Reimann, R. M., Walsh, D. A., & Krebs, E. G. (1971) *J. Biol. Chem. 246*, 1986-1995.
Rossi, P., Giorgi, M., Geremia, R., & Kincaid, R. L. (1988) *J. Biol. Chem. 263*, 15521-15527.
Sharma, R. K. (1990) *J. Biol. Chem. 265*, 1152-1157.
Sharma, R. K., & Wang, J. H. (1979) *Adv. Cyclic Nucleotide Res. 10*, 187-198.
Sharma, R. K., & Wang, J. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2603-2607.
Sharma, R. K., & Wang, J. H. (1986a) *J. Biol. Chem. 261*, 14160-14166.
Sharma, R. K., & Wang, J. H. (1986b) *J. Biol. Chem. 261*, 1322-1328.
Sharma, R. K., & Wang, J. H. (1986c) *Biochem. Cell. Biol. 64*, 1072-1080.
Sharma, R. K., Wang, T. H., Wirch, E., & Wang, J. H. (1980) *J. Biol. Chem. 255*, 5916-5923.
Sharma, R. K., Taylor, W. A., & Wang, J. H. (1983) *Methods Enzymol. 102*, 210-219.
Sharma, R. K., Adachi, A.-M., Adachi, K., & Wang, J. H. (1984) *J. Biol. Chem. 259*, 9248-9254.
Sharma, R. K., Mooibroek, M., & Wang, J. H. (1988) *Mol. Aspects Cell. Regul. 5*, 265-295.
Shenolikar, S., Thompson, W. J., & Strada, S. J. (1985) *Biochemistry 24*, 672-678.
Vandermeers, A., Vandermeers-Piret, M.-C., Rathe, J., & Christophe, J. (1983) *Biochem. J. 211*, 341-347.
Wang, J. H., Sharma, R. K., & Mooibroek, M. (1990) *Wiley Ser. Mol. Pharmacol. Cell Regul. 2*, 19-59.

Cyclic Parathyroid Hormone Related Protein Antagonists: Lysine 13 to Aspartic Acid 17 [*i* to (*i* + 4)] Side Chain to Side Chain Lactamization

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ABSTRACT: Cyclization of parathyroid hormone related protein (7-34)amide [PTHrP(7-34)NH_2] via covalent bond formation between the ϵ-amino of Lys^{13} and the β-carboxyl of Asp^{17} yielded a 20-membered ring lactam. This analogue, [Lys^{13},Asp^{17}]PTHrP(7-34)NH_2, was 5-10-fold more potent than the linear parent peptide (K_b = 15 and 18 nM in PTH receptor binding assays, and K_i = 130 and 17 nM in PTH-stimulated adenylate cyclase assays in bovine renal cortical membrane and in human bone derived B10 cells, respectively). In contrast, a linear analogue in which charges in positions 13 and 17 were eliminated and other stereoisomers of the above-mentioned lactam in which either Lys^{13} and/or Asp^{17} were replaced by the corresponding D-amino acids were much less potent with regard to antagonist bioactivity than the parent peptide. The rationale for the design of the lactam as well as the conformational implications for the PTHrP sequence in light of reported models suggested for the 1-34 peptide are described. The potential use of conformationally constrained analogues for elucidating the "bioactive conformation" of antagonists and for the design of substantially simplified molecular structures for antagonists is discussed.

The purification, cloning, and structural elucidation of parathyroid hormone related protein (PTHrP) represents the successful conclusion of a long search for a tumor-secreted factor responsible for many cases of humoral hypercalcemia of malignancy (Moseley et al., 1987; Stewart et al., 1987; Mangin et al., 1988; Thiede et al., 1988). The new hormone, termed parathyroid hormone related protein (PTHrP), is structurally homologous to PTH in only a limited domain comprised of 8 out of 13 residues at the N-terminus (Suva et al., 1987) (see Figure 1). However, PTHrP interacts with what has been regarded conventionally as PTH receptors and stimulates actions similar to those caused by PTH with a potency comparable, for the most part, to that of PTH (Rodan et al., 1983; Stewart et al., 1983; Strewler et al., 1983).

Thus, PTH and PTHrP provide an unusual opportunity to conduct structure-activity studies based on two endogenous nonhomologous peptide hormones which interact with the same receptor to produce a very similar array of biological effects.

Like PTH, the synthetic N-terminal peptide, PTHrP(1-34)NH_2, is a full agonist in bone-derived (rat osteosarcoma cells, ROS 17/2.8 and UMR-106) and kidney-derived assays [bovine renal cortical membranes (BRCM) and opossum kidney (OK) epithelial cells (Horiuchi et al., 1987; Strewler et al., 1987; Juppner et al., 1988; Nissenson et al., 1988; Shigeno et al., 1988; Kemp et al., 1987; Rabbani et al., 1988; Pizurki et al., 1988)].

[Tyr^{34}]bPTH(7-34)NH_2 was the first PTH antagonist shown to be effective in vitro and in vivo (Mahaffey et al., 1979; Rosenblatt et al., 1988). Following the same rationale, truncation of 6 amino acid residues from the N-terminus of the PTHrP(1-34) agonist generated a potent antagonist, PTHrP(7-34)NH_2 (McKee et al., 1988). This peptide inhibited binding of radiolabeled PTH to receptors and anta-

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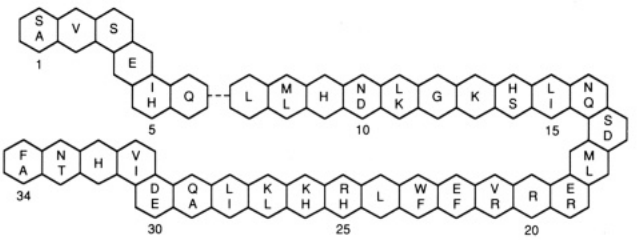

FIGURE 1: Sequence homology of the N-terminal 1–34 sequence of human PTH and human PTHrP. Common residues for both sequences are given in single-letter notation in the center of the hexagon. Sites that differ in their amino acid sequence are marked by two single letters in the upper and lower corners of the hexagon. The letter in the upper corner corresponds to the PTH sequence, and the letter in the lower corner corresponds to the PTHrP sequence. Deletion of six amino acid residues from the N-terminus eliminates a considerable portion of homology shared by the two hormones.

gonized PTH-stimulated adenylate cyclase in both kidney- and bone-based assays. Substitution of D-Trp for Gly at position 12 in the 7–34 sequences of both PTH and PTHrP resulted in substantial enhancement of antagonist activity (Chorev et al., 1990a). However, in a specialized bone-based assay designed to increase responsiveness to PTH-stimulated adenylate cyclase, PTHrP(7–34)NH_2 and [D-Trp^{12}]PTHrP(7–34)NH_2 were found to possess partial agonist activity, as distinguished from the corresponding PTH(7–34)NH_2-derived analogues, which were pure antagonists (McKee et al., 1990; Nutt et al., 1990) devoid of agonist-like properties. This difference between the PTH- and PTHrP-derived 7–34 sequences was also observed in vivo: the PTHrP analogue produced a phosphaturic effect in thyroparathyroidectomized rats (Horiuchi et al., 1989), whereas the PTH peptide lacked this activity.

Recently, we were able to show that the nonhomologous amino acid residues in the 7–13 sequences of PTH and PTHrP play a major role in determining the pharmacological profile of PTH- and PTHrP-derived peptides (Nutt et al., 1990). Substitution of Asp^{10} and Lys^{11} in the PTHrP(7–34)NH_2 sequence with the corresponding amino acids from the native PTH sequence, namely, Asn and Leu, respectively, resulted in hybrid analogues that were pure antagonists. The reciprocal effect was obtained upon substituting Asn^{10} and Leu^{11} in [Tyr^{34}]bPTH(7–34)NH_2 with the corresponding amino acid residues from the native PTHrP sequence: [Asp^{10},Lys^{11},Tyr^{34}]bPTH(7–34)NH_2 was 6-fold less potent as an antagonist than the parent compound, and partial agonist properties were revealed (Nutt et al., 1990).

Taken together, these observations and others (Chorev et al., 1990a–c) suggest that the activation domain is not confined to the 1–6 N-terminal region, as was previously suggested, but extends at least to the 10–12 region. Chou–Fasman analysis predicts that, for PTH(1–34), the regions 1–9 and 16–28 are present as α-helices, residues 12–15 form a β-turn, and the remaining residues adopt a β-sheet structure (Chorev et al., 1990a). However, Lee and Russell could not detect any secondary structural elements for hPTH(1–34) in aqueous solutions employing ^{1}H NMR techniques (Lee & Russell, 1989). For PTHrP(1–34) the Chou–Fasman alogarithm predicts that residues 1–9, 13–19, and 29–34 form α-helices, β-turns are present between residues 9 and 12 and between residues 19 and 22, and the remaining sequence forms β-sheet structure (Chorev et al., 1990a). On the basis of ^{1}H NMR and CD studies, Barden and Kemp proposed that the secondary structure of PTHrP in water is composed of an α-helical 3–9 segment and two type I β-turns, comprising residues 10–13 and 16–19, which are further stabilized by two salt bridges between the ϵ-amino group of Lys^{13} and the β-carboxyl group of Asp^{10} and between the δ-guanido NH_2 function of Arg^{21} and the β-carboxyl group of Asp^{17} (Barden & Kemp, 1989).

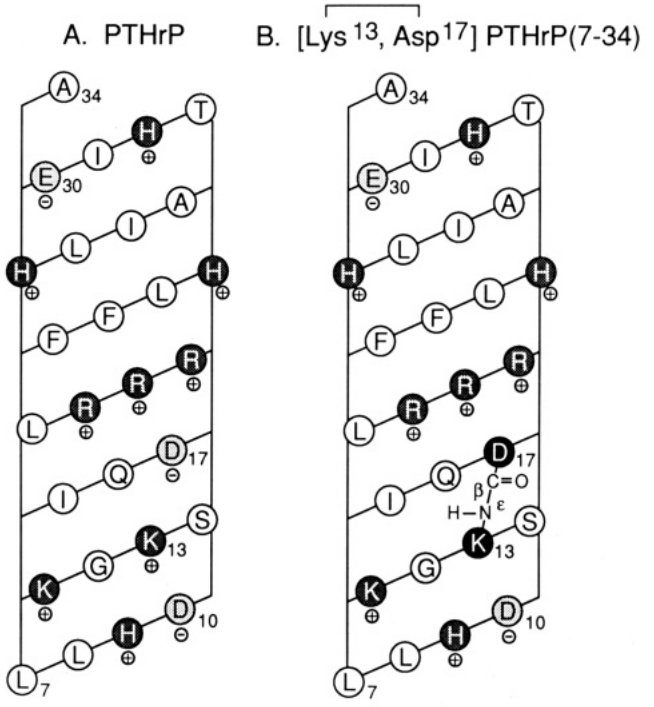

FIGURE 2: Linear PTHrP(7–34) schematically presented in an α-helical conformation (A). Cyclic lactam formed between Lys^{13} and Asp^{17} in an α-helical conformation (B). Key amino acids are highlighted: postively charged (heavily shaded) and negatively charged residues (lightly shaded) and residues participating in the cyclization (black).

Molecular rigidification reduces the conformational variations available to a particular molecule. This is especially true if the molecule to be studied is a linear peptide in which numerous degrees of conformational freedom in the backbone and side chains exist. Various studies have found that molecular rigidification via cyclization of a linear peptide may result in enhanced potency, improved selectivity, increased metabolic stability, and prolonged bioactivity. Cyclization can contribute toward structural simplification and provide insight into the bioactive conformations of a peptide (Deber et al., 1976; Kessler, 1982; Hruby, 1982a, 1985; Veber & Freidinger, 1985).

In this paper we have used molecular rigidification by lactam formation between residues 13 and 17 of PTHrP(7–34)NH_2 to address whether the conformations suggested for PTHrP(1–34) are relevant to antagonist activity. This specific modification was chosen because it should stabilize and extend an α-helical element predicted to be present on the basis of Chou–Fasman analysis (see Figure 2). The results indicate that locking the side chains of Lys^{13} and Asp^{17} together, via lactam formation, generates a more potent antagonist. In addition, this result suggests that the salt bridges proposed for the agonist peptide by Barden and Kemp (1989) are not im-

portant for the bioactive conformation of the antagonist.

Materials and Methods

Materials. Ultrapure-grade [Nle^{8,18},Tyr^{34}]bPTH(1–34)-NH_2, *N*-Boc-L-Asp(α-OBZl), *N*-Boc-L-Asp(β-cHex)-OH, *N*-Boc-D-Asp(α-OBzl), *N*-Boc-L-Lys(N^{ϵ}-Fmoc)-OH, *N*-Boc-D-Lys(N^{ϵ}-Fmoc)-OH, *N*-Boc-L-Lys(N^{ϵ}-Ac)-OH, and *N*-Boc-N^{π}-Bom-L-His-OH were obtained from Bachem Inc. (Torrence, CA). The rest of the *N*-Boc-protected amino acid derivatives, *p*-methylbenzhydrylamine resin hydrochloride (1% cross-linked, 0.64 mmol of nitrogen/g), *N*,*N*′-dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotryazole, diisopropylethylamine (DIPEA), piperidine, and trifluoroacetic acid (TFA) were purchased from Applied Biosystems Inc. (Foster City, CA). Dichloromethane (DCM) and *N*,*N*-dimethylformamide (DMF), both B&J brand, were purchased from Baxter Healthcare Co. (Muskegon, MI). Hydrogen fluoride was purchased from Matheson (Secaucus, NJ). *p*-Cresol was purchased from Aldrich Chemical Inc. (Milwaukee, WI). Bovine serum albumin, Tris-HCl, phosphocreatine, creatine phosphokinase, GTP, 3-isobutyl-1-methylxanthine, and Mg-ATP were obtained from Sigma (St. Louis, MO). (Benzotriazol-1-yl-oxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) was purchased from Reichelieu Biotechnologies (St.-Hyacithe, QC, Canada). Bovine kidneys were the gift of Baums Meat Packing Inc. (Hatfield, PA).

SaOS-2/B10 Cell Cultures. SaOS-2/B10 cells were grown in RPMI 1640 media (Mediatech, Washington, DC), 0.1 ng/mL glutamine, and kanamycin supplemented with 10% fetal bovine serum as described by Chorev et al. (1990b).

PTH Receptor Binding and Adenylate Cyclase Assays. Kidney-based assays were performed with bovine renal cortical membranes (BRCM) following the procedures of Goldman et al. (1988).

Bone-based assays were performed with human osteosarcoma SaOS-2/B10 cell cultures. Cyclic AMP was measured according to the procedure described by Rodan et al. (1983), including modifications reported by Chorev et al. (1990b). Testing for partial agonist activity in analogues included in this study was performed in a specialized, sensitive assay using bone-derived ROS 17/2.8 cells treated with dexamethasone and pertussis toxin as described by McKee et al. (1990). Receptor binding affinities for PTHrP analogues were obtained by using cells plated in 24-well plates (Costar, Cambridge, MA) in RPMI 1640 medium supplemented with 1% BSA, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, and 0.1% sodium azide. [Nle^{8,18},mono-^{125}I-Tyr^{34}]bPTH(1–34)NH_2 (50 000 cpm/well) was added to confluent monolayers in the absence or presence of varying amounts of analogue in a final volume of 0.25 mL. Cultures were incubated at room temperature for 2 h. The binding reaction was terminated by placing the cultures on ice and washing four times with ice-cold phosphate-buffered saline. Radioactivity associated with the cells was recovered by solubilizing the cells in 1 mL of 1 N NaOH. In general, 6% of total counts added bound, and specific binding was between 65% and 75%.

Data Analysis. Inhibition constants for binding (K_b) and adenylate cyclase (K_i) were calculated according to Cheng and Prusoff (1973).

Peptide Synthesis and Analytical Procedures. The peptides were synthesized on an Applied Biosystems 430A automated peptide synthesizer using version 1.2 of the software and a modification of Merrifield's solid-phase procedure (Merrifield, 1969). The synthesis of sequences 7–12 and 18–34 of PTHrP followed the procedure outlined by Chorev et al. (1990a) including the following modifications (see Figure 3): after recoupling of each of the three arginines (residues 18–21) and histidines (residues 25 and 26) and prior to the removal of the N^{α}-Boc protecting group, an acetylation of the residual free α-amino groups was carried out by means of a DCC-mediated acetic acid (114 μL, 2 mmol) coupling. The formation of the lactam during assembly of the peptide chain on the resin utilized the methodology introduced by Felix and co-workers in the synthesis of cyclic human growth hormone releasing factor (Felix et al., 1988a,b). This approach enables most of the synthesis to be conducted with the standard N^{α}-Boc protection/TFA deprotection. Only during the incorporation of residues 17–13 is a modified procedure required. This modification utilizes a 9-methylfluorenyl ester on the β-carboxyl of Asp^{17} and an N^{ϵ}-Fmoc group on Lys^{13}, both of which are labile when exposed to the high concentration of base regularly used to neutralize the α-amino TFA salt prior to coupling. Therefore, the synthesis of the lactam portion (residues 13–17) required the following modifications: Neutralization of the TFA salts, prior to coupling of each of the residues comprising the lactam portion, was carried out with 5% DIPEA in DMF. Asp^{17} and Lys^{13} were incorporated as Boc-L-Asp(β-OFm) and Boc-L-Lys(N^{ϵ}-Fmoc)-OH derivatives (Bolin et al., 1989). Deprotection of side chain protecting groups N^{ϵ}-Fmoc and β-OFm from Lys^{13} and Asp^{17}, respectively, included the following steps (15 mL of solvent per each step): 20% piperidine/DMF (2 × 1 min and 1 × 20 min), wash with DMF (2 × 1 min), treatment with 20% DIPEA/DMF (2 × 1 min), and wash with DMF (2 × 1 min). The cyclization manipulation included two cycles of the following steps (quantities correspond to 900 mg of resin-bound peptide (≈260 μmol): addition of BOP (345 mg, 780 μmol) followed by 1.5% DIEA/DMF (60 mL for 2 h) and washes with DMF (1 × 2 min), DCM (4 × 1 min), and DMF (2 × 1 min). Following ring closure acetylation of any residual ϵ-amino groups were performed by using DCC-mediated acetic acid coupling. The completed side chain protected resin-bound lactam was cleaved from the resin with simultaneous removal of side chain protecting groups by treatment with 10% cresol/HF for 75 min at 0 °C. The purification of the crude peptides followed the procedure described by Chorev et al. (1990a).

Results

Analysis of Peptides. The purified peptides were analyzed by analytical reversed-phase HPLC (RP-HPLC) on Vydac protein C_{18} columns: k' values and elution conditions are shown in Table I. Amino acid analysis data were also obtained for each analogue (data not shown).[1] Molecular weights of the analogues were obtained by fast-atom-bombardment mass spectrometry (FAB-MS) (Table I). These data are in full agreement with the expected values. The purity of analogues based on analytical RP-HPLC and amino acid analyses was >98%.

Biological Activities of Peptides. The inhibition of PTH binding by the analogues to BRCM and SaOS-2/B10 cells and the antagonism of [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH_2-stimulated adeylate cyclase activity in both assay systems are summarized in Table II. Dose-dependent inhibition of PTH-stimulated adenylate cyclase in SaOS-2/B10 cells by the parent (nonmodified) antagonist, PTHrP(7–34)NH_2, and by the lactam analogues **2** and **4** is depicted in Figure 3A. Dose-dependent inhibition of PTH binding to B10 cells by the same analogues is depicted in Figure 3B.

[1] The amino acid analysis data were submitted to the scrutiny of the reviewers and will be furnished upon request made directly to the authors.

Table I: Physicochemical Characterization of [Lys[13],Asp[17]-Modified]PTHrP(7–34)NH_2 Analogues

analogue:	1 [Lys[13](Nε-Ac),Asn[17]]	2 [Lys[13],Asp[17]]	3 [D-Lys[13],Asp[17]]	4 [Lys[13],D-Asp[17]]	5 [D-Lys[13],D-Asp[17]]
		FAB-MS			
formula	$C_{163}H_{260}N_{50}O_{37}$	$C_{162}H_{255}N_{49}O_{36}$			
calculated MW	3407	3347	3347	3347	3347
$[M]^+$ found	3408	3347	3346	3345	3346
		RP-HPLC[a]			
k'	18.1	18.5	13.5	12.6	12.1
(% B_0)–(% B_{30})[b]	15–30	15–35	15–35	20–35	20–35

[a] The analogues were analyzed on a Vydac protein C_{18} column (15 × 0.42 cm) at a flow rate of 1.5 mL/min and monitored at 214 nm. [b] The linear gradient used was of the following composition: A = 0.1% TFA in acetonitrile/H_2O (1:19); B = 0.1% TFA in acetonitrile.

Table II: Biological Activities of PTHrP(7–34)NH_2 Lactam Analogues[a]

PTHrP(7–34)NH_2 and derivatives	BRCM (nM)		B10 cells (nM)	
	K_b[b]	K_i[c]	K_b[b]	K_i[d]
no modification	100 ± 30	610 ± 130	170 ± 8	80 ● 10
1, [Lys[13](Nε-Ac), Asn[17]]	240 ± 30	2000 ● 430	210 ± 4	120 ± 20
2, [Lys[13],Asp[17]]	15 ± 2	130 ± 20	18 ● 2	17 ± 6
3, [D-Lys[13],Asp[17]]	420 ● 60	1300 ± 240	360 ± 25	350 ± 35
4, [Lys[13],D-Asp[17]]	1100 ± 170	>10000	1400 ± 130	900 ± 60
5, [D-Lys[13],D-Asp[17]]	2000 ± 500	>10000	1400 ± 340	550 ± 30

[a] Values are the mean ± SEM from at least three experiments. [b] Inhibiting binding of 25000 cpm of [Nle[8,18],mono-[125]I-Tyr[34]]bPTH(1–34)NH_2 to 50 μg of BRCM membranes SaOS2 or B10 cells. [c] Antagonist activity reflects inhibition of 3 nM [Nle[8,18],Tyr[34]]bPTH(1–34)NH_2. [d] Antagonist activity reflects inhibition of 0.25 nM [Nle[8,18],Tyr[34]]bPTH(1–34)NH_2.

In analogue **1**, the charged functions on Lys^{13} and Asp^{17} were modified by acetylation of the ε-amino and amidation of the β-carboxyl, respectively. Elimination of these charges resulted in a 2–3-fold reduction in antagonist potency in both BRCM assays compared to the nonmodified linear parent peptide, PTHrP(7–34)NH_2. Reduced potency of this analogue was also observed in the SaOS-2/B10 assays (about 70% compared to the parent peptide). Cyclization via lactamization through amide bond formation between the side chains of amino acids 13 and 17, [Lys[13],Asp[17]]PTHrP(7–34)NH_2 (analogue **2**), resulted in an analogue with substantially increased binding affinities and antagonistic activities for PTH-stimulated adenylate cyclase than the linear parent peptide in BRCM and SaOS-2/B10 systems.

Changes in the chirality of Lys^{13} and/or Asp^{17} resulted in lactam stereoisomers (analogues **3**–**5**) which were very weak antagonists. Inversion of chirality at Asp^{17} was the most detrimental, either together with or separated from the inversion of chirality at Lys^{13}. Analogues [Lys[13],D-Asp[17]]- and [D-Lys[13],D-Asp[17]]PTHrP(7–34)NH_2 (analogues **4** and **5**, respectively) were 1–2 orders of magnitude less potent than the L,L-lactam, analogue **2**. The effects of introducing D-Lys in position 13 of the lactam upon biological potencies closely resembled the effects seen following charge elimination in the linear peptide (cf. analogues **3** and **1**, respectively, in Table II). Thus, [D-Lys[13],Asp[17]]PTHrP(7–34)NH_2 (analogue **3**) was 10–30-fold less active than the most active lactam, analogue **2**.

The potency of the linear and cyclic antagonists analogues **1**–**5** in the BRCM–PTH binding assay is about 1 order of magnitude higher than their potency in inhibition of PTH-stimulated adenylate cyclase in the same system. This corresponds to previous observations for other series of linear PTH-derived antagonists in the BRCM system (Goldman et al., 1988; Chorev et al., 1990a). This characteristic discrepancy in potencies in the BRCM system does not occur in the SaOS-2/B10 system (Table II).

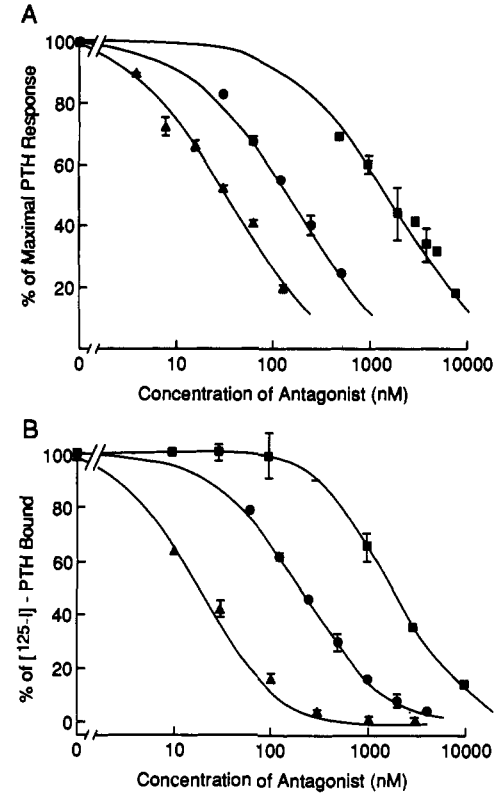

FIGURE 3: Effects of PTHrP(7–34)NH_2 and the corresponding lactams on (A) inhibition of PTH-stimulated adenylate cyclase and (B) binding of radiolabeled PTH ligand, [Nle[8,18],mono-[125]I-Tyr[34]]bPTH(1–34)-NH_2, in B10 cells. Assays were performed as described under Materials and Methods. Analogues: PTHrP(7–34)NH_2 (●); [Lys[13],Asp[17]]PTHrP(7–34)NH_2, analogue **2** (▲); [Lys[13],D-Asp[17]]-PTHrP(7–34)NH_2, analogue **4** (■).

Since the parent linear analogues of PTHrP, PTHrP(7–34)NH_2 and [D-Trp[12]]PTHrP(7–34)NH_2, when tested in a sensitive bone-based assay system designed to detect weak bioactivity, demonstrated partial agonism (McKee et al.,

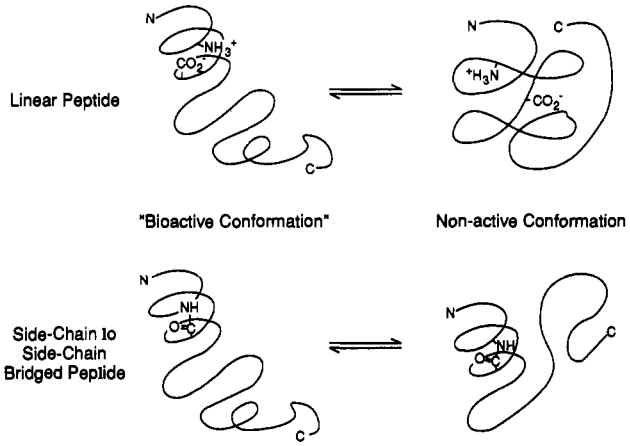

FIGURE 4: Schematic presentation of conformational equilibrium of linear and side chain to side chain lactam-bridged peptide. In the latter case locking the α-helical motif essential for the "bioactive conformation" prevents complete randomization of the cyclic structure.

1990), the effect of these novel structural modifications on agonist activity was examined in the specialized assay system. The most active lactam, [Lys[13],Asp[17]]PTHrP (analogue **2**), was devoid of any partial agonist-like activity at concentrations up to 10 μM (data not shown). The less active analogues, the linear analogue **1** and the lactam **3**, did show some marginal partial agonist-like activity at 10 μM (data not shown). The degree of agonism observed was similar to that found for 10 μM [Tyr[34]]bPTH(7–34)NH_2 under identical conditions (McKee et al., 1990).

Discussion

One of the most successful means for identifying a "bioactive conformation" for a hormone and to subsequently design highly potent hormone analogues is to introduce conformational constraint. Side chain to side chain cyclization, yielding cyclic heterodetic peptides, is only one of several modes of cyclization available in peptides which will result in conformational constraint. The premise of such an approach is the assumption that the side chains and their functions involved in cyclization are not essential for interaction with the macromolecular target (e.g., receptor). Such an approach also anticipates that the conformational constraints introduced via cyclization will preferentially "lock" the molecule in the putative "bioactive conformation" or in a closely related structure. In the enkephalin (DiMaio & Schiller, 1980; DiMaio et al., 1982; Mosberg et al., (1983) and somatostatin (Veber et al., 1978, 1984; Kessler et al., 1986, 1988) systems, cyclization was utilized to elucidate bioactive conformations and to achieve higher potency and other desired properties.

PTHrP(7–34)NH_2 is a linear peptide of 28 amino acids. In order to facilitate the elucidation of the bioactive conformation, we constrained the conformation, attempting to lock structural features in a position favorable for receptor interaction. In a helical structure, side chains of two residues separated by three intervening residues, namely, residues i and $(i + 4)$, are positioned in close proximity across a single helical pitch. Figure 4 depicts schematics of a putative conformational equilibrium for the linear PTHrP(1–34) sequence and the corresponding side chain to side chain, Lys[13]-to-Asp[17], lactam in their low-energy conformations. Although the putative helical segment may be stabilized by a salt bridge, once the bridge is disrupted, the whole structure may randomize. On the other hand, once the ϵ-amino and β-carboxyl groups are locked via a covalent bond, the parts of the molecule proximal to this structural constraint will be forced into maintaining to a greater extent the preferential low-energy α-helical organization. Thus, the cyclic molecule will spend more time (statistically) in the α-helical conformation than the linear peptide and will never be subjected to an absolute randomization. Preliminary circular dichroism spectrometry studies (CD) suggest that the most bioactive lactam analogue **2** has the highest helical content in the presence or absence of detergent micelles (unpublished results). To examine the effects of charge elimination which occurs when the lactam bridge is formed, and to abolish salt bridge formation between residues Lys[13] and Asp[17] without establishing a covalent bond (lactam), we synthesized the linear peptide analogue **1**, substituted with a neutral side chain containing amino acid residues Lys(N^{ϵ}-Ac) and Asn, respectively. The high potency of the lactam [Lys[13],Asp[17]]PTHrP(7–34)NH_2 (analogue **2**) compared to either nonmodified PTHrP(7–34)NH_2 or the charge-eliminated [Lys[13](N^{ϵ}-Ac),Asn[17]]PTHrP(7–34)NH_2 (see Table II) (analogue **1**) strongly suggests that electrostatic charge attraction between opposite charges in positions 13 and 17 contributes to the antagonist-relevant "bioactive conformation" and that these residues may indeed be part of a helical structure. However, the contribution of the charges per se to receptor interaction is negligible.

The explanation for lower potencies of lactam analogues **3**–**5**, in which either one or both residues in positions 13 and 17 are in the D-configuration, compared to the L,L-lactam analogue **2** is less straightforward. Molecular modeling indicates that a helical structure should be able to accommodate all combinations of D- and L-configurations when the amino acids are separated by at least five residues (inclusive). The 20-membered ring formed by Lys[13]-to-Asp[17] cyclization should be sufficiently large to tolerate substitution of different chiralities at the above-named positions. Yet the data indicate that a D-residue in position 17 is much less favored than a D-residue in position 13 (Table II). This configuration dependence for bioactivity may reflect unusual steric requirements imposed by the receptor. Another possible explanation for the surprising stereochemical dependence of bioactivity may derive from different degrees of steric hindrance imposed on Lys[13] and Asp[17] by their closest neighboring residues in the α-helical conformation. Lys[13] is located between residues with either no or a very small side chain (Gly[12] and Ser[14]). In contrast, Asp[17] is located between two more bulky residues, Gln[16] and Leu[18]. Moreover, it faces a triad of arginines (residues 19–21) located across the α-helical pitch. This stretch of positive ions is subject to internal electrostatic repulsion and therefore must either be allowed significant spatial separation or be provided with opposing charges in close proximity. In either case, the net result is that much less space is available to the residue in position 17 compared to that in position 13. This may explain why replacement of L- with D-Asp in position 17 leads to substantial loss in bioactivity in the lactams [Lys[13],D-Asp[17]]- and [D-Lys[13],Asp[17]]PTHrP(7–34)NH_2 (analogues **4** and **5**, respectively).

Interestingly, cyclization via side chains of residues 13 and 17 in PTHrP(7–34)NH_2 results in removal of the partial agonist activity observed for the linear analogue. The absence of residual agonist-like activity cannot simply be attributed to the elimination of charges in positions 13 and 17, since the removal of charges by blocking (as in analogue **1**) or via cyclization (as in analogue **3**) showed residual agonism, albeit reduced compared to the parent compound. This suggests that the overall conformational constraint resulting from the ring formation between side chains of L-Lys[13] and L-Asp[17], together

with the removal of charge, contributes to the elimination of minor conformations capable of inducing PTH agonist activity.

This structure–activity study does not support the conformational features proposed by Barden and Kemp (1989) based on a ^{1}H NMR study of PTHrP(1–34) in water. They suggest that only residues 3–9 are involved in an α-helical structure, while salt bridges between the pairs Asp^{10}–Lys^{13} and Asp^{17}–Arg^{21} enclose two β-type turns. By covalent bond formation between Lys^{13} and Asp^{17}, locking in a cyclic structure, formation of both the salt bridges suggested by Barden and Kemp is precluded. Since cyclic analogue **2** displayed higher affinity and enhanced inhibitory activity than the linear molecule, we presume that the bioactive conformation of the PTHrP-derived antagonist must be different than that suggested for the agonist by Barden and Kemp. Nevertheless, the conformation they propose may represent the bioactive conformation of the agonist form, which conceivably could be very different from the antagonist-relevant "bioactive conformation". A similar hypothesis has been put forward to explain structure–activity–conformation relationships for oxytocin (Hruby et al., 1979, 1983; Hruby, 1982b).

Felix and co-workers also performed an i to $(i + 4)$ side chain to side chain cyclization of a linear hormone analogue in the growth hormone releasing factor system and generated highly potent, metabolically stable lactams possessing prolonged duration of action in vivo (Felix et al., 1988b, 1989; Madison et al., 1989). Recently, along similar lines, cyclic cholecystokinin (CCK-8) and neuropeptide Y were prepared and found to have interesting bioactivities (Danho et al., 1990; Bouvier et al., 1990).

This report suggests that the 13–19 segment in PTHrP(7–34)NH_2 may be present in the α-helical conformation predicted by Chou–Fasman analysis. Ring closure between residues 13 and 17 may not only lock this antagonist in a "bioactive conformation" but also extend the α-helical region beyond the 13–19 segment.

We now plan to undertake detailed conformational analysis of the potent antagonist, analogue **2**, which may provide insight regarding the predominant conformation in solution. Since the cyclic antagonist [$\overline{\text{Lys}^{13},\text{Asp}^{17}}$]PTHrP(7–34)$NH_2$ (analogue **2**) is highly potent and conformationally constrained, its conformation in solution may better reflect the conformation interacting with the receptor. We also intend to apply similar conformational constraints to other parts of PTHrP(7–34)NH_2 in an attempt to further reduce molecular flexibility and lock extended regions of the antagonist in predetermined conformations.

References

Barden, J. A., & Kemp, B. E. (1989) *Eur. J. Biochem. 184*, 379.

Bolin, D. R., Wang, C.-T., & Felix, A. M. (1989) *Org. Prep. Proc. Int. 21*, 67.

Bouvier, M., Taylor, J. W., & Osapay, G. (1990) In *Abstracts of the 21st European Peptide Symposium*, Platja d'Aro, Spain, P249, p 210 ESCOM, Leiden.

Cheng, Y.-C., & Prusoff, W. H. (1973) *Biochem. Pharmacol. 22*, 3099.

Chorev, M., Goldman, M. E., McKee, R. L., Roubini, E., Levy, J. J., Gay, C. T., Reagan, J. E., Fisher, J. E., Caporale, L. H., Golub, E. E., Caulfield, M. P., Nutt, R. F., & Rosenblatt, M. (1990a) *Biochemistry 29*, 1580.

Chorev, M., Roubini, E., Goldman, M. E., McKee, R. L., Gibbons, S. W., Reagan, J. E., Caulfield, M. P., & Rosenblatt, M. (1990b) *Int. J. Peptide Protein Res.* (in press).

Chorev, M., Roubini, E., McKee, R. L., Gibbons, S. W., Reagan, J. E., Goldman, M. E., Caulfield, M. P., & Rosenblatt, M. (1990c) *Science* (submitted for publication).

Danho, W., Makofske, R., Swistok, J., Michalewsky, J., Gabriel, T., Nelson, D., Triscari, J., Fry, D., & Madison, V. (1990) In *Abstracts of the 21st European Peptide Symposium*, Platja d'Aro, Spain, L24, p 70 ESCOM, Leiden.

Deber, C. M., Madison, V., & Blout, E. R. (1976) *Acc. Chem. Res. 9*, 106.

DiMaio, J., & Schiller, P. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7162.

DiMaio, J., Nguyen, T. N.-D., Lemieux, C., Schiller, P. W. (1982) *J. Med. Chem. 25*, 1438.

Felix, A. F., Wang, C.-T., Heimer, E. P., & Fournier, A. (1988a) *Int. J. Peptide Protein Res. 31*, 231.

Felix, A. F., Heimer, E. P., Wang, C.-T., Lambros, T. J., Fournier, A., Mowles, T. F., Maines, S., Campbell, R. M., Wegrzynski, B. B., Toome, V., Fry, D. C., & Madison, V. S. (1988b) *Int. J. Peptide Protein Res. 32*, 441.

Felix, A. F., Wang, C.-T., Heimer, E. P., Campbell, R. M., Madison, V. S., Fry, D. C., Toome, V., Downs, T. R., & Frohman, L. A. (1990) in *Peptides, Proceedings of the 11th American Peptide Symposium 1989* (Rivier, J. E., & Marshall, G. R. Eds.) p 226, ESCOM, Leiden.

Goldman, M. E., Chorev, M., Reagan, J. E., Nutt, R. F., Levy, J. J., & Rosenblatt, M. (1988) *Endocrinology 123*, 1468.

Horiuchi, N., Caulfield, M. P., Fisher, J. E., Goldman, M. E., McKee, R. L., Reagan, J. E., Levy, J. J., Nutt, R. F., Rodan, S. B., Schofield, T. L., Clemens, T. L., & Rosenblatt, M. (1987) *Science 238*, 1566.

Horiuchi, N., Caulfield, M. P., Nutt, R. F., Levy, J. J., Rosenblatt, M., & Clemens, T. L. (1989) *Program of the 71st Annual Meeting of the Endocrine Society*, Seattle, WA, p 177 (Abstract), Byrd Publishing, Fairfax.

Hruby, V. J. (1982a) *Life Sci. 31*, 189.

Hruby, V. J. (1982b) in *Topics in Molecular Pharmacology* (Roberts, G., & Burgen, A., Eds.) p 99, Elsevier/North-Holland, Amsterdam.

Hruby, V. J. (1985) *Trends Pharmacol. Sci. 6*, 259.

Hruby, V. J., Deb, K. K., Yamamoto, D. M., Hadley, M. E., & Chan, W. Y. (1979) *J. Med. Chem. 22*, 7.

Hruby, V. J., Rockway, T. W., Viswanatha, V., & Chan, W. Y. (1983) *Int. J. Protein Res. 21*, 24.

Jueppner, H., Abou-Samra, A.-B., Uneno, S., Gu, W.-X., & Potts, J. T., Jr. (1988) *J. Biol. Chem. 263*, 8557.

Kemp, B. E., Moseley, J. M., Rodda, C. P., Ebeling, P. R., Wettenhall, R. E. H., Stapelton, D., Diefenbach-Jagger, H., Ure, F., Michelangeli, V. P., Simmons, H. A., Raisz, L. G., & Martin, T. J. (1987) *Science 238*, 1568.

Kessler, H. (1982) *Angew. Chem., Int. Ed. Engl. 21*, 512.

Kessler, H., Klein, M., Muller, A., Wagner, K., Bats, J. W., Ziegler, K., & Frimmer, M. (1986) *Angew. Chem., Int. Ed. Engl. 25*, 997.

Kessler, H., Klein, M., & Wagner, K. (1988) *Int. J. Peptide Protein Res. 31*, 481.

Lee, S. C., & Russell, A. F. (1989) *Biopolymers 28*, 1115.

Madison, V. S., Fry, D. C., Greeley, D. V., Toome, V., Wegrzynski, B. B., Heimer, E. P., & Felix, A. M. (1990) in *Peptides, Proceedings of the 11th American Peptide Symposium 1989* (Rivier, J. E., & Marshall, G. R., Eds.) p 575, ESCOM, Leiden.

Mahaffey, J. E., Rosenblatt, M., Shepard, G. L., & Potts, J. T., Jr. (1979) *J. Biol. Chem. 254*, 6496.

Mangin, M., Webb, A. C., Dreyer, B. E., Posillicio, J. T., Ikeda, K., Weir, E. C., Stewart, A. F., Bander, N. H.,

Milostone, L., Barton, D. E., Francke, U., & Broadus, A. E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 597.
McKee, R. L., Goldman, M. E., Caulfield, M. P., DeHaven, P. A., Levy, J. J., Nutt, R. F., & Rosenblatt, M. (1988) *Endocrinology 122*, 3008.
McKee, R. L., Caulfield, M. P., & Rosenblatt, M. (1990) *Endocrinology 127*, 76.
Merrifield, R. B. (1969) *Adv. Enzymol. Subj. Biochem. 32*, 221.
Mosberg, H. I., Hurst, R., Hruby, V. J., Gee, K., Yamamura, H. I., Galligan, J. J., & Burks, T. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5871.
Mosely, J. M., Kubota, M., Difenbachen-Jagger, H., Wettenhall, R. E. H., Kemp, B. E., Suva, L. J., Rodda, C. P., Ebeling, P. R., Hudson, P. J., Zajac, J. T., & Martin, T. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5048.
Nissenson, R. A., Diep, D., & Strewler, G. J. (1988) *J. Biol. Chem. 263*, 12866.
Nutt, R. F., Caulfield, M. P., Levy, J. J., Gibbons, S. W., Rosenblatt, M., & McKee, R. L. (1990) *Endocrinology 127*, 491.
Pizurki, L., Rizzoli, R., Moseley, J., Martin, T. J., Caverzasio, J., & Bonjour, J.-P. (1988) *Am. J. Physiol. 255*, F957.
Rabbani, S. A., Mitchell, J., Roy, D. R., Hendy, G. N., & Goltzman, D. (1988) *Endocrinology 123*, 2709.
Rodan, S. B., Insogna, K. L., Vignery, A. M., Stewart, A. F., Broadus, A. E., D'Sousa, S. M., Bertolini, D. R., Mundy, G. R., & Rodan, G. A. (1983) *J. Clin. Invest. 72*, 1511.
Rosenblatt, M., Segre, G. V., Tyler, G. A., Shepard, G. L., Nussbaum, S. R., & Potts, J. T., Jr. (1980) *Endocrinology 107*, 545.
Shigeno, C., Yamamoto, I., Kitamura, N., Noda, T., Lee, K., Sone, T., Shiomi, K., Ohtaka, A., Fujii, N., Yajima, H., & Konishi, J. (1988) *J. Biol. Chem. 263*, 18369.
Stewart, A. F., Insogna, K. L., Goltzman, D., & Broadus, A. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1454.
Stewart, A. F., Wu, T., Goumas, D., Burtis, W. J., & Broadus, A. E. (1987) *Biochem. Biophys. Res. Commun. 146*, 672.
Strewler, G. J., Williams, R. D., & Nissenson, R. A. (1983) *J. Clin. Invest. 71*, 769.
Strewler, G. J., Stern, P. H., Jacobs, J. W., Eveloff, J., Klein, R. F., Leung, S. C., Rosenblatt, M., & Nissenson, R. A. (1987) *J. Clin. Invest. 80*, 1803.
Suva, L. J., Winslow, G. A., Wettenhall, R. E. H., Hammonds, R. G., Moseley, J. M., Diefenbach-Jagger, H., Rodda, C. P., Kemp, B. E., Rodriguez, H., Chen, E. Y., Hudson, P. J., Martin, T. J., & Wood, W. I. (1987) *Science 237*, 893.
Thiede, M. A., Strewler, G. J.; Nissenson, R. A., Rosenblatt, M., & Rodan, G. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4605.
Veber, D. F., & Freidinger, R. M. (1985) *Trends. Neurosci. 8*, 392.
Veber, D. F., Holly, F. W., Paleveda, W. J., Nutt, R. F., Bergstrand, S. J., Torchiana, M., Glitzer, M. S., Saperstein, R., & Hirschmann, R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2636.
Veber, D. F., Saperstein, R., Nutt, R. F., Freidinger, R. M., Brady, S. F., Curley, P., Perlow, D. S., Paleveda, W. J., Colton, C. D., Zacchei, A. G., Tocco, D. J., Hoff, D. R., Vandlen, R. L., Gerich, J. E., Hall, L., Mandarino, L., Cordes, E. H., Anderson, P. S., & Hirschmann, R. (1984) *Life Sci. 34*, 1371.

Solvent Denaturation and Stabilization of Globular Proteins†

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ABSTRACT: Statistical thermodynamic theory has recently been developed to account for the stabilities of globular proteins. Here we extend that work to predict the effects of solvents on protein stability. Folding is assumed to be driven by solvophobic interactions and opposed by conformational entropy. The solvent dependence of the solvophobic interactions is taken from transfer experiments of Nozaki and Tanford on amino acids into aqueous solutions of urea or guanidine hydrochloride (GuHCl). On the basis of the assumption of two pathways involving collapse and formation of a core, the theory predicts that increasing denaturant should lead to a two-state denaturation transition (i.e., there is a stable state along each path separated by a free energy barrier). The denaturation midpoint is predicted to occur at higher concentrations of urea than of GuHCl. At neutral pH, the radius of the solvent-denatured state should be much smaller than for a random-flight chain and increase with either denaturant concentration or number of polar residues in the chain. A question of interest is whether free energies of folding should depend linearly on denaturant, as is often assumed. The free energy is predicted to be linear for urea but to have some small curvature for GuHCl. Predicted slopes and exposed areas of the unfolded states are found to be in generally good agreement with experiments. We also discuss stabilizing solvents and compare thermal with solvent denaturation.

Proteins can be denatured in the presence of certain small-molecule solutes in high concentrations. Examples of such solutes are urea and guanidine hydrochloride. Other solutes in high concentration stabilize proteins. Examples of these are sugars, glycerol, polyols, and poly(ethylene glycol) (Arakawa, 1982; Back, 1979; Gekko & Timasheff, 1981; Lee & Lee, 1981; Lee & Timasheff, 1981). One problem in understanding the molecular mechanism of nonspecific solute action on protein stability has been the absence of a molecular

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