C-Terminal Analogues of Parathyroid Hormone: Effect of C-Terminus Function on Helical Structure, Stability, and Bioactivity

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ABSTRACT: We have studied the effects of C-terminal group modifications (amide, methylamide, dimethylamide, aldehyde, and alcohol) on the conformation, adenylyl cyclase stimulation (AC), or binding of parathyroid hormone (hPTH) analogues, hPTH(1−28)NH_2 and hPTH(1−31)NH_2. hPTH(1−31)NH_2 has a C-terminal α-helix bounded by residues 17−29 [Chen, Z., et al. (2000) *Biochemistry 39*, 12766]. In both cases, relative to the natural analogue with a carboxyl C-terminus, the amide and methylamide had increased helix content whereas the dimethylamide forms had CD spectra more similar to the carboxyl one. Conformational effects were more pronounced with hPTH(1−28) than with hPTH(1−31), with increases in helix content of ∼30% in contrast to 10%. Stabilization of the C-terminal helix of residues 1−28 seemed to correlate with an ability of the C-terminal function to H-bond appropriately. None of the analogues affected the AC stimulating activity significantly, but there was an up to 15-fold decrease in the level of apparent binding of the carboxyl hPTH(1−28) analogue compared to that of the methylamide and a 4-fold decrease in the level of binding of the aldehyde or dimethylamide. There was no significant change in binding activities for the 1−31 analogues. These observations are consistent with previous studies that imply the importance of a region of the hormone's C-terminal α-helix for tight binding to the receptor. They also show that modulation of helix stability does have an effect on the binding of the hormone, but only when the C-terminus is at the putative end of the helix. The similarity of AC stimulation even when binding changed 10-fold can be explained by assuming greater efficacy of the weaker binding PTH−receptor complexes in stimulating AC.

The α-helix is a common structure in bioactive peptides, and an amphiphilic α-helix appears at or very near the C-terminus of most hormones reacting with class II GPCRs.[1] This class includes receptors activated by parathyroid hormone (PTH), secretin, glucagon-releasing hormone, calcitonin, and vasoactive intestinal peptide. These hormones are all approximately 30 residues long, and the natural fragment for most of them has a C-terminal carboxyl that is at or near the end of the amphiphilic helix (*1*). We reported earlier that replacement of the carboxyl with a carboxamide group led to increased adenylyl cyclase (AC) activating activity for hPTH(1−30), when tested against rat osteosarcoma 17/2 cells (*2*), and suggested that hPTH(1−31)NH_2 could have greater activity in vivo than hPTH(1−31)OH, on the basis of reports that other bioactive peptides are more stable to carboxypeptidases when in the amide form and could thus have improved properties for transport from the site of application (*3*).

Numerous studies have demonstrated the presence of two functional domains in PTH. The N-terminal portion reacts with the receptor's juxtamembrane domain, critical for AC activating activity, and the C-terminal region, containing the helix, reacts with the receptor's N-terminal extracellular sequence, essential for high-affinity binding [reviewed by Gensure et al. (*4*)]. Although both hPTH(1−28)NH_2 and hPTH(1−31)NH_2 are similar in receptor activation, as measured by AC stimulating activity (*2*), only hPTH(1−31)-NH_2 is fully active as an anabolic agent in vivo (*5*). However, hPTH(1−28)NH_2 can be made almost fully active in vivo by stabilizing its C-terminal α-helix by side chain lactam formation between Glu-22 and Lys-26 and substitution of Lys-27 with Leu (*6*). We and others have also reported that certain lactam analogues within the C-terminal region of PTH or parathyroid hormone-related peptide (PTHrP) that stabilize its α-helix also increase AC stimulating activity (*7*−*11*). There have also been periodic reports of receptor activity associated with the C-terminal region of residues 28−34 of

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[1] Abbreviations: AC, adenylyl cyclase; CD, circular dichroism; CH_2-OH, C-terminal alcohol; DIEA, *N*,*N*-diisopropylethylamine; DCM, dichloromethane; GPCR, G protein-coupled receptor; H, C-terminal aldehyde; hPTH, human parathyroid hormone; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo(4,5-bipyridin-1-ylmethylene)-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOBt, 1-hydroxybenzotriazole; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; $N(CH_3)_2$, C-terminal dimethylamide; $NHCH_3$, C-terminal methylamide; NH_2, C-terminal amide; OH, C-terminal carboxyl; OSu, *N*-hydroxysuccinimide ester; P1R, human PTH receptor type 1; PKC, protein kinase C; TBTU, 2-(1*H*-benzotriazolyl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid.

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hPTH(1−34), including an in vivo chick hypercalcemia activity (*12*) and an in vitro membrane-bound PKC-stimulating activity in some cell lines (*13*), and these could conceivably be explained by appropriate stabilization or destabilization of this α-helix. Consistent with this idea, we observed earlier that constraining hPTH(20−34)NH_2 by lactam formation between either residues 22 and 26 or 27 and 30 caused loss of membrane-bound PKC activity in comparison with that of either the linear hPTH(20−34)NH_2 or another lactam-constrained analogue, cyclo(Lys-26−Asp-30)NH_2 (*14*). These data suggest that any modification that modulates the C-terminal region, particularly its helical conformation, could have an effect on its in vitro or in vivo bioactivities.

At least two possibilities for a PTH C-terminal group effect on receptor activation exist. One is a correlation of α-helix stability with receptor activation if the C-terminal region binds as an α-helix. NMR studies of hPTH(1−34) (*15*) and hPTH(1−31)NH_2 (*16*) under near-physiological solvent conditions showed the α-helix is bounded by Ser-17 and Gln-29. Analogue studies have implied that this region binds to the receptor with the hydrophobic face of the amphiphilic α-helix toward the receptor's large N-terminal extracellular sequence (*17*, *18*). A second possibility is that the C-terminal amide H-bonds to the receptor, mimicking the peptide bond present in the natural circulating hormone fragment.

The parathyroid hormone analogues hPTH(1−31)and hPTH(1−28) make up a good system for studying effects of the C-terminal group on structure and function. hPTH-(1−28) is terminated at the end of the amphiphilic helix observed in hPTH(1−31)NH_2. Here we report a systematic study of C-terminal modification on the structure, as measured by CD, and bioactivity, as measured by AC stimulation and receptor binding activity. To do this, we have studied the effects of substitution of the amide function with carboxyl, monomethylamide, dimethylamide, aldehyde, and alcohol groups. This has permitted assessment of any effects of the C-terminal function on helix stabilization and removal of potential H-bond NH donors or carbonyl acceptors and may be useful in understanding and predicting effects on similar hormones or other bioactive peptides.

MATERIALS AND METHODS

Materials

2-Chlorotrityl resin was purchased from Biosearch Technologies (0.47 mmol/g). Fmoc-leucine (0.22 mmol/g), valinal (0.23 mmol/g), and leucinal (0.21 mmol) NovaSyn TG resins were obtained from NovaBiochem. N-Fmoc-protected amino acids were purchased from ChemImpex and Peptides International. N-Boc-Ser was from ChemImpex. HATU and diisopropylethylamine were from Applied Biosystems. HOBt was obtained from Quantum Biotechnologies and TBTU from Albatross Chemicals Inc. Methylamine HCl, dimethylamine HCl, *N*,*N*′-dicyclohexylcarbodiimide, and *N*-(3-dimethylamino)-propyl-*N*′-ethylcarbodiimide HCl were purchased from Sigma. $NaBH_4$ was from Aldrich and *N*-hydroxysuccinimide from Fluka.

C-Terminal Analogue Syntheses

hPTH(1−31)NH_2, hPTH(1−28)NH_2, hPTH(1−31)OH, and hPTH(1−28)OH. These C-terminal carboxyl and amide analogues were synthesized by standard Fmoc solid-phase peptide synthesis protocols on a continuous-flow peptide synthesizer with modifications described in detail elsewhere (*10*, *11*). Other syntheses are summarized below with details in the Supporting Information. MS data were collected by MALDI-TOF MS (Voyager-DE STR, Perseptive/Applied Biosystems, Foster City, CA). The MS data and HPLC elution times are given in Table 1.

Table 1: Mass Spectroscopy and HPLC Data for hPTH C-Terminal Analogues

	molecular mass[a] (Da)		HPLC[b]		
analogue	observed	expected	system 1[c]	system 2[d]	purity
(1−31)OH	3719.5	3719.4	34.7	24.1	98
(1−31)NH_2	3718.0	3718.4	32.8	33.3	98
(1−31)$NHCH_3$	3732.8	3732.4	35.6	36.1	92
(1−31)$N(CH_3)_2$	3746.9	3746.5	36.6	35.3	95
(1−31)CH_2OH	3707.0	3705.4	37.4	36.8	95
(1−31)H	3704.0	3703.4	37.2	36.9	99
(1−28)OH	3378.0	3377.0	26.0	23.0	99
(1−28)NH_2	3376.0	3376.1	24.9	33.4	99
(1−28)$NHCH_3$	3390.6	3390.1	25.3	34.3	99
(1−28)$N(CH_3)_2$	3404.8	3404.1	27.1	36.6	97
(1−28)CH_2OH	3364.0	3363.1	27.8	36.8	99
(1−28)H	3362.1	3361.0	26.4	32.5	97

[a] Masses were confirmed by MALDI-TOF MS (Voyager-DE STR, Perseptive/Applied Biosystems). [b] Reverse-phase HPLC was performed on a Gilson chromatograph using a Vydac C18 analytical column (4 mm × 238 mm), using ∼10 mg of sample. Detection was at 214 nm. The elution rate was 1 mL/min. [c] System 1 was solution A (0.1% TFA in H_2O) and solution B (0.1% TFA in acetonitrile). Linear gradients from 3 to 27% B over 15 min, from 27 to 28% B over 30 min, and from 28 to 98% B over 5 min. [d] System 2 was solution A [25 mM Na_2HPO_4 in H_2O (pH 7.2)] and solution B (30% 25 mM Na_2HPO_4 in H_2O and 70% acetonitrile). Linear gradients from 10 to 45% B over 15 min, from 45 to 55% B over 30 min, and from 55 to 98% B over 5 min.

hPTH(1−31)$NHCH_3$ and hPTH(1−28)$NHCH_3$. Fully protected hPTH(1−31)OH, with Boc-Ser1, was synthesized on 2-chlorotrityl resin and released by treatment with an AcOH/TFA/DCM mixture (1:1:3) for 2 h at 25 °C. The side chain-protected hPTH(1−31)OH was then dissolved in DCM; 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide HCl, methylamine HCl, and DIEA were added to the solution, and the mixture was stirred at 25 °C for 19 h. The solvent was removed under reduced pressure and the residue resuspended in ethyl acetate. The solution was washed with 10% aqueous Na_2CO_3, 0.1 M aqueous HCl, and brine. The organic layer was dried over $MgSO_4$ and filtered, and the solvent was removed to yield a white solid. Protecting groups were removed via cleavage with reagent K, precipitated with *tert*-butyl methyl ether, and dried. The sample was purified by HPLC with a 3%/min gradient of acetonitrile in a 0.1% TFA/H_2O mixture.

The side chain-protected hPTH(1−28)OH was prepared as described for hPTH(1−31)NH_2 starting with Fmoc-Leu-NovaSyn TGT resin. The fully protected hPTH(1−28)OH was dissolved in DCM, and methylamine HCl and DIEA were added. The solution was cooled to 0 °C and 1-ethyl-3-[3′-(dimethylamino)propyl]carbodiimide HCl added portionwise. Stirring was continued at 0 °C for 2 h and at room temperature overnight. The reaction mixture was diluted with DCM, washed with 10% aqueous Na_2CO_3, 0.1 M aqueous HCl, and brine, dried over $MgSO_4$, and concentrated in vacuo. Deprotection of the side chains was performed by

treatment with reagent K and purified by HPLC as described above.

hPTH(1−31)N(CH$_3$)$_2$ and hPTH(1−28)N(CH$_3$)$_2$. These dimethylamides were prepared as described above for the monomethylamides using dimethylamine HCl in place of methylamine HCl.

hPTH(1−31)CH$_2$OH and hPTH(1−28)CH$_2$OH. (*A) Acylation of a Modified Amino Acid*. The fully protected hPTH-(1−30)OH, attached to the resin by the γ-carboxyl of Asp-30, was coupled with C-terminal valinol by HATU or *N*-hydroxysuccinimide activation. These reactions were incomplete (64−68% yield), probably due to steric hindrance of the Asp-30 α-carboxyl group.

(B) Method of C-Terminal Modification. The fully protected hPTH(1−31)OH and *N*-hydroxysuccinimide were dissolved in THF, and the solution was cooled to 0 °C. *N*,*N*′-Dicyclohexylcarbodiimide was added and the reaction mixture stirred at 0 °C for 2 h and then shaken at room temperature overnight. The organic solution was filtered and concentrated to dryness in vacuo. The residue of the fully protected hPTH(1−31)OSu was dissolved in DCM and $NaBH_4$ and acetic acid slowly added. The mixture was stirred at room temperature for 5 h. The organic solution was washed with H_2O and brine, dried over $MgSO_4$, and evaporated in vacuo. Deprotection of the side chains was performed by treatment with reagent K. The crude peptide alcohol, hPTH(1−31)CH$_2$OH, was purified by HPLC and analyzed by MALDI-TOF MS (Table 1).

hPTH(1−28)CH$_2$OH was prepared by C-terminal modification as described above but starting with the fully protected hPTH(1−28)OH.

hPTH(1−31)H and hPTH(1−28)H (19). hPTH(1−31)H was synthesized with preloaded aldehyde resin based on the oxazolidine linker (*20*), valinal NovaSyn TG resin as the support, and Fmoc chemistry. The next three amino acids [FmocAsp(OtBu), FmocGln(Trt), and FmocLeu] were attached with HATU and DIEA manually. Completion of the reaction was monitored by a Kaiser test for free amine groups. Remaining amino acids were coupled with TBTU and HOBt on the continuous-flow peptide synthesizer. Side chain deprotection and cleavage from resin were carried out in two stages. Side chain protecting groups were removed with reagent K for 2.5 h at room temperature. The peptide aldehyde resin was washed with TFA and DCM, and then final cleavage was performed by three 30 min treatments with an AcOH/H_2O/DCM/MeOH mixture (10:5:63:21). After evaporation of organic solvents, the aqueous solution was diluted with H_2O and lyophilized. The crude peptide aldehyde was purified by HPLC with 0.1% TFA in a MeCN/H_2O mixture.

hPTH(1−28)H was prepared as described above for the 1−31 analogue, but starting with leucinal NovaSyn TG resin.

Peptide Purification

The crude peptides were dissolved in H_2O and purified with 0.1% TFA in a MeCN/H_2O mixture on a Gilson RP-HPLC system linked to a Vydac C18 semipreparative column (10 mm × 218 mm) at a flow rate of 2 mL/min with detection at 214 nm. Fractions containing only the desired peptide were combined and lyophilized. Product identities were confirmed by MALDI-TOF MS. HPLC and MALDI-TOF data for the purified C-terminally modified analogues are given in Table 1.

Cell Cultures

HKRK-B7 cell lines were derived from porcine kidney cell line LLC-PK1 and were stably transformed to express full-length human P1R at a level of ∼950000 receptors/cell (*21*).

Adenylyl Cyclase Activity

AC activities were measured using a direct enzyme-linked immunosorbent assay for cAMP (Amersham Biosciences) as described previously (*21*). Reported activities are each the average of three separate experiments.

Receptor Binding

Radioligand binding assays were performed as previously described (*22*). In brief, a ^{125}I-labeled radioligand [∼100000 cycles per minute per well of a 24-well plate of ^{125}I[Nle^{8,21},Tyr^{34}]PTH-(1−34)NH$_2$] was incubated with whole cells expressing wild-type P1R in the absence or presence of varying concentrations (3×10^{-9} to 1×10^{-5} M) 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 and lysed in NaOH, and the entire lysate was counted for γ-irradiation. Data were analyzed using the four-parameter equation $B_c = B_{min} + (B_{max} - B_{min})/[1 + (IC_{50}/c)^{slope}]$, where B_c is the percent bound at concentration c.

Circular Dichroism

CD spectra were obtained on a JASCO J600 spectropolarimeter, operated at ambient temperature (20−22 °C). Peptides were dissolved in H_2O and sodium phosphate buffer (pH 7.2), added to a final concentration of 25 mM. Peptide concentrations were 0.11−0.15 mM, calculated from the absorption at 280 nm, using an extinction coefficient of 5700 M^{-1}. The CD spectrum of hPTH(1−17)NH$_2$ was subtracted from sample spectra to provide an estimate of the CD that can be attributed to the C-terminal α-helix, residues 17−29 (*16*), according to the formula

$$[\theta]^{17-28} = 2.6[\theta]^{1-28} - 1.5[\theta]^{1-17} \text{ or } [\theta]^{17-31} = 2.1[\theta]^{1-31} - 1.1[\theta]^{1-17}$$

RESULTS

Secondary Structures of C-Terminal Analogues

hPTH(1−31) Analogues. CD spectra are highly sensitive to changes in α-helix. The α-helix portion of the CD spectrum of hPTH(1−31) is almost entirely due to a large amphiphilic helix between residue Ser-17 and Gln-29 (*16*, *17*). The N-terminal α-helix between residues Ser-3 and His-9, determined by NMR (*16*), contributes little to the CD signal at 222 nm of hPTH(1−31)NH$_2$ (see the Supporting Information). To show more clearly the effect of the C-terminal modifications on helix structure, we have subtracted the spectrum of hPTH(1−17)NH$_2$, as described previously (*23*). This is permissible because the two regions of hPTH(1−31)NH$_2$ have been shown to have little interac-

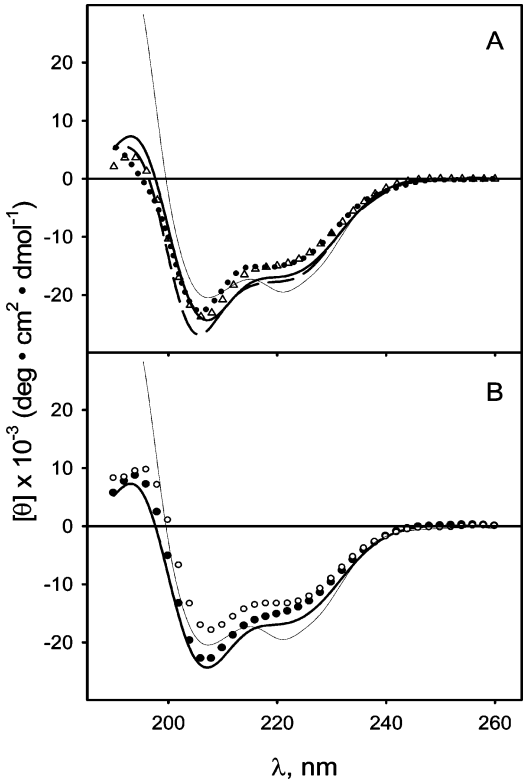

FIGURE 1: CD spectra of hPTH(17−31) C-terminal analogues. The spectra were obtained at room temperature (20−22 °C). The spectra were calculated by subtraction of the hPTH(1−17)NH_2 spectrum from the hPTH(1−31)NH_2 spectrum as described in Materials and Methods. Each graph contains the spectrum of (17−31)NH_2 at 0.12 mM (thick line) and the spectrum of c(Glu-22−Lys-26)hPTH(17−31)NH_2 (thin line) as an example of a helix conformation associated with increased (∼3-fold) AC stimulating and binding activities: (A) 0.12 mM (17−31)OH (△), 0.11 mM (17−31)$NHCH_3$ (− − −), and 0.11 mM (17−31)$N(CH_3)_2$ (●) and (B) 0.13 mM (17−31)CH_2OH (●) and 0.13 mM (17−31)H (○).

Table 2: CD α-Helix Parameters of hPTH(1−31) C-Terminal Analogues[a]

C-terminus	$-[\theta]_{209}$ ($\times 10^{-3}$ deg cm^2 dmol^{-1})	$-[\theta]_{222}$ ($\times 10^{-3}$ deg cm^2 dmol^{-1})	$[\theta]_{222}/[\theta]_{209}$
OH	22.0	14.5	0.66
NH_2	23.5	16.7	0.71
$NHCH_3$	24.5	17.6	0.72
$N(CH_3)_2$	20.8	14.8	0.71
H	17.7	13.4	0.76
CH_2OH	22.1	14.7	0.67
cyclic[b]	20.0	19.4	0.97

[a] After subtraction of the hPTH(1−17)NH_2 spectrum. [b] Cyclic refers to c(Glu-22−Lys-26)hPTH(1−31)NH_2.

tion (*16*) and thus the hPTH(1−17) and hPTH(17−31) regions make independent contributions to the spectrum.

The spectra for the 17−31 regions of hPTH(1−31) C-terminal analogues are shown in panels A and B of Figure 1. The characteristic α-helix values from Figure 1, $[\theta]_{222}$ and $[\theta]_{209}$, are listed in Table 2, along with the $[\theta]_{222}/[\theta]_{209}$ ratios. This value, estimated from poly(L-Lys), is 1.09 for an ideal α-helix (*24*), and these values are included in Tables 2 and 3 to provide some idea about how well the helix structure in hPTH approaches an ideal one. The latter spectrum is a reference for a structure that is presumably closer to the receptor-bound form. To make comparisons clearer, the spectra for the amide analogue and that of a cyclic analogue

Table 3: CD α-Helix Parameters of hPTH(1−28) C-Terminal Analogues[a]

C-terminus	$-[\theta]_{209}$ ($\times 10^{-3}$ deg cm^2 dmol^{-1})	$-[\theta]_{222}$ ($\times 10^{-3}$ deg cm^2 dmol^{-1})	$[\theta]_{222}/[\theta]_{209}$
OH	18.5	9.7	0.52
NH_2	22.1	15.2	0.69
$NHCH_3$	20.6	15.5	0.75
$N(CH_3)_2$	16.6	10.2	0.61
H	21.7	14.3	0.66
CH_2OH	23.8	15.9	0.67
cyclic[b]	20.0	19.4	0.97

[a] After subtraction of the hPTH(1−17)NH_2 spectrum. [b] Cyclic refers to c(Glu-22−Lys-26)hPTH(1−31)NH_2.

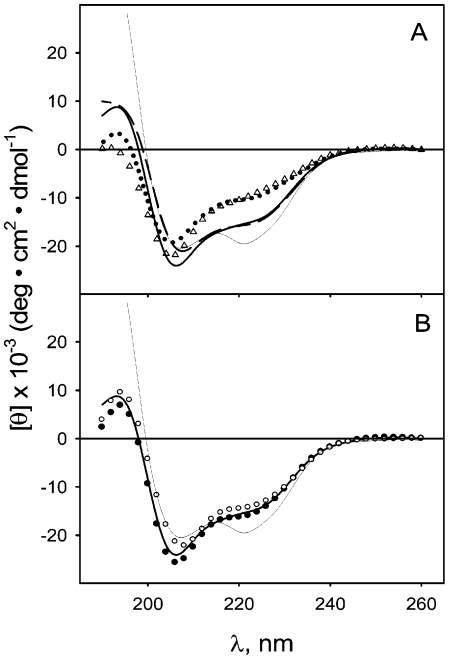

FIGURE 2: CD spectra of hPTH(17−28) C-terminal analogues. The spectra were obtained at room temperature (20−22 °C). The spectra were calculated by subtraction of the hPTH(1−17)NH_2 spectrum from the hPTH(1−28)NH_2 spectrum as described in Materials and Methods. Each graph contains the spectrum of 0.12 mM (17−28)-NH_2 (thick line) and the spectrum of c(Glu-22−Lys-26)hPTH(17−31)NH_2 (thin line) as an example of a helix conformation associated with increased (∼3-fold) AC stimulating and binding activities: (A) 0.12 mM (17−28)OH (△), 0.11 mM (17−28)$NHCH_3$ (− − −), and 0.11 mM (17−28)$N(CH_3)_2$ (●) and (B) 0.13 mM (17−28)CH_2OH (●) and 0.13 mM (17−28)H (○).

with high binding and AC stimulating activity, cyclo(Glu-22−Lys-26)hPTH(1−31)NH_2 (*23*), are included in both parts of the figure. This analogue has a $[\theta]_{222}/[\theta]_{209}$ value of 0.97, the closest to the ideal helix form. The spectra of the C-terminal amide and monomethylamide are very similar except for the 209 nm region (Figure 1A). The spectra of the C-terminal alcohol (Figure 1B), acid, and dimethylamide (Figure 1A) are also almost identical, ∼15% lower in magnitude at 222 nm than that of the amide. The aldehyde exhibited the least α-helix, as measured by $[\theta]_{222}$, but the greatest $[\theta]_{222}/[\theta]_{209}$ value (Table 2).

hPTH(1−28) Analogues. The hPTH(1−28) acid and dimethylamide analogues had magnitudes at 222 nm ∼35% lower than those of the remaining hPTH(1−28) analogues (Figure 2A and Table 3). In contrast, the alcohol and aldehyde analogues had minima similar to that of the amide, but the aldehyde analogue had a $[\theta]_{222}/[\theta]_{209}$ value closer to

that of a helical conformation. These results suggest that the ability of the C-terminal functional group to form a H-bond with a suitably spaced backbone or side chain group was important for the observed helix stabilization. Neither the dimethylamide nor the negatively charged C-terminal carboxyls had such stabilization. An increased macrodipole from the carboxylate might also contribute to the lower stability of hPTH(1−28)OH. Such diminished stability of helices terminating in a carboxyl rather than amide function has been noticed before and is often thought to result from effects on their helix macrodipoles (*25*). However, the dimethylamide C-terminally modified helix does not have an increased macrodipole, and this suggests that this explanation for these differential effects is incomplete.

HPLC Retention Behavior

Peptides with an inducible amphiphilic α-helix have increased retention times relative to similar α-helical sequences that are not amphiphilic (*26*, *27*). The retention behaviors of the various C-terminal analogues are shown in Table 1. At acidic pH (0.1% aqueous TFA), the order of retention times for hPTH(1−31) analogues is as follows: $CH_2OH \cong H > N(CH_3)_2 > NHCH_3 > OH > NH_2$. In contrast, the hPTH(1−28) analogues have the following order: $CH_2OH > N(CH_3)_2 > H \cong OH > NHCH_3 \cong NH_2$. To better compare these retention times with the helix stability from CD, the analogues were eluted with a solvent at neutral pH. At pH 7.2, the hPTH(1−31) retention times are in the following order: $H \cong CH_2OH \cong NHCH_3 > N(CH_3)_2 > NH_2 \gg OH$. Those of hPTH(1−28) are as follows: $CH_2OH \cong N(CH_3)_2 > NHCH_3 > NH_2 > H \gg OH$. Two aberrant behaviors stand out. At acidic pH, the retention data for the carboxyl and amide C-terminal PTH analogues are the opposite of that expected when comparing with the helix stability inferred from CD performed at pH 7.2 (Table 2). At neutral pH, these retentions are much higher than that of the C-terminal acid forms, consistent with the expected behavior from the CD study at neutral pH. This suggests that the strong macrodipole (*25*) found at the neutral, but not acidic, pH has a destabilizing effect on the C-terminal α-helix. We also examined the effect of pH on the CD spectra of the carboxyl and amide analogues of hPTH(1−28) and hPTH(1−31) (Figure 3). Both peptides exhibited substantially decreased helix content, as measured by CD, at pH 2.0 as opposed to pH 7.2. Presumably, this effect comes from the discharge of the acid side chain groups and the resulting loss of possible ion pairs. Ionic side chain pairs are known to contribute to C-terminal α-helix stabilization (*28*), and these are lost at acidic pH. However, at acidic pH, H-bond possibilities remain between Glu-22^0 and Lys-26^+ or Lys-26^+ and Asp-30^0. These nonionic H-bonds have been shown to largely compensate for loss of the ion pairs (*29*).

Bioactivities of C-Terminal hPTH(1−31) Analogues

Both binding and AC stimulating activities were measured against HKRK-B7 cells stably transformed with the receptor P1R. The AC stimulating activities (Table 4) were all similar in magnitude, even for those that had binding activities differing by an order of magnitude. Unlike results previously reported for hPTH(1-30), using ROS 17/2 cells (*2*), both hPTH(1−31) and hPTH(1−28) with carboxyl termini had

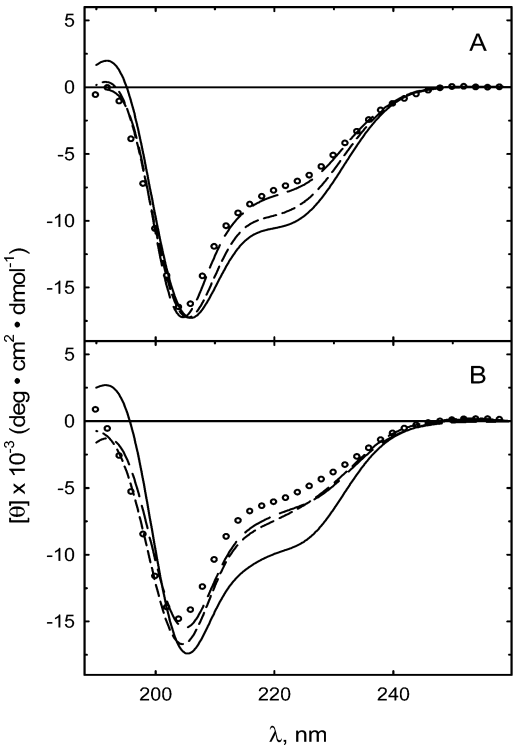

FIGURE 3: CD spectra of hPTH C-terminal analogues at pH 2.0 and 7.2: (A) hPTH(1−31)OH at pH 2.0 (○), hPTH(1−31)NH_2 at pH 2.0 (− − −), hPTH(1−31)OH at pH 7.2 (- - -), and hPTH(1−31)NH_2 at pH 7.2 (—) and (B) hPTH(1−28)OH at pH 2.0 (○), hPTH(1−28)NH_2 at pH 2.0 (− − −), hPTH(1−28)OH at pH 7.2 (- - -), and hPTH(1−28)NH_2 at pH 7.2 (—).

higher activities than with amide termini, but only with hPTH(1−28) (3-fold difference) was the difference in activity outside experimental error. The binding data exhibited larger differences (Figure 4), and these were much more pronounced for hPTH(1−28) than for hPTH(1−31) analogues. In general, the binding data paralleled the α-helix amounts, with the exception of the aldehyde C-terminus. Thus, the observed binding had some correlation to the helix magnitude and conformation. The AC stimulating activity did not correlate well with the binding activity. Such a lack of correlation has been noted previously by other workers (*30*−*32*), and analogues exhibiting relatively weak binding in the competitive assay can still retain almost normal AC stimulating activity (*33*). AC stimulation is a complex phenomenon and is not just a function of the apparent binding but also the exact conformation of the binding complex. It is the latter that ultimately determines the activity of any given PTH analogue against its receptor.

DISCUSSION

C-Terminal group effects on the secondary structure of hPTH, as measured by CD, were much greater with the 1−28 as opposed to the 1−31 analogues. In part, there is an inherently greater helical stability of the 1−31 analogues because of the presence of the Lys-26/Asp-30 ion pair and H-bond. A second reason is that the C-terminal group of Leu-28 may directly provide stability to the helix in contrast to Val-31, known to be external to the helix by two or three residues (*16*). The 1−28 data can be compared to our previous study of backbone-methylated analogues where data for the *N*-methylated Gln-29 in the context of hPTH(1−31)-

Table 4: Summary of Binding and AC Stimulation Activities of C-Terminal PTH Analogues

	binding in HKRK-B7 cells[a] IC_{50}[b] (nM)		AC activity in HKRK-B7 cells[c] ED_{50}[d] (nM)	
C-terminus	1−31	1−28	1−31	1−28
OH	70 ± 18 (14)	10507 ± 2736 (9)	2.9 ± 1.7 (3)	0.5 ± 0.2 (3)
NH_2	63 ± 11 (15)	1019 ± 346 (9)	4.9 ± 1.5 (6)	1.7 ± 0.4 (3)
$NHCH_3$	49 ± 13 (3)	268 ± 22 (3)	2.8 ± 0.9 (6)	1.8 ± 0.4 (3)
$N(CH_3)_2$	456 ± 138 (3)	1256 ± 144 (3)	2.8 ± 1.0 (6)	1.2 ± 0.6 (3)
H	65 ± 1.0 (3)	1284 ± 244 (3)	2.4 ± 0.8 (3)	1.7 ± 0.9 (3)
CH_2OH	48 ± 4 (3)	581 ± 60 (3)	5.7 ± 1.6 (3)	1.8 ± 0.7 (3)

[a] Binding assays were performed with ^{125}I[Nle^{8,21},Tyr^{34}]PTH(1−34)NH_2 as a tracer ligand. [b] IC_{50} values were calculated by nonlinear regression analysis. Values are means ± the standard error of the mean of data compiled from *N* separate experiments, each performed in duplicate. [c] AC activities were obtained by a direct ELISA of cAMP. [d] ED_{50} values were calculated by a fit of data with the sigmoidal function. Values are means ± the standard error of the mean of data compiled from *N* separate experiments.

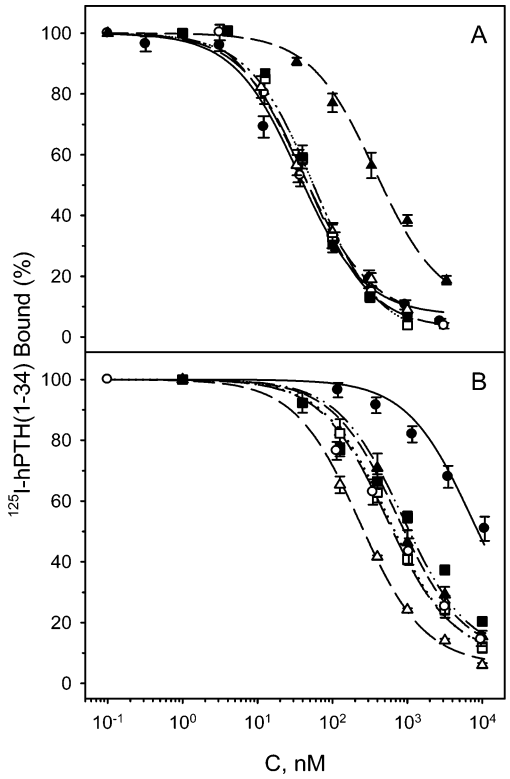

FIGURE 4: Binding curves of hPTH C-terminal analogues. (A) hPTH(1−31): OH (●), NH_2 (○), $NHCH_3$ (△), $N(CH_3)_2$ (▲), CH_2-OH (□), and H (■). (B) hPTH(1−28): −OH (●), NH_2 (○), $NHCH_3$ (△), $N(CH_3)_2$ (▲), CH_2OH (□), and H (■).

NH_2 were presented. There was an approximately 8-fold decrease in the level of binding, but AC stimulating activity was the same as that of the unmethylated sequence. The parallel data for the 1−28 C-terminal analogues are a comparison of the dimethylamide and monomethylamide, in which the amide nitrogen has an environment similar to that of these methylated and unmethylated hPTH(1−31)NH_2 analogues. The ratio of the binding constants is similar, being ~6-fold less for the dimethylamide. The corresponding AC stimulating activities were the same in both cases. Overall, is there a correlation between secondary structure and the binding activity of the 1−28 analogues? The answer to that is a qualified yes. The most tight binding analogue also had the greatest helicity as measured by $[\theta]_{222}$ and was most α-helix-like, as measured by the $[\theta]_{222}/[\theta]_{209}$ ratio. In contrast, the COOH analogue clearly was the lowest in terms of these parameters. The other analogues especially paralleled the $[\theta]_{222}/[\theta]_{209}$ ratios but also to a lesser extent the $[\theta]_{222}$ values. The obvious anomaly is the CHO terminal analogue that has a weaker binding but has relatively high helix parameters. This might be explained after a detailed study of the NMR structure of the C-terminal 1−28 analogues. The CHO analogue is much more reactive than the others and likely is reacting with a nearby side chain.

We have attempted to gain some insight into this by modeling the 17−28 region as an ideal helix and then performing a geometry minimization to the lowest-energy form, using an AMBER force field (Figure 5). These calculations show that, except for the carboxylate and dimethylamide group termini, there are helix-stabilizing interactions involving the C-terminal group and/or the backbone NH group of Leu-28. The carboxylate analogue has a helix-destabilizing interaction with the side chain of Arg-25 and Lys-26, resulting in a distortion of the C-terminal end of the α-helix. The dimethylamide group, on the other hand, does not form a strong stabilizing H-bond with any group. The C-terminal aldehyde is reactive compared to other substitutions and can hydrate in solution to form a *gem*-diol.

HPLC retention times are a complex function of the tendency to form an α-helix, the pH conditions, temperature, and the amphiphilicity of the helix, but the last is the dominant determinant of retention (*35*). As with the CD, the greatest effects occurred with the 1−28 analogues, and all 1−28 analogues had lower retention times at pH 2.0 than at pH 7.2 (Table 1). This can be explained by the loss of the helix-stabilizing ion pair between the side chains of Glu-22 and Lys-26. At pH 7.2, the 1−31 or 1−28 hPTH analogues with a C-terminal carboxyl were found to have much lower retention times and less helix than the corresponding amides. At neutral pH, the gain of a C-terminal charge, and an accompanying macrodipole (*25*), partly offsets the increase in helix stability resulting from an ion pair from Glu-22 and Lys-26. (Tables 2 and 3). However, the retention times did not correlate well with the order of helix stability, as measured by CD. The strong retention of the dimethylamide suggests that the hydrophobic nature of this group contributes to the HPLC behavior.

Mixed effects on structure or bioactivity have been reported for other bioactive peptides. Not only PTH but many other hormones [including secretin (*36*) and glucagon (*37*)] of the class II receptor family have an α-helix terminating near the C-terminus. Changing the natural C-terminal amide of secretin to a carboxyl group had no effect on in vivo bioactivity (*38*, *39*). The glucagon-like peptide was unaffected at the receptor level by changing the natural amide to

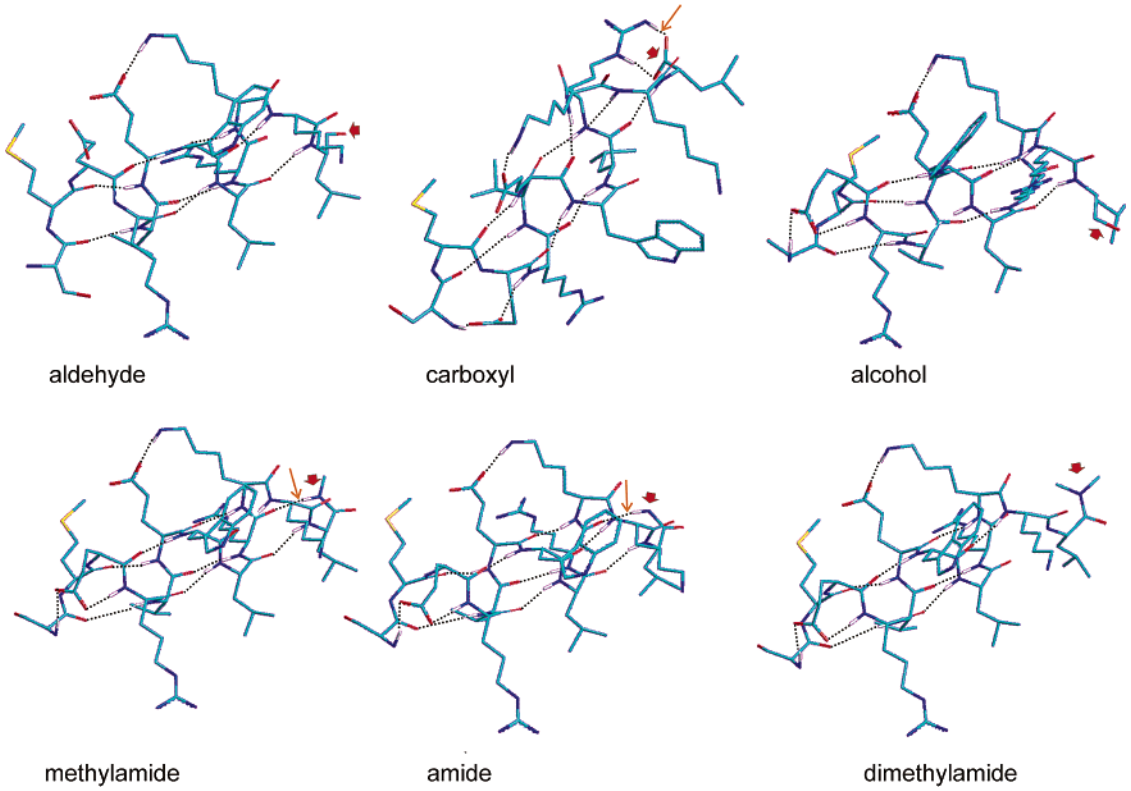

FIGURE 5: Hydrogen bonding of the 17−28 helical region of C-terminal analogues of hPTH(1−28). Models of the 17−28 regions were formed by geometry minimizing an initial α-helix of each analogue, using an AMBER force field (*34*). In addition to $i-i+4$ backbone interactions and a side chain Glu-22···Lys-26 H-bond, the following involving C-terminal groups are indicated with an orange arrow: OH, multiple H-bonds of the carboxylate group with the side chain of Arg-25 and Lys-26; $NHCH_3$, C-terminal N with the backbone NH group of Arg-25; and NH_2, C-terminal N with the backbone NH group of Arg-25. The positions of the C-terminal groups are indicated with red arrowheads.

carboxyl, but its in vivo plasma lifetime was shortened (*40*).

In contrast, insect neuropeptides, kinins, were reported to have greatly diminished in vivo bioactivity when the natural C-terminal amide was substituted with a carboxyl or dimethylamide but retained activity if the group was a methylamide (*41*). Replacement of the C-terminal amide of a wasp venom resulted in a large decrease in bioactivity that was ascribed to destabilization of of a C-terminal backbone H-bond necessary for α-helix in that region (*42*). The carboxyl group in comparison to the amide was reported to be critical for a pentapeptide inhibition of a phosphatase (*43*). Peptide aldehydes have particular functional significance in some cases (*20*, *44*). Geraghty et al. in a systematic study of C-terminal modification similar to our study reported that the activity of a molluscan FMRFamide, both in vitro and in vivo, was unaffected by a methylamide or dimethylamide substitution but was diminished ∼30-fold by an alcohol one (*45*). Thus, to date, there is no pattern in the effects of C-terminal modification of peptides on their bioactivities. However, for hormones activating class II receptors, there appears to be no effect on receptor activation but some on binding if the C-terminal end is also the termination of the α-helix, as observed here for hPTH(1−28) analogues. In most cases, the C-terminal end of the hormone is actually several residues removed from the C-terminal end of the helix, and therefore, the effects in vitro on receptor binding and activation are minimal. In vivo effects can be more marked, in contrast, because they depend on the metabolic stability of the hormone and also on transport properties that are still poorly understood. C-Terminal amides are more resistant to some carboxypeptidases (*46*), but not all (*47*), in comparison to the carboxyl C-termini. There is some evidence that some amphiphilic helical peptides are transported more efficiently. hPTH(1−31)NH_2 appeared more rapidly in the circulation when the C-terminal helix was stabilized by lactam formation between residues 22 and 26 (*10*).

Accumulated data show that the PTH C-terminal helix binds with its hydrophobic face to the long N-terminal extracellular sequence of its receptor and that residues Arg-20, Trp-23, Leu-24, and Leu-28 are critical for the binding (*4*, *48*, *49*). Furthermore, in the context of hPTH(1−31)-NH_2, the backbone nitrogen of Leu-28, but not Gln-29, is critical. The data presented in this paper provide further support for this mode of binding.

The observation, especially obvious with the 1−28 analogues, that activity and binding do not necessarily have a direct correspondence has been observed previously, both with PTH and with other hormones. Kenakin and Onaran (*50*) have addressed this seeming paradox. The reason is likely that a ligand may bind preferentially to any of a large number of receptor conformations that have varying degrees of bioactivity efficacy, in this case the ability to bind a Gα complex and thus eventually stimulate adenylyl cyclase activity. Thus, although a PTH analogue might compete relatively poorly against the binding of the particular PTH tracer used for the binding assay, the PTH−receptor complex that forms can be much more efficient in AC stimulation. To make the comparison between the 1−28 analogues in terms of such an analysis, we have recast the data in Table

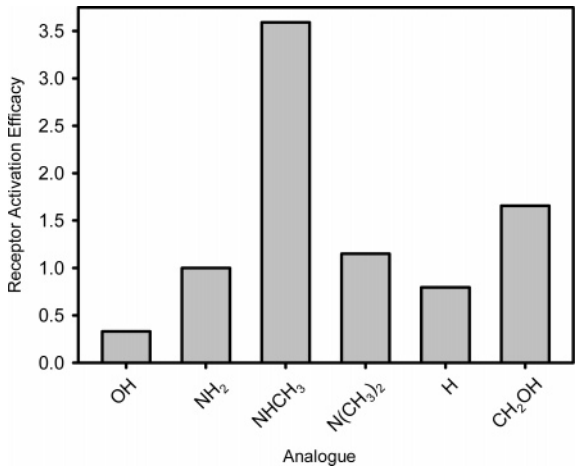

FIGURE 6: Bioactivity data from Figure 4 and Table 4 cast in terms of an efficacy, defined here as the ratio of relative AC stimulating to binding activities.

4 in terms of an arbitrary efficiency function. The function is defined as the relative bioactivity divided by the relative binding, where the reference analogue is hPTH(1−28)NH_2 (Figure 6). In these terms, the analogues with the lower binding affinities actually form the most efficient complex with the receptor, simply because all of the analogues exhibit quite similar AC stimulating activities. Thus, in terms of affinities, the analogues are in the following order: $NHCH_3$ > CH_2OH > NH_2 > $N(CH_3)_2 \approx$ H > OH. In terms of AC stimulation alone, only the carboxyl analogue has an activity significantly higher than the remaining ones. However, in terms of efficiency as defined above, the C-terminal analogues are in the following order: $NHCH_3$ > CH_2OH > $N(CH_3)_2$ > NH_2 > H > OH (Figure 6). This sort of analysis implies that the effectiveness of an analogue at the receptor level is a complex function of its binding and the exact conformation of the complex when bound. A tight-binding analogue may function better than a weak one even if the weaker-binding one generates a complex with more activity.

SUPPORTING INFORMATION AVAILABLE

Synthesis of C-terminally modified hPTH analogues (Table 1), CD spectra of hPTH analogues (Figure 1), and CD spectra of C-terminally modified hPTH analogues (Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Whitfield, J. F., Morley, P., and Willick, G. E. (1998) in *The Parathyroid Hormone: An Unexpected Bone Builder for Treating Osteoporosis*, Landes, Austin, TX.
2. Neugebauer, W., Barbier, J. R., Sung, W. L., Whitfield, J. F., and Willick, G. E. (1995) Solution structure and adenylyl cyclase stimulating activities of C-terminal truncated human parathyroid hormone analogues, *Biochemistry 34*, 8835−8842.
3. Lee, V. H. (1988) Enzymatic barriers to peptide and protein absorption, *Crit. Rev. Ther. Drug Carrier Syst. 5*, 69−97.
4. Gensure, R. C., Gardella, T. J., and Juppner, H. (2005) Parathyroid hormone and parathyroid hormone-related peptide, and their receptors, *Biochem. Biophys. Res. Commun. 328*, 666−668.
5. Rixon, R. H., Whitfield, J. F., Gagnon, L., Isaacs, R. J., Maclean, S., Chakravarthy, B., Durkin, J. P., Neugebauer, W., Ross, V., Sung, W., and Willick, G. E. (1994) Parathyroid hormone fragments may stimulate bone growth in ovariectomized rats by activating adenylyl cyclase, *J. Bone Miner. Res. 9*, 1179−1189.
6. Whitfield, J. F., Morley, P., Willick, G. E., Isaacs, R. J., MacLean, S., Ross, V., Barbier, J. R., Divieti, P., and Bringhurst, F. R. (2000) Lactam formation increases receptor binding, adenylyl cyclase stimulation and bone growth stimulation by human parathyroid hormone (hPTH(1−28)NH_2), *J. Bone Miner. Res. 15*, 964−970.
7. Condon, S. M., Morize, I., Darnbrough, S., Burns, C. J., Miller, B. E., Uhl, J., Burke, K., Jariwala, N., Locke, K., Krolikowski, P. H., Kumar, N. V., and Labaudiniere, R. F. (2000) The bioactive conformation of human parathyroid hormone. Structural evidence for the extended helix postulate, *J. Am. Chem. Soc. 122*, 3007−3014.
8. Condon, S. M., Darnbrough, S., Burns, C. J., Bobko, M. A., Morize, I., Uhl, J., Jariwala, N. U., Burke, K., and Labaudiniere, R. F. (2002) Analogues of human parathyroid hormone (1−31)-NH_2: Further evaluation of the effect of conformational constraint on biological activity, *Bioorg. Med. Chem. 10*, 731−736.
9. Bisello, A., Nakamoto, C., Rosenblatt, M., and Chorev, M. (1997) Mono- and bicyclic analogs of parathyroid hormone-related protein. 1. Synthesis and biological studies, *Biochemistry 36*, 3293−3299.
10. Barbier, J. R., Neugebauer, W., Morley, P., Ross, V., Soska, M., Whitfield, J. F., and Willick, G. (1997) Bioactivities and secondary structures of constrained analogues of human parathyroid hormone: Cyclic lactams of the receptor binding region, *J. Med. Chem. 40*, 1373−1380.
11. Barbier, J. R., MacLean, S., Morley, P., Whitfield, J. F., and Willick, G. E. (2000) Structure and activities of constrained analogues of human parathyroid hormone and parathyroid hormone-related peptide: Implications for receptor-activating conformations of the hormones, *Biochemistry 39*, 14522−14530.
12. Tregear, G. W., Van Rietschoten, J., Greene, E., Keutmann, H. T., Niall, H. D., Reit, B., Parsons, J. A., and Potts, J. T., Jr. (1973) Bovine parathyroid hormone: Minimum chain length of synthetic peptide required for biological activity, *Endocrinology 93*, 1349−1353.
13. Jouishomme, H., Whitfield, J. F., Chakravarthy, B., Durkin, J. P., Gagnon, L., Isaacs, R. J., MacLean, S., Neugebauer, W., Willick, G., and Rixon, R. H. (1992) The protein kinase-C activation domain of the parathyroid hormone, *Endocrinology 130*, 53−60.
14. Neugebauer, W., Gagnon, L., Whitfield, J., and Willick, G. E. (1994) Structure and protein kinase C stimulating activities of lactam analogues of human parathyroid hormone fragment, *Int. J. Pept. Protein Res. 43*, 555−562.
15. Klaus, W., Dieckmann, T., Wray, V., Schomburg, D., Wingender, E., and Mayer, H. (1991) Investigation of the solution structure of the human parathyroid hormone fragment (1−34) by ^{1}H NMR spectroscopy, distance geometry, and molecular dynamics calculations, *Biochemistry 30*, 6936−6942.
16. Chen, Z., Xu, P., Barbier, J. R., Willick, G., and Ni, F. (2000) Solution structure of the osteogenic 1−31 fragment of the human parathyroid hormone, *Biochemistry 39*, 12766−12777.
17. Neugebauer, W., Surewicz, W. K., Gordon, H. L., Somorjai, R. L., Sung, W., and Willick, G. E. (1992) Structural elements of human parathyroid hormone and their possible relation to biological activities, *Biochemistry 31*, 2056−2063.
18. Gardella, T. J., Wilson, A. K., Keutmann, H. T., Oberstein, R., Potts, J. T., Jr., Kronenberg, M., and Nussbaum, S. R. (1993) Analysis of parathyroid hormone's principal receptor-binding region by site-directed mutagenesis and analog design, *Endocrinology 132*, 2024−2030.
19. Potetinova, Z. V., Milgotina, E. I., Makarov, V. A., and Voiushina, T. L. (2001) Synthesis of modified peptides with C-terminal α-amino aldehydes, *Russ. J. Bioorg. Chem. 27*, 141−150.
20. Ede, N. J., Eagle, S. N., Wickham, G., Bray, A. M., Warne, B., Shoemaker, K., and Rosenberg, S. (2000) Solid phase synthesis of peptide aldehyde protease inhibitors. Probing the proteolytic sites of hepatitis C virus polyprotein, *J. Pept. Sci. 6*, 11−18.
21. Takasu, H., Guo, J., and Bringhurst, F. R. (1999) Dual signaling and ligand selectivity of the human PTH/PTHrP receptor, *J. Bone Miner. Res. 14*, 11−20.
22. Shimizu, N., Guo, J., and Gardella, T. J. (2001) Parathyroid hormone (PTH)-(1−14) and -(1−11) analogs conformationally constrained by α-aminoisobutyric acid mediate full agonist responses via the juxtamembrane region of the PTH-1 receptor, *J. Biol. Chem. 276*, 49003−49012.
23. Barbier, J. R., Gardella, T. J., Dean, T., Maclean, S., Potetinova, Z., Whitfield, J. F., and Willick, G. E. (2005) 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, *J. Biol. Chem. 280*, 23771−23777.
24. Yang, J. T., Wu, C.-S., and Martinez, H. M. (1986) Calculation of protein conformation from circular dichroism, *Methods Enzymol. 130*, 208−269.
25. Munoz, V., and Serrano, L. (1995) Elucidating the folding problem of helical peptides using empirical parameters. II. Helix macrodipole effects and rational modification of the helical content of natural peptides, *J. Mol. Biol. 245*, 275−296.
26. Buttner, K., Pinilla, C., Appel, J. R., and Houghten, R. A. (1992) Anomalous reversed-phase high-performance liquid chromatographic behavior of synthetic peptides related to antigenic helper T cell sites, *J. Chromatogr. 625*, 191−198.
27. Zhou, N. E., Mant, C. T., and Hodges, R. S. (1990) Effect of preferred binding domains on peptide retention behavior in reversed-phase chromatography: Amphipathic α-helices, *Pept. Res. 3*, 8−20.
28. Bodkin, M. J., and Goodfellow, J. M. (1995) Competing interactions contributing to α-helical stability in aqueous solution, *Protein Sci. 4*, 603−612.
29. Scholtz, J. M., Qian, H., Robbins, V. H., and Baldwin, R. L. (1993) The energetics of ion-pair and hydrogen-bonding interactions in a helical peptide, *Biochemistry 32*, 9668−9676.
30. Peggion, E., Mammi, S., Schievano, E., Schiebler, L., Corich, M., Rosenblatt, M., and Chorev, M. (2003) Structure−function relationship studies of bovine parathyroid hormone (bPTH(1−34)) analogues containing α-amino-iso-butyric acid (Aib) residues, *Biopolymers 68*, 437−457.
31. Cohen, F. E., Strewler, G. J., Bradley, M. S., Carlquist, M., Nilsson, M., Ericsson, M., Ciardelli, T. L., and Nissenson, R. A. (1991) Analogues of parathyroid hormone modified at positions 3 and 6. Effects on receptor binding and activation of adenylyl cyclase in kidney and bone, *J. Biol. Chem. 266*, 1997−2004.
32. Shimizu, N., Dean, T., Khatri, A., and Gardella, T. J. (2004) Amino-Terminal Parathyroid Hormone Fragment Analogs Containing α,α-di-alkyl Amino Acids at Positions 1 and 3, *J. Bone Miner. Res. 19*, 2078−2086.
33. Schievano, E., Mammi, S., Carretta, E., Fiori, N., Corich, M., Bisello, A., Rosenblatt, M., Chorev, M., and Peggion, E. (2003) Conformational and biological characterization of human parathyroid hormone hPTH(1−34) analogues containing *β*-amino acid residues in positions 17−19, *Biopolymers 70*, 534−547.
34. Cornell, W. D., Cieplak, P., Bayly, C. I., Gould, I. R., Merz, K. M., Jr., Ferguson, D. M., Spellmeyer, D. C., Fox, T., Caldwell, J. W., and Kollman, P. A. (1995) A Second Generation Force Field for the Simulation of Proteins, Nucleic Acids, and Organic Molecules, *J. Am. Chem. Soc. 117*, 5179−5197.
35. Wieprecht, T., Rothemund, S., Bienert, M., and Krause, E. (2001) Role of helix formation for the retention of peptides in reversed-phase high-performance liquid chromatography, *J. Chromatogr., A 912*, 1−12.
36. Wray, V., Nokihara, K., and Naruse, S. (1998) Solution structure comparison of the VIP/PACAP family of peptides by NMR spectroscopy, *Ann. N.Y. Acad. Sci. 865*, 37−44.
37. Chang, X., Keller, D., Bjørn, S., and Led, J. J. (2001) Structure and folding of glucagon-like peptide-1-(7−36)-amide in aqueous trifluoroethanol studied by NMR spectroscopy, *Magn. Reson. Chem. 39*, 477−483.
38. Solomon, T. E., Walsh, J. H., Bussjaeger, L., Zong, Y., Hamilton, J. W., Ho, F. J., Lee, T. D., and Reeve, J. R., Jr. (1999) COOH-terminally extended secretins are potent stimulants of pancreatic secretion, *Am. J. Physiol. 276*, G808−G816.
39. Sumi, S., Nagawa, F., Hayashi, T., Amagase, H., and Suzuki, M. (1984) Overproduction of a gastrointestinal hormone, secretin, in *Escherichia coli* cells and its chemical characterization, *Gene 29*, 125−134.
40. Wettergren, A., Pridal, L., Wojdemann, M., and Holst, J. J. (1998) Amidated and non-amidated glucagon-like peptide-1 (GLP-1): Non-pancreatic effects (cephalic phase acid secretion) and stability in plasma in humans, *Regul. Pept. 77*, 83−87.
41. Nachman, R. J., Coast, G. M., Holman, G. M., and Beier, R. C. (1995) Diuretic activity of C-terminal group analogues of the insect kinins in *Acheta domesticus*, *Peptides 16*, 809−813.
42. Sforca, M. L., Oyama, S., Jr., Canduri, F., Lorenzi, C. C., Pertinhez, T. A., Konno, K., Souza, B. M., Palma, M. S., Ruggiero Neto, J., Azevedo, W. F., Jr., and Spisni, A. (2004) How C-terminal carboxyamidation alters the biological activity of peptides from the venom of the eumenine solitary wasp, *Biochemistry 43*, 5608−5617.
43. Core, L. J., Ishikawa, S., and Perego, M. (2001) A free terminal carboxylate group is required for PhrA pentapeptide inhibition of RapA phosphatase, *Peptides 22*, 1549−1553.
44. Woo, J.-T., Sigeizumi, S., Yamaguchi, K., Sugimoto, K., Kobori, T., Tsuji, T., and Kondo, K. (1995) Peptidyl aldehyde derivatives as potent and selective inhibitors of cathepsin l, *Biorg. Med. Chem. Lett. 5*, 1501−1504.
45. Geraghty, R. F., Irvine, G. B., Williams, C. H., and Cottrell, G. A. (1994) Biological activity and receptor binding properties of some C-terminally modified analogues of FMRFamide, *Peptides 15*, 73−81.
46. Stennicke, H. R., Mortensen, U. H., and Breddam, K. (1996) Studies on the hydrolytic properties of (serine) carboxypeptidase Y, *Biochemistry 35*, 7131−7141.
47. Mortensen, U. H., Raaschou-Nielsen, M., and Breddam, K. (1994) Recognition of C-terminal amide groups by (serine) carboxypeptidase Y investigated by site-directed mutagenesis, *J. Biol. Chem. 269*, 15528−15532.
48. Morley, P., Whitfield, J. F., and Willick, G. E. (1999) Design and applications of parathyroid hormone analogues, *Curr. Med. Chem. 6*, 1095−1106.
49. Barbier, J. R., MacLean, S., Whitfield, J. F., Morley, P., and Willick, G. E. (2001) Structural requirements for conserved arginine of parathyroid hormone, *Biochemistry 40*, 8955−8961.
50. Kenakin, T., and Onaran, O. (2002) The ligand paradox between affinity and efficacy: Can you be there and not make a difference? *Trends Pharmacol. Sci. 23*, 275−280.

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