accelrys, Inc., San Diego, CA, USA). We utilized the high-resolution crystal structure of an engineered human beta2-adrenergic G-protein-coupled receptor (pdb id: 2rh1 [9]) as the template for the topological orientation and the arrangement of the seven membrane-spanning helices. In addition, human PTH1R (pdb id: 3c4m [10]) were used as the templates for the topological orientation and the arrangement of extracellular domain.

Interaction between hPTH′ and Receptor

Mapping receptor–ligand interaction was performed with the usage of Molecular Operating Environment (MOE) 2009 (Chemical Computing Group Inc., Montreal, Canada). The method was initially described by Alex M. Clark [11]. The information content displayed in the ligand interactions panel consists of the selected ligand and the receptor-interacting entities, hydrogen bonds (HB), solvent interactions, metal ligation, and nonbonded residues. The solvent-accessible surface area and the ligand proximity outline were also estimated. The HB scores were expressed in percentage, and the HB directionality was noted. The ligand and residue solvent accessibility metrics were estimated by measuring the exposed surface area once each of the atoms had been assigned a van der Waals'-like radius of +1.4 Å (water solvent). The solvent exposure of receptor residues was calculated by examining the difference between the solvent-exposed surface area of the receptor with and without the presence of the ligand. For the ligands, the surface accessibility calculation was carried out on the ligand + receptor complex. The default settings were applied for the definition of hydrogen-bonded and proximity interactions.

Results and Discussion

hPTH′ Structure Determination by NMR

The NMR structure for hPTH′ was calculated by means of the experimental restraints obtained from 2D-NOESY spectra. From this procedure, two helical regions were suggested for the hPTH′ in aqueous buffer solution: from Ile5 to Met8 and from Glu22 to Gln29. Helix typical medium-range $d_{\alpha N}(i, i+3)$ and $d_{\alpha\beta}(i, i+3)$ NOESY cross peaks corroborate the existence of two helical regions for hPTH′. These two helical regions were connected in a highly flexible region. The highly flexible region in the crystal structure of PTH was found to form a regular helix. These crystal structures support the propensity of the peptides to form α-helical structures, but their physiological significance is difficult to interpret because of the limited solvent exposure and extensive protein–protein interactions within the crystals [12]. Figure 1 illustrates that ten NMR structures of hPTH′ were superimposed for best fit of the backbone heavy atoms of residues 22–29 and the Ramachandran plot of the first model (upper right). The restraints used for the structural calculations are summarized in Table 1.

The helix–turn–helix is the principal component of the conformational ensemble of the PTH in solution [13–18]. The secondary structure of hPTH′ was similar to PTH (PDB ID 1FVY [13], 1ZWA [15], and 1HPY [15]) because of their similar helices (Figure 2). The main differences of these structures compared with those of PTH existed in the form of a shorter N-terminal helix and longer flexible region. In specific comparison with 1FVY, 1ZWA, and 1HPY, the N-terminal hPTH′ helix appears from the residues Ile5 to Met8 rather than from Val2 to Asn10. From a structural point of view, proline extensions did not affect the interaction with the receptor. Experiments have shown that the hPTH′ with proline extensions at both termini and Arg at position 11 increased blood calcium faster and more efficiently [5,19].

Modeling of Ligand–Receptor Interactions between hPTH′ and PTH Receptor

Structurally, the hPTH′ receptor PTH1R belongs to the family of class B G-protein-coupled receptors. The receptors comprise an extracellular N-terminal extracellular domain (of ~−160 residues, termed the N-domain) and a juxtamembrane domain (termed the J-domain) of seven membrane-spanning α-helices with intervening loops. The PTH1R structure of the N-domain has been determined by NMR spectroscopy [10,20,21]. At the same time, the structure of the J-domain remains unknown, although the putative homology models based on the rhodopsin crystal structure have been developed [22–24]. The N-terminal helices of the hPTH′ interact with the isolated J-domain of the PTH1R, and C-terminal helices of the hPTH′ bind the N-domain. The effort to monitor ligand interaction with the J-domain of the PTH1R domains by NMR is ongoing.

Although potency was reduced from that of PTH(1–34), the analogs of the N-terminal 1–14 fragment of PTH is fully active both *in vitro* and *in vivo*, and it can reproduce all biological

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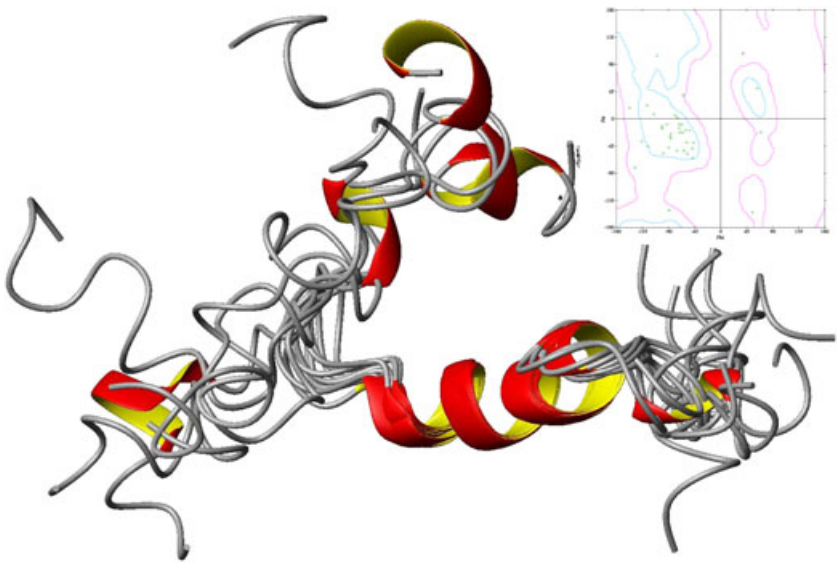

Figure 1. Superimposed ten NMR structures of hPTH′. All the structure models were superimposed using the backbone heavy atoms of residues 22–29. The upper right shows the Ramachandran plot of the first model.

Table 1. Structure statistics from NMR analysis

Quantity	Value
Total unambiguous distance restraints	457
Intraresidual	301
Sequential ($\|i-j\|=1$)	83
Medium ($2\leq\|i-j\|\leq 4$)	72
Long range ($\|i-j\|\geq 5$)	1
Root mean square deviation from the average atomic coordinates (22–29)	
Backbone atoms	0.31 ± 0.13
All heavy atoms	1.07 ± 0.35
Overall root mean square deviation for covalent bonds relative to the standard dictionary	
Bond (Å)	0.009
Angles (°)	1.3

response characteristics of the native intact PTH [25–27]. The N-terminus of PTH is very important to its activity. The NMR structures of N-terminal hPTH′ showed that residues Ile5 to Met8 from the helix interacted with the hydrophobic pockets of the receptor formed by K240, A284, V285, and F447 of PTH1R (peptide residues are denoted with a three-letter code to distinguish them from one-letter-code receptor residues). Residue Met8 was located on the same side of the helix as Ile5. We hypothesize that these two residues and the Val2 residue projected toward the third extracellular loop and are still required for the activation of PTH1R. From the NMR structures of hPTH′, even though the prolines were not excised, they did not impede the hydrophobic interactions between hPTH′ and PTH1R. After the substitution of Leu11 with arginine, hPTH′ not only fitted the receptor cavity better but also formed HB with I190 and V193 (Figure 3). These HB may differentiate PTH receptor agonists from antagonists (paper to be published).

The C-terminus of hPTH′ has demonstrated a stable amphiphilic α-helix between residues 22 and 29. Just like PTH, Leu24, Leu28, and Val31 on the hydrophobic face of this helix can strongly affect the result. We then calculated the interactions

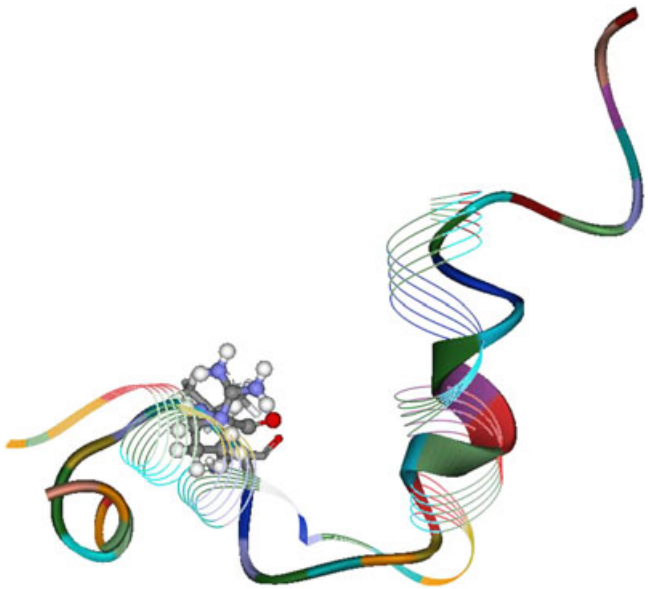

Figure 2. Structures of PTH (id: 1fvy, line ribbon) and hPTH′ (id: 2l1x, solid ribbon). Mutation at 11 is shown with the line and stick. The secondary structure of hPTH′ is similar to the PTH structures 1FVY and 1HPY.

in the N-domain of PTH1R with MOE 2009, as shown in Figure 4. The positively charged residue Arg20 of hPTH′ interacted with the negatively charged residues D137 and D29 (scored 80% and 40%, respectively, by MOE), whereas the HB between carbonyl and amine groups existed between Arg20 and D137, M32, D29 of PTH1R. So the hPTH′ plugged into the PTH1R pocket formed by D137 and D29. This may be assumed by the fact that substituted Arg11 has made the N-terminal helix shorter and the flexibility of the middle region larger. The hinge region around the Arg20 anchored the PTH1R to keep the interaction of the N and J domain-binding sites in the receptor sandwich hPTH′. Augen's studies also suggest that Arg20 forms an intermolecular salt bridge with D137, an intermolecular HB to the backbone carbonyl of M32, and an intramolecular HB with the side chain

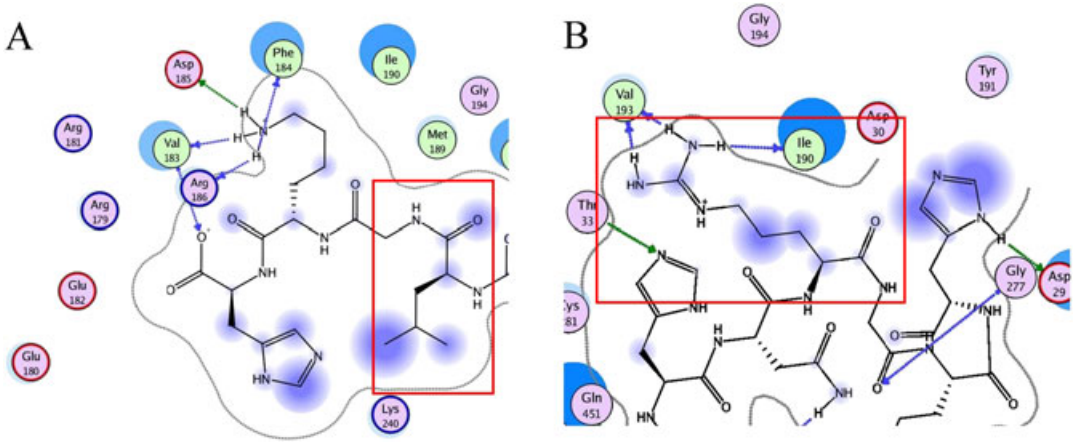

Figure 3. Interactions at 11 residues. (A) Leu11 in PTH interacts with PTH1R (B); the mutated Arg11 in hPTH' interacts with PTH1R. The hPTH' not only fitted the receptor cavity better but also formed hydrogen bonds with Ile190 and Val193.

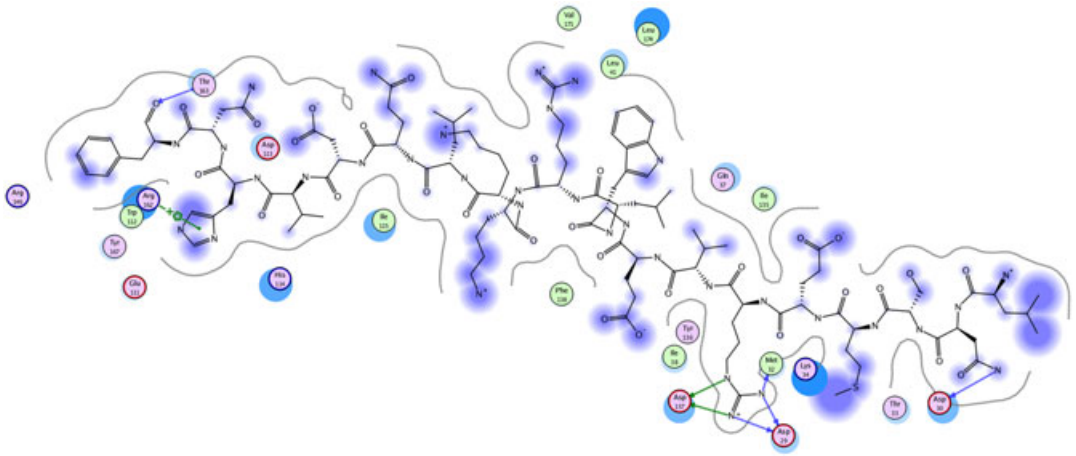

Figure 4. Interactions between the N-domain of PTH1R and the C-terminus of hPTH'. His34 interacts with R162 of PTH1R via arene–cation interaction. Asn16 forms an intermolecular hydrogen bond with D30. The hinge region around the Arg20 anchors the hPTH' with PTH1R on residues D29 and D137 from PTH1R.

of D17 [21]. From these results, we believe that the hinges are important structural elements for bioactivity in PTH/PTHrP analogs [10]. In addition, His34 interacts with R162 of PTH1R via arene–cation interaction. Asn16 forms an intermolecular HB with D30.

The PTH as an anabolic agent has been used for accelerating bone repair [28]. The development of improved therapeutics, particularly the orally available small-molecule analogs, will benefit from understanding the structural activity relationships between PTH analogs and PTH receptor. Efforts in finding the small-molecule analogs are currently under way in our laboratory.

The atomic coordinates and structure factors (PDB code: 2l1x, RCSB ID: RCSB101853) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatic Rutgers University, New Brunswick, NJ (http://www.rcsb.org).

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