The structure of human parathyroid hormone-related protein(1–34) in near-physiological solution

Marcus Weidler[a], Ute C. Marx[a,b,*], Gabi Seidel[a], Wolfgang Schäfer[c], Eike Hoffmann[c], Angelika Eßwein[c], Paul Rösch[a]

[a]*Lehrstuhl für Biopolymere, Universität Bayreuth, D-95440 Bayreuth, Germany*
[b]*Niedersächsisches Institut für Peptid-Forschung (IPF), Feodor-Lynen-Straße 31, D-30625 Hannover, Germany*
[c]*Boehringer Mannheim, Sandhofer Str. 116, D-68298 Mannheim, Germany*

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Abstract Parathyroid hormone-related protein plays a major role in the pathogenesis of humoral hypercalcemia of malignancy. Under normal physiological conditions, parathyroid hormone-related protein is produced in a wide variety of tissues and acts in an autocrine or paracrine fashion. Parathyroid hormone-related protein and parathyroid hormone bind to and activate the same G-protein-coupled receptor. Here we present the structure of the biologically active NH_2-terminal domain of human parathyroid hormone-related protein(1–34) in near-physiological solution in the absence of crowding reagents as determined by two-dimensional proton magnetic resonance spectroscopy. An improved strategy for structure calculation revealed the presence of two helices, His-5–Leu-8 and Gln-16–Leu-27, connected by a flexible linker. The parathyroid hormone-related protein(1–34) structure and the structure of human parathyroid hormone(1–37) as well as human parathyroid hormone(1–34) are highly similar, except for the well defined turn, His-14–Ser-17, present in parathyroid hormone. Thus, the similarity of the binding affinities of parathyroid hormone and parathyroid hormone-related protein to their common receptor may be based on their structural similarity.

Key words: Parathyroid hormone-related protein; Parathyroid hormone; Nuclear magnetic resonance; Circular dichroism; Peptide structure

1. Introduction

Human parathyroid hormone-related peptide (hPTHrP) was originally identified through its causative role in human humoral hypercalcemia of malignancy (HHM) [1]. The hPTHrP gene is thought to belong to the same family as the parathyroid hormone (PTH) gene [1]. Unlike PTH, PTHrP is a product of almost all normal tissues, both fetal and adult, and plays various physiological roles in a paracrine/autocrine fashion [2], including regulation of cellular proliferation and differentiation [3], fetus development [4], reproduction system maintenance [5], transepithelial (renal, placental, oviduct, mammary gland) calcium transport regulation [6,7], relaxation of vascular and non-vascular smooth muscles [8,9], calcium and phosphate homeostasis [10] and decrease of systemic blood pressure [11]. The expression of the PTHrP gene in normal tissues is tightly regulated by a number of factors, involving both transcriptional and post-transcriptional mechanisms [12–14]. PTHrP overproduced and secreted by certain malignancies enters the circulation, interacts with a receptor common for PTHrP and PTH, and causes hypercalcemia in cancer patients [1,15]. The PTH-like biological activities of the 141 amino acid PTHrP, such as stimulation of intracellular cAMP formation, its influence on the serum calcium level and relaxation of smooth muscle cells, are located within the NH_2-terminal 34 amino acids [1,8,16,17], in close analogy to PTH itself. Other fragments of PTHrP, for example from the COOH-terminus or the midregion, exert diverse functions [1,8,16,18–22]. Effects of PTHrP(1–34) on its target cells are mediated by adenylate cyclase and/or by protein kinase C [15,23,24]. Recent studies imply an interaction of the 1–14 and 15–34 domains of PTHrP(1–34) on binding to the PTH/PTHrP receptor and direct or indirect involvement of His-5, Arg-19, and Arg-21 in this interaction [25]. The two peptides PTH and PTHrP share eight identical amino acids in the region 1–13, but only three identical amino acids in the region 14–34.

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SVSEIQLMHN LGKHLNSMER VEWLRKKLQD VHNF
AVSEHQLLHD KGKSIQDLRR RFFLHHLIAE IHTA
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The COOH-terminal domain, Ile-15–Ala-34, of hPTHrP(1–34) is thought to be involved in receptor binding, while the NH_2-terminal domain, Ala-1–Ser-14, is suggested to trigger the signal transduction cascade [8], analogous to the situation in hPTH. In view of the multiple functions of hPTHrP mediated by its NH_2-terminal fragment 1–34, particularly its major role in pathogenesis of humoral hypercalcemia of malignancy, the determination of the three-dimensional structure of this peptide is a problem of considerable biochemical and medical interest.

Most of the hPTHrP structures were determined in solution in the presence of 2,2,2-trifluoroethanol (TFE), and the results of these structure determinations differ in important aspects [26–29]. Earlier work in TFE-free solution had to be partly dismissed on grounds of misassigned proton resonances [30].

It is generally accepted that NH_2-terminal fragments of human PTH such as hPTH(1–37), hPTH(1–34), hPTH(2–37), hPTH(3–37), hPTH(4–37), and the NH_2-terminal fragment of bovine PTH, bPTH(1–37), as well as several derivatives obtain not only secondary structure, but also show tertiary interactions in TFE-free solution ([31–34], Marx et al., unpublished). Due to the flexibility of the loop region around Gly-12, however, the relative location of the two helices is not fixed and no overall tertiary structure is preferred ([32–34], Marx et al., unpublished). Structural features of hPTH(1–34) were also suggested to depend on salt concentration [34].

As the only reliable structural data of native wild-type

*Corresponding author.

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hPTHrP(1–34) were determined in the presence of 50% TFE [30], we decided to determine the structure of hPTHrP(1–34) in near-physiological aqueous solution in the absence of crowding reagents.

2. Materials and methods

PTHrP(1–34) was obtained as a commercial product (Neosystems, Strasbourg).

Circular dichroism (CD) spectra were recorded at 298 K in 0.1 mm cells from 250 to 190 nm at 20 nm/min on a Jasco 600A CD spectropolarimeter with a solution of 1 mg/ml (~250 μM) peptide in 50 mM potassium phosphate, pH 5.1, 250 mM NaCl, and averaged over 16 scans. The reference sample contained buffer without protein and the instrument was calibrated with ammonium D-camphorsulfonate.

For proton NMR spectroscopy, hPTHrP(1–34) was dissolved in 50 mM potassium phosphate buffer with 250 mM NaCl, pH 5.1, to a final concentration of 1.5 mM. All NMR experiments were carried out at 277 K or 298 K on a Bruker AMX600 spectrometer. Homonuclear double quantum filtered COSY [35], clean TOCSY with an MLEV-17 spin-locking sequence [36], and NOESY [37] experiments were performed in H_2O/D_2O (9:1) employing standard methods [38]. NOESY mixing times were 100 ms and 200 ms, and clean TOCSY mixing times were 60 ms and 80 ms. All spectra were acquired in phase-sensitive mode with quadrature detection in both dimensions using the time-proportional phase incrementation technique in ω_1 [39]. The solvent signal was suppressed by continuous coherent irradiation prior to the first excitation pulse and during the mixing time of the NOESY experiments. 4096×512 data points were collected with a spectral width of 6024 Hz in both dimensions. Baseline correction up to sixth order was performed for all two-dimensional spectra in both dimensions. A π/4-shifted sinebell-squared filter was used prior to Fourier transformation. Zero-filling resulted in a data size of 4096×1024 data points in the frequency domain. The NDEE 2.0 software package (Software Symbiose, Bayreuth, Germany) was used for data processing on Unix workstations and PCs. Chemical shift values are reported relative to 2,2-dimethyl-2-silapentane sulfonate.

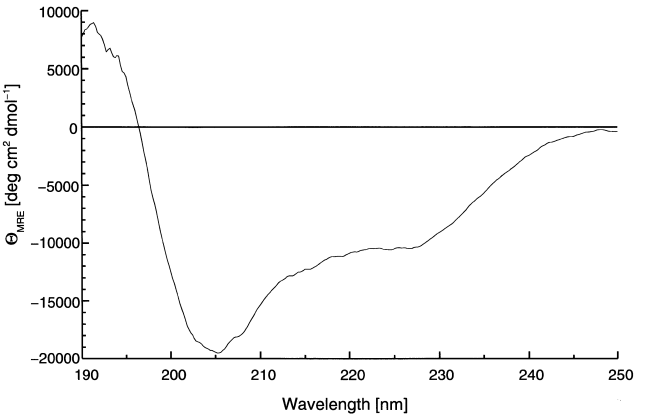

Fig. 1. CD spectrum of hPTHrP(1–34) at 298 K, 1 mg/ml (~250 μM) peptide in 50 mM potassium phosphate, pH 5.1, 250 mM sodium chloride.

For structure calculations, only NOEs visible in the 277 K spectra with 200 ms mixing time were taken into account. Identical calculations combining information obtained at the two different temperatures resulted in virtually identical structures.

An estimate of secondary structure elements can be obtained from

Table 1
^{1}H chemical shifts and assignments for PTHrP(1–34), 1.5 mM, at 277 K in 50 mM potassium phosphate, pH 5.1, 250 mM NaCl relative to DSS as an external standard, ±0.01 ppm

Residue	NH	αH	βH	γH	Others
Ala-1		4.15	1.52		
Val-2	8.66	4.18	2.08	0.97	
Ser-3	8.64	4.45	3.92/3.85		
Glu-4	8.68	4.23	1.99/1.92	2.26/2.21	
His-5	8.64	4.65	3.25/3.18		8.60(2); 7.28(4)
Gln-6	8.49	4.26	2.07/2.00	2.35	7.71/7.01 (NH_2)
Leu-7	8.47	4.30	1.64	1.53	0.93/0.87(δ)
Leu-8	8.30	4.28	1.63/1.55	1.47	0.91/0.86(δ)
His-9	8.54	4.66	3.28/3.17		8.62(2); 7.29(4)
Asp-10	8.50	4.59	2.73/2.68		
Lys-11	8.53	4.28	1.91/1.83	1.47	3.01(ε); 1.71(δ)
Gly-12	8.59	3.95			
Lys-13	8.23	4.36	1.88/1.79	1.48/1.41	3.00(ε); 1.69(δ)
Ser-14	8.59	4.47	4.01/3.95		
Ile-15	8.40	4.05	1.91	1.52/1.25/0.93	0.88(δ)
Gln-16	8.40	4.17	2.06	2.41	7.66/7.00(NH_2)
Asp-17	8.36	4.53	2.74		
Leu-18	8.30	4.15	1.63		0.92/0.87(δ)
Arg-19	8.10	4.20	1.90/1.71	1.61	3.22(δ); 7.33(ε); 7.00/6.56(guanidino)
Arg-20	8.15	4.13	1.76/1.60	1.48	3.14(δ); 7.29(ε); 7.00/6.56(guanidino)
Arg-21	8.25	4.18	1.92/1.77	1.67	3.19(δ); 7.42(ε); 6.92/6.55(guanidino)
Phe-22	8.20	4.49	3.09/3.03		7.34(2,6); 7.25(3,5); 7.19(4)
Phe-23	8.25	4.48	3.20/3.06		7.34(2,6); 7.26(3,5); 7.07(4)
Leu-24	8.20	4.13	1.62		0.91/0.87(δ)
His-25	8.29	4.59	3.23/3.13		8.58(2); 7.25(4)
His-26	8.35	4.60	3.21/3.01		8.53(2); 7.18(4)
Leu-27	8.35	4.29	1.62	1.54	0.90/0.84(δ)
Ile-28	8.26	4.12	1.84	1.17/0.85	0.90(δ)
Ala-29	8.41	4.28	1.36		
Glu-30	8.39	4.23	1.96/1.90	2.28/2.20	
Ile-31	8.32	4.11	1.80	1.43/1.15	0.85(δ)
His-32	8.85	4.86	3.27/3.17		8.59(2); 7.28(4)
Thr-33	8.42	4.32	4.22	1.21	
Ala-34	8.26	4.12	1.36		

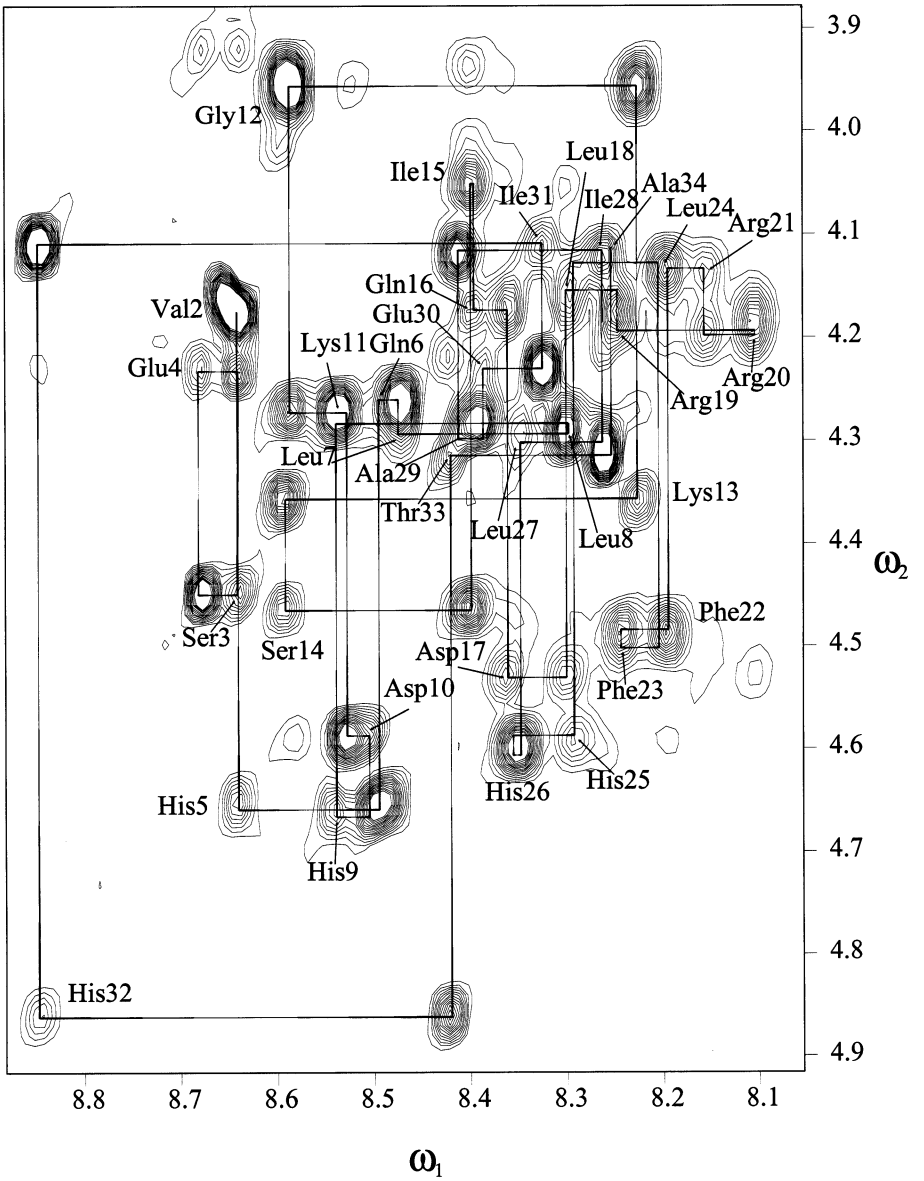

Fig. 2. Fingerprint region of the NOESY spectrum of hPTHrP(1–34), 1.5 mM, at 277 K in 50 mM potassium phosphate, pH 5.1, 250 mM sodium chloride.

the Cα proton chemical shifts [40,41]. CαH resonances shifted to high field relative to the corresponding random coil values [42] indicate local α-helical structure, whereas downfield shifted resonances are typical for local β-sheet conformation. Elements of regular secondary structure are assumed to be present if a deviation from the random coil value of more than 0.1 ppm is observed. To get a more reliable picture it is suggested that only resonances with the same sense chemical shift deviation for a stretch of more than three sequential residues are taken into account [41].

Simulated annealing (SA) and distance geometry calculations were carried out with the XPLOR 3.840 program package [43]. Intensities of NOESY cross-peaks were estimated from the number of contour lines in the spectrum and divided into three distance interval classes: strong, 0.18–0.27 nm; medium, 0.18–0.40 nm; weak, 0.18–0.55 nm. The pseudoatom approach [44–46] was used for distances involving unresolved resonances from methyl or methylene groups, that is, 0.05 nm was added to the upper distance limit. Frequency degenerated NOESY cross-peaks were incorporated into the structure calculations as 'ambiguous' [43]. Proton-proton distances in the calculated structures were determined using the program 'BackCalc_db 2.0' (Software Symbiose, Bayreuth, Germany) and compared with the combinations of distances possible for each frequency degenerated NOESY cross-peak. The distance information was used in further structure calculations only if one of the possible distance combinations was fulfilled in more than 50% of the calculated structures. This procedure was repeated several times.

Elements of regular secondary structure were deduced by chemical shift analysis (cf. above) and the inspection of the NOE pattern. In addition, the structures were checked for the existence of secondary structural elements by MOLMOL 2.5.1 [47].

3. Results

The evaluation of the far-UV CD spectrum of the peptide (Fig. 1) by standard methods [48,49] yielded an average helix content of hPTHrP(1–34) of 27–37%.

Sequence-specific assignments of the spin systems identified in the COSY and TOCSY spectra could be performed with standard techniques [38] as the spectra were well resolved (Fig. 2, Table 1). Chemical shift data of the CαH resonances were analyzed according to the chemical shift index strategy

Table 2
NOE restraints, energy contributions and deviations from standard geometry

No. of NOEs	
Total	761
$\|i-j\|=0$	424
$\|i-j\|=1$	185
$\|i-j\|=2, 3, 4, 5$	149
$\|i-j\|>5$	3
Rmsd from ideality after SA refinement with X-PLOR	
NOEs (nm)	0.00516
Angles (deg)	0.66706
Bonds (nm)	0.00062
Impr (deg)	0.62933
Average energies (kJ/mol) after SA refinement with X-PLOR	
$E_{overall}$	981.57
E_{bonds}	93.71
E_{angles}	296.38
E_{NOE}	424.59
Rmsd among backbone structures (nm) after SA refinement with X-PLOR	
Ser-14–Leu-27	0.043
Gln-16–His-26	0.022
Whole protein	0.401

NOE violations: three of the 30 lowest energy structures show a single NOE violation.

[41,42], yielding an estimate of elements of regular secondary structure (Fig. 3A). An α-helical stretch from Ile-15 to Leu-27 is clearly suggested, and a short helix around Glu-6 may also be deduced (Fig. 3A). This preliminary estimate was confirmed and refined by analysis of NOESY cross-peak patterns (Fig. 3B). According to this data, hPTHrP(1–34) forms two helical stretches, a short helix near the NH_2-terminus, His-5–Asp-10, and a longer COOH-terminal helix, Ser-14–Ala-29. 424 intraresidual and 337 interresidual NOE connectivities (Table 2) were used in an SA protocol with subsequent refinement to generate 100 structures from an elongated starting conformation. The 30 structures with lowest total energies were superimposed, and the local root mean square deviation of the backbone atoms was calculated with a seven residue window. The two regions representing helices as judged from the chemical shift and NOE data, His-5–Asp-10 and Ser-14–Ala-29, were well defined (Fig. 3C) and connected by a flexible linker. The short NH_2-terminal helix, His-5–Leu-8, is present in 60% of the structures, and COOH-terminal α-helix is present in all calculated structures. The core sequence of this helix is formed by residues Gln-16–His-26, but the helix extends from Ser-14 to Leu-27 in some structures (Fig. 4). NOESY cross-peaks could be observed between the terminal methyl groups of Ile-15 and the guanidine group of Arg-21. These, however, proved to be insufficient to define even a locally stable tertiary fold.

4. Discussion

The hPTHrP(1–34) structure determined here and the structure of hPTH(1–37) as well as hPTH(1–34) in TFE-free solution ([32], Marx et al., unpublished) are highly similar, ex-

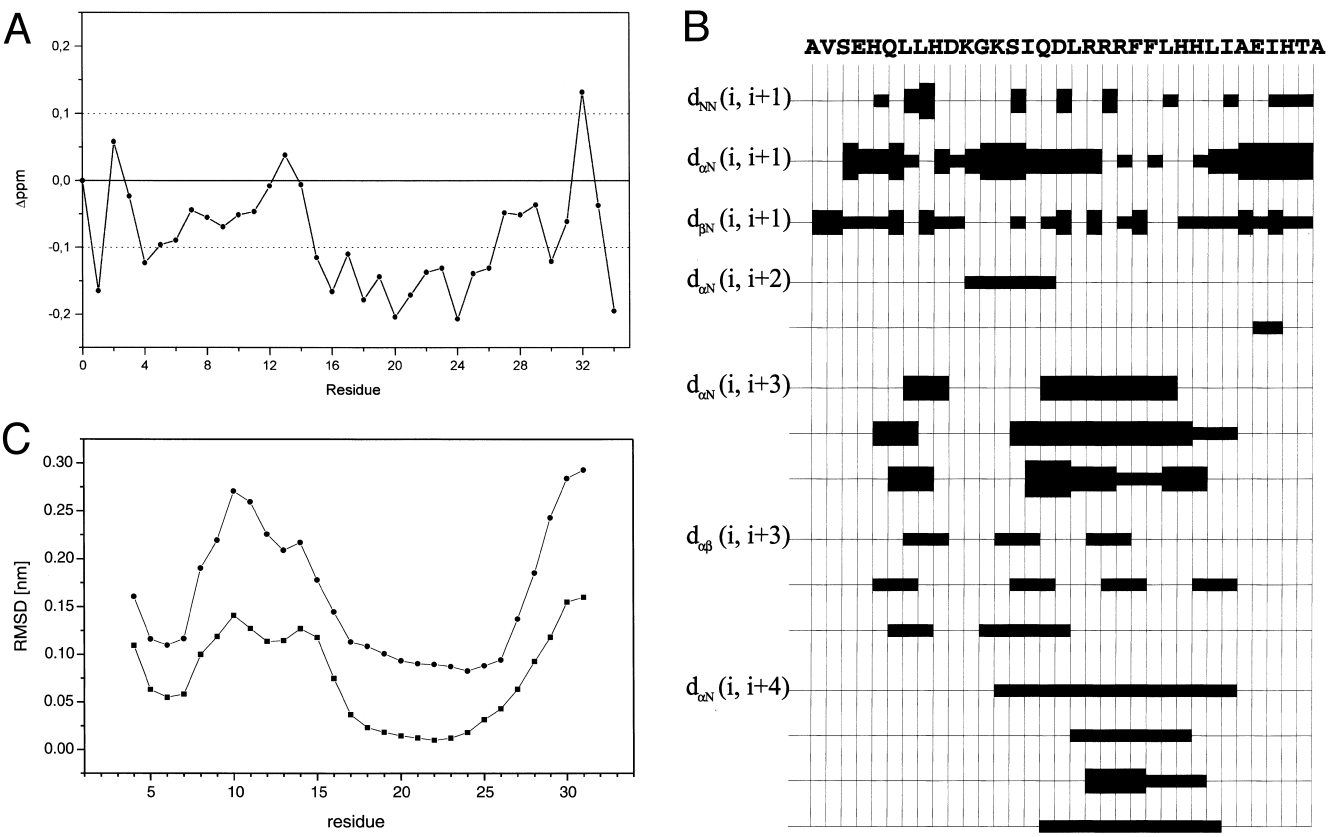

Fig. 3. A: Chemical shift plot of δ(CαH, peptide)/δ(CαH, random coil) according to Wishart et al. [41,42]. B: Pattern of sequential and medium range NOESY connectivities versus peptide sequence. The height of the bars corresponds to the relative intensity of the NOESY cross-peaks. C: Local RMSD values calculated with a seven amino acid window. The plot was calculated on the basis of the 30 final structures of PTHrP(1–34). Upper trace: all heavy atoms. Lower trace: backbone atoms.

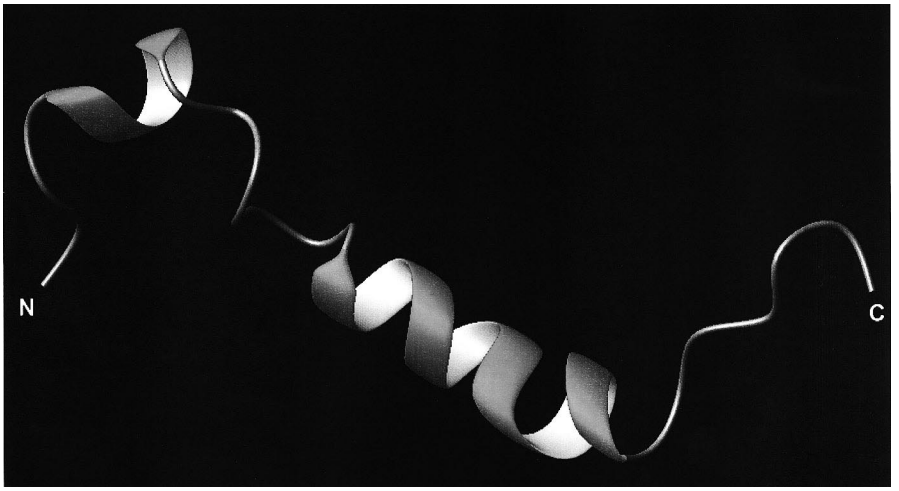

Fig. 4. MOLMOL 2.5.1 [47] cartoon of the lowest internal energy structure. The amino- and carboxy-termini are indicated.

cept for the well defined turn, His-14–Ser-17, that in combination with a flexible link connects the two α-helices in hPTH. This is compatible with the observation that the two peptides are able to bind to the same receptor with comparable affinities [50]. As hPTH and hPTHrP show only 15.8% homology in the stretch Leu-15–Phe-34 that is thought to be responsible for receptor binding, this might indicate structure-based peptide-receptor recognition. No indication for interactions between the two helices of hPTHrP(1–34), His-5–His-9 and Ser-14–Leu-27 according to the MD calculations, was observed. In contrast, a hydrophobic core and a flexible hinge are present between these helices in (Ala-26)hPTHrP(1–34) in the presence of TFE [27]. Hydrophobic interactions should be more favorable under the present, TFE-free solution conditions than in the presence of TFE [32,51–53], but the salt bridge between Arg-20 and Glu-30 [27] may be stabilized in TFE-containing buffer as a consequence of the reduced dielectric constant of the solution. This salt bridge, not observable under the current near physiological conditions, results in spatial proximity of the residues suggested to form the hydrophobic core.

In contrast to hPTH NH_2-terminal fragments, hPTHrP(1–34) does not show tertiary interactions between residues Ile-15 and Phe-23. Whereas the COOH-terminal helix in the hPTH fragments starts at position 17, this helix starts at position 14/15 in the hPTHrP fragment. Thus, in hPTHrP, Ile-15 is involved in a stable regular secondary structure that does not allow interaction with Phe-23. The COOH-terminal helix in hPTHrP(1–34) starts close to Ser-14, which may well serve as a helix cap. The analogous residue in hPTH is Ser-17, rendering a shift of the helix start of three residues from the NH_2-terminus.

The absence of any indication for interactions between the two helices does not rule out the possibility that such an interaction is induced or stabilized upon binding to the receptor, as proposed recently [25]. Indeed, the structure presented here would enable the two helices to interact upon receptor binding as they are only connected via the flexible loop around Gly-12. PTHrP(14–34) is still able to bind to the PTH/PTHrP receptor, albeit weakly [54], whereas peptide fragments containing only the NH_2-terminal amino acids do not show any biological activity [55]. The COOH-terminal helix is essential for receptor binding, while the signal transduction cascade is triggered by the NH_2-terminal part [56]. It may thus be speculated that initial receptor binding of the COOH-terminal helix facilitates receptor interaction with the NH_2-terminal helix. Alternatively, interaction between the two helices of the peptide may be necessary for activity and may be induced upon receptor binding [25].

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