Solution Structure of Parathyroid Hormone Related Protein (Residues 1–34) Containing an Ala Substituted for an Ile in Position 15 (PTHrP[Ala15]-(1–34))

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The structure of human parathyroid hormone (PTH) related protein (residues 1–34) containing an Ala substituted for an Ile in position 15 was studied by two-dimensional proton nuclear magnetic resonance spectroscopy. This mutant retains quite high levels of adenylate cyclase activity based on slightly reduced PTH receptor binding capacity. Three segments of helix were revealed extending from His5 to Lys13, Lys13 to Arg19, and from Phe24 to Thr28/Ala24, with a decided kink in the first two helices around Gly12. N- and C-terminal helices were stabilized by charged and hydrophobic side chain interactions between His5 and Glu30, Asp17 and both His9 and His25, and between Leu6 and Ala29, resulting in a globular molecule occupying a single conformation. While the structure of the entire mid-molecule region differed greatly from the structure of the native peptide, the structure of both N- and C-terminal regions remains essentially unaltered. The residues responsible for initiating signal transduction in the mutant are located in the vicinity of the residues responsible for receptor binding. The C-terminal amphipathic helix forming the receptor binding site exhibits reduced binding as a result of the closely applied N-terminal signal transduction-activating region. Although not contributing directly to receptor binding, the N-terminal region can sterically affect hormone binding through modifications to certain N-terminal side chains.

Parathyroid hormone related protein (PTHrP) was discovered because of its expression by cancers, commonly of squamous origin, giving rise to the syndrome of humoral hypercalcemia of malignancy (HHM) (for reviews, see Refs. 1 and 2). In these cancer patients, PTHrP is secreted into the circulation with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PTHrP, parathyroid hormone related protein; hPTHrP, human PTHrP; PTH, parathyroid hormone; PTHrP[Ala15]-(1–34), parathyroid hormone related protein (residues 1–34) containing an Ala substituted for an Ile in position 15; F3EtOH, trifluoroethanol; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; r.m.s., root mean square; TOCSY, total correlation spectroscopy.

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increases helix content in those peptides that already have a propensity to form helices in water (23), which is the case with PTHrP (13) and PTH polypeptides (16, 17, 24). However, the mild hydrophobicity can have the effect of weakening hydrophobically stabilized regions of polypeptide structures (25). Thus, any interaction between the N- and C-terminal helices in PTH/PTHrP-(1–34) would be expected to be weakened under these conditions.

In an attempt to disrupt the relatively strong and consistent hydrophobic packing found between residues 15 and 23 in both PTH (16, 21) and PTHrP (18, 20), a new active analog of PTHrP-(1–34)-amide was synthesized with Ile15 replaced by an Ala. The structure was examined to check for any changes to the hydrophobic packing that could then be quantitated from involvement in direct receptor binding. The intention was to determine the part or parts of the structure remaining unaltered that could be responsible for receptor binding. Since signal transduction is separate from receptor binding and since the design of agonist-specific sites could have been well separated. A comprehensive understanding of the structure-activity relationship of the PTHrP/PTH pair of molecules requires a detailed knowledge of their structure and mobility. In particular, the design of antagonists useful for the control of hypercalcemia of malignancy firstly requires that the stabilized structure of the receptor binding domain be determined. We have thus studied the stabilized structure of the active mutant PTHrP[Ala15]-(1–34)-amide in 8% F3EtOH-d4 and compared the principal structural features of the hormone with the tertiary solution structures of other homologous hormones to identify the residues responsible for receptor binding and their spatial relationship with residues responsible for signal transduction.

MATERIALS AND METHODS

Peptide Synthesis—Human PTHrP[Ala15]-(1–34)-amide was synthesized on an Applied Biosystems 430A peptide synthesizer using t-butyloxy carbonyl amino acid derivatives (26). The Glu, Ser, and Thr side chains were protected using dinitrophenol while the Lys side chains had chlorobenzyl protection, and Arg side chains were protected using dinitrophenol while the Lys side chain was amidated. The structure was examined to check for any changes to the hydrophobic packing that could then be quantitated from involvement in direct receptor binding. Since signal transduction is separate from receptor binding and since the design of agonist-specific sites could have been well separated. A comprehensive understanding of the structure-activity relationship of the PTHrP/PTH pair of molecules requires a detailed knowledge of their structure and mobility. In particular, the design of antagonists useful for the control of hypercalcemia of malignancy firstly requires that the stabilized structure of the receptor binding domain be determined. We have thus studied the stabilized structure of the active mutant PTHrP[Ala15]-(1–34)-amide in 8% F3EtOH-d4 and compared the principal structural features of the hormone with the tertiary solution structures of other homologous hormones to identify the residues responsible for receptor binding and their spatial relationship with residues responsible for signal transduction.

Biological Activity—Adenylate cyclase activation, in response to PTHrP[Ala15]-amide, was assessed in PTH-receptor positive osteogenic sarcoma cells UMR-106–01 by measurement of cAMP produced per million cells (4) using a cAMP radioimmunoassay (28). PTHrP[Ala15]-amide was compared with human PTH-(1–34)-amide in the same assay over the concentration range 0.1–100 nM. Potency in the assay (EC50) is the peptide concentration producing half-maximal enzyme activation.

Specific Binding—PTH-receptor binding of PTH/PThrP peptides to PTH-receptor positive UMR-106–01 cells was assessed as previously described (29) by competition of increasing amounts of PTHrP-(1–34)-amide or PTHrP[Ala15]-amide with iodinated PTHrP[Tyr36] or PTHrP-(1–34) and the Ic50 recorded.

Proton NMR Spectroscopy—PTHrP[Ala15]-(1–34)-amide was dissolved in 92% H2O, 8% (v/v) 2H2O to give a final concentration of 2.0 mM at pH 4.1 and 294 K in 5-mm precision tubes. Chemical shifts of the resonances remained unaltered over the concentration range 0.5–3.0 mM, indicating the molecule remained monomeric at this pH and concentration as found in earlier studies of PTH and PTHrP (5, 16, 30–31). Sample pH was adjusted with dilute HCl and NaOH and measured with an Activaion B5J1130 thin stem micro-electrode.

NMR spectra were obtained on a Bruker AMX-600 spectrometer operating in the Fourier transform mode with quadrature detection in both directions without sample spinning and with the use of a calibrated external temperature control unit. One-dimensional spectra were obtained using a spectral width of 600–1000 Hz with a 150-μs acquisition delay of 2 s. A total of 256 summed free induction decays were collected in a data block of 32 K. Signal-to-noise ratio was 670 with the dedicated proton probe.

Signal Assignments—Spin systems were assigned using standard procedures (32). The complete spin systems from all residues were identical using two-dimensional TOCSY and double-quantum filtered correlation spectroscopy spectra. The TOCSY spectra were obtained with a MLEV-17 pulse sequence (33, 34) using a mixing time of 100- and 2.5-ms trim pulses, which enabled connectivities from the amino acid backbone NH resonances to all the coupled side chain proton resonances to be measured. Spin-lock field strength was 9 kHz. Suppression of the water resonance was achieved using continuous low power (65 dB) 0/180 coherence irradiation throughout the relaxation (1.8 s) and mixing periods (35) with a stimulated cross-peaks under bleached α modification to reduce cross-peak suppression under the solvent (36). Two-dimensional phase-sensitive NOESY experiments (37, 38) were used to make sequence-specific resonance assignments as well as for estimations of proton-proton distance constraints. These were recorded using the time-proportional phaseincrements method (39) also using the stimulated cross-peaks under bleached α modification. A mixing time of 200 ms was used to provide the distance constraints table since it provided sufficient cross-peak intensity without spin-diffusion effects becoming problematic. The sweep widths in both ω1 and ω2 were 6024 Hz. 4 K data points were acquired in ω1 with 512–1024 data points collected in ω2, with each free induction decay consisting of 128–160 scans. The data were zero-filled to 4–8 K × 1–2 K and then apodized using Gaussian multiplication in ω2 and shifted sine-bell in ω1 prior to Fourier transformation. Third-order polynomial functions were employed to correct base lines. Data were analyzed using XWIN NMR on a Silicon Graphics Indy workstation. Chemical shifts were referenced to trimethylsilylpropanesulfonic acid.

Distance Geometry Calculations—The 365 measured NOE cross-peaks, of which 195 were structure-determining interresidues, were separated into three conservative distance categories depending on contour intensity. Strong NOE were given an upper distance constraint of 0.30 nm, medium NOE a value of 0.40 nm, and weak NOE a value of 0.45 nm. Corrections for pseudatoms were applied wherever stereospecific identifications were not obtained. A total of 2000 distance geometry structures were calculated from random starting configurations using the program DIANA 2.8 (40) on a Silicon Graphics Indy workstation. This technique utilizes least squares minimization in torsion angle space incorporating a variable target function, which considers progressively longer range constraints as the global structure is generated.

Dynamic Simulated Annealing—The best 20 distance geometry structures possessing the lowest penalty values were refined in X-PLOR (32) using a dynamic simulated annealing protocol (42) run on Silicon Graphics Indigo R4000 and Indy workstations. Non-bonded interactions were not considered in the DIANA distance geometry structures. Consequently, they possessed poor potential energy values. 500 cycles of computationally inexpensive conjugate gradient energy minimization were applied in the initial stage of the simulation keeping the atom co-ordinates fixed to improve the distance geometry starting structures. Covalent geometry was constrained with parameters hνbonds = 20 kJ mol-1 \( \cdot \) nm-2, hangles = 2000 kJ mol-1 \( \cdot \) rad-2, hpropper = 200 kJ mol-1 \( \cdot \) rad-2, and kimbond = 0 kJ mol-1 \( \cdot \) rad-2 (41). The non-bonded interactions were modelled with the function repel \( z = \frac{v}{k} \) mol-1 rad-1 z with asymptote \( z = \frac{v}{k} \) mol-1 rad-1 z and \( z = \frac{v}{k} \) mol-1 rad-1 z (41). The non-bonded interactions were modelled with the function repel \( z = \frac{v}{k} \) mol-1 rad-1 z with asymptote \( z = \frac{v}{k} \) mol-1 rad-1 z (41). The non-bonded interactions were modelled with the function repel \( z = \frac{v}{k} \) mol-1 rad-1 z with asymptote \( z = \frac{v}{k} \) mol-1 rad-1 z (41). The non-bonded interactions were modelled with the function repel \( z = \frac{v}{k} \) mol-1 rad-1 z with asymptote \( z = \frac{v}{k} \) mol-1 rad-1 z (41).
minimization. Molecular graphics were processed using Insight II operating on a Silicon Graphics Indigo 2.

RESULTS

The IC\(_{50}\) was measured for both native human PTHrP-(1–34)-amide and the Ala\(^{15}\) analog used in the structure analysis with values of 0.7 and 4.5 nM obtained, respectively, from the data in Fig. 1. The PTH-like activities of the peptides were assayed for stimulation of \(^{3}H\)cAMP formation in intact UMR-106–01 cells pre-labeled with \(^{3}H\)adenine (4). The PTHrP analog exhibited only slightly lower relative adenylate cyclase activity than that of native PTHrP based on reduced binding (Fig. 1). Points are means of at least triplicates, and basal activity is reduced only slightly lower relative adenylate cyclase activity that little has changed in the binding site structure.

The numerous sequential NH resonances in Fig. 3 reveal the likely presence of a substantial quantity of helix. Connectivities from Phe\(^{22}\) through to Ala\(^{34}\) at the C terminus are certainly indicative of the presence of a long stretch of continuous helix within this segment. The overall pattern of sequential and non-sequential inter-residue backbone and side chain NOEs is summarized in Fig. 4. The relative intensity of the inter-residue NOE is represented by the line thickness. The shortest inter-proton distances are those with the strongest NOE and are represented by the thickest lines. Degenerate NOEs which are probably present are shown by the asterisks and dotted lines. These were used only for qualitative purposes and remained excluded from structure calculations. Weak or absent sequential H\(_{\alpha}/NH\) cross-peaks combined with strong sequential NH/NH\(_{i+1}\) cross-peaks are indicators of the presence of \(\alpha\)-helix (43), a conclusion supported by the presence of several medium range NOEs, such as H\(_{\alpha}/NH\) and H\(_{\alpha}/H\beta_{\alpha+3}\). On the basis of the patterns of these medium range NOEs, a large region in the sequence appears to contain helix. However, the pattern of long range NOEs indicates that the helix is bent at one point, at least enabling the ends to interact. Some of these interactions are between side chains of His\(^{6}\)-His\(^{26}\), Leu\(^{8}\)-Ala\(^{23}\), His\(^{3}\)-His\(^{25}\), His\(^{8}\)-Ile\(^{26}\), His\(^{3}\)-Ala\(^{29}\), and Ser\(^{14}\)-His\(^{26}\).

All 385 constraints were used in the distance geometry algorithm DIANA to generate 2000 structures from random starting conformations. These calculations yielded 20 structures, which satisfied all distance constraints within 5 pm. Each member of the family of 20 structures possessing the very lowest penalty functions displayed very good covalent geometry. These low penalty structures were refined further using
dynamic simulated annealing (X-PLOR 3.1) to include non-bonded interactions. A mean r.m.s. deviation from ideal bond lengths of 0.215 ± 0.011 pm was determined. The mean r.m.s. deviation from ideal bond angles was 0.311 ± 0.011°. A mean Lennard-Jones potential equal to 2748 ± 58 kJ mol⁻¹ reinforced the conclusion that non-bonded contacts remained good following dynamic simulated annealing. The complete family of refined structures was superimposed, and the r.m.s. deviation of backbone atoms was measured throughout the molecule. An average structure was calculated from which the r.m.s. deviation of each structure was determined. When all atoms were included, an r.m.s. value of 1.55 ± 0.15 Å (mean ± S.D.) was obtained while the backbone atoms alone yielded an r.m.s. value of 0.88 ± 0.19 Å. The N-terminal three residues were

### TABLE I

| Residue | NH  | δH  | βH  | Others                       |
|---------|-----|-----|-----|------------------------------|
| Ala¹    | 8.53 | 4.15 | 1.56 | CH₃ 0.99, 0.99               |
| Val²    | 8.45 | 4.47 | 3.97, 3.89 | CH₂ 2.30, 2.28 |
| Ser³    | 8.59 | 4.23 | 2.02, 1.97 | CH₂ 2.39, 2.37; NH 7.62, 6.88 |
| Glu⁴    | 8.52 | 4.60 | 3.26, 3.19 | CH 1.53; CH₂ 0.93, 0.87    |
| His⁵    | 8.30 | 4.26 | 2.13, 2.11 | CH 1.75; CH₂ 0.94, 0.84    |
| Gln⁶    | 8.27 | 4.26 | 1.68, 1.66 | CH 1.75; CH₂ 0.94, 0.84    |
| Leu⁷    | 8.34 | 4.07 | 1.78, 1.53 | CH 1.75; CH₂ 0.94, 0.84    |
| His⁸    | 8.20 | 4.41 | 3.26, 3.21 | CH 1.75; CH₂ 0.94, 0.84    |
| Asp⁹    | 8.43 | 4.28 | 1.95, 1.84 | CH 1.59, 1.59; CH₂ 1.68, 1.68; CH₃ 3.03, 3.03; NH 7.50 |
| Lys¹⁰   | 8.48 | 3.97, 3.97 | 1.91, 1.83 | CH 1.53, 1.48; CH₂ 1.72, 1.72; CH₃ 3.03, 3.03; NH 7.50 |
| Ser¹¹   | 8.47 | 4.43 | 4.03, 3.97 | CH 1.49                     |
| Ala¹²   | 8.39 | 4.21 | 2.14, 2.11 | CH 2.44, 2.42; CH₂ 1.67, 1.67; CH₃ 0.95, 0.90 |
| Glu¹³   | 8.25 | 4.13 | 2.81, 2.79 | CH 1.67; CH₂ 0.95, 0.90    |
| Asp¹⁴   | 8.21 | 4.52 | 1.84, 1.84 | CH 1.67; CH₂ 0.95, 0.90    |
| Leu¹⁵   | 8.12 | 4.11 | 1.84, 1.84 | CH 1.67; CH₂ 0.95, 0.90    |
| Arg¹⁶   | 8.08 | 4.11 | 1.94, 1.94 | CH 1.67; CH₂ 0.95, 0.90    |
| Arg¹⁷   | 8.05 | 4.07 | 1.88, 1.88 | CH 1.67; CH₂ 0.95, 0.90    |
| Arg¹⁸   | 8.05 | 4.17 | 1.98, 1.98 | CH 1.67; CH₂ 0.95, 0.90    |
| Phe¹⁹   | 8.34 | 4.62 | 3.29, 3.17 | CH 1.67; CH₂ 0.95, 0.90    |
| Leu²⁰   | 8.11 | 4.40 | 1.82, 1.82 | CH 1.67; CH₂ 0.95, 0.90    |
| His²¹   | 8.24 | 4.40 | 3.19, 3.19 | CH 1.67; CH₂ 0.95, 0.90    |
| His²²   | 8.13 | 4.42 | 3.26, 3.26 | CH 1.67; CH₂ 0.95, 0.90    |
| Leu²³   | 8.13 | 4.16 | 1.62, 1.62 | CH 1.67; CH₂ 0.95, 0.90    |
| Ile²⁴   | 8.05 | 3.94 | 1.87  | CH 1.67; CH₂ 0.95, 0.90    |
| Ala²⁵   | 7.90 | 4.23 | 1.38  | CH 1.67; CH₂ 0.95, 0.90    |
| Glu²⁶   | 8.06 | 4.23 | 2.04, 1.96 | CH 1.67; CH₂ 0.95, 0.90    |
| Ile²⁷   | 8.06 | 4.02 | 1.87  | CH 1.67; CH₂ 0.95, 0.90    |
| His²⁸   | 8.51 | 4.78 | 3.35, 3.23 | CH 1.67; CH₂ 0.95, 0.90    |
| Thr²⁹   | 8.14 | 4.23 | 4.29  | CH 1.67; CH₂ 0.95, 0.90    |
| Ala³⁰   | 8.29 | 4.29 | 1.45  | CH 1.67; CH₂ 0.95, 0.90    |

**FIG. 3.** A section of the two-dimensional NOESY spectrum in Fig. 2 showing sequential HN-HN, HN-HN, and sequential HN-HN connectivities throughout the molecule.
clearly disordered, and so comparing the 20 best structures with the average structure over the backbone atoms for residues 4–34 yielded a much smaller r.m.s. value of 0.44 ± 0.11 Å. The segment 4–33 reveals quite low local average r.m.s. deviation in backbone atoms, whereas for all non-hydrogen atoms, the value was higher with the Arg cluster 19–21 being particularly unrestrained. In contrast, the residues around 8 and 28 are particularly well constrained as expected from the number of NOE constraints involving them. The φψ dihedral angle pairings in the segments Glu4-His9/Asp10, Lys13-Arg19, and Phe22-Ala34 give the appearance of helical conformations.

A view of the backbone of 20 structures superimposed over the segment Glu4-Ala34 is shown in Fig. 5. As was deduced from the dihedral angles, three segments of helix are present in the regions mentioned above. The N-terminal helices are separated by a kink around Gly12 and ends in a distinct turn at the Arg cluster (residues 19–21). A single stretch of helix then continues to the C terminus. The whole structure is stabilized by interactions between side chains in the N- and C-terminal helices. The N terminus appears devoid of any regular structure, but the bulk of the molecule occupies a well defined conformational space under these solution conditions. This is best revealed in Fig. 6. As was deduced from the average structure is shown with a ribbon and the several interacting side chains are shown represented in ball-and-stick form. The side chains shown include His5, Leu8, His9, Ser14, Leu18, His25, His26, Ile28, and Ala29.

**DISCUSSION**

A single major conformer of hPTHrP[Ala15]-(1–34)-amide is revealed with no evidence of hinges found at residues 13/14 and 20/21 in different analogs (15–16, 18). The axis of the C-terminal helix in Fig. 6 forms an angle of 80° with respect to the axis of the N-terminal helix. The two helices appear to be stabilized primarily through electrostatic interactions between the side chains of Asp17, near the bend at the end of the central helix, and the side chain of His25, some 3 Å away, as well as an Asp17 to His25 interaction, about 0.5 Å further removed. Other interactions include Glu30 separated from His5 by 2.5 Å, and Glu30 to His26 along the same helix, nearly 3.5 Å away. The methyl groups of Leu8 and Ala29 form an apparently tight interaction that helps stabilize the molecule. Thus a total of eight residues, six of them charged, have inwardly oriented side chains while all others can be considered more surface accessible. The C-terminal helix exhibits a distinct hydrophobic face comprising the side chains of Phe22, Phe23, Leu24, Leu27, Ile28, and Ile31.

The three Arg side chains 19–21 are clustered on the outer surface of the molecule at the tight bend separating the C-terminal helix from the central helix.

A fundamental purpose of the investigation was to determine whether alterations in the key hydrophobic interaction sites responsible for stabilizing the peptide affect receptor binding properties. Binding of the stabilized conformer to PTH receptors was found to be reduced about 6-fold with respect to native PTHrP-(1–34) or only about 3-fold with respect to PTH-(1–34) as a result of substituting Ala for Ile15 even though the local structure encompassing the mutation site was found to be dramatically altered and all hinges were eliminated. The structure of the putative receptor binding site formed by the C-terminal helical domain remained unaffected. Thus, receptor binding must depend principally on the maintenance of the structure of the C-terminal helix 22–34 and the availability of
The N terminus, vital for signal transduction, is located adjacent to the essential C-terminal support to the results in this paper which show that the locus maintaining strong receptor binding but modifications of side 
residues barely affects receptor binding while essentially abolish-
by 3 log. However, simple deletion of the N-terminal six resi-
dues in PTH-(1–34)-amide reduces binding capacity to PTH recep-
tors in both bone cells and kidney membranes by 2 log to about 100 nm (47) while even PTHrP(14–34) still binds at 10 μM (48). The structure in Fig. 6 reveals how the absence of the bulk of the N-terminal helix would destabilize the C-terminal helix, thus reducing binding affinity by 2 log while agonist activity is reduced about 7 log (10). Structural comparisons between PTH-
(1–34) and all the PTHrP(1–34) analogs reveal that the major-
ity of segment 22–34 in all the hormones forms an α-helix. The N-terminal part of this C-terminal helical segment inter-
acts closely with residue 15 in the putative secondary binding 
region (16, 20, 21), and so the sites are immediately adjacent to 
one another. Since the C-terminal segment can, by itself, fully 
displace PTH(1–34) and PTHrP(1–34) from the receptor, it is 
more likely that segment 14–24 simply stabilizes the C-termi-
mal helical structure without being directly involved in receptor 
binding. Certainly, the 3-fold lower binding exhibited by the 
the Ala15 analog compared with PTH indicates the central 
portion of the molecule plays, at best, a miniscule role in direct 
receptor binding.

Progressive C-terminal deletions reduce biological activity in 
PTHrP(1–34)-amide by progressively reducing the length of the C-terminal helix and thus the stability of the receptor 
binding site. Removal of segment 30–34 results in the osteo-
ergic sarcoma cell cAMP and the chicken kidney adenylate 
cyclase activities declining by 90% (4) and the loss of all mito-
genic activity in PTH (11, 12). A much larger reduction (100-
fold) in binding affinity was observed in PTH (7). Additional 
deletion of residues His30 to Ala34 essentially abolishes agonist 
activity and binding (4). The structure presented here reveals 
that the site of agonist activity must be adjacent to the primary 
receptor binding site so that modifications to residues at the N 
terminus can cause steric blocking effects on the binding of the 
C-terminal helix to the receptor (45). The residues responsible 
for signal transduction do not possess any regular structure in 
the absence of interactions with the receptor. The mid-portion 
of the molecule accommodates dramatic structural differences 
while maintaining significant receptor binding capacity and 
agonist activity provided the N- and C-terminal helices are 
conserved and able to stabilize each other in the required 
conformation. This helps provide a structural basis for the 
design of effective PTHrP and PTH antagonists. We conclude 
that these must primarily mimic the structure of the conserved 
hydrophobic face of the C-terminal amphipathic α-helix.

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