Backbone-methylated Analogues of the Principle Receptor Binding Region of Human Parathyroid Hormone

EVIDENCE FOR BINDING TO BOTH THE N-TERMINAL EXTRACELLULAR DOMAIN AND EXTRACELLULAR LOOP REGION* [8]

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We have used backbone N-methylations of parathyroid hormone (PTH) to study the role of these NH groups in the C-terminal amphiphilic α-helix of PTH (1–31) in binding to and activating the PTH receptor (P1R). The circular dichroism (CD) spectra indicated the structure of the C-terminal α-helix was locally disrupted around the methylation site. The CD spectra differences were explained by assuming a helix disruption for four residues on each side of the site of methylation and taking into account the known dependence of CD on the length of an α-helix. Binding and adenyl cyclase-stimulating data showed that outside of the α-helix, methylation of residues Asp20 and Val31 had little effect on binding or structure. Within the α-helix, disruption of the structure was associated with increased loss of activity, but for specific residues Val21, Leu24, Arg28, and Leu38 there was a dramatic loss of activities, thus suggesting a more direct role of these NH groups in correct P1R binding and activation. Activity analyses with P1R-delNT, a mutant with its long N-terminal region deleted, gave a different pattern of effects and implicated Ser17, Trp23, and Ser26 as important for its PTH activation. These two groups of residues are located on opposite sides of the helix. These results are compatible with the C-terminal helix binding to both the N-terminal segment and also to the looped-out extracellular region. These data thus provide direct evidence for important roles of the C-terminal domain of PTH in determining high affinity binding and activation of the P1R receptor.

Parathyroid hormone (PTH)† is a major regulator of extracellular calcium, functioning mainly through kidney, bone, and intestinal receptors (1, 2). The hormone and certain analogues strongly stimulate bone growth in both animals (3) and humans (4, 5). PTH and its paracrine equivalent, parathyroid hormone-related peptide (PTHrP), bind to a G-protein-linked seven-transmembrane receptor (P1R) (6) and activate both adenylyl cyclase (AC)/cAMP-dependent protein kinase and phospholipase-C (PLC)-dependent and -independent protein kinase C pathways (7, 8). Deletion of even two N-terminal residues leads to a total loss of AC and PLC signaling, as well as the anabolic activities (9). In contrast, the PLC-independent protein kinase C activity requires the C-terminal region of hPTH (1–34) (10, 11).

The principal receptor-binding domain of PTH-(1–34) (12) and PTH-(1–31)NH2 is a C terminus α-helix bounded by residues 17–29 (13); this domain (14) interacts with the long extracellular N terminus sequence of P1R (8, 15). A deletion analogue of the P1R (P1R-delNT) that lacks the N-terminal domain (Ref. 16 and citations, therein) can activate the AC and PLC signaling pathways on stimulation with PTH-(1–34) (17), although with much reduced potency, whereas N-terminal PTH analogues as short as PTH-(1–11) exhibit equivalent potencies on P1R-delNT and the intact P1R (16, 17). The presence of PLC-independent protein kinase C activity determinants in the C-terminal region of PTH-(1–34) implies that this region also binds to the extracellular loop/transmembrane domain region of the receptor, but only one report directly supports such an interaction (18).

The osteogenic activity of a C terminus-truncated PTH analogue, hPTH-(1–31)NH2 (19), is similar to that of the well studied PTH-(1–34) (20). This analogue has a helix-bend-helix structure in aqueous media at near-physiological pH and ionic strength (14), similar to that found in previous studies of PTH-(1–34) (13). A C-terminal amphiphilic α-helix, extending from Ser17 to Glu29, is the major structure within this receptor-binding region, and this structure is thought to bind via its hydrophobic face to the receptor (21–23). Thus, binding and AC-stimulating activities are very sensitive to replacement of residues on the nonpolar face of this helix, including Leu24, Leu28, and to a lesser extent Val31, whereas residues on the polar face are less sensitive to substitution (22, 24).

In general, the specificity of hormone binding to and activation of the receptor is likely determined not only by side-chain interactions between the two molecules but also by specific H-bonding of backbone CO and NH groups (25, 26). Thus a complex pattern of H-bonding involving side-chain and backbone groups is expected to be important for the strength and specificity of the overall interaction (27). Backbone methylation has been used to probe the role of backbone amide nitrogens in...
Backbone-methylated PTH Analogues

Peptide Synthesis—All methylated analogues were based on the sequence of hPTH-(1–31)NH2 (SVSEIQLMHNGLHLSMVRVELRKKQLQD-VH-NH2). Peptides were synthesized using a Fmoc protocol as described previously (32). Fmoc-α-methyl-Leu and Fmoc-α-methyl-Asp were purchased from Chem-Impex and Fmoc-α-methyl-Val from NovaBiochem (La Jolla, CA). The remaining chemicals were purchased from Aldrich. The remaining methylations were done during synthesis by slight modification of the method of Miller and Scmidt (33). Briefly, after addition of the amino acid to be methylated and removal of the Fmoc, synthesis was stopped and the α-amino group was then protected with o-nitrobenzenesulfonylmide by treatment with the corresponding nitrobenzenesulfonate for 3 h. The o-nitrobenzenesulfonyl protection was selectively removed by treatment with β-mercaptopetanol and 1,8-diaza-bicyclo-(5.4.0)undec-7-ene in N,N-dimethylformamide for 3 h. Completion of methylation was monitored by the Kaiser test for free amine. If methylation was incomplete, as indicated by a positive Kaiser test, the o-nitrobenzenesulfonyl was added again and methylation repeated. For coupling of the next amino acid, HATU was used in place of 2-[1H-benzotriazol-1-yl]-1,1,3,3-tetramethyluronium tetrafluoroborate. The main problems observed occurred at this step and were especially serious for the coupling of Fmoc-Glut(Bu) after methylation of the Arg20 residue.

The synthesis of [N-α-Me-Leu24]Gln29-hPTH-(1–31)NH2 was performed as described earlier for [Leu24]Glu22-Lys26-hPTH-(1–31)NH2 (21), instead using N-α-Me-Leu at residue 24. Coupling of the next amino acid, Fmoc-Trp(Boc), was done using HATU at 48 °C until a negative Kaiser test was obtained. Removal of allyl- and acetyl-protecting groups and cyclization were performed after the addition of the final Boc-Sert(Bu).

Peptides were purified by elution from a semipreparative C18 silica column (Vydac, 10 µ, 1 × 25 cm), using gradients of 0.1% trifluoroacetic acid/acetone in 0.1% trifluoroacetic acid/water. Peptide purities were verified by analytical high pressure liquid chromatography (Vydac C18, 10 µ, 4 × 238 mm) using two different trifluoroacetic acid/water/acetoniitrile elution systems (for details, see supplemental data). Masses were confirmed by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (Voyager-DE STR, Perseptive/Applied Biosciences, CA). Purities were 94–99% for all peptides except the methylated Ser17 (78%) and Val31 (75%).

Circular Dichroism—Spectra were obtained on a JASCO J-600 spectropolarimeter at 20 °C. Four spectra were averaged and the data smoothed by the JASCO software. The instrument was calibrated with ammonium (½)-10-carboxylsulfonate. Peptide concentrations were calculated from the absorbance at 280 nm, using an extinction coefficient of 5700 M⁻¹ for the single tryptophan.

RESULTS

Circular Dichroism—To obtain the spectrum of the Ser17, Gln29 helix within hPTH-(1–31)NH2 and other analogues described here, we subtracted the spectrum for hPTH-(1–17)NH2. The only structure, based on NMR data, within residues 1–17 of hPTH-(1–31)NH2 is a short α-helix between residues 3–9 (14), and this short helix contributes little to the α-helix of hPTH-(1–31)NH2. Previous CD and NMR data has also shown no or weak contributions from the tertiary structure of PTH; thus the N- and C-terminal portions of the molecule behave independently (13, 14, 39). The resulting spectrum for hPTH-(17–31)NH2 has the expected form of an α-helix, with a [θ]222 of −31,400 degrees cm²dmol⁻¹ (Fig. 1). This compares to a value of −28,000 degrees cm²dmol⁻¹ for a fully populated α-helix of 12 residues (35).

The CD spectra suggested major losses of α-helix in some methylated analogues, particularly for those with a methylation near the center of the Ser17-Gln29 α-helix. Fig. 2 shows three of the spectra obtained after subtraction of that of hPTH-(1–17)NH2, and these clearly show that the apparent α-helix, when methylation is near the center of the helix at Leu24, is much less than for Ser17 and Gln29 at the N- and C termini of the helix. Fig. 3 shows the helix parameter [θ]222 for hPTH-(1–31)NH2 for each methylated residue position, after subtraction of the spectrum for hPTH-(1–17)NH2, expressed as the ratio to the [θ]222 for the unmethylated form, −11,880 degrees cm²dmol⁻¹. Minimum values are seen near the center of the helix, with up to 80% loss at Trp23, and progressively smaller effects when the methylation is at either end of the helix. When the methylation is external to the helix, at residues Gln29 and Val31, no loss of helix was observed, but ~34% of the helix was lost when methylation was at Aasp30. Similarly, when the methylation was at the N terminus of the helix, the apparent loss of helix was ~15%, based on the [θ]222 values.

Backbone methylation leads to disruption of the helix around the immediate point of methylation, although the exact extent

Data Are Expressed per Bond—The ellipticities at 222 nm for o-helices of different lengths were extrapolated from the data of Chin et al. (34) for short fixed Ala o-helices, using a quadratic equation to fit their data as follows: [θ]222 = 6448–5844N₁ – 252N₁², where N₁ = number of helical peptide bonds. This is compared with evaluation of the mean residue ellipticity of a helix of N₁ peptide units from the empirical formulation of Yang et al. (35) as follows: [θ]222 = [θ]222 – xN₁, where x is a wavelength-dependent constant that has the value 4 at 222 nm and [θ]222 = −43,000 degrees cm²dmol⁻¹ (36). In the Chin et al. (34) formulation, the x is effective dependent on N₁ and results in ellipticities for helical regions with as little as two residues.

Cell Cultures—HKK-B7 cell lines were derived from the porcine kidney cell line LLC-PK1 and were stably transfected to express full-length human P1R to ~950,000 receptors/cell (37). P1R-Def, a human P1R construct with most (residues 24–181) of the N-terminal extracellular domain deleted (38) was used to transiently transfected COS-7 cells and was used to assay interactions that occur specifically to the extracellular loop/transmembrane domain region of the receptor. Adenylyl Cyclase Activity—AC activities were measured using a direct enzyme-linked immunosorbent assay for cAMP (Amersham Biosciences) as described elsewhere (37). The CAMP experiments comparing the effect of methylation at Leu24 in the linear and lactamized (cyclic) PTH-(1–31) scaffold were performed using radioimmunoassay to quantify CAMP, as described earlier (38). Activi­ties reported were each the average of three separate experiments.

Receptor Binding—Radioiodinated binding assays were performed as previously described (38). In brief, a 125I-labeled radioligand (~100,000 cycles/well of a 24-well plate of 125I-[Nle8,21,Tyr34]PTH-(1–34)NH2) was incubated with whole cells expressing the wild-type P1R in the presence of varying concentrations of unlabeled peptide. After incubation for 4 h at 15 °C, the binding mixture was removed by aspiration, the cells were rinsed three times with binding buffer, lysed in NaOH, and the entire lysate was counted for γ-irradiation.
residue α-helix (Fig. 3). This model resulted in predicted ellipticities of zero for Val21 to Lys26, contrary to the observed presence of the helix after these residues were N-methylated. The more recent model of Chin et al. (34), using a dependence of \( \alpha \) on helix length (34), resulted in a profile much more similar to the experimentally observed one. This suggests that, even in the presence of backbone N-methylation, short helical regions are present in the 12-residue-long helix in hPTH-(1–31)NH2.

Binding of N-Methylated Analogues to HKRRK-B (P1R)—The methylations fell into three broad categories with respect to their effect on analogue binding to P1R (Table I). The first category included four methylations that had little effect on the binding, those on Arg20, Glu22, Asp30, and Val31. The second category included methylations that resulted in analogues with moderate to weak binding behavior, those on Ser17, Met18, Trp23, Glu22, Lys26, and Lys27. The last category, and perhaps the most interesting, included those that resulted in peptides that had lost almost all capacity to bind the receptor with binding constants >100,000 nM. These included methylations at Val21, Leu24, Arg25, and Leu28. The severe reductions in apparent binding affinity seen with these methylations suggest that the NH groups of these amino acids may be involved in critical H-bonds with the receptor.

Adenylyl Cyclase Activities—The adenylyl cyclase activities of the analogues (Table I), in general, paralleled their binding activities. The exceptions to this included those with methylations at Glu19, Glu22, and Lys26, which had substantial activities despite rather weak binding affinities. The reasons for these discrepancies are not clear but may be related to the fact that the binding assays utilized a heterologous radioligand ([125I]-[Nle8,21,Tyr34]PTH-(1–34)NH2) (43–45).

The high signaling activity of the N-methylated Arg20 analogue is particularly noteworthy, because the side chain of this arginine is critical for PTH bioactivity, tolerating very little modification (46). The essential loss of activities of analogues with methylations of Val21, Leu24, Arg25, Lys27, and Leu28 suggests that the α-amino groups of these residues are involved in critical contact with the receptor. The lack of effect of methylation of Val21, Asp30, and Glu22 is not surprising given that there is little difference between the AC-stimulating activities of hPTH-(1–28)NH2 and hPTH-(1–31)NH2 (47).

Adenylyl Cyclase Activities with P1R-delNT—This truncated receptor construct (17) lacks nearly all of the long, N-terminal sequence of the P1R that has been associated with binding to the C-terminal region of PTH (1–34) (48). The assumption that the C-terminal helix of hPTH-(1–31) binds only to the extracellular N-terminal domain of P1R implies that the effect of individual N-methylation of the residues in this region would have no effect on interaction with delNT-P1R. However, the results of Fig. 4 clearly show that some of the methylations diminished cAMP signaling responses induced by the analogues, tested at a single high concentration (1 μM) on this truncated receptor. Furthermore, although the absolute AC responses measured on this receptor cannot be directly compared with those determined on the intact receptor (ED50 values were not determined for the truncated receptor, and different cAMP assay formats were used in the two experiments, radioimmunoassay versus enzyme-linked immunosorbent assay), clear differences in the patterns of effects that the methylations had on activity at the two receptors could nevertheless be discerned. Thus, three methylated analogues, Ser17, Trp23, and Lys26, show particularly low activity with P1R-delNT but have moderate to high activities with the intact P1R. This may indicate that the NH groups of these residues form H-bonds with the extracellular loops or transmembrane domains of the receptor (P1R and P1R-delNT). None of these residues is asso-

![FIG. 1. Circular dichroism spectra of hPTH-(1–31)NH2 (solid line), hPTH-(1–17)NH2 (dashed line), and the calculated spectra of hPTH-(1–31)NH2 (dotted line). The spectrum of hPTH-(17–31)NH2 was obtained by subtracting the spectrum of hPTH0-(1–17)NH2 from that of hPTH-(1–31)NH2 using the formula \( \theta [\text{deg}] = 2.1/[\text{deg}] - 1.1/10^{-17} \).](image1)

![FIG. 2. Circular dichroism spectra of backbone N-methyl analogues of hPTH-(1–31)NH2. Shown are the calculated spectra for the Ser17-Val21 portion of [Nle8,21,Tyr34]PTC-(1–34)NH2 (solid line), [Nle8,21,Tyr34]PTC-(1–31)NH2 (long-dashed line), and [Nle8,21,Tyr34]PTC-(1–17)NH2 (short-dashed line). Calculated spectra are as described in the legend to Fig. 1. deg, degrees.](image2)
FIG. 3. Observed and predicted ellipticities at 222 nm for backbone methylated analogues. All are expressed as the ratio of the methylated to the unmethylated hPTH-(1–31)NH₂. Shown are the observed CD ratios (gray bars), ratios predicted from length dependence of Yang et al. (35) (black bars), and ratios predicted from length dependence of Chin et al. (34) (white bars). See “Materials and Methods” for details.

TABLE I

| Methylated residue¹ | Binding (IC₅₀)b | AC ED₅₀c |
|---------------------|----------------|----------|
| Wild-type (hPTH-(1–31)NH₂) | 78 (8) | 14.5 (0.9) |
| Ser27 | 1400 (200) | 61.0 (7.0) |
| Met18 | 7800 (800) | 57.9 (8.3) |
| Glu19 | 18,000 (1000) | 80.5 (6.5) |
| Arg20 | 280 (90) | 24.8 (0.2) |
| Val21 | 105,000 (39,000) | >500 |
| Glu22 | 14,000 (4,000) | 150.3 (10.1) |
| Trp23 | 12,000 (500) | 124.0 (2.0) |
| Leu24 | >100,000 | >500 |
| Arg25 | >100,000 | >500 |
| Lys26 | 56,000 (5000) | 49.9 (5.9) |
| Lys27 | 52,000 (2000) | >500 |
| Leu28 | >100,000 | >500 |
| Gln29 | 620 (36) | 14.5 (2.2) |
| Asp30 | 56 (5) | 18.4 (0.3) |
| Val31 | 100 (6) | 20.0 (2.3) |

¹ Binding assays were performed with ¹²⁵I-[Nle⁸,2¹,Tyr³⁴]PTH-(1–31)NH₂ as tracer ligand.
² IC₅₀ values were calculated by non-linear regression analysis.
³ Values are means (±S.E.) of data compiled from three separate experiments, each performed in duplicate.
⁴ AC activities were obtained by direct enzyme-linked immunosorbent assay of cAMP.
⁵ ED₅₀ values were calculated by fit of data with sigmoidal function.

The backbone methylations described here led to two main conclusions. First, the CD data indicated a general loss of structure that could be explained in large measure by a simple model assuming localized disruption near the N-methylated residue. Second, although there was a general loss of activity throughout the region of the helix, there were specific methylations that resulted in a dramatic loss of binding and AC activity, and the patterns of losses differed depending on whether the intact receptor or delNT-P1R was used for the measurements.

We know that there is disruption of helical structure at the site of a specific backbone methylation and that this disruption affects neighboring peptide bonds (40, 49). These effects were incorporated into our analysis of the CD data. There is a paucity of data concerning the extent of helix disruption caused by a backbone NH methylation, but the effects on the hormone-receptor complex caused by some of our methylations appeared to be more severe than what would result from just the loss of one H-bond, and numerous other stabilizing interactions were expected to be lost. This is important, because the loss of only one H-bond would contribute, from the known free energy

helicity and to increase binding affinity and potency by severalfold (21, 32). The CD spectrum of the analogue, compared with that of N-Me-Leu²⁴-PTH-(1–31), showed that, although there was little increase of [θ]²₂₂, the spectrum clearly had a more helical structure but not with the same intense helical minima of 209 and 222 nm and the classical helical shape seen with the unmethylated analogue cGlu²²-Lys²⁶)₅₇-hPTH-(1–31)NH₂ (Fig. 5). It suggested a more classical helix but with a disruption at the point of methylation.

Comparison of the activities of the analogues in HKRK-B7 cells indicated that the lactam greatly increased the AC-stimulating activity of the N-Me-Leu²⁴ analogue. This increase in signaling potency was accompanied by at least some increase in binding affinity, in that inhibition of radioligand binding could be detected (Table I). The data on these analogues thus suggest that a large portion of the loss of binding and AC-stimulating activity caused by the Leu²⁴ methylation is directly related to the loss of α-helical structure.

DISCUSSION

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change, an approximate 10-fold loss of $K_a$ (50), far less than what we observed for several of the methylations. Our CD analysis was also limited to the effects of chain length on the CD signal and could not take into account the known effect of distortion of the $\alpha$-helix from the classical $\varphi, \psi$ angles of $-60^\circ, -40^\circ$ (42), and the relaxation of the trans conformation of the peptide bond at the point of methylation to a cis-trans equilibrium (28). We also cannot be certain that PTH binds to the receptor in a conformation very similar to that which occurs in solution, in particular for the C-terminal domain studied here. However, a transfer nuclear Overhauser effect study of pituitary adenyl cyclase activating polypeptide (PACAP) provides evidence that this hormone binds to the receptor with a conformation very much like the average solution one in its C-terminal domain (51). The receptors for PACAP and PTH are related, and the hormones share similar structural features, thus suggesting that PTH would likely behave similarly.

The model in Fig. 6 shows the location of effects of methylation on binding and activity for P1R and delNT-P1R. Previous data implicates the C-terminal amphiphilic $\alpha$-helical domain of PTH-(1–31) in binding to the long, extracellular N terminus of P1R. Deletion of the P1R extracellular N-terminal sequence drastically limits PTH binding. Substitution of a photoactivatable group for Trp23 identifies a close proximity to the N-terminal P1R segment, residues 23–40 (52). The dramatic (≈150-fold) effects on P1R-binding affinity seen with NH methylations near Trp23 at Leu24 and Arg25 provide evidence to support an important role for this interaction in the overall stability of the complex.

Relatively little evidence has implicated the same region of PTH in binding to the extracellular looped-out region of the receptor. Other photochemical cross-linking data implicate Lys27 of the hydrophobic face being proximal to Leu261 of EC-1 (18) and Glu19 as proximal to the extracellular end of transmembrane-2 (53). The data presented here with P1R-delNT, together with these chemical cross-linking studies (18, 52), strongly suggest that the C terminus of PTH-(1–34) does indeed interact with the extracellular looped-out region of the receptor. It is striking that the NH substitutions that most strongly affect interaction with P1R-delNT occur on the opposite face of the helix from those that most strongly affect interaction with the intact P1R (Fig. 6). This suggests that the polar face of the helix binds to the extracellular loops, whereas the
nonpolar face binds to the N-terminal domain. It is also noteworthy that, of the 13 possible backbone H-bond donors in the 17–29 helical segment, seven are implicated in binding to some part of the receptor. A recent Ala scan study has identified Arg\(^{20}\), Val\(^{21}\), Trp\(^{23}\), Leu\(^{24}\), and Arg\(^{25}\) as important residues in PTH-P1R recognition.\(^{2}\) Collectively, these results and those of other previous reports (22, 24, 46) identify a hot spot from the C-terminal region of the receptor.

FIG. 6. **Model views of hPTH-(1–31)NH\(_2\) Ser\(^{17}\)-Gln\(^{29}\) helix with critical residues.** The helix is viewed along the long axis from the N terminus (A) and from the side (B). Backbone amide hydrogens that, when methylated, result in greatly diminished activities with P1R (those of Val\(^{21}\), Leu\(^{24}\), Arg\(^{25}\), and Leu\(^{28}\)) are shown in green, and the corresponding ones that diminish activity with P1R-delNT (those of Ser\(^{17}\), Trp\(^{23}\), and Lys\(^{28}\)) are highlighted in orange.

**Table II**

| Methylated analogue\(^a\) | Binding (IC\(_{50}\))\(^b\) | AC ED\(_{50}\)\(^c\) |
|--------------------------|--------------------------|--------------------------|
| hPTH-(1–31)NH\(_2\) | 80 (18)\(^e\) | 5.4 (1.6) |
| [N-\(\text{Me-Leu}^{24}\)]hPTH-(1–31)NH\(_2\) | >100,000 | 463 (70) |
| [N-\(\text{Me-Leu}^{24}\)\(\text{eGlu}^{25}\)\(\text{Lys}^{28}\)]hPTH-(1–31)NH\(_2\) | 91,057 (8,448) | 13 (4.0) |

\(^a\) Binding assays were performed with \(\text{[Nle}^{8,21},\text{Tyr}^{34}\]\)PTH-(1–34)NH\(_2\) as tracer ligand.

\(^b\) IC\(_{50}\) values were calculated by non-linear regression analysis.

\(^c\) Values are means (± S.E.) of data compiled from three separate experiments, each performed in duplicate.

\(^d\) AC activities were obtained by radioimmunoassay of cAMP as described previously (38).

\(^e\) ED\(_{50}\) values were calculated by fit of data by nonlinear regression (38).

Combined with an earlier implication of extracellular loop binding to PTH (18), the data in this paper suggests that the principal binding domain C terminus of PTH binds in a cooperative manner to both the N terminus region of the receptor and one or more of the extracellular loops. Binding of PTH to either P1R-delNT (54) or the extracellular N terminus sequence alone of P1R (15) is very weak (\(~\mu\)M). The experiments reported here thus help to delineate the nature of the overall binding and activation process.

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