# Solution Structure and Adenylyl Cyclase Stimulating Activities of C-Terminal Truncated Human Parathyroid Hormone Analogues†

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*Received December 29, 1994; Revised Manuscript Received March 16, 1995*⊗

ABSTRACT: Analogues of human parathyroid hormone (hPTH) truncated at the C-terminal end have been studied for adenylyl cyclase (AC) activity and for solution conformation by circular dichroism (CD) spectroscopy. Analogues of hPTH-(1−34)-$NH_2$, containing the first 28−31 residues, had only a slightly diminished ability to stimulate AC in rat osteosarcoma (ROS) cells as compared to that of the parent analogue. CD data on hPTH-(16−34)-$NH_2$ and C-terminal deletion mutants of hPTH-(1−34)-$NH_2$ supported the presence of a partially stable α-helix over residues 17−28. A carboxyl-terminal mutant, hPTH-(1−30)-OH, showed both reduced helix and greatly reduced AC-stimulating activity as compared to the corresponding amide analogue. In contrast, both of these analogues, in the presence of palmitoyloleoylphosphatidylserine (POPS) vesicles, showed an equal stabilization of α-helix. All other analogues showed at least some enhancement of α-helix in the presence of POPS. However, both in neutral, aqueous buffer and in POPS, the relative amount of α-helix decreased greatly as the peptide was shortened below the 1−28 sequence. These data provide additional support for an amphiphilic α-helix over residues 21−28 being the conformation for receptor binding of hPTH for stimulation of AC activity. Modeling human parathyroid hormone-related peptide as an α-helix over this same region, and comparison to hPTH, suggests that both may bind via the hydrophobic face to the receptor.

Parathyroid hormone (PTH)[1] is a major regulator of calcium metabolism by means of its action on osteoblast and renal epithelial cell receptors (Rosenblatt et al., 1989). Almost all of the biological activities of the complete 84-residue peptide are found associated with the amino-terminal 34 residues (Jüppner, 1989). Thus, binding of PTH-(1−34) to the receptor results in increased activities of adenylyl cyclase (AC) (Rosenblatt et al., 1989) and protein kinase C (PKC) (Cosman et al., 1989). We have recently shown that the minimum sequence required to stimulate PKC activity is hPTH-(29−32)-$NH_2$ (Jouishomme et al., 1994) whereas that for AC activity was reported earlier to be hPTH-(1−27)-OH (Tregear et al., 1973).

Efforts have been made to dissect the PTH-(1−34) peptide into regions critical for binding and for signalling activities and to relate these to any solution structure. Its principal binding region has been localized to within residues 14−34 (Caulfield et al., 1990). In contrast to the C-terminal end, deletion of the N-terminal residue of either peptide leads to an almost complete loss of AC agonist activity (Tregear et al., 1973). There is also a weak binding activity associated with residues 1−6 (Nussbaum et al., 1980). Parathyroid hormone-related peptide, a hormone which binds to the same receptor as PTH and effects the same responses (Jüppner et al., 1991), has little sequence similarity to PTH in the principal binding region. However, as in PTH-(1−34), several C-terminal residues of PTHrP-(1−34) can be removed with little loss of activity (Kemp et al., 1987).

The lack of well-defined structure by PTH has made correlation of solution structure to receptor-bound structure difficult. CD (Zull et al., 1990) and NMR (Smith et al., 1987) data suggested the presence of some ordered structure within PTH-(1−34). NMR studies in the presence of 10−40% TFE have pointed to the presence of two α-helical regions within PTH-(1−34) linked by a disordered region (Barden & Cuthbertson, 1993; Barden & Kemp, 1993; Strickland et al., 1993; Wray et al., 1994). As a result of a CD study of hPTH-(1−84) and subfragments, in the presence of increasing concentrations of TFE, Neugebauer et al. (1992) concluded that the most stable helical region was contained within residues 17−29. A recent NMR study of fragments of hPTH-(1−84) led to a similar conclusion (Wray et al., 1994). The NMR data have shown no evidence for interaction of the two helices with one another. Thus, there is no evidence that PTH, free in solution, adopts a stable, folded configuration where the hydrophobic residues of the two helices pair with one another, such as postulated earlier (Cohen et al., 1991). In addition, Neugebauer et al. (1992) demonstrated the presence of an amphiphilic helix, included within residues 21−34 of hPTH, that could be important for receptor binding. Consistent with this, Gardella et al. (1993) have shown that Leu-24, Leu-28, and Val-31 are especially important for optimal stimulation of AC activity.

Although NMR, in principle, provides much more detail on molecular structure than other spectroscopic techniques, such as CD, its application to short peptides, such as these PTH analogues, is limited by their lack of specific structure. Thus, most NMR experiments on PTH have been carried out in solvents that induce structure, such as TFE, or at a

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⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1995.

[1] Abbreviations: hPTH, human parathyroid hormone; hPTHrP, human parathyroid hormone-related peptide; CD, circular dichroism; TFE, trifluoroethanol; POPS, 1-palmitoyl-2-oleoylphosphatidylserine.

0006-2960/95/0434-8835$09.00/0 Published 1995 by the American Chemical Society

nonphysiological pH. CD, in contrast, is highly limited in providing detailed structure, but can readily be performed on these analogues at much lower concentrations than the NMR and at physiological pH values. The CD induced by α-helical structure is intense and has a relatively large and almost unique contribution near 220 nm. Thus, CD is a valuable tool in estimating the amount of helical structure in peptides, since the signal from this structure stands out from the other conformations that the peptide may adopt.

In this paper, we examine the effect of deletion of C-terminal residues of hPTH-(1−34)-$NH_2$ on AC activity and use CD to monitor the corresponding α-helix content of the analogues. We show that the loss of activity is coincident with destabilization of the helix associated with residues 17−29 and also that substitution of the free carboxyl group for the amide at the C-terminus of hPTH-(1−30) results in a substantial loss of AC-stimulating activity and of α-helix. Assuming that the hydrophobic face of this α-helix, in either hPTH or hPTHrP, binds to the receptor, we show that there is a similarity between the binding regions of the two molecules which may explain their ability to bind to the same receptor.

## MATERIALS AND METHODS

*Synthetic PTH Amide Analogues.* The following peptides were synthesized: hPTH-(1−34)-$NH_2$, hPTH-(1−31)-$NH_2$, hPTH-(1−30)-$NH_2$, hPTH-(1−29)-$NH_2$, hPTH-(1−28)-$NH_2$, hPTH-(1−27)-$NH_2$, and hPTH-(1−26)-$NH_2$. All were synthesized with a MilliGen 9050 continuous-flow peptide synthesizer using TentaGel S RAM (Rapp Polymere, Tübingen, Germany) as the support and FMOC chemistry (Fields & Noble, 1990). Amino acids were activated by 2-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate/*N*-hydroxybenzotriazole in dimethylformamide. The following side-chain protection groups were used: *tert*-butyloxycarbonyl (Lys), 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Arg), *O*-*tert*-butyl (Asp, Glu), *tert*-butyl (Ser), and trityl (Asn, Gln, His). The resulting peptide amides were purified by HPLC and their identities confirmed by mass spectrometry (SCIEX, Mississauga, ON; or VG Quattro, Manchester, U.K.).

*Recombinant Expression of hPTH-(1−30).* hPTH-(1−30) was expressed in *Escherichia coli* and purified from the resulting inclusion bodies (W. Sung, in preparation).

*Bioassays.* AC and membrane-associated PKC activities of rat osteosarcoma (ROS 17/2) cells were determined as described previously (Jouishomme et al., 1994). AC activities are expressed as a percent of the stimulation by hPTH-(1−34)-$NH_2$ observed in a concurrent experiment.

*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 bring the final concentration to 25 mM. Peptide concentrations were calculated from the absorption at 280 nm, using an extinction coefficient of 5700 $M^{-1}$ for the single tryptophan. Spectra were measured at peptide concentrations between 0.1 and 0.2 mg/mL. Data are expressed per peptide bond.

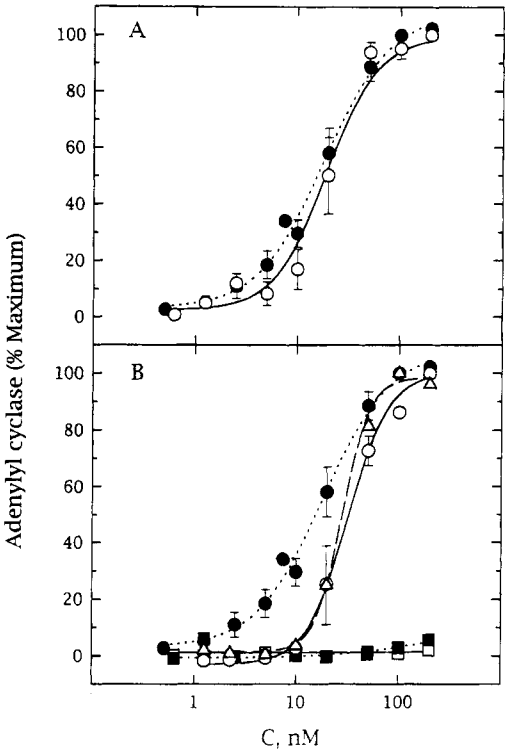


FIGURE 1: Adenylyl cyclase activities of hPTH analogues. (A) hPTH-(1−34)-$NH_2$ (●- - -●) and hPTH-(1−31)-$NH_2$ (○—○); (B) hPTH-(1−34)-$NH_2$ (●- - -●), hPTH-(1−29)-$NH_2$ (△- - -△), hPTH-(1−28)-$NH_2$ (○—○), hPTH-(1−27)-$NH_2$ (■- - -■), and hPTH-(1−26)-$NH_2$ (□—□).

*Geometry Optimization of α-Helices.* Sequences were geometry-optimized for an α-helical conformation using an AMBER force field and Hyperchem software.

*Preparation of Palmitoyloleoylphosphatidylserine (POPS) Vesicles.* Small unilamellar POPS vesicles (Avanti Polar Lipids) were prepared by suspending the lipid in buffer (25 mM sodium phosphate, pH 7.2) to a concentration of about 15 mg/mL. The suspension was sonicated in an ice bath for about 15 min, using a probe-type sonifier, and then centrifuged in a microfuge for 10 min. Vesicles were stored at room temperature and used within a few hours after preparation.

## RESULTS

*Biological Activities.* Except for hPTH-(1−34)-$NH_2$, none of the analogues were able to stimulate the activity of membrane-bound PKCs in the osteoblast-like ROS 17/2 cells. This was expected, since the minimal sequence required for hPTH stimulation of these activities includes residues (29−32)-$NH_2$ (Jouishomme et al., 1994). In contrast, hPTH-(1−31)-$NH_2$ was fully active, as compared to hPTH-(1−34)-$NH_2$, when its AC-stimulating activity was measured (Figure 1A). Three other analogues, hPTH-(1−30)-$NH_2$, hPTH-(1−29)-$NH_2$, and hPTH-(1−28)-$NH_2$ showed slightly diminished activities (Figure 1B and Figure 2). The inflections of the AC-stimulating activity curves of the latter appeared to be sharper than those for hPTH-(1−31)-$NH_2$ or hPTH-(1−34)-$NH_2$. Of the remaining analogues, hPTH-(1−27)-$NH_2$ had a greatly diminished activity, and hPTH-(1−26)-$NH_2$ had no activity at all concentrations tested (Figure 1B).

Although hPTH-(1−30)-$NH_2$ was observed to be still highly active in the AC assay, the activity of hPTH-

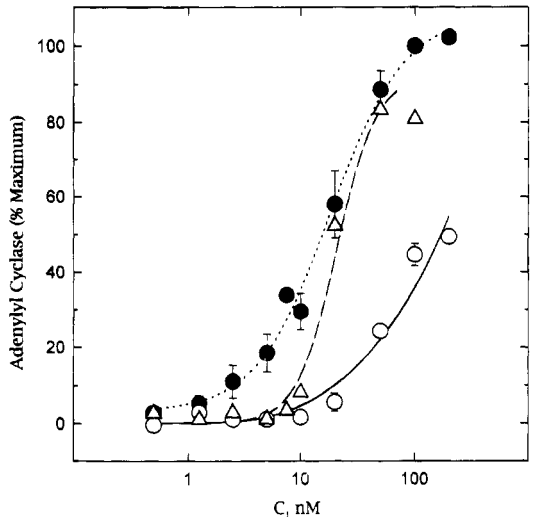


FIGURE 2: Adenylyl cyclase activities of hPTH analogues. Shown are hPTH-(1−34)-$NH_2$ (●- - -●), hPTH-(1−30)-$NH_2$ (△—△), and hPTH-(1−30)-OH (○—○).

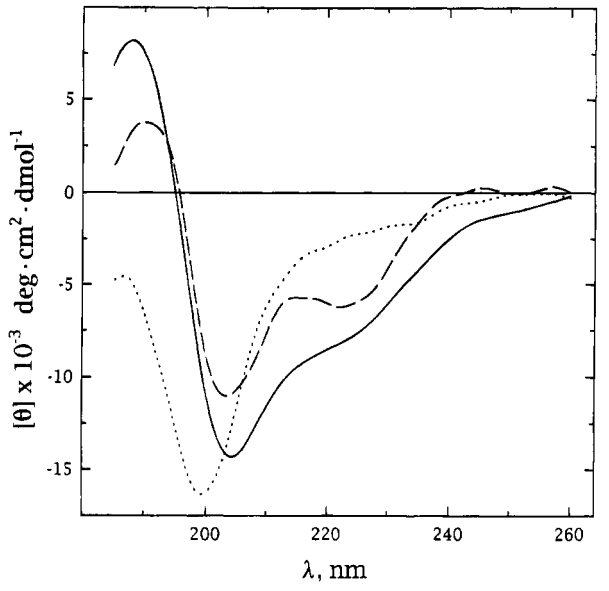


FIGURE 3: CD spectra of subfragments of hPTH-(1−34)-$NH_2$ in 25 mM sodium phosphate, pH 7.2. Peptide concentrations were 0.15 mg/mL. Shown are hPTH-(16−34)-$NH_2$ (—), hPTH-(13−34)-$NH_2$ (− −), and hPTH-(20−34)-$NH_2$ (•••).

(1−30)-OH was found to be greatly diminished (Figure 2). Thus, hPTH-(1−30)-$NH_2$ had a half-maximal activity of about 20 nM, as opposed to about 16 nM for the reference analogue hPTH-(1−34)-$NH_2$. In contrast, hPTH-(1−30)-OH had a half-maximal activity of 174 nM, about 8-fold less than that of the corresponding amide.

*CD Spectra of Analogues with Truncated N-Terminal Ends.* We have previously argued that the enhanced elipticity at 222 nm ($[\theta]_{222}$) observed in hPTH and some subsequences of it is due to a partially stable α-helix extending from residues 17−29 (Neugebauer et al., 1992). In part, this conclusion was derived from the spectra observed in fragments 13−34, 1−34, 1−19, and 20−34. Thus, fragments 1−34 and 13−34 had substantial α-helix, while fragments 1−19 and 20−34 were observed to have none. To establish further the location of this α-helix, we synthesized hPTH-(16−34)-$NH_2$ and examined its CD spectrum (Figure 3). It is noteworthy that this analogue has a strong contribution

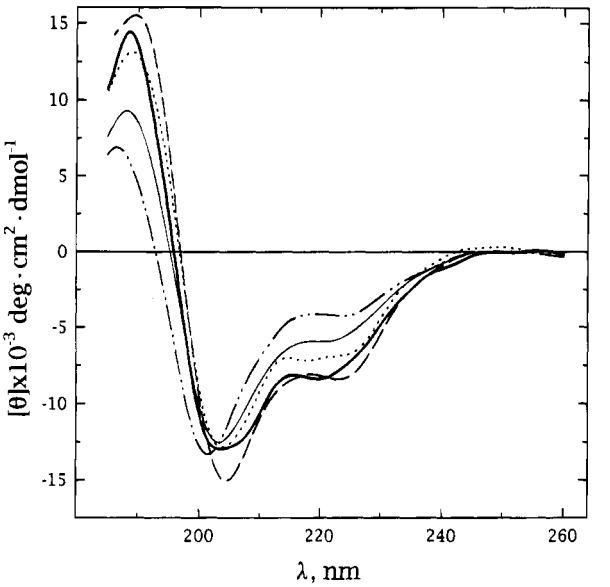


FIGURE 4: CD spectra of C-terminus deletion analogues of hPTH-(1−34)-$NH_2$ in 25 mM sodium phosphate, pH 7.2. Peptide concentrations were 0.15 mg/mL. Shown are hPTH-(1−26)-$NH_2$ (− •• −), hPTH-(1−27)-$NH_2$ (—), hPTH-(1−28)-$NH_2$ (•••), hPTH-(1−29)-$NH_2$ (− −), and hPTH-(1−31)-$NH_2$ (thick solid line).

of α-helix to its CD signal, as indicated by the intensity of the signal at 222 nm, as compared to the 20−34 fragment.

The expected CD spectra for α-helices are both chain length dependent and dependent on the precise geometry of the helix (Yang et al., 1986; Manning & Woody, 1991). Ignoring the effect of fluctuating geometry, it is not possible to positively estimate the number of residues in a peptide that is in a helical conformation due to the chain length dependence of the ellipticity per residue (Yang et al., 1986). We have analyzed the data for α-helical content based on the observed elipticity at 222 nm and a value of −28 000 deg·cm$^2$·dmol$^{-1}$ for a helix of 10 residues (Yang et al., 1986). Subject to the above considerations, our calculations indicated that about six residues are, on average, in an α-helical configuration in hPTH-(16−34)-$NH_2$ (Neugebauer et al., 1992). In the presence of 40% trifluoroethanol (TFE), the $[\theta]_{222}$ value increased to −21 400 deg·cm$^2$·dmol$^{-1}$, which corresponds to an α-helix of about 14 residues (data not shown). Similar CD spectra were obtained by Gardella et al. (1993) for [Tyr$^{34}$]-hPTH-(15−34)-$NH_2$ and [Glu$^{24}$,Tyr$^{34}$]-hPTH-(15−34)-$NH_2$. Both ours and the data of Gardella et al. (1993) are consistent with a postulated helix between residues Ser-17 and Gln-29. These present results support the notion that the helix requires Ser-17 for stabilization, which is compatible with the observation that Ser has a high probability of occurrence at the N-terminus of an α-helix (Richardson & Richardson, 1989).

*CD Spectra of hPTH-(1−34)-$NH_2$ Analogues Truncated at the C-Terminus.* The loss of adenylyl cyclase activities by the 1−27 and 1−26 analogues suggested that the putative α-helix between residues 17 and 29 might be important to receptor binding. The CD spectra of the hPTH analogues including residues 1−26, 1−27, 1−28, 1−29, and 1−30 are shown in Figure 4. A diminution of the ellipticity at 222 nm is seen as the peptide is shortened. This is particularly marked with hPTH-(1−26)-$NH_2$. In this analogue, the minimum near 202 nm in the longer peptides is obviously shifted toward a lower wavelength, which corresponds to a

Table 1: Helical Residues in hPTH-$NH_2$ Analogues

| | number of residues in α-helix[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| % TFE | 1−26 | 1−27 | 1−28 | 1−29 | 1−30 | 1−30[b] | 1−31 |
| 0 | 4 | 6 | 7 | 8 | 7 | 6 | 9 |
| 40 | 20 | 19 | 20 | 24 | 24 | 23 | 25 |

[a] α-Helix was estimated from $[\theta]_{222}$ values, using the equation $n = ([\theta]_{222}^{obs}/[\theta]_{222}^{10})N$, where $[\theta]_{222}^{obs}$ = observed ellipticity at 222 nm, $[\theta]_{222}^{10}$ = ellipticity value of 28 000 deg·cm²·dmol⁻¹ for a helix of length 10 at 222 nm (Yang *et al.*, 1986), $N$ = peptide length, and $n$ = number of residues in helix. [b] Peptide has free carboxyl terminus.

loss of structure. A pronounced minimum at about 199 nm is seen in those analogues which have little structure, such as seen in Figure 3 for hPTH-(20−34)-$NH_2$ (Neugebauer et al., 1992). Analysis of the $[\theta]_{222}$ data indicated that the number of helical residues had dropped from an average of 8 in hPTH-(1−28)-$NH_2$ through hPTH-(1−31)-$NH_2$ to 4 in hPTH-(1−26)-$NH_2$ (Table 1). This is compatible with the previous postulate that the α-helix in hPTH-(1−34) in neutral, aqueous solution is within residues 17−29 (Neugebauer et al., 1992). The loss of helix as the peptide is shortened from 1−28 to 1−26 suggests that the helix, averaging 7 residues in hPTH-(1−28)-$NH_2$, is most stable near the C-terminus of the 13-residue 17−29 sequence. Analysis of hPTH-(1−34) in terms of α-helical tendencies of amino acids (O'Neill & DeGrado, 1990) indicated that the helix of residues 17−29 is most stable toward its C-terminal end (Neugebauer et al., 1992). Thus, it is expected that shortening of the helix from the C-terminal end of the peptide would have the most effect on the CD of the peptide, when observed in neutral, aqueous buffer. This is what was observed here.

In contrast, peptides containing residues 1−29 to 1−31 had about 25 residues in α-helical conformation, when in the presence of 40% TFE (Table 1). This is close to the estimate of 26 helical residues under the same solvent conditions previously estimated for hPTH-(1−34)-$NH_2$ (Neugebauer et al., 1992). This TFE concentration is the level at which the maximum amount of α-helix is obtained in these PTH analogues (Neugebauer et al., 1992). The additional helix in the presence of TFE comes partly from stabilization of a 9-residue helical sequence near the N-terminus of the peptide and partly from full stabilization of the 13-residue helix in the 17−29 sequence (Klaus et al., 1992; Neugebauer et al., 1992; Wray et al., 1994). Under these conditions, we observed a loss of α-helix when four residues were removed from hPTH-(1−31)-$NH_2$.

*CD Spectra of hPTH-(1−30)-$NH_2$ and hPTH-(1−30)-OH.* The ability to stimulate AC activity by hPTH-(1−30)-$NH_2$ was much greater than that of hPTH-(1−30)-OH (Figure 2). Therefore, we compared the CD spectra of the two in neutral, aqueous buffer to see if this activity difference was reflected in a difference in solution structure (Figure 5). A decrease in the intensity of the ellipticity at 222 nm, coupled with a blue-shift of the minimum at 202 nm, was observed in hPTH-OH as compared to hPTH-(1−30)-$NH_2$. This implies that the α-helix in hPTH-OH is slightly less stable in solution than that of hPTH-(1−30)-$NH_2$, resulting in an apparent loss, on average, of one residue in an α-helical conformation (Table 1).

*Amphiphilic Helices of Truncated Analogues.* We have previously pointed out that hPTH-(1−34) has within it a

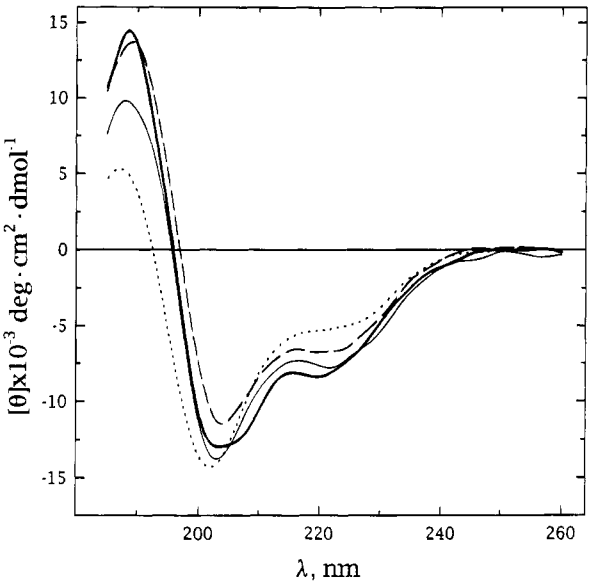


FIGURE 5: Comparison of CD spectra of hPTH-(1−30)-$NH_2$ and hPTH-(1−30)-OH in 25 mM sodium phosphate, pH 7.2. Peptide concentrations were 0.15 mg/mL. Shown are hPTH-(1−30)-$NH_2$ (− −), hPTH-(1−30)-OH (•••), hPTH-(1−31)-$NH_2$ (thick solid line), and hPTH-(1−34)-$NH_2$ (—).

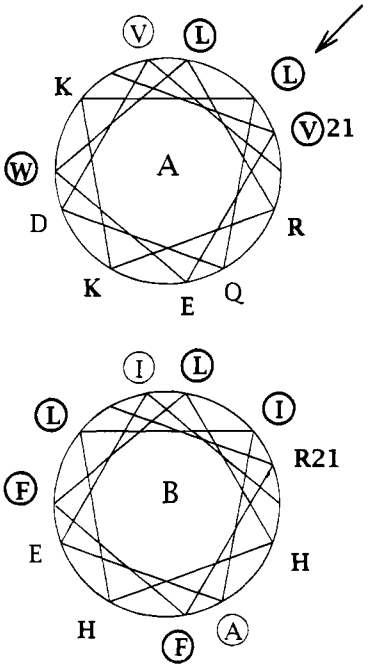


FIGURE 6: Helical wheel presentation of (A) hPTH-(21−31). Residues critical for AC activity are in boldface, and hydrophobic residues are circled. Removal of Leu-28, marked with an arrow, from hPTH-(1−28)-$NH_2$ results in an almost total abolition of AC-stimulating activity. Hydrophobic residues are circled. For comparison, hPTHrP-(21−31) is shown in (B), and residues 21−28 are also shown in boldface.

putative amphiphilic α-helix bounded by residues Val-21 and Phe-34. Experimental evidence for its presence was provided by the observation of an increase in α-helix in analogues containing this sequence when in the presence of lipid (Neugebauer et al., 1992). Removal of residues from the C-terminus results in some removal of this amphiphilic structure. The amphiphilicity of a sequence can be viewed as a helical wheel, and Figure 6 shows the amphiphilic regions, 21−28 and 21−31, within hPTH-(1−28)-$NH_2$ and hPTH-(1−31)-$NH_2$. Leu-28 is shown by an arrow; removal

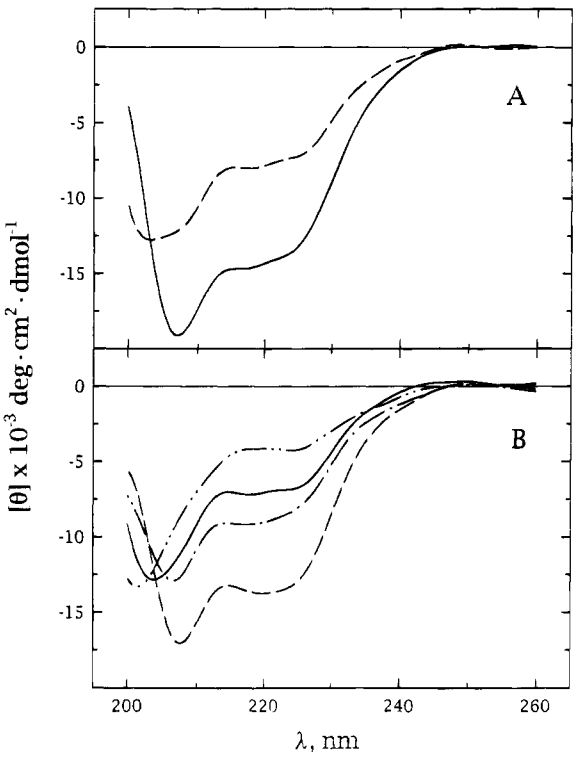


FIGURE 7: Effect of lipid vesicles on CD spectra of subfragments of hPTH-(1−34)-$NH_2$. (A) hPTH-(1−31)-$NH_2$ minus (− −) and plus POPS (—); (B) hPTH-(1−26)-$NH_2$ minus (− •• −) and plus POPS (− • −), hPTH-(1−28)-$NH_2$ minus (—) and plus (− −) POPS.

Table 2: Induction of α-Helix by POPS

| | number of residues in α-helix[a] | | | | | |
|---|---|---|---|---|---|---|
| ±POPS[b] | 1−26 | 1−27 | 1−28 | 1−30 | (1−30)-OH[c] | 1−31 |
| − | 4 | 6 | 7 | 7 | 6 | 8 |
| + | 8 (9)[d] | 10 (10) | 13 (11) | 14 (13) | 14 (13) | 16 (14) |

[a] α-Helix was estimated as described in Table 1. [b] POPS, palmitoyloleoylphosphatidylserine, 14 mg/mL, in 25 mM sodium phosphate, pH 7.2. [c] Except as noted, all peptides have terminal amides. [d] Total number of residues if the putative helix in sequence bounded by residues 17−29 is fully stabilized.

of this residue results in a dramatic loss of AC-stimulating activity. For comparison, the same sequence regions within hPTHrP are also shown in the figure.

*Induction of α-Helical Structure in Lipid.* Amphiphilic helices often show an enhancement of α-helix on complexing with a phospholipid (Epand et al., 1983; Neugebauer et al., 1992; Willis, 1994) such as POPS, an acidic phospholipid. The CD spectra of hPTH-(1−31)-$NH_2$, hPTH-(1−28)-$NH_2$, and hPTH-(1−26)-$NH_2$ in the presence and absence of POPS are shown in Figure 7. The relative enhancement of α-helix in each analogue is consistent with the assumption that the entire helix from Ser-17 to Leu-28, in each of peptides 1−28 and 1−30, has been stabilized in the presence of the lipid (Table 2). The value for the maximum number of residues in the helical conformation, based on this assumption, is shown in parentheses in Table 2 beside each value for the estimated number of residues in α-helical conformation. The relative enhancement of the spectrum in POPS drops off sharply between hPTH-(1−28)-$NH_2$ and hPTH-(1−26)-$NH_2$. This reflects the removal of Leu-28, which leaves a sequence only weakly amphiphilic as an α-helix. Thus, the amphiphilic helix and that critical to receptor binding of the peptide for AC-stimulating activity coincide. Unlike the situation in buffer alone, no difference was observed in the CD spectra of hPTH-(1−30)-$NH_2$ and hPTH-(1−30)-OH when in the presence of POPS (Figure 8).

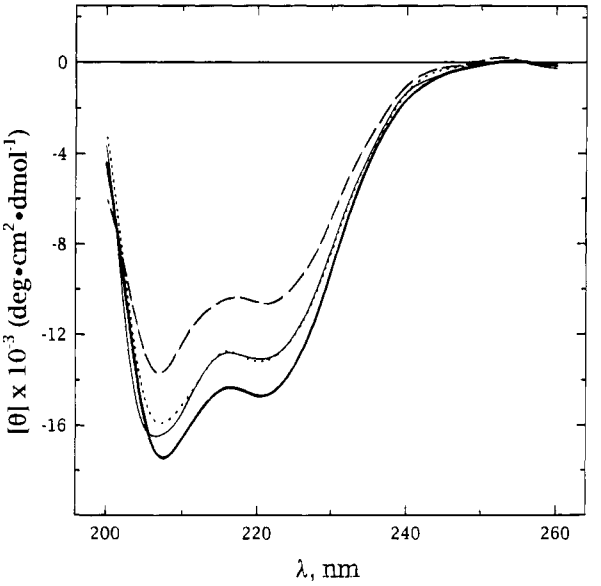


FIGURE 8: CD spectra of hPTH deletion analogues in the presence of POPS. Shown are hPTH-(1−30)-$NH_2$ (•••) and hPTH-(1−30)-OH (—). For comparison, hPTH-(1−27)-$NH_2$ (− −) and hPTH-(1−31)-$NH_2$ (thick solid line) have also been included in this figure.

## DISCUSSION

Parathyroid hormone shows both catabolic and anabolic effects on bone. We have been interested in establishing the minimal structure which has an anabolic effect, i.e., is able to promote bone growth in an ovariectomized rat model. We have recently shown that hPTH-(1−31)-$NH_2$ is very active in this assay, and has full AC-stimulating activity but is unable to stimulate membrane-bound PKCs (Rixon et al., 1994). Since the principal binding region of PTH has been shown to reside within the 14−34 region, progressive truncation from the C-terminal end should aid in determining the exact sequence requirement for its binding. Our present results clearly show that the minimum sequence for essentially full AC-stimulating activity is hPTH-(1−28)-$NH_2$. The activities of progressively shortened C-terminal fragments showed little loss, with $C^{50\%}$ increasing from 16 nM to 27 nM, until removal of Leu-28. This resulted in a dramatic loss of AC-stimulating activity. An earlier study of Tregear et al. (1973) gave similar results, although their AC activity for hPTH-(1−28)-OH had dropped to 10% of the level observed with hPTH-(1−34)-OH. Their results are not directly comparable to those reported here, since their analogues terminated with the COOH group. We have shown here that the C-terminal group, as free acid or amide, can have a substantial effect on the functional binding of an analogue to its receptor, with hPTH-(1−30)-OH having about 12% of the activity of hPTH-(1−30)-$NH_2$. PTHrP has been shown to bind to the same receptor as PTH (Jüppner et al., 1991). As with PTH, truncation at the C-terminal end results in a loss of activity. hPTHrP-(1−29)-$NH_2$ was reported to have about 10% of the activity of the 1−34 fragment when assayed for AC-stimulating activity in the osteogenic sarcoma cell line UMR 106-01 (Kemp et al., 1987). Fragments 1−25 and shorter had no activity.

Nussbaum et al. (1980) have studied the binding of bPTH fragments truncated at the N- and C-termini using canine

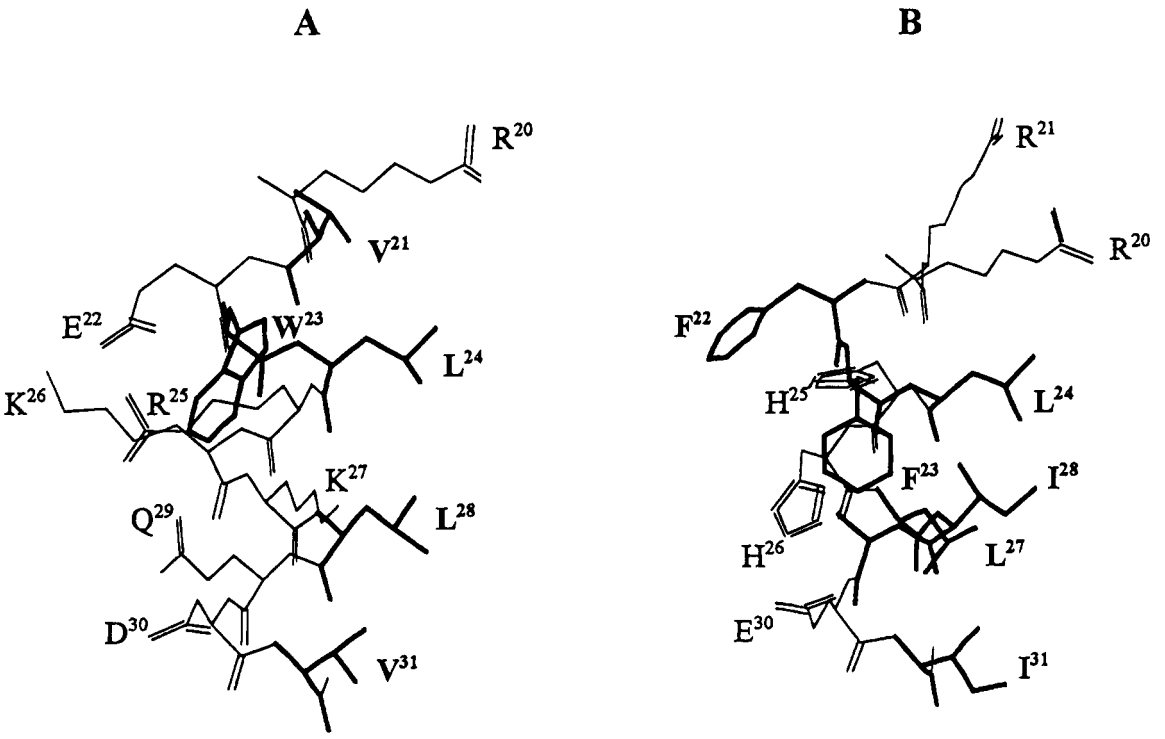


FIGURE 9: Comparison of binding regions of hPTH-(20−31)-$NH_2$ (A) and hPTHrP-(20−31)-$NH_2$ (B). The 17−31 region of each peptide was assembled in an α-helical configuration, and geometry-optimized. The helical axis is in the plane of the page with the N-terminus at the top. The hydrophobic residues are shown in boldface. The residues on the hydrophobic face are either conserved or replaced by similar residues.

renal cortical membranes. From their data and from previous work (Mahaffey et al., 1979), they concluded that an essential receptor binding region was included within bPTH-(25−34), because the association constant of the 25−34 fragment was 20-fold greater than the 10−27 one. Segre et al. (1979), using the same system, reported that bPTH-(1−28)-$NH_2$ had 8% of the AC activity of their parent analogue, [$Nle^8$, $Nle^{18}$, $Tyr^{34}$]-bPTH-(1−34)-$NH_2$. On the basis of a comparison of the abilities of [$Tyr^{34}$]-hPTH-(14−34)-$NH_2$ and [$Tyr^{34}$]-hPTHrP-(14−34)-$NH_2$ to inhibit PTH binding and AC-stimulating activities in bovine renal cortical membrane and ROS 17/2.8 assays, Caulfield et al. (1990) concluded that the 14−34 region of these sequences was most important for receptor binding. Our work is generally consistent with these earlier findings. However, we did not observe substantial loss of activity until six residues were removed from the C-terminus of hPTH-(1−34)-$NH_2$. Thus, we conclude that the critical region for AC-stimulating activity is included within the 14−28 sequence.

We and others have previously argued that hPTH in solution has a partially stable helix, in neutral, aqueous solution or on the addition of 10% TFE, that is bounded approximately by residues 17−29 (Klaus et al., 1991; Neugebauer et al., 1992). Further studies in acidic (Barden & Kemp, 1993) or neutral media containing high percentages of TFE (Strickland et al., 1993; Wray et al., 1994) have provided evidence for the presence of this α-helix in solution. Data presented here on the CD spectrum of hPTH-(16−34)-$NH_2$ (Figure 3) provide additional evidence that the helix observed in hPTH in neutral, aqueous solution is bounded at the N-terminus by Ser-17. The substitution of a COOH group for the amide terminus gives rise to an unfavorable interaction with the macrodipole associated with the helix, and thus this substitution is expected to destabilize the α-helix at the C-terminal end (Cohen et al., 1991). Our observed loss of helix of hPTH-(1−30)-OH as compared to hPTH-(1−30)-$NH_2$ provides experimental evidence for the theoretically expected loss of helix in the analogue with the carboxyl function as compared to the amide one. All other things being equal, this loss of α-helical structure in solution would be expected to result in a lower binding affinity if the structure of the peptide in the peptide−receptor complex was also an α-helix. This is consistent with the observed lower AC-stimulating activity of hPTH-(1−30)-OH relative to hPTH-(1−30)-$NH_2$. It is also compatible with the earlier observation that PTH-(1−34)-$NH_2$ and [$Tyr^{34}$]-PTH-(1−34)-$NH_2$ have enhanced biological activity in comparison with the same analogues having the carboxyl group in place of the amide (Parsons et al., 1975).

Relating the solution structure of linear peptides with a high degree of conformational freedom, such as PTH, to the receptor-bound structure is not straightforward. Nonetheless, one can argue that the PTH-(17−31)-$NH_2$ sequence is most likely to be bound as an α-helix to the PTH receptor, since this structure already exists, at least in part, in this sequence in solution. Assuming that the binding structure in the 20−31 region is in an α-helical configuration, the question as to the way in which this helix binds to the receptor is important. Gardella et al. (1993) have studied numerous mutants in this region, and came to the conclusion that the hydrophobic face of the putative amphiphilic α-helix was most sensitive to substitutions. In particular, Leu-24, Leu-28, and Val-31 were intolerant to substitutions. We have also shown that replacement of Lys-27, a polar residue on the hydrophobic face of the helix, with a Leu resulted in an increase in AC-stimulating activity (Surewicz et al., 1993). Thus, the data point to the hydrophobic face of an α-helix being in direct contact with the receptor.

Our present data also point to C-terminal residues up to, and including, Leu-28 as being critical for functional binding to the receptor. The remaining residues, to residue 34, appear to contribute little to the binding in terms of the AC-stimulating activity. Thus, the concentrations of analogue for 50% full-stimulatory activity are about 16, 19, 20, and 27 nM for analogues (1−34)-$NH_2$, (1−31)-$NH_2$, (1−30)-$NH_2$, and (1−29)-$NH_2$, respectively. We have proposed that the ability of PTH analogues to restore bone mass in an osteoporotic rat model is coupled to its AC-stimulating as opposed to its PKC-stimulating activity (Rixon et al., 1994). However, we have more recently determined that hPTH-(1−30)-$NH_2$ has little activity in the osteoporotic rat model (J. Whitfield, unpublished data). Closer examination of the activity curves of Figures 1 and 2 reveals that the apparent breadth of these transitions from nonstimulation to full stimulation is broader for (1−34)-$NH_2$ and (1−31)-$NH_2$ than for (1−30)-$NH_2$ and (1−29)-$NH_2$. Thus, the former two have an activity between 1 and 10 nM concentrations that is missing in the latter two. This suggests the presence of a second, as yet unidentified receptor for PTH, which has a requirement for a longer sequence, up to and including Val-31. The presence of a second receptor has previously been suggested by Seitz et al. (1990), based on data for binding of rat PTH-(1−34) to ROS cells.

Any model for binding of this 20−31 region to the receptor should take into account the fact that PTHrP also binds to the same receptor (Jüppner et al., 1991) and has at least as much AC-stimulating activity as PTH (Kemp et al., 1987). It has been reported to have the same receptor binding region as PTH (Caulfield et al., 1990) and to have a putative α-helix within that region (Barden & Kemp, 1989; Ray et al., 1993). Willis (1994) demonstrated that hPTHrP-(1−34) bound to lipid micelles and gave an enhancement of α-helix similar to that observed with hPTH. This hPTHrP sequence need not bind to the receptor with the same backbone conformation, but it is likely the case, given its propensity to form an α-helix in this same region. The two binding regions, within residues 20−31, are shown in Figure 9. When modeled as α-helices, and viewed along their predominantly hydrophobic faces, there are some interesting similarities. Residues Arg-20 and Leu-24 are shared in the two sequences. Leu-28 in hPTH becomes an Ile in hPTHrP. Trp-23 is replaced by another aromatic residue, Phe, in hPTHrP. Lys-27 becomes a Leu in hPTHrP. However, replacement of Lys-27 by a Leu in hPTH-(1−34)-$NH_2$ resulted in an increase in AC-stimulating activity (Surewicz et al., 1993). The residues on the opposite face show only some general similarities, such as a predominance of polar residues. That this face is likely facing the solvent is supported by the mutagenesis studies of Gardella et al. (1993), wherein the residues on this face of the helix were shown to be relatively tolerant of substitution.

A common receptor for both PTH and PTHrP has been isolated and found to be part of a G-protein-linked receptor family (Jüppner et al., 1991; Segre & Goldring, 1993). This receptor has not only been found to stimulate AC activity but also to couple to a phospholipase-C-dependent hydrolysis of membrane-associated phosphatidylinositol 4,5-bisphosphate. The latter leads to a generation of diacylglycerol (Abou-Samra et al., 1992), which results in translocation of PKCs from the cytosol to the cell membrane. This PKC stimulation is independent of the AC-stimulating activity since it only requires the (29−32)-$NH_2$ fragment (Jouishomme et al., 1994). Given that the 25−34 sequence competes, albeit weakly, with the binding of the hormone for AC stimulation (Nussbaum et al., 1990), it is likely that the receptor binding region for PKC stimulation is an extension of that for AC simulation. While the binding region for AC stimulation very likely accommodates the hormone as an α-helix, there is no evidence that this is so for the remaining PKC-stimulating sequence. It is interesting to note, however, that this sequence is an extension of that shown in PTH to form an amphiphilic α-helix (Neugebauer et al., 1992). This bifunctional nature of PTH is similar to that reported for secretin, a hormone with a receptor in the same family as PTH (Jüppner et al., 1991). Glucagon release by the pancreatic islets required the entire hormone, but a shorter C-terminal fragment brought about insulin release (Kofod et al., 1991).

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BI943009K