Through the use of oligonucleotide-directed mutagenesis we have generated variants of a recombinant human parathyroid (PTH) hormone-(1–34)-homoserine (RPTH) in which a positively charged residue (Arg or Lys), a negatively charged residue (Glu), or a neutral residue (Gly) has been substituted at every position throughout the peptide. These 106 PTH analogs have been tested for their ability to stimulate cAMP production in the rat osteosarcoma cell line, UMR106. Analysis of these peptides led to the construction of several analogs containing multiple substitutions at sites of potential structural importance. Several of these analogs were shown to have 3-5-fold enhanced activity and receptor affinity. Circular dichroism (CD) and lipid binding studies were then performed on these analogs. Circular dichroism demonstrates enhanced helical content in the presence of lipid vesicles, particularly anionic lipid. The [Arg15,19,22, Lys29]RPTH (+6RPTH) analog requires higher concentrations of trifluoroethanol to attain enhanced helicity. The intrinsic tryptophan fluorescence of the peptides are blue shifted more in the presence of the anionic lipid dimyristoyl phosphatidylglycerol (DMPG) than with the zwitterionic lipid dimyristoyl phosphatidylcholine (DMPC). Effects of the peptides on the phase transition behavior of DMPC shows that +6RPTH has less effect on the lipid than does RPTH. This difference in lipid interaction is also exhibited with isothermal titration calorimetry, in which RPTH reacts exothermally with DMPG, while +6RPTH shows little or no heat change. The pH dependence of binding of the hydrophobic probe 1,1’-bis(4-anilino)naphthalene-5,5’-trisulfonic acid, also shows a difference in exposure of hydrophobic sites between RPTH and +6RPTH. The +6RPTH has about a 5-fold greater affinity for receptor binding. We suggest that this enhanced activity is a consequence of the altered lipid interaction of +6RPTH, combined with increased conformational flexibility, particularly in the carboxyl-terminal region of the molecule.

Peptide hormones interact with specific receptor proteins on cell surfaces. This specific interaction may be modulated by the target cell surface which can cause the accumulation of the hormone near the site of the receptor as well as inducing a particular folding and orientation of the peptide (1). An interesting example to study in this regard is parathyroid hormone. This hormone plays an important biological role in calcium metabolism (2). Intact parathyroid hormone is an 84-amino acid peptide, but a 34-amino acid fragment, comprising the amino-terminal segment of the hormone, maintains virtual activity (2). While the biological activity of this peptide will be determined by its interaction with its receptor, certain conformational and membrane-binding properties of the peptide will likely be required for maximal biological activity. This 34-amino acid peptide has regularly spaced hydrophobic amino acid residues which could form either a 310 helix or an α-helix with a hydrophobic domain that twists around the helix axis. This peptide can interact with phospholipids to attain a more structured conformation (3, 4). There also have been several NMR studies on the conformation of the 34-amino acid fragment of the human parathyroid hormone (hPTH-(1–34)). 1 These studies demonstrated an increased α-helical content in trifluoroethanol with the formation of two helical segments, one in the region of residues 3–13 and the other around residues 16–27 (5–7). More recently, a detailed NMR and molecular dynamics study of hPTH-(1–37) has appeared (8). This work concluded that this peptide in buffer contained an α-helical region between residues 5 and 10, a flexible link at residues 12 and 13 followed by a well-defined turn comprising residues 14 to 17. There is another α-helical segment between residues 17 and 28. Results from a series of hPTH-(1–34) analogs indicate that a number of amino acid residues can be substituted without loss of biological activity and in fact, in several cases there is enhanced activity. Some of the modified forms of the peptide hormone with enhanced activity have non-conserved substitutions. We wished to study how these alterations in sequence affected the interaction of these hormone analogs with both phospholipids and the PTH receptor and to determine if there is a relationship between changes in biological activity and differences in structural or lipid binding properties of these peptides.

MATERIALS AND METHODS

Reagents—Restriction enzymes, mammalian cell culture media, and Escherichia coli cell line DH10B (F− mcra Δ(mrr− hsdRMS− mcrBC) Δ807lacZΔM15 ΔlacX74 deor recA1 araD139 Δ(ara,leu)7697 galU galK λ− rpsL endA1 nupG) were purchased from Life Technologies, Inc./BRL.

1The abbreviations used are: hPTH, human parathyroid hormone; DMPC, dimyristoyl phosphatidylcholine; DMPG, dimyristoyl phosphatidylglycerol; bis-ANS, 1,1’-bis(4-anilino)naphthalene-5,5’-trisulfonic acid; LUV, large unilamellar vesicles; DSC, differential scanning calorimetry; TFE, trifluoroethanol; RPTH, recombinant hPTH-(1–34)-homoserine; +6RPTH, [Arg15,19,22, Lys29]RPTH homoserine; Arg15,22,30 [Arg15,22,30]RPTH-homoserine; Arg15, [Arg15]RPTH-homoserine; Lys29, [Lys29]RPTH-homoserine; PIPES, 1,4-piperazinediethanesulfonic acid; HPLC, high performance liquid chromatography.
Studies on Analogs of Recombinant Parathyroid Hormone

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(Gaithersburg, MD). UMR106 rat osteosarcoma cells were obtained from the American Type Culture Collection (ATCC). Tαq polymerase was from Perkin Elmer. His-bind resin was purchased from Novagen (Madison, WI) and used according to the manufacturer’s instructions. DNase and lysozyme were purchased from Boehringer Mannheim. Cyanogen bromide was purchased from Aldrich. Sep-Pak columns were from Millipore (Bedford, MA). Oligonucleotides were synthesized on an Applied Biosystems Inc. model 394 DNA synthesizer using ABI chemicals. Synthetic human parathyroid hormone-(1–34) (bPTH) and synthetic bovine parathyroid hormone-(1–34) (bPTH) were purchased from Bachem (Torrance, CA). pBAD18 was a kind gift from Dr. Luz-Maria Guzman. Dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) were purchased from Avanti Polar Lipids (Pelham, AL). The lipids showed a single spot on TLC at a 50-μg load, using I2 vapor for detection.

The bis-ANS was purchased from Molecular Probes (Eugene, OR).

PHT Expression Plasmid—The expression vector consisted of a modified form of the ara B-containing plasmid, pBAD18 (9), with the following sequence inserted downstream from the ara B promoter.

5′-GCT CGG GCT AGG TAA CTA ATG GAG GAT NheI SdI
ACA TAA ATG AAA GCT TAC TTC TrpL leader

GTT CTG AAA GGT TCC CTG GAC CGT GAC CCG GAA TTC

GTC CGC ATG ATC AAC CAG ATC TCC CAC CAC CAC CAT CAC CAT

His-bind

AAG CTG TCC CAG CAC CAT GAC CAG NheI

His-Le leader

His-Asn-Leu-Gly Lys-His-Le leader

SalI BglII

His-Asn-Leu Met-Ile-Asn-Met-

Val-Asp-Glu-Ile-Gln-Leu-Leu

Sequence I

The sequence contains a Shine-Dalgarno ribosome binding site (SSD) and TrpL leader peptide. This 17-amino acid leader sequence has previously been shown to enhance expression of small proteins and may promote the sequestering of fusions into inclusion bodies (10, 11). The polyhistidine site allows for rapid purification of recombinant parathyroid hormone (RPTH), and RPTH analogs via nickel chelation chromatography.

A RPTH gene, with the following coding sequence, was designed using high-use codons and constructed from partially overlapping oligonucleotides.

Val-Asp-Met-Ile-Asn-Met-

Ser-Val-Ser-Glu-Ile-Gln-Leu-Leu

His-Asn-Leu-Gly Lys-His-Asn-Ser-

Val-Asp-Met-Ile-Asn-Met-

Arg-Lys-Lys-Leu-Gln-Val-His-Asn-Pyr-

Tyr-Met-Gln-Ile-Ser

Sequence II

This RPTH gene was cloned into the Sall and BglII sites of the modified pBAD vector.

RPTH analog genes were constructed in a manner similar to that described for the RPTH gene. Each amino acid codon was individually replaced with (A/G)A(A/G), which codes for lysine, arginine, glutamate, or glycine. Transformants expressing the desired PTH variant genes were selected by plasmid DNA sequence analysis.

PHT Analog Expression and Purification—PHT analogs were expressed and purified as described previously (12) with the following modifications. Analogs were expressed in groups with concurrent expression of RPTH as a control. Following elution from His-bind resin, analogs were precipitated with 4 volumes of absolute ethanol. This precipitate was dissolved in 1 ml of 70% formic acid and a 200-fold molar excess of CNBr was added. The reaction was allowed to proceed for 2 h at room temperature in the dark under argon after which time the sample was loaded onto a Sep-Pak column, washed with 5 volumes of 20% acetonitrile, 0.1% trifluoroacetic acid, and then eluted with 3 ml of 60% acetonitrile, 0.1% trifluoroacetic acid. The RPTH analog was then lyophilized and stored at –20°C.

cAMP Assay—UMR106 cells were seeded at 2.5 × 10^5/well in 48-well microtiter plates and allowed to grow to confluence. Media (Dulbecco’s modified Eagle’s medium/F12, l-Glu, penicillin-streptomycin, 5% fetal bovine serum) was replaced daily. Assays were performed on cells 3–5 days post-confluence. Media was removed and replaced with 0.5 ml of fresh media containing PTH (or an analog) at the indicated concentration. cAMP was measured at 1, 10, 30, and 60 min. 3-Isothyl-1-methylxanthine. After 5 min of incubation, cells were washed once with ice-cold phosphate-buffered saline and the cAMP was extracted twice with 1 ml of absolute ethanol. The extractions were combined and ethanol removed by evaporation under vacuum. The dry residue was resuspended in 1 ml of scintillation proximity assay (“SPA”) buffer (Amersham), and cAMP concentration determined by using a commercially available SPA kit (Amersham).

PHT Iodination and Receptor Binding—PHT iodination and receptor binding were performed as described previously.

Preparation of Sonicated Vesicles—Lipids in powder form were suspended in PIPES buffer and vortexed vigorously; then placed in a bath-type sonicator (Cale Parmer Ultrasonic Model 8849–00) and sonicated to clarity.

Circular Dichroism—The circular dichroism (CD) spectra of the peptides were recorded on an AVIV model 61 DS solid-state CD instrument (AVIV Associates, Lakewood, N J). The instrument was interfaced with a computer, which was used for all data mathematical calculations. A 1-mm sample cell, maintained at 25°C with a thermostated cell holder, was used for all spectral studies. Peptide concentrations in solution, prior to the addition of lipid, were determined from the ultraviolet absorption at 280 nm and by the Pierce BCA Protein assay. The CD spectra were corrected for baseline and normalized per mol of amino acid residue. These spectra were then used for estimation of secondary structure by the procedure of Chang et al. (15) and reported as percent of α-helix (%H), β-structure (%β), turns (%T), and random coil or other structures (%R) present, respectively.

Intrinsic Fluorescence Spectra—Tryptophan fluorescence emission spectra were recorded on an SLM-Aminco Series 2 luminescence spectrometer, using 1-cm path length quartz cuvettes and 4-nm slits.

Differential Scanning Calorimetry (DSC)—Lipid films were made from DMPC or DMPG dissolved in chloroform/methanol (2/1, v/v) to which varying quantities of peptide dissolved in methanol were added. After solvent evaporation with nitrogen, final traces of solvent were removed in a vacuum chamber for 90 min. The lipid films were suspended in buffer by vortexing at 45°C for 30 s. The final lipid concentration was 1.5 μM at a lipid to peptide ratio of about 15, in buffer at pH 7.40. Two different buffers were used, one at physiological salt concentration was composed of 20 mM PIPES, 0.15 mM NaCl, 1 mM EDTA, 0.002% NaN3 and the other at low ionic strength was 10 mM sodium phosphate. The lipid suspensions were put through three freeze-thaw cycles and degassed under vacuum before being loaded into an MC-2 high sensitivity scanning calorimeter (Microcal Co, Amherst, MA). A heating scan rate of 39 K/h was employed. The bilayer to hexagonal phase transition was fitted to a single van’t Hoff component and the transition temperature reported as that for the fitted curve.

Titration Calorimetry—Isothermal heats of reaction were measured using the Omega cell of a Microcal titration calorimeter (16). Solutions were degassed under vacuum prior to use. A 0.1 mM peptide solution was placed in the 1.3-ml reaction cell and thermally equilibrated at 20°C. DMPC or DMPG as small (or sonicated) unilamellar vesicles were made in PIPES buffer or in 10 mM sodium phosphate, pH 7.4. A solution of 100 mM lipid was used to fill a 100-μl motor-driven syringe and 2.5-, 5-, or 10-μl aliquots were delivered at 5-min intervals. The peptide solution in the reaction vessel was stirred at 400 rpm and maintained at 35°C. The observed enthalpies were corrected for the small heat of dilution of lipid into buffer. The calorimeter was calibrated electromagnetically. The data were analyzed using the OMEGA v1.1 EMM Scientific Software (1987) from Microcal or ORIGIN™ Scientific Software for Microcal Omega data. Data were fitted to a one independent binding site model.

Bis-ANS Titration—The fluorescence study of the pH dependence of bis-ANS binding was performed by a procedure similar to that described by Butko et al. (17). Fluorescence was excited at 395 nm and emission was observed at 533 nm, with a 420-nm emission cut-off filter. Measurements were made in PIPES buffer containing either 1.5 or 3 μM peptide and 15 μM bis-ANS. The pH was changed by adding HCl or NaOH and the fluorescence intensity at 533 nm recorded at each pH.
RESULTS

Native PTH-(1–34) contains methionine residues at positions 8 and 18. Oxidation of methionine at position 8 results in substantial changes in secondary structure as determined by circular dichroism whereas oxidation of Met18 has little effect on secondary structure (18). The mono- or dioxidized forms of the peptide display significantly reduced affinity and biological activity (19). Previous reports (20) indicated that substitution of norleucine for methionine decreases the in vitro activity of the peptide to approximately 43% of the unsubstituted peptide. However, a peptide containing the phenylalanine to tyrosine substitution at position 34 in combination with Nle at positions 8 and 18 has an in vitro activity only slightly less (76%) than the unmodified peptide. Given the necessity of avoiding internal methionine residues to prevent fragmentation of the peptide following cyanogen bromide cleavage of the fusion protein, the methionine residues were changed to leucine and Phe has been changed to Tyr in the recombinant form of the peptide. Cleavage of the fusion peptide with cyanogen bromide results in the addition of a homoserine/homoserine lactone (Hse) residue at the carboxyl-terminal of the peptide (21). These substitutions, Leu, Tyr, Hse35, when combined into a synthetic PTH-Hse peptide (peak 2) comprises greater than 80% of the total protein and is RPTH. Comparison of the “crude” Sep-Pak-purified sample with an HPLC purified RPTH showed no significant difference in adenylate cyclase stimulatory activity (data not shown).

Dual point cAMP assays were performed using the crude Sep-Pak purified samples. Dose-response and receptor binding curves were performed on RPTH analogs which were purified to >95% purity by reverse phase HPLC on a Vydac C18 column. Purity and composition were determined by electrospray mass spectroscopy.

The ability to stimulate adenylate cyclase activity was tested in the rat osteosarcoma cell line, UMR106. Initially, 106 RPTH analogs (a positive, negative, and neutral charge at each position) were functionally tested at a concentration of 5 nM, slightly higher than the EC<sub>50</sub> of hPTH (EC<sub>50</sub>; 2.8 nM). Those analogs with activities greater than 50% of wild type were then retested at a peptide concentration of 1 nM. Analyzing peptides at two concentrations ensured that, for active peptides, one of the concentrations would lie on the linear portion of the dose-response curve and thereby yield an accurate measure of the relative activity.

Substitution of any of the four amino acids (Lys, Arg, Glu, and Gly) in positions 1–8 in all cases decreased the activity of the peptide to less than 20% of wild type activity and in many cases to below detectable levels (Fig. 3A). Positions 9 and 10 were somewhat more tolerant of substitution. Leu<sup>11</sup> may play a role in ligand receptor interaction since the Arg<sup>11</sup> analog, which

Fig. 1. Analogs were expressed and CNBr cleaved as described under “Materials and Methods” and HPLC purified to >95% purity. A shows the cAMP-stimulating abilities of the four analogs. Gln<sup>29</sup>-Lys (H) EC<sub>50</sub> ~ 1.2 nM, Glu<sup>19</sup>-Arg (B) EC<sub>50</sub> ~ 1.3 nM, Glu<sup>22</sup>-Arg (F) EC<sub>50</sub> ~ 2.6 nM, and Leu<sup>15</sup>-Arg (J) EC<sub>50</sub> ~ 1.7 nM. B shows the inhibition of receptor binding of [125I-(Nle<sup>8,18,34</sup>Tyr)<sub>34</sub>-bPTH-(1–34)-NH<sub>2</sub> Activities and receptor binding of synthetic bPTH, synthetic hPTH, RPTH, and +6RPTh are shown in C and D. Values are the mean of three experiments all performed in triplicate.
has a side chain backbone similar to leucine but which may also form ionic interactions retains complete activity but Glu¹¹ with its negative charge is inactive.

Chou-Fasman (22) calculations suggest a disordered region at positions 10–13 of hPTH-(1–34) while Garnier-Robson calculations predict a turn. Barden and Kemp (23) have shown by NMR the presence of a type-I β-turn at positions 10–13 and 16–19 in PTH-related peptide (PTHRP). NMR studies by Klaus et al. (24) using slightly different solution conditions, however, suggest that this region in hPTH is disordered and does not form a β-turn. A more recent study (8) suggests a flexible link at positions Gly¹² and Lys¹³ and a well-defined turn from His¹⁴ to Ser¹⁷. Substitutions at Gly¹² (Fig. 3B) either eliminate or significantly reduce (Lys < 10%) activity. This loss in activity could be due to the disruption in peptide structure. Chorev et al. (25) have shown, however, that substitutions of Ala, o-Ala, or α-aminoisobutyric acid at position 12 are well tolerated, suggesting that some flexibility at this position is tolerated but substitution with Pro decreases receptor binding by 2 orders of magnitude. Substitution at Lys¹³ is well tolerated. The Arg¹³ and Glu¹³ analogs are fully active and the Gly¹³ analog retains greater than 40% activity. Since substitutions at this position have little effect on CAMP stimulation, it is unlikely that this residue interacts with the receptor. Deletion of this amino acid, however, results in an analog with about 5% of the activity of that found in the complete peptide (26) and NMR analysis of this analog supports the hypothesis that it is important in the folding of the peptide. With the exception of Leu¹⁵, the remainder of the loop region, positions 14 through 17 (Fig. 3B), is tolerant of substitution with all analogs showing some activity. At position Leu¹⁵, the positively charged amino acids, Lys and Arg, retain greater than 50% activity while the negative and neutral substitutions, Glu and Gly, are completely inactive.

Positions 18–34 (Fig. 3, B and C), have been reported to have an amphipathic helical structure in the presence of TFE or lipid vesicles (27). Mutations which alter the hydrophobic face of the amphipathic helix, positions 21, 24, 28, and 31, dramatically reduce the activity of the peptide. Leu¹⁸ is predicted to initiate the helix, but substitution of either Glu or Arg at this position has only a moderate effect on the activity suggesting that this position is not critical to the overall structure. The hydrophilic surface of the helix has a highly charged character with 5 positively charged and three negatively charged residues, as well as a critical Trp at position 23. Conversion of the negatively charged residues, Glu¹⁹, Glu²², or Asp³⁰, to positively charged residues either maintains or slightly enhances the activity of the peptide. Amino acid substitution at Trp²³ dramatically decreases bioactivity of the peptide suggesting that this residue makes a critical hydrophobic contact. There is also good evidence to suggest (26) that positions 19 and 21 may play a significant role in intramolecular stabilization between the amino and carboxyl-terminals of the peptide.

Based on NMR analysis, Klaus et al. (24) have suggested that Arg²⁵ is involved in a salt bridge with Glu⁴. Substitution of any of the amino acids (Lys, Arg, Glu, Gly) at either of these positions significantly reduces the CAMP stimulatory ability of the peptide. If the side chains at these two positions are primarily involved in salt bridge formation, then one might expect that reversing them would have little effect on the structure and activity of the peptide. The peptide [Arg²⁵,Glu⁴]RPTH at 500 nM failed to stimulate a CAMP response suggesting that either salt bridge formation alone is not sufficient or that one or both of these amino acids also plays a direct role in ligand-receptor interaction.

Based on studies with model peptides (28), a salt bridge has also been postulated to occur between Glu²² and Arg²⁵ or Lys²⁶, which could help stabilize the amphipathic helix. Since the charge reversal analog E22R shows full activity, this potential salt bridge is unlikely to be important for activity. Charge reversal at positions Arg²³, Lys²⁶, Lys²⁷, or His²⁵, decrease the peptide activity to less than 10% of wild type. In NMR studies of PTHRPr, Barden and Kemp (23) have suggested the possibility that His³² forms a salt bridge with Glu⁴ thereby helping to stabilize the peptide structure. Since these two residues are highly conserved in both PTH and PTHRPR through a variety of species, they may be necessary for proper folding of the peptide. Our data are not completely consistent with this supposition since Lys³² or Gly³² analogs are completely inactive while Arg³² and Gly³² retain activity. Since the Gly³² analog retains...
nearly complete activity, it is unlikely that His$^{32}$ is involved in a salt bridge with Glu$^3$. We cannot rule out the possibility, however, that additional structural changes in the Gly$^{32}$ analog may allow other biologically active structures. Position 25 is particularly sensitive to substitution in that even a conservative substitution, R25K, decreases the activity by over 80%.

Fig. 3. A, B, and C, activity values of each analog are from three to six experiments each performed in triplicate. Activities are presented as a percentage of RPTH activity (mean ± S.D., n = 4–9).
Arg<sup>25</sup> may take part in a charge-charge interaction with the receptor whereas Lys<sup>25</sup> may be sterically hindered from performing the same function. Positions Asn<sup>33</sup> and Tyr<sup>34</sup> do not have dissociation constants slightly lower than either hPTH or RPTH. The +6RPTH analog has significantly enhanced bioactivity with an EC<sub>50</sub> = 0.9 nM and a K<sub>d</sub> = 1.5 nM. These values are approximately 3- and 5-fold lower, respectively, than the corresponding values for either hPTH or RPTH and are comparable to the values for bPTH.

In separate experiments, all possible combinations of the mutations comprising the +6RPTH analog were constructed along with several which contained substitutions at Asp<sup>30</sup>. Expression levels of these analogs ranged from 1 to 10 mg/mliter as compared to ~100 mg/mliter for either RPTH or +6RPTH. After HPLC purification, dose-response curves were generated as in Fig. 1 for each analog and EC<sub>50</sub> values determined by linear regression best fit from the curves (Table I). Three of these analogs, [Arg<sup>15,19,22</sup>]RPTH, [Arg<sup>22</sup>,Lys<sup>29</sup>]RPTH, and [Arg<sup>22</sup>,30]RPTH had EC<sub>50</sub> values comparable to that of the +6RPTH analog. The Arg<sup>22</sup> substitution was contained within each of these peptides yet, when assayed individually, it displayed an EC<sub>50</sub> value significantly lower than that observed for RPTH. While the EC<sub>50</sub> values of these three analogs appeared to be enhanced, the receptor affinity was not significantly changed. Other analogs of this series which contain the Arg<sup>22</sup> substitution are only slightly less active than the +6RPTH, with EC<sub>50</sub> values of approximately 1.8 nM. This suggests that within this series of substitutions, the Glu<sup>22</sup> to Arg substitution is necessary but not sufficient for enhanced activity. The E22R substitution does not appear to have as dramatic of an effect when combined in other series. It has been previously reported (30) that an D30R substitution significantly enhanced the adenylate cyclase stimulatory ability of the peptide. However, when both Glu<sup>22</sup> and Asp<sup>30</sup> are converted to arginine, the EC<sub>50</sub> values are comparable to or slightly lower (2-fold) than for RPTH. In this case the E22R substitution had very little effect on the bioactivity of the peptide.

Other substitutions had a much more modest effect on the adenylate cyclase activity. For example, unless combined with the Arg<sup>22</sup> substitution, the Arg<sup>15</sup>, Lys<sup>29</sup> combination appears to only slightly enhance the adenylate cyclase activity of the peptide. All combinations yielded peptides with adenylate cyclase activation activities intermediate between RPTH and the +6RPTH analog. Interestingly, only the +6RPTH peptide had both an EC<sub>50</sub> value and a K<sub>d</sub> value comparable to that observed with bPTH. The cause of this observation is currently unknown and warrants further investigation.

Qualitatively the RPTH and RPTH analogs behave similarly. They exhibit more structure in the presence of lipid than in its absence and DMPG promotes more structure than DMPC. The secondary structure content of the various peptides in the presence and absence of lipid has been calculated from the CD spectra (Table I) as described under “Materials and Methods.” The β-structure content is least accurately assessed by this criterion. Furthermore, β-structure aggregates may be formed by peptide-peptide interactions in the presence of lipid, rather than by lipid effects on the conformation of the peptide. We will therefore limit our discussion to changes in α-helical content under different conditions. Some differences among the various cases are that the unmodified RPTH has greater helical structure in the presence of DMPC, compared with the other peptides. In addition, the Arg<sup>15</sup> and the Lys<sup>29</sup> analogs are the only ones which exhibit a marked dependence of structure on peptide concentration, both in buffer and in the presence of DMPG. In other cases there is essentially no dependence on
peptide concentration and data at only one peptide concentration is shown.

TFE titrations were carried out in mixtures of TFE with 10 mM phosphate buffer, pH 7.4. RPTH shows an increased α-helical structure at a lower TFE concentration than +6RPTH, but both peptides achieve a full helical conformation by 50% TFE (Fig. 4), at 25°C. At this concentration of TFE, when heating up to 50°C, the RPTH retains, almost entirely, its helical structure. In contrast, the +6RPTH analog loses part of its α-helix to only 60% helicity.

The peptides have a Trp emission at or close to 350 nm in buffer, indicative of this residue being exposed to solvent in aqueous solution. Addition of small (or sonicated) unilamellar vesicles of DMPC did not greatly affect this emission maximum (not shown). Thus, although CD and DSC results indicate that some of these peptides interact with DMPC, this interaction does not lead to a sequestering of the Trp residue into a less polar environment. Addition of DMPG, however, caused a marked blue shift in the fluorescence emission (Table III), indicating that the tryptophan side chain (residue 23) becomes partially buried in a hydrophobic environment upon binding to DMPG. This observation is of particular interest because of the important role this residue has for receptor binding. There was no time dependence of the shifts observed. It has recently been suggested that Trp23 interacts hydrophobically with Leu15 (8).

DSC of mixtures of DMPC and the peptides was studied in two buffers, one of low ionic strength and the other of higher, physiological ionic strength. High ionic strength would suppress the ionic interactions between the cationic peptides and anionic charges on the lipid. In the presence of the low ionic strength buffer, 10 mM sodium phosphate, pH 7.4, the RPTH and the analogs greatly broaden the phase transition of the phospholipid, except for the +6RPTH and the Arg25 mutant which have little effect on the phase transition properties of this lipid (Fig. 5). When the DSC was done in PIPES buffer, pH 7.4, at the higher salt concentration of 0.15 M NaCl, none of the peptides had any effect on the phase transition of DMPC. In comparison (Fig. 5), all the peptides studied broaden the phase transition of DMPG in PIPES buffer.

Titrating a solution of peptide with sonicated vesicles of DMPG gave markedly different results for the RPTH and the +6RPTH analog (Fig. 6). The RPTH gave an exothermic reaction at 35°C. This process can be characterized by a binding constant of $2.8 \times 10^4$ M$^{-1}$ for the peptide binding to a cluster of 25 lipid molecules in PIPES buffer, pH 7.4, and $2.6 \times 10^4$ M$^{-1}$ for the peptide binding to a cluster of 10 lipid molecules in phosphate buffer, pH 7.4. A similar result was obtained with Lys25, which gave a binding constant of $2.7 \times 10^4$ M$^{-1}$ for the

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**Table II**

Estimated secondary structure content from circular dichroism

CD at 25°C in 10 mM phosphate buffer pH 7.4, 1-mm path length. (#) %T refers to percent of turns and %R to the remainder of the structure, not accounted for by the regular structures.

| Peptide | Lipid | Peptide concentration | Lipid/peptide | %α | %β | %δ | %R |
|---------|-------|-----------------------|---------------|----|----|----|----|
| RPTH    | None  | 53                    | 0             | 24 | 31 | 3  | 43 |
|         | DMPC  | 53                    | 14            | 32 | 20 | 10 | 38 |
| +6RPTH  | None  | 53                    | 14            | 54 | 6  | 36 | 9  |
|         | DMPC  | 57                    | 0             | 6  | 36 | 9  | 49 |
| Lys25   | None  | 57                    | 13            | 33 | 19 | 31 | 16 |
|         | DMPC  | 57                    | 13            | 33 | 19 | 31 | 16 |
| Arg19,22,30 | None | 50                  | 15            | 17 | 38 | 7  | 39 |
| Arg25   | None  | 50                    | 15            | 38 | 29 | 13 | 20 |
|         | DMPC  | 50                    | 15            | 38 | 29 | 13 | 20 |
|         | DMPG  | 50                    | 15            | 38 | 29 | 13 | 20 |
|         | Lys29 | 50                    | 15            | 38 | 29 | 13 | 20 |
|         | Arg15 | 50                    | 15            | 38 | 29 | 13 | 20 |
|         | DMPG  | 50                    | 15            | 38 | 29 | 13 | 20 |

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**Table III**

Intrinsic fluorescence emission

Spectra obtained at 25°C, with 1-cm path length quartz cuvettes, in 10 mM phosphate buffer pH 7.4. The excitation wavelength was 280 nm and 4-nm slits were used.

| Peptide | Lipid | Peptide concentration | Lipid/peptide | Emission maximum |
|---------|-------|-----------------------|---------------|------------------|
| RPTH    | None  | 52                    | 0             | 348              |
| +6RPTH  | None  | 52                    | 14            | 342              |
| Lys25   | None  | 57                    | 0             | 352              |
| Arg19,22,30 | None | 57                  | 13            | 338              |
| Arg25   | None  | 50                    | 15            | 348              |
|         | DMPC  | 50                    | 15            | 348              |
|         | DMPG  | 50                    | 15            | 348              |
|         | Lys29 | 50                    | 15            | 348              |
|         | Arg15 | 50                    | 15            | 348              |

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**Fig. 4.** TFE titration at 25°C in 10 mM phosphate buffer pH 7.4. ◉, 50 μM RPTH. ○, 50 μM +6RPTH. Path length was 1 mm.
peptide binding to a cluster of 10 lipid molecules, in phosphate buffer. In contrast, little or no enthalpy was observed under these conditions for the reaction of DMPG with the $RPTH_{16}$ mutant. The binding of either RPTH or the $RPTH_{16}$ mutant to DMPC by titration calorimetry showed only small heats of reaction, which could not be readily fitted into a binding isotherm. No corrections for the effective charge of the peptides at the lipid interphase were made.

The fluorescent probe bis-ANS has been used to identify hydrophobic binding sites on proteins since it exhibits an increased quantum yield in a less polar environment (31). Bis-ANS is negatively charged and therefore tends to bind better to proteins at low pH (17) where the protein is more cationic. This is observed for RPTH and two of the mutants but not with the $RPTH_{16}$ or the Arg$_{19,22,30}$ analogs which exhibit an anomalous pH dependence of the fluorescence of bis-ANS (Fig. 7).

DISCUSSION

A number of mutants of RPTH (net charge +1), with additional positive charges placed at key positions of potential structural importance, were studied. The amino acid substitutions in the analogs studied in this work did not greatly affect the conformational properties of the peptides in buffer, as exhibited by CD, and the presence of anionic lipid enhanced the helical content of all of the peptides in a similar manner (Table II). In addition, all of the peptides inserted into phospholipid bilayers to a similar extent as measured with shifts in the fluorescence emission spectra of Trp (Table III). However, there are marked differences in the nature of the interaction of the $RPTH_{16}$ analog (net charge +7) with lipid, compared with the other peptides.

It is interesting to compare the equilibrium constants for dissociation of the hormone from its receptor, for the various derivatives (Table I). The $RPTH_{16}$ mutant stands out as having the highest affinity for the receptor (lowest $K_D$). A priori one would expect an analog with greatly altered charge and sequence to be less active than the native hormone. We would like to speculate that the enhanced receptor binding affinity observed for the $RPTH_{16}$ derivative is related to its altered
conformational property. This may allow it to insert into membranes in a different manner, facilitated by its structure. This altered structure is not primarily a consequence of a difference in secondary structure.

Some of the altered features observed with +6RPTH as compared to RPTH are that the +6RPTH has: (a) no effect on the phase transition of DMPC; (b) no heat of reaction with DMPG as detected by titration calorimetry; (c) an anomalous pH titration with bis-ANS fluorescence; (d) increased hydrophilicity in the region between residues 11–18 as seen in hydrophathy plots (data not shown); (e) decreased structure in DMPC, observed with CD; and (f) decreased structural stability in TFE, observed with CD.

In the Arg15 mutant (net charge +2), some of these altered features were also present. This analog has a small effect on the phase transition of DMPC and its hydrophilicity between residues 11 and 18 is increased, but its activity is similar to that of the native RPTH. This indicates that a single charge replacement in the turn region, which would confer altered conformational properties in the surrounding areas by increasing its hydrophilicity, is by itself not sufficient for enhanced activity. If the charge replacement is made in the second hinge region only, around residues 29–30 (7), we obtain the mutant Lys29 (net charge +2) that behaves structurally like RPTH and does not show enhanced receptor affinity.

In the mutant Arg9,22,30 (net charge +7), three negatively charged residues were replaced by positively charged ones. These substitutions would greatly affect the stability of the carboxyl-terminal helix, retaining the integrity of the turn. However, this mutant behaved conformationally like RPTH including anomalous pH titration with bis-ANS and did not show any enhanced receptor binding affinity, indicating that the changes in charge groups by themselves are not sufficient for altered activity and that the conformation of the molecule is not greatly altered. Concomitant changes seem to be necessary in more than one region of the molecule for increased activity. This result is consistent with reports by Avnur et al. (32) that complete substitution of positions 24–34 in PTHRP with a model amphipathic helix yields a peptide which has only a slightly enhanced bioactivity.

Biotinylations of Lys26 or Lys27 (33) had no effect on receptor binding affinity and single site mutations of K26Q (34), H32R, or D30Y (35) had no effect on either receptor binding affinity or adenylate cyclase activity. Taken together, this data suggests that the hydrophilic surface of the amphipathic helix in native PTH may play a limited role in ligand-receptor interactions, but that this interaction can be enhanced by the further addition of positively charged residues to the surface. This additional positive charge may allow the peptide to bind more tightly to a negatively charged surface on the receptor or with negatively charged lipid head groups in the cellular membrane. The data also suggests a possible long range effect on the amino-terminal domain of the peptide.

One of the predominant features of the +6RPTH mutant is its decreased affinity for binding to lipid. This is likely to be a consequence of its conformational properties since lipid affinity is not correlated simply with the overall charge on the peptide. The critical factor determining the overall conformation for this series of analogs is likely to be the conformation near the middle of the molecule which permits the hydrophobic areas in the helices at the amino end and near the carboxyl terminus to become sequestered, preventing their interaction with lipids. The Arg15 mutant by itself does not show enhanced activity indicating that the carboxyl-terminal substitutions present in +6RPTH, may lead to enhanced activity through alternative conformational arrangements. The conformational alteration seen in +6RPTH is also indicated by the temperature sensitivity of the CD of this peptide in 50% TFE. The Arg19,22,30 mutant by itself, with 3 Arg substitutions in the carboxyl-terminal region, has somewhat lower affinity for lipid than RPTH as exhibited by the broadening seen on the phase transition of DMPC; but this broadening is much greater than that exhibited by +6RPTH or Arg15, which hardly affect the DMPC main transition. Thus, the extent of interaction of Arg19,22,30 is intermediate between the RPTH and the +6RPTH mutant. This is likely to be a consequence of differences in the bend region.

Generally the ability to bind lipid has been associated with increased hormone binding potency. In the case of RPTH, the decrease in lipid binding affinity may limit nonspecific binding to the membrane and therefore be a factor favoring partitioning into the receptor.

Functions other than receptor binding and adenylate cyclase activation may also be mediated by PTH. Jouishomme et al. (36) and Sonjen et al. (37) have suggested that the carboxy-terminal of the peptide may play a role in the stimulation of intracellular Ca2+/protein kinase C pathway leading to both increased DNA synthesis and creatine kinase activity. The analogs described here have not yet been tested for their ability to stimulate the protein kinase C pathway.

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