Role of Amino Acid Side Chains in Region 17–31 of Parathyroid Hormone (PTH) in Binding to the PTH Receptor*

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The principal receptor-binding domain (Ser17–Val31) of parathyroid hormone (PTH) is predicted to form an amphiphilic α-helix and to interact primarily with the N-terminal extracellular domain (N domain) of the PTH receptor (PTHR). We explored these hypotheses by introducing a variety of substitutions in region 17–31 of PTH-(1–31) and assessing, via competition assays, their effects on binding to the wild-type PTHR and to PTHR-delNt, which lacks most of the N domain. Substitutions at Arg20 reduced affinity for the intact PTHR by 200-fold or more, and altered affinity for PTHR-delNt by 4-fold or less. Similar effects were observed for Glu substitutions at Trp23, Leu24, and Leu28, which together form the hydrophobic face of the predicted amphiphilic α-helix. Glu substitutions at Arg22, Lys26, and Lys27 (which forms the hydrophilic face of the helix) caused 4–10-fold reductions in affinity for both receptors. Thus, the side chains of Arg22, together with those composing the hydrophobic face of the ligand’s putative amphiphilic α-helix, contribute strongly to PTHR-binding affinity by interacting specifically with the N domain of the receptor. The side chains projecting from the opposite helical face contribute weakly to binding affinity by different mechanisms, possibly involving interactions with the extracellular loop/transmembrane domain region of the receptor. The data help define the roles that side chains in the binding domain of PTH play in the PTH-PTHR interaction process and provide new clues for understanding the overall topology of the bimolecular complex.

Parathyroid hormone (PTH)2 plays a key role in calcium and phosphate homeostasis and has potent effects on the bone-remodeling process. PTH interacts with a Class 2 G protein–coupled receptor that is prominently expressed in bone osteoblasts and in cells located in the proximal and distal portions of the renal convoluted tubules. The PTH receptor (PTHR) is also expressed in the primordia of developing long bones, heart, mammary glands, and other tissues, where it mediates the morphogenetic actions of PTH-related protein (PTHRP) (1).

For both PTH and PTHR, the bioactive portions of the molecule reside within the first 34 amino acids of the processed polypeptides. Within region 1–34, the principal determinants of receptor-binding affinity and receptor-signaling activity map to the C- and N-terminal domains, respectively (2, 3). Solution-phase NMR studies of PTH-(1–34)-based ligands typically show a well formed α-helix in the region of the C-terminal binding domain (4, 5). This C-terminal α-helix, extending approximately from Ser17 to Val31 (5), exhibits strong amphiphilic character (6–8). PTH analog substitution studies have shown that the side chains of Trp23, Leu24, and Leu28, which form the hydrophobic face of this α-helix, are particularly important for efficient interaction with the receptor (9–11).

The mechanism by which PTH interacts with its receptor has been investigated via the approaches of ligand analog design, receptor mutagenesis, and photochemical cross-linking (reviewed in Ref. 12). The view that has emerged from these studies is that the overall mechanism consists of two principal and, to some extent, autonomous components: 1) an interaction between the C-terminal helical domain of the ligand and the N-terminal extracellular domain (N domain; spanning Tyr23 to approximately Ile198) of the mature receptor and 2) an interaction between the N-terminal portion of the ligand and the juxtamembrane domain (J domain) of the receptor containing the extracellular loops and seven transmembrane helices. The N domain component of the interaction is thought to provide the major portion of binding energy and stability to the complex, and the J domain component is thought to mediate the conformational changes involved in receptor activation (13). It now seems likely that most, if not all, of the 15 or so other Class 2 G protein–coupled receptors utilize a similar two-site binding mechanism for interacting with their cognate peptide ligands (14–16).

Consistent with such a two-site binding mechanism for PTH and the PTHR, we have shown that N-terminal PTH peptide fragments, such as PTH-(1–14), bind only extremely weakly to the receptor, but can nevertheless induce at least measurable increases in cAMP levels in PTHR-expressing cells (17). The potency of such N-terminal PTH fragments can be significantly enhanced by introducing substitutions that improve the affinity...
of the ligand for the PTHR J domain (18–20). Thus, the analog [Alb1,3,Gln10,Har11,Trp14]PTH-(1–14)-NH₂ is equipotent to PTH-(1–34) in stimulating cAMP as well as inositol phosphate (IP) production in PTHR-expressing cells. Moreover, such optimized N-terminal PTH analogs retain full potency in cells expressing PTHR-delNt, a PTHR construct that lacks most (Ala24–Arg181) of the N domain, whereas unmodified PTH-(1–34) exhibits at least 100-fold reductions in potency and affinity for PTHR-delNt compared with its actions on the intact PTHR (18–20).

The large reduction in affinity/potency that unmodified PTH-(1–34) exhibits for PTHR-delNt can be attributed, according to the two-site interaction model, to the loss of the binding interactions that normally occur between the C-terminal helical domain of the ligand and the N domain of the receptor. The cross-linking approach has indeed established spatial proximities between PTH residues 23, 27, and 28, when substituted with para-benzoyl-l-phenylalanine (Bpa), and the N domain of the receptor (21, 22). On the other hand, [Lys27(Bp)2]PTH-(1–34)-NH₂, which contains the photoreactive Bp moiety attached to the Lys27 side chain amino groups, was shown to cross-link to the first extracellular loop of the PTHR (23). This observation raised the possibility that the C-terminal binding domain of PTH can functionally interact with the receptor J domain. Consistent with this possibility, we recently showed that methylation of several backbone nitrogen atoms in region 17–31 of PTH-(1–31) impairs, albeit modestly, the capacity of the ligand to stimulate cAMP formation in cells expressing PTHR-delNt (8). Taken together, these observations point to the uncertainty that exists in our understanding of the specific mechanisms by which the C-terminal domain of PTH contributes to the PTHR-binding process.

This study was undertaken to examine further the mode of action used by the C-terminal binding domain of PTH. We sought to address the roles that the side chains in this domain play in the PTHR-binding process, the general functional importance of amphiphilicity, and the potential for binding interactions with the receptor J domain. Our strategy was to introduce a variety of conservative and non-conservative substitutions in region 17–31 of PTH-(1–31)-NH₂ and to assess their effects on binding to the intact PTHR and to PTHR-delNt. The overall results indicate a dominant role for specific interactions between side chains on the hydrophobic face of the C-terminal helix and the receptor N-terminal domain. They also provide evidence for weak interactions between side chains projecting from the hydrophilic face of the helix and the PTHR J domain. The data also shed new light on the overall topology of the bimolecular complex, as they support folding of the bound ligand and proximity of the binding sites in the N and J domains of the receptor.

**MATERIALS AND METHODS**

**Peptide Synthesis**—Peptides were based on the human PTH-(1–31)-NH₂ sequence (SVSEIQLMHNGLKHLNSMERVEW-LRKKLQDV-NH₂). The alanine substitutions were incorporated into this otherwise unmodified PTH-(1–31)-NH₂ scaffold. Subsequently, cyclohexylalanine (Cha) and Glu substitutions were incorporated into the [Ala₁,Arg₁⁰]PTH-(1–31)-NH₂ scaffold, which, because of the Ser₁ → Ala and Glu₁⁰ → Arg substitutions, exhibits improved affinity for PTHR-delNt (19, 24). These Ala-, Cha-, and Glu-substituted PTH-(1–31)-NH₂ and [Ala₁,Arg₁⁰]PTH-(1–31)-NH₂ peptides and their corresponding parental controls were synthesized by the Massachusetts General Hospital Biopolymer Core facility using conventional methodologies as we described previously (20). Additional analogs of PTH-(1–31)-NH₂ with substitutions at position 20 were prepared as part of a previous study (25). All peptides were verified by analytical HPLC, matrix-assisted laser desorption ionization mass spectrometry, and amino acid analysis, and peptide concentrations of stock solutions were established by amino acid analysis. The radioligands ¹²⁵I-[Nle₈,2¹,Trp₃⁴]rPTH-(1–34)-NH₂ and ¹²⁵I-[Alb₁,³,Gln₁₀,Har₁¹,Ala₁²,Trp₁⁴, Tyr₁⁵]-rPTH-(1–15)-NH₂ (henceforth referred to as ¹²⁵I-[Alb₁,³,M]-rPTH-(1–15)-NH₂) were prepared by the oxidative chloramine-T procedure using Na¹²⁵I (specific activity of 2200 Ci/mmol; PerkinElmer Life Sciences) and purified by reversed-phase HPLC.

**Circular Dichroism**—CD spectra were obtained on a Jasco J-600 spectropolarimeter at 20 °C. Four spectra were averaged, and the data were smoothed by the Jasco software. The instrument was calibrated with ammonium (+)-10-camphorsulfonate. Data are expressed as the number of helical residues/peptide chain as calculated from −θ ±222 X 30/28,000, where θ±222 is the mean residue ellipticity at 222 nm, as described previously (8).

**Cell Culture**—Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone, Logan UT), 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate (Invitrogen). For binding and cAMP experiments performed with the intact PTHR, the HKRK-B7 and ROS 17/2.8 cell lines were used. HKRK-B7 cells are derived from the porcine kidney cell line LLC-PK₁ and express, via stable DNA transfection, the wild-type human PTHR at an approximate surface density of 950,000 PTHR-binding sites/cell (26). ROS 17/2.8 cells are rat osteosarcoma cells and express the endogenous PTHR at an approximate surface density of 70,000 PTHR-binding sites/cell (27). The cells were plated and assayed in 24-well plates.

PTH-delNt was derived from the human PTHR by site-directed mutagenesis and lacks most (Ala24–Arg181) of the N domain (28). PTHR-delNt was expressed in COS-7 cells via transient DNA transfection. For binding assays, cells membranes were prepared from the transfected COS-7 cells. To increase the maximum binding of ¹²⁵I-[Alb₁,³,M]-rPTH-(1–15)-NH₂ to PTHR-delNt in these membranes, the cells were cotransfected with a negative-dominate mutant Ga₃ protein (Ga₃(αβγ/3,βGly₂²⁸ → Ala/Ala²⁶⁶ → Ser); hereafter referred to as Ga₃(ND)). This mutant Ga₃ subunit is thought to couple to coganate receptors, and thus stabilize high affinity receptor conformations more efficiently than does wild-type Ga₃ without increasing basal cAMP levels (29). We recently used a precursor of this Ga₃ mutant, Ga₃(αβγ/3,γ), to increase the binding of ¹²⁵I-[Alb₁,³,M]-rPTH-(1–15)-NH₂ to PTHR-delNt in COS-7 cell membranes (18). We subsequently found that Ga₃(ND), which contains the same five-amino acid replacement of the corre-
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—The capacities of the ligands to stimulate formation of cAMP were assessed in COS-7 cells transfected with the intact human PTHR as we described (18). In brief, cell membranes (20 μg/well) were incubated in binding buffer containing radioligand (~30,000 cpm/well) with or without unlabeled peptide ligand (3 × 10^{-5} to 1 × 10^{-6} M) for 90 min at 21 °C (reaction volume of 200 μl). The plates were then subjected to rapid vacuum filtration, and the filters were washed once with buffer, air-dried, detached from the plate, and counted for radioligand.

Data and Statistical Calculations—Binding and cAMP data were processed for curve fitting and derivation of IC_{50} and EC_{50} values using least-squares nonlinear regression analysis and the following equation: y = y_{\text{min}} + (y_{\text{max}} - y_{\text{min}})/[1 + (IC_{50}/x)^{n}].

RESULTS

Alanine Scan of Region 17–31 of PTH—We first individually replaced each residue in region 17–31 of PTH-(1–31)-NH_{2} with alanine and assessed the effects of the substitutions on binding to the intact human PTHR stably expressed in HKRK-B7 cells. Binding was assessed by competition methods using 125I-[Nle^8,21,Tyr^34]rPTH-(1–34)-NH_{2} as a tracer radioligand. The parental PTH-(1–31)-NH_{2} peptide fully inhibited the binding of this tracer with an IC_{50} of 68 ± 10 nM (Fig. 1A and Table 1). The various alanine substitutions had a range of effects on this binding. Most dramatic was that of the Arg substitution, which abolished detectable binding (Fig. 1A).

The alanine substitutions at Trp^23 and Leu^24 reduced the apparent binding affinity by 2-fold or less (Fig. 1, A and B; and Table 1). The alanine substitutions at Glu^19, Glu^22, and Gln^29 each produced a small increase in binding affinity (2-fold) enhancement of the apparent binding affinity, as did the Ala^366, Gly^226, and Ala^366 substitutions at Val^21, Arg^25, Lys^27, Leu^28, and Val^31 reduced affinity by ~3-fold, and the remaining alanine substitutions altered affinity by 2-fold or less (Fig. 1, A and B; and Table 1). The alanine substitutions at Glu^19, Glu^22, and Gln^29 each produced a small (≤2-fold) enhancement of the apparent binding affinity, as did the Ala^366 substitution, which abolished detectable binding (Fig. 1A). We then assessed the effects of the alanine substitutions on binding to PTHR-delNt. For these experiments, we used membranes prepared from COS-7 cells transfected with PTHR-delNt as a tracer radioligand, which we have shown binds exclusively to the PTHR J domain (30). To increase the total specific binding of 125I-[Aib^1,3,M]rPTH-(1–15)-NH_{2} to these membranes, the cells were cotransfected with a negative-domain mutant Go_{i} subunit (Go_{i}^{ND}), which promotes ligand binding presumably by stabilizing high affinity receptor conformations (29).

Unlabeled [Aib^1,3,M]rPTH-(1–15)-NH_{2} (used as a control peptide) fully inhibited the binding of 125I-[Aib^1,3,M]rPTH-(1–15)-NH_{2} to these membranes with high apparent affinity...
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![Graphs and diagrams](Image)

FIGURE 1. Alanine scan of region 17–31 of PTH-(1–31)-NH₂. PTH-(1–31)-NH₂ and analogs thereof with individual alanine substitutions in region 17–31 were assessed by competition methods for binding to the intact PTHR (A and B) and to PTHR-delNt (C and D). Intact HKRK-B7 cells stably transfected with the PTHR and the 125I-[Nle8,21,Tyr34]rPTH-(1–34)-NH₂ tracer radioligand were used in the assays in A and B; membranes prepared from COS-7 cells transiently transfected with PTHR-delNt and the 125I-[Aib1,3,M]rPTH-(1–15)-NH₂ tracer radioligand were used in C and D. To increase the total binding of 125I-[Aib1,3,M]rPTH-(1–15)-NH₂, the COS-7 cells were cotransfected with a negative-dominant Gα mutant. For the experiments with PTHR-delNt, unlabeled [Aib1,3,M]rPTH-(1–15)-NH₂ (dotted line) was used as a control and to determine nonspecific binding, to which the curves for the PTH-(1–31) analogs were extrapolated. Data for the parental PTH-(1–31) peptide (●) and dotted line) are shown in each panel. Data are the means ± S.E. of three or more experiments, each performed in duplicate. SB, specific binding.

(IC₅₀ = 2.2 ± 0.5 nM) (Fig. 1, C and D; and Table 1). As expected from the absence of ligand interactions with the PTHR N domain, unmodified PTH-(1–31)-NH₂ bound to PTHR-delNt with relatively low affinity (IC₅₀ = 3700 ± 400 nM) (Fig. 1, C and D; and Table 1). This binding was nevertheless sufficient to assess the effects of the alanine substitutions on the capacity of the ligand to interact with PTHR-delNt. None of the alanine substitutions altered binding to PTHR-delNt by >5-fold, including the Arg²⁰ → Ala substitution, which had strongly diminished binding to the intact PTHR (Fig. 1, A versus C).

Similarly, the Ala substitutions at Trp²³ and Leu²⁴, which reduced affinity for the PTHR by 19- and 12-fold, respectively, reduced affinity for PTHR-delNt by only ~2-fold. These findings indicate that the mechanisms by which the Ala substitutions at Arg²⁰, Trp²³, and Leu²⁴ impair binding to the intact PTHR are largely independent of interactions with the PTHR J domain.

None of the alanine substitutions had a major impact on the secondary structure of the peptide as revealed by CD spectroscopy analysis. Thus, the CD spectrum of each analog exhibited clear negative deflections in the regions at 209 and 222 nm, which are indicative of α-helical structure (Fig. 2). The number of helical residues/peptide chain (calculated from the CD signal at 222 nm) was between 7 and 10 for each peptide (Table 1). These findings are consistent with a preservation of α-helical structure in region 17–31 of the ligand (6, 8) as well as with the known helix-forming propensity of alanine (31).

Substitutions with Cha—The strong effects that the alanine substitutions at Trp²³, Leu²⁴, and Leu²⁸ had on binding to the intact PTHR (Fig. 1, A and B) suggested that hydrophobicity per se in region 17–31 of PTH might play a key role in determining the affinity of ligand for the receptor. To evaluate this further, we substituted each residue in region 17–31 of PTH with Cha, an amino acid analog that would preserve bulk hydrophobicity, yet alter the specific chemistry and topology of the side chain of the substituted site. We used [Ala³,Arg¹⁹]PTH-(1–31)-NH₂ as a scaffold peptide in these experiments, as we wanted to augment our capacity to assess binding to PTHR-delNt, and the Glu¹⁹ → Arg and Ser¹ → Ala substitutions were known to improve interaction with this truncated receptor (19, 24).

The effects of the Cha substitutions on the intact PTHR in HKRK-B7 cells generally paralleled those of the corresponding alanine substitutions. Thus, relative to the parental [Ala¹,Arg¹⁹]PTH-(1–31)-NH₂ peptide, the Cha substitution at Arg²⁰ had the strongest effect on binding and reduced affinity by 120-fold (p = 0.005). Substitutions at Trp²³ and Leu²⁴ reduced affinity by 14- and 11-fold, respectively (p < 0.002), and those at the remaining positions altered PTHR-binding affinity by 6-fold or less (Fig. 3, A and B; and Table 2). The deleterious effects that the Cha substitutions at Trp²³ and Leu²⁴ had on PTHR-binding affinity indicate that hydrophobicity per se is not the main physicochemical property of these two side chains that underlies their contributions to PTHR-binding affinity.

CD analyses again indicated that none of the Cha substitutions disrupted the α-helical content of the peptide (Table 2 and supplemental Fig. 1B). The Cha²³ and Cha²⁷-substituted analogs exhibited enhanced negative deflections at 209 and 222 nm, which resulted in calculated helical content values that were ~2-fold higher than that of the parental peptide. The basis for these enhanced CD signals (which were not accompanied by parallel changes in our receptor assays) is not clear at present. The Cha substitutions at Arg²³ and Lys²⁰ reduced affinity for PTHR-delNt by ~5–7-fold, and the remaining Cha substitu-
Substitutions were introduced into PTH-(1–31)-NH₂. The helical residue values were calculated from the mean residue ellipticity ([θ]_222) observed in the CD spectra at 222 nm. Competition binding studies with the wild-type PTHR (PTHR-WT) were performed in intact HKRK-B7 cells using [125I]-[Nle⁸,2¹, Tyr³⁴]rPTH(1–34)-NH₂ as a tracer radioligand. Those with PTHR-delNt were performed in membranes prepared from COS-7 cells transiently transfected with PTHR-delNT and a negative-dominant Gαmutant. [125I]-[Alb¹⁷, M]rPTH(1–15)-NH₂ was used as a tracer radioligand, and unlabeled [Alb¹⁷, M]rPTH(1–15)-NH₂ was used as a control. Binding IC₅₀ values are the means ± S.E. of data from the number of experiments indicated in parentheses. Cit, citralline, Orn, ornithine; ND, not determined.

The data so far suggested that certain residues in the C-terminal domain of PTH-(1–31), especially Trp²³, Leu²⁴, and Leu²⁶, contribute to PTHR-binding affinity via mechanisms that are not dependent on interactions with the receptor N domain. To test this hypothesis further, we sought to

| CD helical residues | PTHR-WT (HKRK-B7 cells) | PTHR-delNt (COS-7 cells) |
|--------------------|--------------------------|--------------------------|
| PTH-(1–31)-NH₂     | 8                        | 3666 ± 431 (13)          |
| Ser¹⁷ → Ala        | 10                       | 4678 ± 386 (4)           |
| Met¹⁸ → Ala        | 10                       | 8799 ± 2334 (4)          |
| Glu¹⁹ → Ala        | 9                        | 2940 ± 833 (4)           |
| Arg²⁰ → Ala        | 10                       | 19,343 ± 8110 (4)        |
| Val²¹ → Ala        | 10                       | 5686 ± 486 (4)           |
| Glu²² → Ala        | 9                        | 5857 ± 2237 (4)          |
| Trp²³ → Ala        | 7                        | 7357 ± 1832 (4)          |
| Leu²⁴ → Ala        | 7                        | 8764 ± 1167 (4)          |
| Arg²⁵ → Ala        | 7                        | 7177 ± 1083 (4)          |
| Lys²⁶ → Ala        | 7                        | 6210 ± 948 (4)           |
| Leu²⁷ → Ala        | 9                        | 7288 ± 615 (4)           |
| Gln²⁸ → Ala        | 9                        | 3459 ± 482 (4)           |
| Arg²⁹ → Ala        | 10                       | 1931 ± 303 (4)           |
| Val³¹ → Ala        | 9                        | 1545 ± 215 (4)           |
| Glu¹⁹ → Ala/Glu²² → Ala | 9 | 49 ± 9 (4) | 619 ± 75 (4) |
| Glu¹⁹ → Arg/Glu²² → Arg | 8 | 54 ± 13 (4) | 360 ± 19 (4) |
| Glu¹⁹ → Arg        | 8                        | 1226 ± 332 (4)           |
| Arg²⁰ → Gln        | 7                        | 10,284 ± 121 (3)         |
| Arg²⁰ → Glu        | 7                        | 14,747 ± 324 (3)         |
| Arg²⁰ → Lys        | 8                        | 7105 ± 1275 (3)          |
| Arg²⁰ → Nle        | 12                       | 10,688 ± 1033 (3)        |
| Arg²⁰ → Cit        | 9                        | 10,624 ± 1612 (3)        |
| Arg²⁰ → Orn        | 6                        | 5530 ± 279 (3)           |
| Arg²⁰ → Apa        | ND                       | 5740 ± 775 (3)           |
| Arg²⁰ → Gph        | ND                       | 2047 ± 442 (3)           |
| Arg²⁰ → PipGly      | 8                        | 6605 ± 2407 (5)          |
| [Alb¹⁷, M]rPTH-(1–15)-NH₂ | ND | 2.2 ± 0.5 (8) |

FIGURE 2. CD spectroscopy of alanine-substituted PTH-(1–31)-NH₂ analogs. The parental PTH-(1–31)-NH₂ peptide and derivatives thereof altered by a single alanine substitution in region 17–31 were analyzed by CD spectroscopy. The negative deflections in the mean residue ellipticity ([θ]) in the regions at 209 and 222 nm of the spectra are indicative of α-helical structure. The CD spectra obtained for other peptides used in this study are shown in supplemental Fig. 1. For each peptide, the number of helical residues/peptide chain was calculated from [θ]_222 and the resulting values are reported in Tables 1 and 2.
introduce substitutions that would more strongly disrupt binding to the intact PTHR than did the alanine or Cha substitutions. If the hypothesis were correct, then such substitutions would have little or no effect on binding to PTHR-delNt. We therefore introduced, as a non-conservative substitution, Glu at each position in the C-terminal segment of [Ala1,Arg19]PTH-(1–31)-NH2 that was otherwise occupied by a hydrophobic or positively charged amino acid. The analogs were again assessed for binding to the intact PTHR and to PTHR-delNt. As predicted, several of the Glu substitutions caused severe reductions in binding affinity for the intact PTHR. Thus, the Glu substitutions at Arg20 and Leu24 abolished detectable binding to the intact PTHR, and those at Leu28 and Trp23 reduced apparent affinity by 270- and 160-fold, respectively (Fig. 4, A and B; and Table 2). Modest 2–12-fold reductions in PTHR-binding affinity occurred with the Glu substitutions at Arg19, Val21, Arg25, Lys26, and Lys27. None of the Glu substitutions substantially altered the CD profile of the peptide (Table 2 and supplemental Fig. 1C).

Each of the Glu substitutions had only a mild impact on binding to PTHR-delNt. Thus, Glu substitutions at Arg20, Trp23, Leu24, and Leu28, which severely reduced affinity for the PTHR, reduced affinity for the truncated PTHR by 3-fold (Fig. 4, C and D; and Table 2). The Glu substitutions at Val21 and Arg25

![Table 2](image)

**Table 2**

Helical contents and PTHR-binding properties of [Ala1, Arg19]-PTH-(1–31)-NH2 analogs

| CD helical residues | PTHR-WT (HKRK-B7 cells) | PTHR-delNt (COS-7 cells) |
|---------------------|-------------------------|--------------------------|
| Ser17 → Cha         | 38 ± 8 (7)              | 397 ± 40 (4)             |
| Met18 → Cha         | 112 ± 13 (3)            | 179 ± 41 (4)             |
| Arg19 → Cha         | 233 ± 57 (3)            | 780 ± 83 (4)             |
| Arg20 → Cha         | 4738 ± 333 (3)          | 632 ± 79 (4)             |
| Val21 → Cha         | 74 ± 15 (5)             | 564 ± 172 (4)            |
| Glu22 → Cha         | 75 ± 9 (3)              | 126 ± 45 (4)             |
| Trp23 → Cha         | 546 ± 70 (3)            | 867 ± 402 (4)            |
| Leu24 → Cha         | 436 ± 10 (3)            | 283 ± 68 (4)             |
| Arg25 → Cha         | 103 ± 27 (3)            | 271 ± 1306 (4)           |
| Lys26 → Cha         | 172 ± 27 (3)            | 1872 ± 281 (4)           |
| Lys27 → Cha         | 91 ± 17 (3)             | 629 ± 72 (4)             |
| Leu28 → Cha         | 65 ± 10 (3)             | 226 ± 39 (4)             |
| Gln29 → Cha         | 61 ± 14 (3)             | 319 ± 57 (4)             |
| Asp30 → Cha         | 54 ± 7 (3)              | 305 ± 126 (4)            |
| Val31 → Cha         | 66 ± 20 (3)             | 466 ± 298 (4)            |
| Arg32 → Glu         | 125 ± 29 (4)            | 18,856 ± 9649 (5)        |
| Arg33 → Glu         | >10,000 (3)             | 776 ± 380 (5)            |
| Val34 → Glu         | 447 ± 172 (4)           | 1615 ± 516 (5)           |
| Trp35 → Glu         | 6044 ± 2636 (3)         | 434 ± 84 (5)             |
| Leu36 → Glu         | >10,000 (3)             | 1941 ± 197 (5)           |
| Arg37 → Glu         | 189 ± 53 (4)            | 1629 ± 274 (5)           |
| Lys38 → Glu         | 60 ± 19 (3)             | 832 ± 215 (5)            |
| Lys39 → Glu         | 156 ± 50 (3)            | 1266 ± 293 (5)           |
| Leu40 → Glu         | 10,360 ± 3983 (3)       | 974 ± 252 (5)            |

**FIGURE 3.** Cha scan of region 17–31 of [Ala1,Arg19]PTH-(1–31)-NH2. Residues in region 17–31 of [Ala1,Arg19]PTH-(1–31)-NH2 (A, R) were individually replaced with Cha, and the effects on binding to the intact PTHR (A and B) and to PTHR-delNt (C and D) were assessed as described in the legend to Fig. 1. Data are the means ± S.E. of three or more experiments, each performed in duplicate. 125I-PTH(1–34) SB. 125I-[Nle8,21,Tyr34]rPTH-(1–34)-NH2-specific binding.
The replacement of Arg20 in PTH-(1–31)-NH₂ with Gln, Glu, Lys, (S)-2-amino-4-((2-amino)pyridinyl)butanoic acid (Apa), or 1-(4-guanidinophenyl)alanine (Gph) resulted in a complete loss of binding to the intact PTHR, and the substitutions with PipGly, Nle, and citrulline reduced affinity by ~200-fold relative to the parental peptide (Fig. 5, A and B; and Table 1). None of the position 20 substitutions altered binding to PTHR-delNt by >5-fold (Fig. 5, C and D; and Table 1). These results thus indicate that the effects of the substitutions at position 20 on binding to the PTHR are largely independent of interactions with the PTHR J domain.

Effects on cAMP and IP Signaling—Selected analogs of [Ala³, Arg¹⁹]PTH-(1–31)-NH₂ with Cha or Glu substitutions that markedly impaired binding to the intact PTHR were assessed for their capacity to stimulate cAMP production in ROS 17/2.8 cells. These cells endogenously express the rat PTHR at a moderate level (~70,000 PTHR/cell) and were thus considered more useful for correlating effects on binding affinity and cAMP-signaling potency compared with HKRK-B7 cells, which express ~13-fold higher levels of receptor. Competition binding assays confirmed that the selected substitutions strongly impaired binding to ROS 17/2.8 cells and revealed effects on affinity that paralleled those seen in HKRK-B7 cells (Fig. 6A and Table 3). These effects on affinity in ROS 17/2.8 cell were accompanied by parallel reductions in cAMP-stimulating potency (Fig. 6B and Table 3). Although potency was reduced, each substituted analog (at the highest concentration) produced approximately the same maximum cAMP response as did the parental peptide.

We also assessed the alanine-substituted PTH-(1–31)-NH₂ analogs (at a single concentration of 1 x 10⁻⁶ M) for the capacity to stimulate IP production in COS-7 cells transfected with the PTHR. (ROS 17/2.8 and HKRK-B7 cells produce only barely detectable IP responses to PTH analogs.) In these assays, each analog produced the same ~4-fold increase in IP levels that was observed for the parental peptide (supplemental Fig. 2). Thus, the PTH-(1–31) analogs with C-terminal substitutions that strongly diminished PTHR-binding affinity could nevertheless mediate, at sufficiently high concentrations, robust cAMP- and IP-signaling responses. These findings are consistent with the notion that the principal ligand determinants of receptor activation, at least in terms of the Go₆₃-mediated cAMP- and Go₈₂-mediated IP₃-signaling responses, reside in the N-terminal portion of the ligand (19, 26) and are not directly perturbed by the tested substitutions in region 17–31.

Reduced affinity for PTHR-delNt by ~4-fold (p < 0.01), similar to their effects on binding to the intact PTHR. The Arg¹⁹ → Glu substitution reduced affinity for PTHR-delNt by ~50-fold; this effect is consistent with the potency-enhancing effect seen for the reciprocal Glu¹⁹ → Arg substitution in PTH-(1–20) and PTH-(1–34) analogs in COS-7 cells expressing PTHR-delNt (24). The overall data obtained for these Glu substitutions support the view that the C-terminal domain of PTH-(1–31) interacts predominantly with the PTHR N domain, but can also contribute modestly to binding affinity via mechanisms that are independent of the N domain.

Analysis of Arg²⁰—The arginine at position 20 is one of the most conserved residues in PTH and PTHrP ligands and has been shown to play a key role in the receptor interaction process (10, 11, 25). Little has been revealed, however, about the mechanistic basis for this role. Barbier et al. (25) showed that none of 11 different amino acid analog substitutions at this position in PTH-(1–31)-NH₂ fully preserve cAMP-stimulating potency in ROS 17/2.8 cells. Thus, even the close arginine homologs of citrulline, (S)-4-piperidyl-(N-amidino)glycine (PipGly), and 4-piperidyl-(N-amidino)alanine reduce potency by 8-, 5-, and >21-fold, respectively, and lysine abolishes activity (25). To further dissect the functional role of Arg²⁰, we examined the same position 20-substituted PTH-(1–31)-NH₂ analogs from the study of Barbier et al. for the capacity to bind to the intact PTHR and to PTHR-delNt.

**FIGURE 4. Effects of Glu substitutions in region 19–28 of [Ala³, Arg¹⁹]PTH-(1–31)-NH₂.** Residues in region 19–28 of [Ala³, Arg¹⁹]PTH-(1–31)-NH₂ (A⁹,R¹⁹)PTH(1–31)] were replaced with Glu, and the effects on binding to the intact PTHR (A and B) and to PTHR-delNt (C and D) were assessed as described in the legend to Fig. 1. Data are the means ± S.E. of three or more experiments, each performed in duplicate. ²¹²⁵I-PTH(1–34) SB. ²¹²⁵I-[Nle₂¹,Tyr²⁶]PTH-(1–34)-NH₂-specific binding.

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This study was designed to gain further information on the functional roles that the amino acid side chains in the principal receptor-binding domain of PTH play in the receptor interaction process. The experiments were based on the use of PTH-(1–31)-NH₂ or [Ala₁,Arg₁₉]PTH-(1–31)-NH₂ analogs containing a variety of substitutions in region 17–31 and the assessment of the effects of the substitutions on binding to the PTHR as well as to PTHR-delNt. Analysis of the binding of the analogs to PTHR-delNt expressed in COS-7 cell membranes was a key and novel aspect of our study, as it enabled us, for the first time, to assess the extent to which a targeted amino acid in the C-terminal domain of a relatively unmodified PTH ligand interacts with the receptor N domain (absent in PTHR-delNt) versus the receptor J domain containing the extracellular loops and transmembrane helices. Overall, our findings are largely consistent with the two-site model of the PTH-PTHR interaction mechanism outlined in the Introduction in that they suggest that the C-terminal domain of PTH-(1–31) interacts predominantly with the N domain of the receptor to contribute a large proportion of the overall binding energy to the complex. The data also suggest, however, that the C-terminal domain of the ligand can interact with the receptor J domain to gain a modest increase in the overall stability/affinity of the complex.

The greatest impact on the binding of our PTH-(1–31) peptides to the intact PTHR occurred with the non-conservative Glu substitutions at Arg₂₀, Trp₂₃, Leu₂₄, and Leu₂₈, as each reduced apparent affinity by 150-fold or more. Such effects are consistent with previous PTH substitution studies showing important roles for these four residues in binding to the intact PTHR (6, 9–11). The same Glu substitutions had little or no effect on binding to PTHR-delNt.

Our binding experiments performed with PTHR-delNt utilized membranes prepared from COS-7 cells that were cotransfected with PTHR-delNt and G₅/H₉₂₅₁. (The negative-dominant G₅/H₉₂₅₁ mutant was utilized to improve the total binding of the ¹²⁵I-[Aib₁,₃,M]rPTH-(1–15)-NH₂ radioligand to the membrane preparations.) To assess the possibility that G₅/H₉₂₅₁ per se could account for the lack of effect that the substitutions had on binding to PTHR-delNt, we performed control experiments in COS-7 cell membranes to evaluate the effects of several key substitutions (Cha₂₀, Glu₂₀, Glu₂₃, and Glu₂₄) on binding to the intact wild-type PTHR in the presence of G₅/H₉₂₅₁. The experiments clearly showed that the substitutions again caused large decreases in binding affinity for the intact PTHR, even in the presence of G₅/H₉₂₅₁ (supplemental Fig. 3).
The lack of an effect of the substitutions on binding to PTHR-delNt must therefore be due to the absence of the receptor N-domain. More specifically, both the C-terminal (region 20–31) and N-terminal (region 1–19) domains of the ligand interact with the PTHR J-domain, and the N-domain of the receptor: regions 20–31 and 1–19 of PTHR, respectively.

We observed subtle but consistent effects of substitutions at positions 21 and 25–27 in our PTH-(1–31) peptides on interaction with PTHR-delNt. These effects were accompanied by approximately proportional effects on interaction with the intact PTHR. Such findings suggest that the side chains of these residues, although not making major contributions to overall binding energy, can promote binding via mechanisms that involve interactions with the PTHR J-domain.

The cationic side chains of Arg, Lys, and Lys form the hydrophilic face of the amphiphilic α-helix predicted to reside within region 17–31 of PTH (6, 7). Our data predict that this hydrophilic helical face contributes to the PTHR-binding process by interacting with the N-terminal domain of the receptor. Our findings do not support a mechanism by which these hydrophilic segments interact predominantly with the N-domain of the receptor (24). That residue 19 of the bound ligand is in spatial proximity to the PTHR J-domain is further suggested by the cross-linking of Bpa-containing PTH analogs to the extracellular end of transmembrane helix 2 of the PTHR (34). When considered together, the data for residues 19 and 20 suggest that the 19/20-position in the ligand marks a point of divergence for the ligand segments that interact predominantly with the N and J domains of the receptor: regions 20–31 and 1–19 of PTHR, respectively.

Unlike Arg, residue 19 of PTH appears to interact predominately with the PTHR J-domain (24). This can be seen in our present data by the 90-fold reduction in binding affinity for PTHR-delNt caused by the Arg substitution (25). Our data now imply that these functional groups of the Arg side chain fit within a highly specific binding pocket within the N-domain of the receptor.

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If both the C-terminal (region 20–31) and N-terminal (region 1–19) domains of the ligand interact with the PTHR J-domain, then a bend would most likely be required between the two domains of the receptor-bound ligand. The tertiary structure of receptor-bound PTH has been a matter of some debate, with linear and folded structures supported (4, 35). A folded helix-turn-helix structure for PTH-(1–34) was indeed pre-

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### Table 3

Activities in ROS 17/2.8 cells

| [Ala{sup 1},Arg{sup 3,19}]PTH-(1–31)-NH₂ and analogs thereof containing the indicated substitutions were assessed in intact ROS 17/2.8 cells. Competition binding was assessed in intact cells using [sup 35]I-[Nle{sup 8,21},Tyr{sup 34}]-rPTH-(1–34)-NH₂ as a tracer radioligand. Values are the means ± S.E. of data from three experiments, each performed in duplicate. |
|-----------------------------|
| **Binding IC₅₀** |
| **cAMP EC₅₀** |
| [Ala{sup 1},Arg{sup 3,19}]PTH-(1–31)-NH₂ | 1.6 ± 0.2 | 0.78 ± 0.24 |
| Arg{sup 20} → Cha | 290 ± 130 | 7.5 ± 3.0 |
| Trp{sup 23} → Cha | 19 ± 7 | 0.82 ± 0.11 |
| Leu{sup 24} → Cha | 7.3 ± 0.8 | 0.83 ± 0.17 |
| Arg{sup 20} → Glu | 5800 ± 1200 | 59 ± 31 |
| Trp{sup 23} → Glu | 220 ± 60 | 4.0 ± 2.0 |
| Leu{sup 24} → Glu | 29,000 ± 5000 | 250 ± 70 |
| Leu{sup 28} → Glu | 3100 ± 2100 | 11 ± 2 |

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**Receptor-binding Domain of PTH**

The lack of an effect of the substitutions on binding to PTHR-delNt must therefore be due to the absence of the receptor N-domain and not to the presence of the Go{sub N} mutant.

Our CD analyses indicated that the substitutions did not cause major perturbations in the helical structure of the peptide. It thus seems clear from the data that the side chains of Arg, Trp, Leu{sup 24}, and Leu{sup 28} contribute to the PTHR-binding process by mechanisms that are largely, if not completely, dependent on interactions with the receptor N-terminal domain.

The side chains of Trp, Leu{sup 24}, and Leu{sup 28} form the hydrophobic face of the amphiphilic α-helix predicted to reside within region 17–31 of PTH (6, 7). Our data predict that this hydrophilic helical face contributes to the PTHR-binding process by interacting with the N-terminal domain of the receptor. Our findings do not support a mechanism by which this hydrophilic face contributes to the PTHR-binding process by interacting nonspecifically with the lipid component of the cell membrane, as has been discussed for PTH (32) and for amphiphilic peptide ligands in general (33). If this were the case, then the substitutions would have impacted equally the binding of the ligand to the intact PTHR and to PTHR-delNt, which did not occur. Moreover, the hydrophobic Cha substitutions at Trp and Leu{sup 24} reduced binding to the intact PTHR by ~12-fold and again had little or no effect on binding to PTHR-delNt (Table 2). Thus, the mechanisms by which the side chains of Trp and Leu contribute to the PTHR-binding process are not likely to be based simply on nonspecific hydrophobic interactions with the lipid bilayer, but instead involve additional spatial and chemical features of the side chains and their specific interactions with cognate functional groups in the receptor.

It is clear from our data and that presented elsewhere (10, 11, 25) that Arg{sup 20} of PTH plays a key role in the PTHR-binding process. Each of 10 substitutions tested at this position in PTH-(1–31)-NH₂ reduced affinity for the intact PTHR by at least ~200-fold, and most (seven) abolished detectable binding. Each of these substitutions had only a minor effect on binding to PTHR-delNt. The side chain of Arg, like those of Trp, Leu{sup 24}, and Leu{sup 28}, must therefore contribute to the PTHR-binding process via a mechanism that primarily involves interactions with the PTHR N-domain. The molecular nature of these interactions is not clear at present, but likely involves multiple components of the arginine side chain, including the cationic and H-bonding nitrogen atoms of the guanidino group and the three methylenes of the linker (25). Our data now imply that these functional groups of the Arg side chain fit within a highly specific binding pocket within the N-domain of the receptor.
Receptor-binding Domain of PTH

dicted early on based on modeling and structure-activity data (36), and solution-phase NMR studies of PTH and PTHrP ligands generally reveal mid-region flexibility or a hinge between the N- and C-terminal domains (4, 5, 37, 38). A bend in receptor-bound PTH-(1–34) has more recently been suggested by the cross-linking of both [Bpa11]PTH-(1–34) and [Bpa21]PTH-(1–34) to the same segment (Ala165–Asn176) of the PTHR N domain (39).

In addition to the tertiary structure of the bound ligand, our data have implications for the topology of the occupied receptor and the spatial relationship of the N and J domains. If the C-terminal α-helical domain (region 20–31) of PTH interacts with both the N and J domains of the receptor via its hydrophobic and hydrophilic faces, respectively, as supported by this study, then the N and J domain-binding sites in the receptor must be near each other. This possibility is again supported by cross-linking data: specifically, the cross-linking of one PTH-(1–34) analog with a photolabile Bp moiety incorporated at position 27 as a Bpa substitution with the receptor N domain (22) and that of another PTH-(1–34) analog with the Bp moiety attached to the distal amino groups of the Lys27 side chain with the first extracellular loop of the receptor (23). Because the spatial positioning of the Bp moieties in these two ligands is likely to be similar, a proximity of the two cross-linked sites in the receptor is also likely.

The available data derived from the functional and cross-linking studies combined thus suggest some intriguing hypotheses regarding the topology and domain architecture of the PTH-PTHrP complex. However, the lack of direct structural information on receptor-bound PTH or on the intact PTHR itself hampers our capacity to assimilate such data into a detailed three-dimensional model of the PTH-PTHrP complex. One important goal that may be facilitated by our work is to identify sites in the receptor that are used by key ligand residues such as Arg20, Trp23, Leu24, and Leu28. Our results predict that such interaction sites will be located in the receptor N-terminal domain. For Trp23, the extreme N-terminal segment (Thr33–Leu40) of the N domain needs to be considered, as it contains the cross-linking site for [Bpa21]PTHrP-(1–36) (34). For the other ligand residues, few clues are available: cross-linking has not been achieved for positions 20 and 24, and [Bpa28]PTHrP-(1–36) cross-links to a nonessential segment (Ser61–Gly105) of the N-terminal domain (34).

The recently reported NMR-derived structure of the N-terminal domain of the related corticotropin-releasing factor receptor (15) may open paths for structure-based analyses of the ligand interaction sites in the N domains of the Class 2 G protein-coupled receptors (14). Even with such an approach, additional functional studies will be needed. The new PTH analogs presented here should be of value in this regard, as they can be used in conjunction with PTHR mutants altered at candidate sites in the N-terminal domain to probe for allele-specific rescue effects. This is a direction that we hope to pursue in future studies.

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