Structure and Activities of Constrained Analogues of Human Parathyroid Hormone and Parathyroid Hormone-Related Peptide: Implications for Receptor-Activating Conformations of the Hormones

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ABSTRACT: Parathyroid hormone (PTH) has a helix−bend−helix structure in solution. Part of the C-terminal helix, residues 21−31, is amphiphilic and forms a critical receptor-binding region. Stabilization of this α-helix by lactam formation between residues spaced *i*, *i* + 4 on the polar face was previously reported to increase adenylyl cyclase-stimulating (AC) activity if between residues 22 and 26 but to diminish it if between residues 26 and 30 [Barbier et al. (1997) *J. Med. Chem. 40*, 1373−1380]. This work reports the effects of other cyclizations on the polar face, differing in ring size or position, on α-helix conformation, as measured by circular dichroism, and on AC-stimulating activity. All analogues cyclized between residues 22 and 26 had at least a 1.5-fold increase in activity, suggesting an α-helical structure between about residues 21 and 26. Cyclization between residues 25 and 29 or residues 26 and 30 diminished activity by 20−30%, despite stabilizing α-helix, suggesting that residues 25−31 bind to the receptor in a helical, but not classical α-helical, conformation. Analogues cyclized between residues 13 and 17 had slightly increased activity. A bicyclic analogue, with lactams between residues 13 and 17 and residues 22 and 26, had about the same activity as that cyclized only between 22 and 26. Parathyroid hormone-related peptide (PTHrP) may bind in a manner similar to the common receptor, but hydrophobic moment calculations suggest that it must bind as a tighter helix in order to optimally present its hydrophobic residues to the receptor. Both PTHrP analogues cyclized between either residues 22 and 26 or residues 26 and 30 had more stable α-helices but reduced AC activities, consistent with this hypothesis.

Parathyroid hormone (PTH)[1] is an endocrine hormone, functioning principally to regulate the extracellular calcium concentration in animals. It can also mimic the various functions of the locally acting parathyroid hormone-related peptide (PTHrP) via their shared PTHR1 (type I PTH/PTHrP) receptor (*1*). This regulation is effected through actions on kidney, intestine, and bone cell receptors. If injected in a pulsatile fashion, PTH has a powerful anabolic effect on bone, an effect that depends on its ability to stimulate adenylyl cyclase in osteoblast receptors (*2*). The N-terminal 28 residues of PTH are required for full AC stimulation (*3*, *4*), with loss of residue 28 resulting in a dramatic loss of activity (*3*, *4*). Full activity of the shorter analogues also requires a C-terminal amide (*4*). The shortest fully osteogenic linear PTH has been identified as hPTH(1−30)NH_2 (*5*), with shorter linear fragments 1−28 and 1−29 being only weakly osteogenic. PTHrP has also been reported to have osteogenic activity (*6*), but its 1−31 fragment, unlike that of PTH, was not active in the ovariectomized rat model of osteoporosis (*7*).

The PTHR1 receptor was identified in opossum kidney in 1991 (*1*), and a closely related one was later isolated from rat osteoblasts (*8*). The major receptor-binding region of PTH was shown to reside in residues 14−34 (*9*), and within this sequence is an α-helical region, residues 17−29, identified in both NMR (*10*−*12*) and CD studies (*4*, *13*). Part of this α-helix, residues 20−34, is amphiphilic, and its hydrophobic face has been postulated to bind to the receptor. In support of this, it has been found that hydrophobic residues in this sequence are especially sensitive to mutation (*14*, *15*), while those on the polar residues can be mutated much more freely without loss of activity. A second short α-helix, between about residues 3 and 9, has been identified in NMR studies of hPTH(1−34) (*10*) and hPTH(1−37) (*12*).

Certain side-chain interactions may be important for stabilizing α-helices, including ion pair formation between residues spaced *i*, *i* + 4 apart (*16*). Natural such ion pairs exist between residues 22 and 26 and residues 26 and 30 in hPTH and between residues 13 and 17 and residues 26 and 30 in hPTHrP (Figure 1). Analogues resulting from the formation of lactams between these residues in hPTH were shown to stabilize an α-helical conformation in the region about the lactam (*17*, *18*) One of these lactam analogues, [Leu^{27}]c(Glu^{22}−Lys^{26})hPTH(1−31)NH_2, has both increased AC-stimulating (*17*) and osteogenic activities (*19*). Furthermore, these same modifications greatly enhance the AC-stimulating activity of hPTH(1−28)NH_2 and make it almost

[1] Abbreviations: CD, circular dichroism; DMF, dimethylformamide; DCM, dichloromethane; GRF, growth hormone-releasing factor; HOBt, hydroxybenzotriazole; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; ROS, rat osteosarcoma; TBTU, *O*-benzotriazolyl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; PyAOP, (7-azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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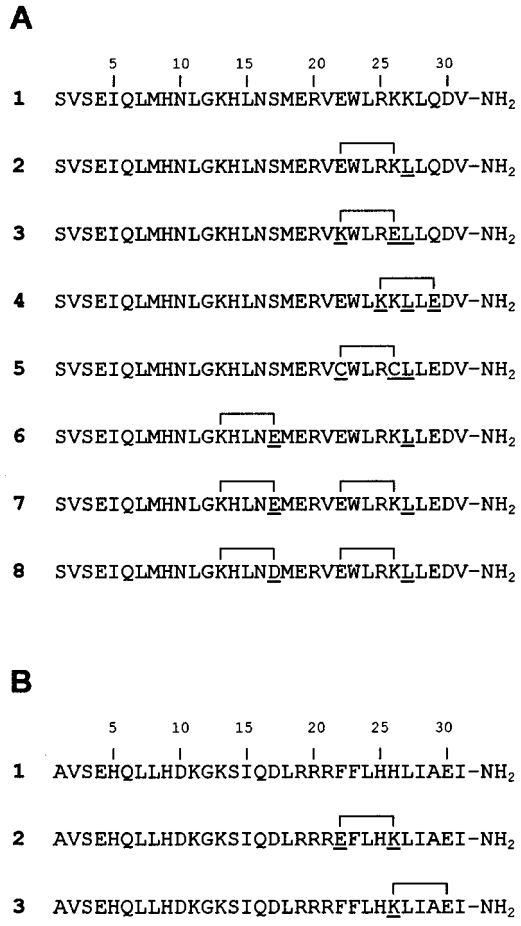

FIGURE 1: Sequences of cyclic analogues described in this paper. Mutated residues are underlined. (A) hPTH analogues: 1, hPTH-(1−31)NH_2; 2, [Leu^{27}]c(Glu^{22}−Lys^{26})hPTH(1−31)NH_2; 3, [Lys^{22},-Glu^{26},Leu^{27}]c(Lys^{22}−Glu^{26})hPTH(1−31)NH_2; 4, [Lys^{25},Glu^{29},-Leu^{27}]c(Lys^{25}−Glu^{29})hPTH(1−31)NH_2; 5, [Cys^{22},Cys^{26},Leu^{27}]c-(Cys^{22}−Cys^{26})hPTH(1−31)NH_2; 6, [Glu^{17},Leu^{27}]c(Lys^{13}−Glu^{17})-hPTH(1−31)NH_2; 7, [Glu^{17},Leu^{27}]c(Lys^{13}−Glu^{17},Glu^{22}−Lys^{26})-hPTH(1−31)NH_2; 8, [Asp^{17},Leu^{27}]c(Lys^{13}−Asp^{17},Glu^{22}−Lys^{26})-hPTH(1−31)NH_2. (B) hPTHrP analogues: 1, hPTHrP(1−31)NH_2; 2, [Glu^{22},Lys^{26}]c(Glu^{22}−Lys^{26})hPTHrP(1−31)NH_2; 3, [Lys^{26}]c-(Lys^{26}−Glu^{30})hPTHrP(1−31)NH_2.

fully osteogenic in the ovariectomized rat model (*20*). We posed the question as to whether all lactams formed between residues on the polar face retain at least major activity, consistent with a postulated mode of receptor binding wherein the region between residues 20 and 31 binds as an α-helix. Aside from those involving natural sequences, such lactams require sequence mutations that must also be analyzed to separate effects arising from those of the linear mutations alone and effects due to conformational restraint imposed by the lactam. The evidence presented here suggests only part of the 21−31 region of hPTH(1−31)NH_2 binds as a classical α-helix, the rest likely binding as some type of distorted helix.

MATERIALS AND METHODS

Chemicals. N-α-Fmoc-(*S*)-*p*-methoxytrityl-L-cysteine [Fmoc-Cys(Mmt)] was obtained from NovaBiochem, and other Fmoc-amino acids were from NovaBiochem or Peptides International.

Analogues of hPTH(1−31)NH_2 or hPTHrP(1−31)NH_2 (Figure 1) were synthesized on a MilliGen 9050 continuous-flow peptide synthesizer, with TentaGel S RAM (Rapp Polymere, Tübingen, Germany) as the support and Fmoc chemistry (*21*), as previously described (*17*). Lactams involving residue pairs in the sequence region 22−31 were formed after residue 18 had been added. The lactam between residues 13 and 17 was synthesized after completion of the entire sequence. They were formed as described previously but with PyAOP/HOBt as the activator. Peptides were cleaved from the support with either TFA/phenol/water/thioanisole/ethanedithiol (18.2/1.125/1.125/1.125/0.56 v/v/v/v/v) or TFA/phenol/water/triisopropylsilane (88/5/5/2 v/v/v/v) and were purified to >95% purity by gradient HPLC (0.1% TFA/water with an acetonitrile gradient of 1%/min on a 1 × 25 cm Vydac C18 column). Masses were confirmed with a PE Sciex electrospray mass spectrometer or a MALDI-TOF mass spectrometer. Correct lactam formation was confirmed by amino acid sequencing. Cys residues were coupled by a modified procedure to avoid racemization. *N*-α-Fmoc-(*S*)-*p*-methoxytrityl-l-cysteine [Fmoc-Cys (Mmt)] was coupled with TBTU/HOBt (0.52 M) and 2.46 M collidine in DMF/DCM (1:1), as described by Han et al. (*22*). Disulfides were formed by iodine oxidation (*23*). The purified reduced peptide in 0.5 mL of HOAc/water (4:1) was treated with I_2 (10 equiv) for 15 h at room temperature. No Met or Trp oxidation was observed under these experimental conditions.

Bioassays. AC-stimulating activities of rat osteosarcoma (ROS 17/2) cells were determined as described previously (*24*). AC activities were expressed as percent of the maximum stimulation by hPTH(1−34)-NH_2 or [Leu^{27}]c-(Glu^{22}−Lys^{26})hPTH(1−31)NH_2 observed in a concurrent experiment. Data were averaged from a minimum of three independent experiments, and the data were fitted with a three-constant sigmoidal function (SigmaPlot, SPSS software). The ED_{50} value, the concentration of analogue that corresponds to the point of half-maximal stimulation, was then evaluated and the error was estimated from the corresponding fit to the error extremities for each data point.

Circular Dichroism Spectroscopy. Spectra were obtained on a Jasco J-600 spectropolarimeter at 20−22 °C. At least four spectra were averaged, and the data were smoothed by the Jasco software. The instrument was calibrated with ammonium (+)-10-camphorsulfonate. Peptides were dissolved in water, and sodium phosphate buffer, pH 7.2, was added to a final concentration of 25 mM. Peptide concentrations were calculated from the absorption at 280 nm, using an extinction coefficient of 5700 M^{-1}. Concentrations of hPTHrP analogues were determined from areas under the HPLC peak, with hPTH(1−31)NH_2 as reference standard. Data are expressed per peptide bond. Residues as α-helix were calculated from the value of θ_{222}, using a value of −28 000 deg·cm^2·$dmol^{-1}$ as the ellipticity per bond (*13*, *25*).

RESULTS

Lactams of [Leu^{27}]hPTH(1−31)NH$_2$. We have described previously the bioactivities of a cyclic analogue of hPTH-(1−31), [Leu^{27}]c(Glu^{22}−Lys^{26})hPTH(1−31)NH_2. This peptide has dramatically improved properties in both AC signaling and increasing of trabecular thickness in an ovariectomized rat model for osteoporosis (*17*, *19*, *26*). Recently, we reported that [Leu^{27}]c(Glu^{22}−Lys^{26})hPTH(1−

Table 1: Mass and AC-Stimulating Activities of Cyclic Analogues of hPTH

analogue	mass (observed)	mass (expected)	activity $ED_{50\%}$ (nM)
$[L^{27}]hPTH(1-31)NH_2$	3702.34[b]	3704.3	11.5 (±5.2)[a]
$[L^{27}]c(E^{22-}K^{26})hPTH(1-31)NH_2$	3685.46 (±0.46)	3685.1	7.5 (±0.6)
$[K^{22},E^{26},L^{27}]hPTH(1-31)NH_2$	3704.19[b]	3703.1	15.8 (±2.9)
$[K^{22},E^{26},L^{27}]c(K^{22-}E^{26})$ $hPTH(1-31)NH_2$	3684.84 (±0.47)	3685.1	6.5 (±0.3)
$[L^{27}]hPTH(1-29)NH_2$	3487.85 (±1.08)	3489.2	13.0 (±0.7)
$[L^{27}]c(E^{22-}K^{26})hPTH(1-29)NH_2$	3470.59 (±0.80)	3471.2	6.8 (±1.4)
$[L^{27}]hPTH(1-28)NH_2$	3360.75 (±0.51)	3361.1	19.1 (±3.1)
$[L^{27}]c(E^{22-}K^{26})hPTH(1-28)NH_2$	3342.63 (±0.50)	3341.8	8.3 (±1.0)
$[C^{22},C^{26},L^{27}]hPTH(1-31)NH_2$	3652.45 (±0.42)	3652.1	26.7 (±5.3)
$[C^{22},C^{26},L^{27}]c(C^{22-}C^{26})$ $hPTH(1-31)NH_2$	3649.71 (±0.61)	3650.1	15.9 (±0.7)
$[L^{27},E^{29}]hPTH(1-31)NH_2$	3704.19[b]	3704.1	21.5 (±1.1)
$[L^{27},K^{25},E^{29}]hPTH(1-31)NH_2$	3675.53[b]	3675.1	29.0 (±3.6)
$[L^{27},K^{25},E^{29}]c(K^{25-}E^{29})$ $hPTH(1-31)NH_2$	3659.3[b]	3658.1	35.8 (±9.0)
$[E^{17},L^{27}]hPTH(1-31)NH_2$	3745.0 (±0.52)	3745.2	13.9 (±2.8)
$[E^{17},L^{27}]c(K^{13-}E^{17})$ $hPTH(1-31)NH_2$	3727.30 (±0.30)	3727.2	12.3 (±2.5)
$[D^{17},L^{27}]c(K^{13-}D^{17},E^{22-}K^{26})$ $hPTH(1-31)NH_2$	3693.09 (±1.98)	3694.1	24.2 (±3.1)
$[E^{17},L^{27}]c(K^{13-}E^{17},E^{22-}K^{26})$ $hPTH(1-31)NH_2$	3709.60 (±0.46)	3710.4	8.0 (±0.7)

[a] Reference *17*. [b] Data from MALDI-TOF mass spectrum.

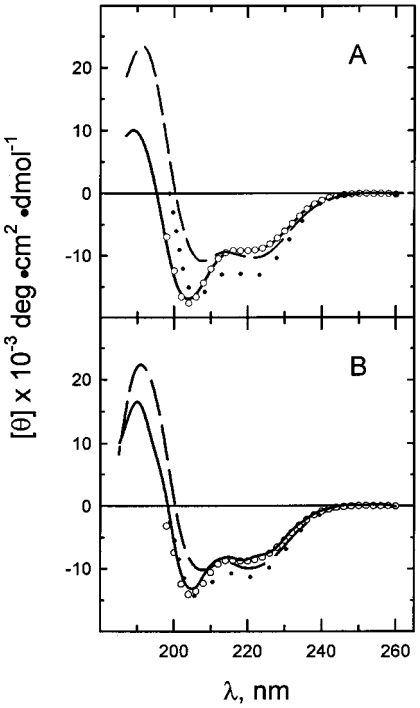

FIGURE 2: CD spectra of C-terminal deletion analogues of $[Leu^{27}]c(Glu^{22-}Lys^{26})hPTH(1-31)NH_2$. (A) Spectra of $hPTH(1-31)NH_2$, 119 μM (—) and 13 μM (○), and of [Leu27]c(Glu22−Lys26)hPTH(1−31)NH2, 123 μM (− −) and 12 μM (●). (B) Spectra of $[Leu^{27}]hPTH(1-28)NH_2$, 81 μM (—), of $[Leu^{27}]hPTH(1-28)NH_2$, 17 μM (○), and of $[Leu^{27}]c(Glu^{22-}Lys^{26})hPTH(1-28)NH_2$, 104 μM (− − −) and 16 μM (●).

$28)NH_2$ has AC-stimulating activity and osteogenic activity in the rat OVX model increased to levels comparable to $hPTH(1-31)NH_2$ or $hPTH(1-34)$ (*20*). The AC-stimulating activities of $[Leu^{27}]c(Glu^{22-}Lys^{26})hPTH(1-29)NH_2$ and $[Leu^{27}]c(Glu^{22-}Lys^{26})hPTH(1-28)NH_2$ are shown in Table 1, and the CD spectrum of $[Leu^{27}]c(Glu^{22-}Lys^{26})hPTH(1-28)NH_2$ is shown in Figure 2. The effect of the cyclization and substitution of Leu-27 is to increase the AC-stimulating activity of the linear 1−28 and 1−29 analogues (*4*) about 5-fold to levels approaching that of $[Leu^{27}]c(Glu^{22-}Lys^{26})hPTH(1-31)NH_2$ (Table 1). It also dramatically increases the binding affinity of the 1−28 analogue for the human receptors expressed in porcine kidney cells (*20*). The corresponding CD spectra (Figure 2) demonstrate a substantial increase in α-helix, similar to that observed with $[Leu^{27}]c(Glu^{22-}Lys^{26})hPTH(1-31)NH_2$ compared to $hPTH(1-31)NH_2$ (*17*). The CD spectra of the cyclic analogues of these 28- and 29-residue analogues and of $[Leu^{27}]c(Glu^{22-}Lys^{26})hPTH(1-31)NH_2$ also show a pronounced concentration dependence, in contrast to the linear ones having a Leu-27 substitution (Figure 2). Thus, the concentration dependency must result from stabilization of the α-helix by the 22−26 lactam. The apparent amount of α-helix of the cyclized form at high concentration (104 μM), 10 residues, is actually less than observed at a lower concentration (16 μM), 11 residues, but the spectrum is much more like that of an ideal α-helix (*25*), as implied by the $\theta_{222}/\theta_{209}$ ratio being closer to 1. These data are consistent with a relatively restricted region of the sequence having a conformation closer to the idealized α-helix. A theoretical study has demonstrated that the CD spectrum is highly sensitive to the φ, ψ angles of the peptide and that a minimum of 7 residues in an α-helical configuration is necessary to give its idealized spectrum (*27*). These considerations suggest that residues 17−28 are stabilized as α-helix by the presence of the lactam. This analysis ignores the weak contribution of the rest of the molecule to $[\theta]_{222}$. Although NMR evidence indicates a short α-helix between residues 3 and 9 in hPTH(1−37) or hPTH(1−34) (*28*), no evidence was found for this structure in the CD spectra of hPTH fragments, particularly hPTH(1−19) (*13*). The lack of CD evidence for this short helix is consistent with the known dependency of helical signal per residue on the number of helical residues (*25*). Helices shorter than about nine residues are not expected to have any characteristic helical spectrum (*27*). The dimerization likely has no importance in terms of PTH function because the concentration at which it is observed is at least 1000-fold higher than biological concentrations. However, it can lead to misinterpreting spectral results in terms of biologically important conformations. Two further possible complications exist in interpreting the CD spectra. These are possible contributions of the single Trp near 225 nm and a contribution from the lactam. In earlier work, we found a strong CD in the 230−300 nm spectral range in the lactam analogue $c(Lys^{26-}Asp^{30})$-

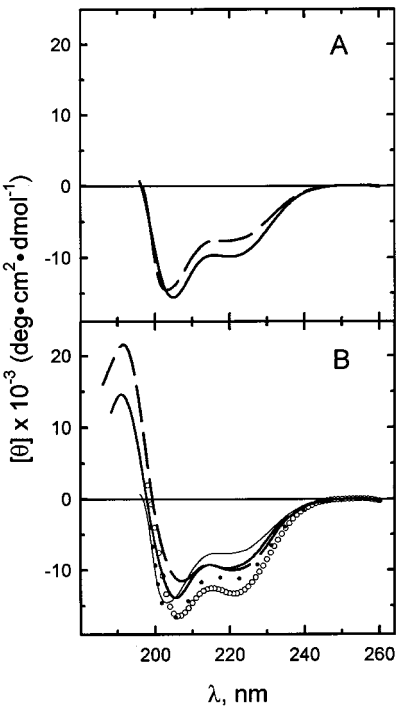

FIGURE 3: (A) CD spectrum of [Lys^{22},Glu^{26},Leu^{27}]hPTH(1−31)-NH_2 at 12 μM (− − −) compared with that of [Leu^{27}]hPTH-(1−31)NH_2 at 13 μM (—). (B) CD spectra of [Lys^{22},Glu^{26},Leu^{27}]c-(Lys^{22}−Glu^{26})hPTH(1−31)NH_2 at 84 μM (− − −) and 12 μM (●) and of [Lys^{22},Glu^{26},Leu^{27}]hPTH(1−31)NH_2 at 82 μM (thick solid line) and 15 μM (thin solid line). The spectrum of [L^{27}]c-(E^{22}−K^{26})hPTH(1−31)NH_2 at 12 μM has also been included for comparison (○).

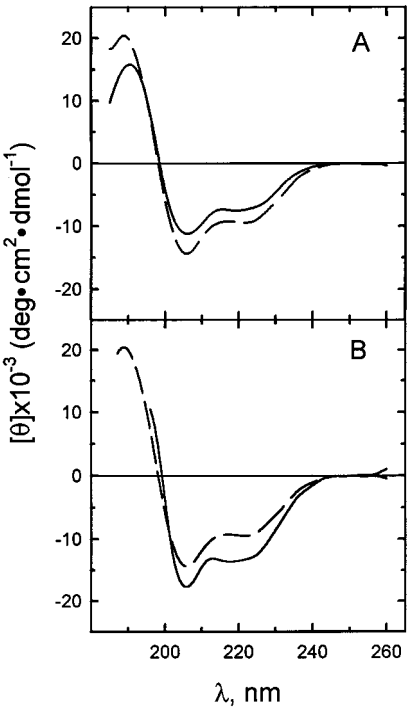

FIGURE 4: (A) CD spectra of [Leu^{27}]hPTH(1−31)NH_2, 95 μM (—), and of [Lys^{25},Leu^{27},Glu^{29}]hPTH(1−31)NH_2, 60 μM (− − −). (B) CD spectra of [Lys^{25},Leu^{27},Glu^{29}]hPTH(1−31)NH_2, 60 μM (− − −), and of [Lys^{25},Leu^{27},Glu^{29}]c(Lys^{25}−Glu^{29})hPTH(1−31)-NH_2 at 47 μM (—).

hPTH(20−34)NH_2 (*18*). However, we did not find a significant spectrum when we later examined [Leu^{27}]c(Glu^{22}−Lys^{26})hPTH(1−31)NH_2. One model for the single lactam contribution is the CD of alkyl β-lactams (*29*). The possible contribution, using this model, is less than 200 deg cm^2/dmol at 220 nm. We have ignored both of these contributions as minor ones that do not affect the conclusions derived from the spectra.

In an earlier study with a multicyclic polypeptide model compound, Ösapay and Taylor (*30*) reported that Lys^i,Glu^{i+4} lactams were only weakly helix-stabilizing, in contrast to Glu^i,Lys^{i+4} ones. The analogue [Lys^{22},Glu^{26},Leu^{27}]c(Lys^{22}−Glu^{26})hPTH(1−31)NH_2 is the same as [Leu^{27}]c(Glu^{22}−Lys^{26})hPTH(1−31)NH_2 but with residues 22 and 26 reversed (Figure 1). This analogue had the same AC-stimulating activity as [Leu^{27}]c(Glu^{22}−Lys^{26})hPTH(1−31)NH_2 (Table 1). When both were measured at similar low concentrations, the CD spectra showed that the linear analogue is less helical, as judged by its θ_{222} value ($\theta_{222} = -7500$) and a blue shift from 205 to 203 nm of the lower wavelength minimum, compared to [Leu^{27}]hPTH(1−31)NH_2 ($\theta_{222} = -9800$) (Figure 3A). In either analogue, ionic interactions between the Glu-22 and Lys-26 side chains are expected to stabilize the α-helix by about 0.5 kcal/mol (*31*). However, the effect of interchanging the Glu-22 and Lys-26 residues is to lose the stabilization of the Ser-17 to Gln-29 α-helix (*12*) resulting from the side-chain interactions of Glu-22 and Lys-26 with the helix macrodipole (*32, 33*). The CD spectra of both the linear and cyclic analogues of [Lys^{22},Glu^{26},Leu^{27}]hPTH(1−31)NH_2 at low and high concentrations indicated a change in conformation to a more helical form at higher concentrations, presumably as a result of dimerization. Comparison of the two 22−26 lactams at the 12 μM concentration showed that the Lys-22 to Glu-26 cyclized analogue had less α-helix (about 12 residues) than the corresponding analogue with the natural sequence (about 14 residues), similar to earlier observations with model peptides (*30*).

Two lactams of *i*, *i* + 4 spaced residues can be formed from the native sequence of PTH, between residues 22 and 26 or residues 26 and 30. Only the analogue with a lactam between residues 22 and 26 had an enhanced AC-stimulating activity (*17*), presumably because it stabilizes a conformation closer to that of the receptor-bound one. If one assumes that residues 21−29 bind with the hydrophobic face of the amphiphilic α-helix interacting with the receptor, then an analogue that has a lactam formed between appropriately substituted residues 25 and 29 of the hydrophilic face should have at least comparable activity to the native sequence. The linear analogue [Lys^{25},Leu^{27},Glu^{29}]hPTH(1−31)NH_2, with an $ED_{50\%}$ of 29 nM, was much less active than [Leu^{27}]hPTH-(1−31)NH_2 ($ED_{50\%}$ 11.5 nM) (Table 1). The lactam analogue [Leu^{27},Lys^{25},Glu^{29}]c(Lys^{25}−Glu^{29})hPTH(1−31)NH_2 had slightly less activity ($ED_{50\%}$ 36 nM) than the linear analogue, similar to the effect of cyclization between residues 26 and 30 (*17*). CD spectra comparing [Leu^{27}]hPTH(1−31)NH_2 and [Lys^{25},Leu^{27},Glu^{29}]hPTH(1−31)NH_2 indicated the latter had more α-helix than the former (Figure 4A), as expected. The mutations Arg25Lys and Gln29Glu are neutral in terms of their single amino acid α-helix stabilizing effects (*34*). However, there are two other competing effects on helical stability resulting from the substitution of Glu for Gln at position 29. The substitution has a negative effect on the stability of the helix resulting from an unfavorable interaction with the helix macrodipole and a positive effect resulting

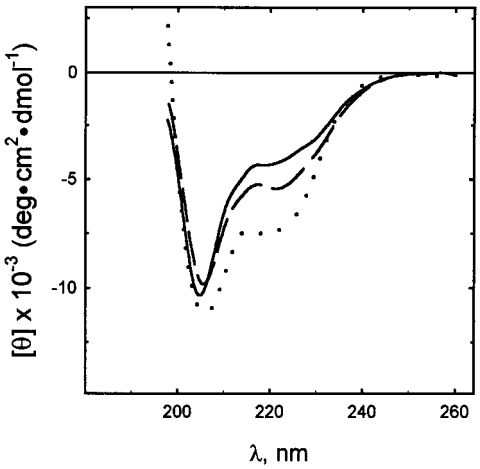

FIGURE 5: CD spectra of [Cys^{22},Cys^{26},Leu^{27}]hPTH(1−31)NH$_2$, 22 μM (− − −), and of [Cys^{22},Cys^{26},Leu^{27}]c(Cys^{22}−Cys^{26})hPTH-(1−31)NH$_2$, 20 μM (—). The spectrum of [Leu^{27}]hPTH(1−31)-NH$_2$ (●) is shown for comparison.

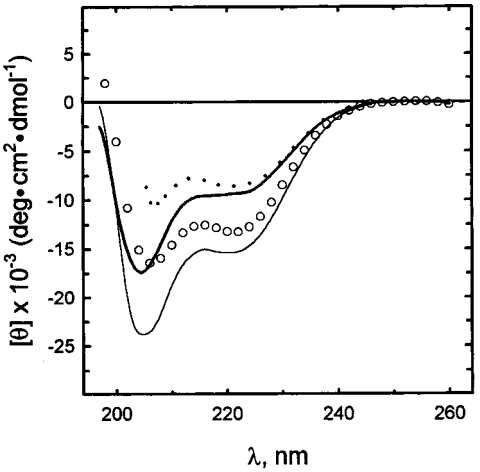

FIGURE 6: CD spectra of [Glu^{17},Leu^{27}]hPTH(1−31)NH$_2$, 12 μM (thin solid line), of [Glu^{17},Leu^{27}]c(Lys^{13}−Glu^{17})hPTH(1−31)NH$_2$, 12 μM (thick solid line), of [Glu^{17},Leu^{27}]c(Lys^{13}−Glu^{17},Glu^{22}−Lys^{26})hPTH(1−31)NH$_2$, 2.6 μM (●), and of [Leu^{27}]c(Glu^{22}−Lys^{26})-hPTH(1−31)NH$_2$, 12 μM (●).

from a favorable ion-pair stabilization between Lys-25 and Glu-29. Ion pairs between *i*, *i* + 4 spaced residues have been reported to stabilize α-helices (*16*) by about −0.5 kcal/mol (*31*, *35*). This latter effect is the most important (*32*), resulting in a net stabilization of the α-helix (Figure 4A). Lactam formation resulted in increased α-helix, with 14 residues estimated as α-helix in the cyclic form and 10 in the linear (Figure 4B). The loss of activity seen in the linear analogue suggests that one or both of residues 25 and 29 have at least some interaction with the receptor, and the similar AC stimulation by the cyclic analogue suggests that the conformational constraint afforded by this cyclization is not specifically favorable to receptor binding. Solution structures from NMR data show that the side chains of Arg-25 and Gln-29 are spaced at least 10 Å apart (*28*). If these conformations reflect the receptor-bound one, then lactam constraint would not be expected to have a positive effect on receptor activation, as was observed (Table 1).

Cystine Analogues of hPTH(1−31)NH$_2$. The lactam analogue [Leu^{27}]c(Glu^{22}−Lys^{26}]hPTH(1−31)NH$_2$ has 21 atoms forming its ring, which, although stabilizing an α-helix in the region of the cyclization, still permits considerable conformational freedom. The corresponding cyclic analogue formed when residues 22 and 26 are mutated to Cys and then cyclized contains only 17 atoms in the ring, thus permitting fewer degrees of freedom. The linear analogue, [Cys^{22},Cys^{26},Leu^{27}]hPTH(1−31)NH$_2$, has less activity than [Leu^{27}]hPTH(1−31)NH$_2$. Cyclization of the molecule by oxidation to give [Cys^{22},Cys^{26},Leu^{27}]c(Cys^{22}−Cys^{26})hPTH-(1−31)NH$_2$ significantly increased its activity, from an ED$_{50\%}$ of about 27 nM to 16 nM (Table 1). Replacement of the Glu-22 and Lys-26 residues with Cys is expected to substantially reduce the α-helix stability, according to a structure-based scale of α-helix propensities of amino acids (*36*). This is in agreement with the relative CD spectra of [Leu^{27}]hPTH(1−31)NH$_2$ and [Cys^{22},Cys^{26},Leu^{27}]hPTH(1−31)NH$_2$, the former having eight residues in α-helix compared to about six residues in the latter. Surprisingly, comparison of the CD spectra of the linear and cyclic Cys-22, Cys-26 analogues indicated little difference in their conformations, the linear form having a slightly greater helical content than the cyclic (Figure 5).

Double Cyclic Analogue of [Leu^{27}]hPTH(1−31)NH$_2$. The monocyclic analogue [Glu^{17},Leu^{27}]c(Lys^{13}−Glu^{17})hPTH(1−31)NH$_2$ had about the same activity compared to the linear analogue, in contrast to the increased activity observed in the monocyclic Glu-22 to Lys-26 analogue (Table 1). The double cyclic analogue [Glu^{17},Leu^{27}]c(Lys^{13}−Glu^{17},Glu^{22}−Lys^{26})hPTH(1−31)NH$_2$ had an activity similar to that of [Leu^{27}]c(Glu^{22}−Lys^{26})hPTH(1−31)NH$_2$. The CD spectra of [Glu^{17},Leu^{27}]hPTH(1−31)NH$_2$, [Glu^{17},Leu^{27}]c(Lys^{13}−Glu^{17})-hPTH(1−31)NH$_2$, [Glu^{17},Leu^{27}]c(Glu^{22}−Lys^{26})hPTH(1−31)-NH$_2$, and [Glu^{17},Leu^{27}]c(Lys^{13}−Glu^{17},Glu^{22}−Lys^{26})hPTH-(1−31)NH$_2$ are shown in Figure 6. [Glu^{17},Leu^{27}]hPTH-(1−31)NH$_2$ is substantially more α-helical than [Leu^{27}]-hPTH(1−31)NH$_2$, having an estimated 16 α-helical residues as opposed to 11. Since the Ser17Glu substitution is expected to be neutral in terms of helix stability (*34*, *36*), we surmise that the source of stability for the additional α-helix may be the ionic interaction from the side chains of Lys-13···Glu-17, which is absent in the natural sequence. The spectrum of [Glu^{17},Leu^{27}]c(Glu^{22}−Lys^{26})hPTH(1−31)NH$_2$ was very similar to that of [Leu^{27}]c(Glu^{22}−Lys^{26})hPTH(1−31)NH$_2$ (data not shown). The spectrum of [Glu^{17},Leu^{27}]c(Lys^{13}−Glu^{17})hPTH(1−31)NH$_2$ is similar in shape to but less intense than the spectrum of its linear form and is clearly less helical, as indicated by its $[\theta]_{222}$ value and $[\theta]_{222}/[\theta]_{209}$, than the corresponding analogue where cyclization is between residues 22 and 26. All spectra are at concentrations from 7 to 12 μM, where there is negligible aggregation. The spectrum of the double cyclic is not the sum of the two monocyclics, suggesting an interaction between the two cyclic regions. Its apparent α-helix content, from the $[\theta]_{222}$ value, is about the same as that of the 13−17 lactam analogue but significantly less than that of the 22−26 lactam analogue. The shape of its spectrum is, however, much more α-helical than that of either analogue with the single lactam. NMR data have indicated that PTH has a bend in the region of Lys-13 to Ser-17 (*37*), and this makes the interpretation of the CD results somewhat complicated. Turn structures can have CD spectra somewhat similar to that observed with α-helices, with a weak negative band at 225 nm but with a positive ellipticity near 209 nm (*38*).

Table 2: Mass and AC-Stimulating Activities of Cyclic Analogues of hPTHrP

analogue	mass (observed)	mass (expected)	activity $ED_{50\%}$ (nM)
hPTHrP(1−31)NH_2	3707.26 (±0.27)	3707.3	21.4 (±1.3)
[E^{22},K^{26}]hPTHrP(1−31)NH_2	3680.38 (±0.31)	3679.1	8.2 (±0.9)
[E^{22},K^{26}]c(E^{22}−K^{26}) hPTHrP(1−31)NH_2	3661.31 (±0.41)	3662.3	10.2 (±2.5)
[K^{26}]hPTHrP(1−31)NH_2	3698.14 (±0.47)	3698.1	7.7 (±2.1)
[K^{26}]c(K^{26}−E^{30}) hPTHrP(1−31)NH_2	3679.93 (±0.78)	3680.1	14.6 (±2.0)

Cyclic Analogues of PTHrP. PTHrP(1−31) also has an amphiphilic helical sequence between residues 21 and 31, which we have postulated binds to the receptor in a manner similar to the binding of the PTH amphiphilic helix (*4*). If so, cyclizations on the polar face could have similar effects on α-helix stabilization and AC stimulation to those observed with PTH. To test this possibility, we synthesized [Glu^{22},-Lys^{26}]hPTHrP(1−31)NH_2 and [Lys^{26}]hPTHrP(1−31)NH_2 and their cyclic analogues, [Glu^{22},Lys^{26}]c(Glu^{22}−Lys^{26})-hPTHrP(1−31)NH_2 and [Lys^{26}]c(Lys^{26}−Glu^{30})hPTHrP-(1−31)NH_2 (Figure 1B). The AC-stimulating activities of these analogues as compared to the native hPTHrP are shown in Table 2. Both of the linear analogues, [Glu^{22},Lys^{26}]-hPTHrP(1−31)NH_2 and [Lys^{26}]hPTHrP(1−31)NH_2, are more active than the natural sequence hPTHrP(1−31)NH_2.Lactam formation diminished the AC-stimulating activity of each of these linear analogues, with [Glu^{22},Lys^{26}]hPTHrP(1−31)NH_2 having about 80% of the activity of the linear form and [Lys^{26}]hPTHrP(1−31)NH_2 having about 50% of the activity of the linear one. Thus, relative activities of the two cyclic analogues were similar to those observed with hPTH, but neither improved the activity of the corresponding linear one.

The CD spectra of both of the cyclic analogues, [Glu^{22},-Lys^{26}]c(Glu^{22}−Lys^{26})hPTHrP(1−31)NH_2 and [Lys^{26}]c(Lys^{26}−Glu^{30})hPTHrP(1−31)NH_2, are clearly more α-helix-like, in terms of the ratios of ellipticities at 222 and 209 nm, than the linear forms (Figure 7). Despite this, the $[\theta]_{222}$ values for the linear and cyclized Glu-22···Lys-26 analogues are very similar. There is no evidence for concentration-dependent dimerization of this Glu^{22}−Lys^{26} cyclic analogue (Figure 7A), but there is evidence for dimerization of [Lys^{26}]c(Lys^{26}−Glu^{30})hPTHrP(1−31)NH_2 (Figure 7B). In this case, the spectrum shows a characteristic increase in the $[\theta]_{222}$ value and approach of the $[\theta]_{209}/[\theta]_{222}$ ratio toward 1, typical of formation of α-helix, as the concentration increases. The linear analogue [K^{26}]hPTHrP-NH_2 has less α-helix than [Glu^{22},Lys^{26}]hPTHrP(1−31)NH_2 but more than its cyclized analogue, [Lys^{26}]c(Lys^{26}−Glu^{30})hPTHrP-NH_2. The spectra suggest cyclization results in a localized α-helix in the region of the lactam but an overall diminishing of helix in the molecule. The data further imply the receptor-binding structure of hPTHrP(1−31) is not α-helical near its C-terminus, similar to hPTH(1−31), but is helical, but perhaps not perfectly α-helical, in the region defined by the 22−26 lactam.

It is noteworthy that whereas hPTH has natural salt bridges that occur between residues 22 and 26 or residues 26 and 30 when this region of PTH is in an α-helical conformation, no such bridges are present in hPTHrP. In fact, hPTHrP-(21−31)NH_2, unlike [Leu^{27}]hPTHrP(21−31)NH_2 or even the wild-type sequence hPTH(21−31)NH_2, is not amphiphilic and α-helical simultaneously. This is shown in Figure 8,

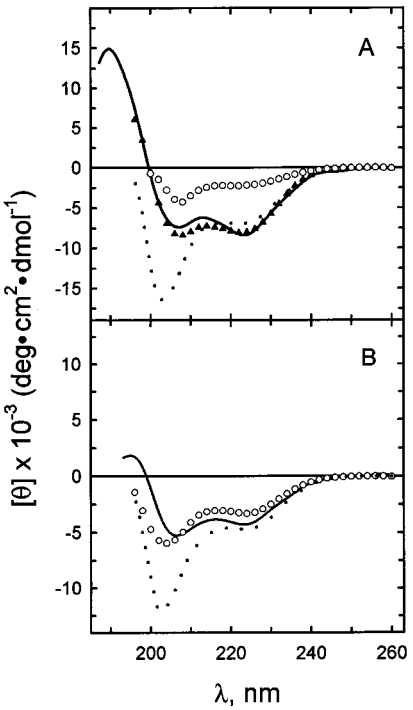

FIGURE 7: CD spectra of cyclic analogues of hPTHrP(1−31)NH_2. (A) Spectra of [Glu^{22},Lys^{26}]hPTHrP(1−31)NH_2, 14 μM (●), of [Glu^{22},Lys^{26}]c(Glu^{22}−Lys^{26})hPTHrP(1−31)NH_2, 68 μM (—) and 14 μM (▲), and of hPTHrP(1−31)NH_2, 14 μM (○). (B) Spectra of [Lys26]hPTHrP(1−31)NH2, 14 μM (●), and of [Lys^{26}]c(Lys^{26}−Glu^{30})hPTHrP(1−31)NH_2, 68 μM (—) and 14 μM (○).

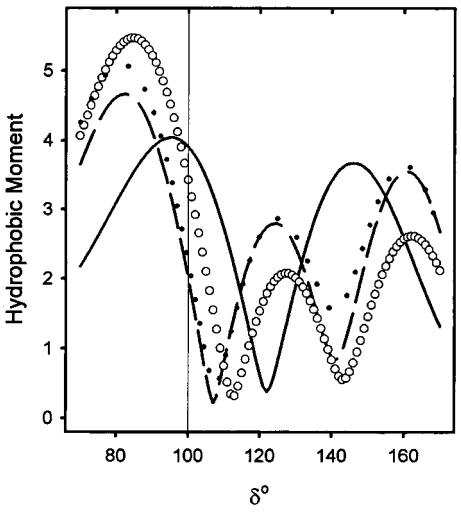

FIGURE 8: Hydrophobic moments (*39*) for C-terminal helical region of hPTH and hPTHrP. For an ideal α-helix, δ is 100°. Calculated moments are shown for the following peptides, with the value of δ for the maximum nearest 100° in parentheses: hPTH(21−31) (95°), (—); hPTHrP(21−31) (82°), (− − −); [Glu^{22},Lys^{26}]hPTHrP-(21−31) (84°), (○); and [Lys^{26}]hPTHrP(21−31) (81°), (●)

where the hydrophobic moment is calculated as a function of helical angle (*39*). For an ideal amphiphilic α-helix, this function has a maximum at 100°. The native sequence of hPTHrP(21−31)NH_2 has a maximum moment at 82°, well displaced from that of 97° for [Leu^{27}]hPTH(21−31)NH_2 or 96° for hPTH(21−31)NH_2.

DISCUSSION

When a peptide, such as PTH, binds to its receptor, there is a large entropic penalty due to loss of translational and

rotational degrees of freedom of the peptide (*40*). The overall loss of entropy is partly diminished by the presence of internal vibrational modes in the complex not present in the unbound peptide (*41, 42*). The remaining contributions to a favorable free energy of binding are assumed to result from a favorable enthalpy of binding and entropic contributions from the hydrophobic effect, including a favorable entropic effect from the release of bound water from the nonpolar side chains that become buried on complex formation. Entropy-driven binding has been observed, for example, in ligand binding to the δ-opoid receptor (*43*). Water release has been shown to result in both favorable enthalpic and entropic contributions, depending on the system (*44*). Detailed studies with human growth hormone and its receptor have shown that both enthalpic and entropic effects can determine favorable binding (*45*).

Regardless of the compensatory mechanisms to overcome the unfavorable entropy change on receptor binding, a suitable constraint of the peptide will increase receptor binding by limitation of the degrees of freedom of the free peptide. Such a cyclization must limit the conformations of the peptide to a subset that includes the productively bound conformations of the hormone. Early work indicated α-helices are stabilized by Glu···Lys^+ *i, i* + 4 spaced salt bridges, although not *i, i* + 3 spaced ones (*16*). Later work with amphiphilic model peptides showed that lactams between residues spaced *i, i* + 4 indeed stabilized the helices, but this depended on the location and orientation of the residues. Thus, Glu···Lys lactams were helix-stabilizing but Lys···Glu ones destabilized the α-helical conformation (*46*). In pioneering work, Felix et al. (*47*) applied lactam stabilization to growth hormone-releasing factor (GRF), where they reported that an analogue containing a lactam between residues 8 and 12 had a long α-helical segment and improved bioactivity. These workers reported the structure of cyclic analogues between residues Asp-8 and Lys-12 and residues Lys-21 and Asp-25. In both instances α-helices near the N-terminus and C-terminus were stabilized by the lactams (*48*). Although GRF and PTH share certain structural features, including size, the location of their helices, and an amphiphilic helical sequence near their C-termini, the two hormones share no sequence similarity. The reason for their apparent structural similarities became clear after their respective receptors had been isolated and shown to belong to a common family (*1*).

Initially, we focused on lactam formation between natural possible ion pairs: residues 22 and 26, 26 and 30, and 27 and 30. Our work on these lactam-containing analogues situated in the osteogenically active fragment [Leu^{27}]hPTH-(1−31)NH_2 demonstrated that only the lactam between residues 22 and 26 led to both increased AC-stimulating and osteogenic activities (*17, 19*). As shown here, the residues can be reversed without significantly affecting AC-stimulating activity. Although it has been reported that Lys···Glu orientations did not induce α-helix in a model peptide (*46*), the effect observed depends on the context of the lactam. Kapurniotu and Taylor (*49*) observed that only a Lys···Asp lactam induced α-helix among the three lactams of calcitonin they examined, and Fry et al. (*48*) reported local helix stabilization in a GRF analogue having a lactam between Lys-21 and Asp-25. We did not observe any difference between the CD spectra of the lactams when the pairs were Glu-22···Lys-26 or Lys-22···Glu-26 at the highest concentrations (about 80 μM). However, spectra taken at a lower concentration (about 15 μM) did show a small difference (Figure 3). One could argue that the situation at the higher concentration, where there is likely dimerization, is perhaps closer to that expected for the receptor-bound hormone. Regardless, helix stabilization is associated with higher agonist activity.

The Cys-22···Cys-26 cyclic analogue was prepared to study the effect of reduction of ring size, in this case from 21 atoms for the Glu···Lys lactam to 17 for the Cys···Cys one. AC-stimulating activitiy was diminished by presence of the double Cys mutation but partially overcome by cyclization. The loss of helix in the linear analogue, observed in the CD spectrum, is expected from known amino acid helix propensities (*50*). There was no helical stabilization, in comparison with either the linear [Cys^{22},Cys^{26},Leu^{27}]hPTH(1−31)NH_2 or [Leu^{27}]hPTH(1−31)NH_2, as a result of ring formation (Figure 5). This interpretation assumes that there is no contribution of the cystine to the far-UV CD. A recent study of bovine pancreatic trypsin inhibitor indicated that no correction to the CD spectrum was necessary in this wavelength region for a disulfide contribution (*51*). However, it should be noted that there are uncertainties in the interpretation of the CD data of our Cys analogues. A theoretical study has demonstrated that the intensity of the CD is quite sensitive to the specific ϕ, ψ angles present (*27*), and the constraint of the smaller ring may yield a helix, but with a different geometry than with the lactam. Loss of activity, similar to that observed here, has been reported with human growth hormone releasing hormone, which activates a PTH-related receptor, on replacing an Asp-25···Orn-29 lactam with a Cys···Cys ring (*52*). Interpretation of these changes in ring size requires that the effect of the substitutions in the linear analogue also be considered, and this has not always been done in studies reported in the literature.

Cyclization with a lactam between residues 13 and 17 of hPTHrP(1−34) was observed to enhance AC stimulation of a human osteosarcoma cell line, SaOS-2/B-10, by a factor of about 3, and this activity enhancement was retained in a double cyclized 13−17/26−30 analogue even though the 26−30 analogue itself was much less active than the linear PTHrP (*53*). Double cyclic analogues of hPTH(1−31)NH_2, having 13−17 and 26−30, 13−17 and 18−22, or 18−22 and 26−30 lactams were described very recently (*54*). Their results suggested only a bicyclic analogue with 18−22 and 22−26 lactams had increased activity relative to the linear analogue. A double cyclic analogue of PTHrP(7−34)NH_2, with side-chain lactams between residues 13 and 17 and residues 26 and 30, has been reported to have increased antagonist activity compared to the linear parent (*53*). In our work, AC stimulation of ROS 17/2 cells by [Glu^{17},Leu^{27}]c-(Lys^{13}−Glu^{17})hPTH(1−31)NH_2 was approximately the same as that of the linear analogue. However, enhancement was increased in [Glu^{17},Leu^{27}]c(Lys^{13}−Glu^{17},Glu^{22}−Lys^{26})hPTH-(1−31)NH_2. The Asp-17 analogue, [Asp^{17},Leu^{27}]c(Lys^{13}−Asp^{17},Glu^{22}−Lys^{26})hPTH(1−31)NH_2, had diminished activity, presumably due to the restriction of the smaller ring size of the first cyclization (Table 1). Our CD results of Figure 6 show that the structure of the double cyclic mutant [Glu^{17},-Leu^{27}]c(Lys^{13}−Glu^{17})hPTH(1−31)NH_2 is not a simple sum of the structures of the two single cyclic analogues. A similar

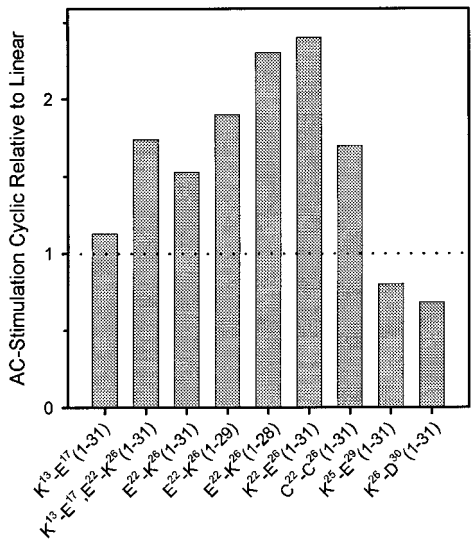

FIGURE 9: Ratios of AC-stimulating activities of cyclic to corresponding linear analogues of hPTH.

observation was reported in an earlier NMR and CD study of mono- and bicyclic analogues of GRF, a hormone that binds to a PTH-related receptor (*48*). Presumably, this is because the peptide regions bounded by the lactams interact with one another.

Although these data indicate that the region bounded by residues 21 and 27 binds to the receptor as an α-helix, the binding conformation of the C-terminal region from about residue 26 to 31 is most likely some type of distorted helix. Analogues that are cyclized in this region either have no increased activity as a result of cyclization, such as [Leu^{27}]c(Lys^{26}−Asp^{30})hPTH(1−31)NH$_2$ (*17*), or have lowered activity, as observed with the analogue [Lys^{25},Leu^{27},Glu^{29}]c(Lys^{25}−Glu^{29})hPTH(1−31)NH$_2$ reported here. From these data, PTH seems to bind to the receptor as an α-helix between about residues 21 and 27 and a more extended helix, or at least a distorted one, from about residue 27 to the C-terminus, residue 31. We observed no enhancement of activity on constraint of residues 13−17 alone. Since there is a bend in linear PTH in this region, interpretation of the CD data is difficult. Figure 9 summarizes the relative activities of constrained versus linear analogues. It shows clearly that only the region from about residues 21 to 27 likely is in a true α-helix conformation.

Our conclusions concerning the effect of cyclization on the AC agonist activity of PTH are largely compatible with the data recently reported by Condon et al. (*54*). They found a modest effect of cyclization between residues 13 and 17 on AC stimulation and a strong effect of a midregion cyclization, between residues 18 and 22, similar to the effect observed by us between residues 22 and 26. There was no additional enhancement of activity, relative to that of the 18−22 monocyclic analogue, with the 13−17/22−26 bicyclic analogue, but there appeared to be some additional activity when the 26−30 region was cyclized in addition to the 18−22 cyclization. However, their conclusions on the receptor-bound conformation of PTH are not quite consistent with our data. They concluded that hPTH(1−31) binds to the receptor as one continuous helix, rather than the helix−bend−helix inferred from numerous NMR studies on the solution structure of PTH. To circumvent the low solubility of the double lactam analogues, they performed their experiments at pH ∼3.6 and in 20% acetonitrile. Under these conditions, they obtained a strong CD signal with the tricyclic analogue, which indicated this molecule was fully α-helical. In addition, their experiments were performed at a concentration of 40 μM, a level at which we observe dimerization leading to increased helix in some cyclic analogues. Solubility of our double cyclic PTH analogues was also a problem, necessitating the use of very dilute solutions. Acetonitrile has been reported to induce α-helix in both model and natural peptides (*55*, *56*), and we suggest this difference in solvent conditions and peptide concentrations likely explains the difference in our results. It should also be noted that their reported spectrum for hPTH(1−31)NH$_2$ is very much more intense, having a $[\theta]_{222}$ of −19 300 compared to our previously reported value of −7500 in neutral, aqueous buffer (17). We cannot absolutely rule out the possibility that PTH binds as a continuous helix, but we believe the preponderance of data at this time is more indicative of it binding with some bend near its midregion.

The activity and CD data reported here on similar analogues of hPTHrP(1−31)NH$_2$ are similar overall to the observations with hPTH. However, there is no clear enhancement of AC stimulation by lactam formation between residues 22 and 26 as is seen with PTH. A recent NMR study of hPTHrP(1−34) indicated that this molecule has two helices, a short one between His-5 and Asp-10 and a longer C-terminal one between residues Ser-14 and Ala-29, connected by a flexible segment (*37*). As shown by their CD spectra, cyclized [Glu^{22},Lys^{26}]hPTHrP(1−31)NH$_2$ had greater α-helix than the linear sequence and cyclized [Lys^{26}]hPTHrP(1−31)NH$_2$ had less helical content than the linear one (Figure 7). In either case, lactam formation constrained a portion of the molecule to α-helix, as evidenced by the θ_{222} values and particularly by the ratios $\theta_{209}/\theta_{222}$. There is evidence from receptor interaction studies of PTH/PTHrP hybrids that the residue 21−34 region of the two species can be substituted independently and activate the receptor and also that their 15−34 domains have similar receptor affinities (*57*). This, along with knowledge that certain residues on the hydrophobic face of the C-terminal α-helix of hPTH are critical for receptor binding and activation, implies that the PTHrP must adopt a somewhat similar configuration to activate the receptor. The AC activation and CD data presented here suggest that the conformation of PTHrP bound to the receptor is likely a somewhat distorted helix that would permit optimal interaction of its C-terminal hydrophobic residues with the receptor and not the perfect one that seems to characterize bound PTH in the 21−27 residue region.

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