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Modification of the Arginines in Parathyroid Hormone: Effect on Biological Activity†

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ABSTRACT: The amino-terminal region 1–34 of parathyroid hormone contains all the structural requirements necessary for full biological activity in vitro and in vivo. Carboxyl- and amino-terminal modifications and substitutions of this region have been studied extensively, leading to the synthesis of analogues of enhanced biological activity and hormone antagonists. Structural alteration of the central region of the active segment has failed to produce large changes in biological activity. Arginine is present at positions 20 and 25. Modification of these arginines was performed without undertaking total synthesis of an arginine-substituted analogue. 1,2-Cyclohexanedione reacts specifically, completely, and reversibly with arginine to form a single addition product. The 1,2-cyclohexanedione-treated analogue of parathyroid hormone, [DHCH-Arg-20, DHCH-Arg-25]hPTH-(1–34), was, at most, 16% as active as the unmodified hormone, hPTH-(1–34). Reversal of the arginine modification completely restored biopotency. Hence, the centrally positioned arginines of the active fragment may serve an important role in receptor interaction. This finding suggests that the preparation of synthetic analogues of parathyroid hormone which contain substitutions for arginine may be important to future structure-activity analyses of this hormone. Specific postsynthetic modification of arginine should prove of general utility in evaluating the role of arginine in the biological activity of peptide hormones.

Structure–activity studies utilizing synthetic fragments and analogues of parathyroid hormone (PTH)[1] have led to the successful preparation of peptides more potent than the native hormone itself (Rosenblatt and Potts, 1977; Rosenblatt et al., 1976) and inhibitors of PTH action (Goltzman et al., 1975; Rosenblatt et al., 1977). These and other studies (Tregear and Potts, 1973; Parsons et al., 1975) have explored extensively the effect on biological activity of structural modification at either the amino or carboxyl terminus of the fully active region, PTH-(1–34), of the 84 amino acid hormone. When attention turned toward other regions of the molecule, it was found that substitutions or modifications in the central portion of the active fragment were remarkable for their relative lack of effect on biological activity: substitution of norleucine for the methionines of positions 8 and 18 was well tolerated in terms of biopotency (Rosenblatt et al., 1976), and modification of the tryptophan at position 23 by attachment of the *o*-nitrophenylsulfenyl group to the indole nitrogen produced no change in the potency of an analogue of enhanced activity (Rosenblatt and Potts, 1977) or a hormone inhibitor (Rosenblatt et al., 1977). Interest continues in finding structural alterations of the central portion of the active region which cause either dramatic quantitative or qualitative effects on biological properties. Such modifications, if combined with modifications at the amino or carboxyl terminus, might augment either biopotency or inhibitory activity of certain analogues. Since the synthesis, purification, chemical characterization, and biological evaluation of a 34 amino acid hormone analogue entail considerable effort, a convenient approach to screening for alterations that cause large changes in biological properties is to selectively modify particular amino acids after synthesis. This approach avoids undertaking an exclusive synthesis for each new analogue in order to determine sequence positions critical for biological activity. In PTH, arginine occurs centrally in the active fragment at positions 20 and 25. Patthy and Smith (1975a) reported a technique for selectively modifying arginine under mild conditions using 1,2-cyclohexanedione. They demonstrated this reagent to react completely with the guanidino function of arginine to form a single addition product, N^7,N^8-(1,2-dihydroxycyclohex-1,2-ylene)arginine (DHCH-Arg). Unmodified arginine could be regenerated quantitatively from DHCH-Arg by another set of mild reaction conditions. The method was then applied to determining the function of arginine in two enzymes, lysosyme and RNase A (Patthy and Smith, 1975b). Our study describes the use of this method to prepare an analogue of human PTH, [DHCH-Arg-20,DHCH-Arg-25]hPTH-(1–34), and the biological evaluation of this compound in vitro to assess the role of arginine in PTH action.

Experimental Procedure

Synthesis of hPTH-(1–34). The active region of human PTH, hPTH-(1–34), was synthesized by a modification (Tregear et al., 1974) of the Merrifield solid-phase technique (1969) using a Beckman Model 990 automated synthesizer. Merrifield 1% cross-linked chloromethylated resin (Lab Sys-

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[1] Abbreviations used: PTH, parathyroid hormone; hPTH, human parathyroid hormone; DHCH-Arg, N^7,N^8-(1,2-dihydroxycyclohex-1,2-ylene)arginine; Boc, *tert*-butoxycarbonyl; MRC, Medical Research Council, United Kingdom.

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TABLE I: Amino Acid Content after Acid Hydrolysis of Synthetic Peptides.[a]

residue	hPTH-(1-34) predicted	hPTH-(1-34) obtained	[DHCH-Arg]-hPTH-(1-34) predicted	[DHCH-Arg]-hPTH-(1-34) obtained	regenerated hPTH-(1-34) predicted	regenerated hPTH-(1-34) obtained
Asp	4	4.3	4	4.2	4	4.4
Ser[b]	3	2.6	3	2.6	3	2.7
Glu	5	5.1	5	4.9	5	5.2
Gly	1	1.0	1	1.1	1	1.0
Val	3	3.0	3	2.8	3	3.0
Met	2	2.0	2	1.9	2	1.9
Ile	1	0.8	1	0.9	1	0.9
Leu	5	5.2	5	5.0	5	5.5
Phe	1	0.9	1	0.8	1	0.9
Lys	3	3.3	3	3.1	3	3.4
His	3	2.8	3	2.7	3	2.8
Arg	2	2.0	<0.4[c]	0.3	2	1.6

[a] All values represent the average of three separate aliquots of peptide after acid hydrolysis in 5.7 N HCl at 110 °C in an evacuated desiccator for 24 h in the presence of 1/2000 (v/v) mercaptoethanol. Tryptophan was not determined. [b] Corrected for degradative losses during acid hydrolysis. [c] During acid hydrolysis, up to 20% of DHCH-Arg may revert to arginine (Patthy and Smith, 1975a).

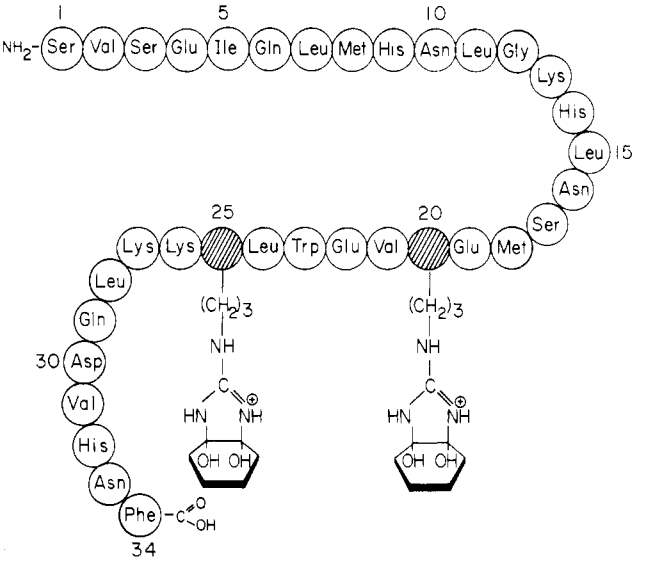

FIGURE 1: Amino acid sequence of [DHCH-Arg-20, DHCH-Arg-25]-hPTH-(1-34); the side chain at positions 20 and 25 has been enlarged to depict structure.

tems, Inc.) was the solid support. The *tert*-butoxycarbonyl (Boc) group protected the α-amino group of each amino acid during coupling, except arginine, which was protected by the amyloxycarbonyl group. Side-function protection was as follows: (a) serine was protected as the *O*-benzyl ether, (b) aspartic and glutamic acid were protected as benzyl esters, (c) histidine was protected by the *p*-toluenesulfonyl group (protected histidine was stored as the dicyclohexylamine salt and desalted immediately before use), and (d) lysine by the 2-chlorobenzyloxycarbonyl group. All amino acids were incorporated using dicyclohexylcarbodiimide as the coupling agent, except glutamine and asparagine, which were coupled as "active" *p*-nitrophenyl esters. All Boc-amino acids were purchased from Beckman Instruments and Peninsula Laboratories, except protected lysine, which was obtained from Bachem Chemicals.

The synthetic peptide was purified by gel-filtration and ion-exchange column chromatography and then chemically characterized by amino acid analysis, thin-layer chromatography, thin-layer electrophoresis, and Edman sequence analysis as previously described (Tregear 1974; Tregear et al., 1975, 1977) and found to be homogeneous and of high purity (>95%).

Preparation of [DHCH-Arg-20,DHCH-Arg-25]hPTH-(1-34). Selective modification of the arginine residues of hPTH-(1-34) was performed according to the method of Patthy and Smith (1975a,b) to produce [DHCH-Arg-20,DHCH-Arg-25]hPTH-(1-34) shown in Figure 1. Nine milligrams of hPTH-(1-34) was dissolved in 300 μL of distilled H_2O. The solution was added to 1.0 mL of sodium borate buffer (0.2 M, pH 8.7). 1,2-Cyclohexanedione (7.5 mg) was dissolved in 300 μL of sodium borate buffer and added to the above mixture, which was then incubated at 37 °C for 2 h. After reacting, the mixture was acidified with 50 μL of glacial acetic acid, followed by 500 μL of 1 M acetic acid. The solution was placed on a polyacrylamide gel (Bio-Gel P-2) (Bio-Rad) column (100 × 1.2 cm) and eluted with 1 M acetic acid for desalting of the modified peptide.

Reversal of Modification of Arginine. Five milligrams of [DHCH-Arg-20,DHCH-Arg-25]hPTH-(1-34) was partially dissolved in 1.25 mL of 0.5 M hydroxylamine buffer (pH 7.0) and incubated at 37 °C overnight. The mixture was centrifuged and the supernatant taken and acidified with 75 μL of glacial acetic acid. The solution was placed on a P-2 column (100 × 1.2 cm) and eluted with 1 M acetic acid for desalting of the peptide.

Bioassay. Assessment of biological activity in vitro was performed using a modification of the rat renal cortical adenylate cyclase assay (Krishna et al., 1968; Marcus and Aurbach, 1969, 1971). [^{32}P]ATP and c[^{3}H]AMP were purchased from New England Nuclear. The bPTH standard used in the assays was Medical Research Council Standard, Lot No.MRC 72/286 (National Institute for Medical Research, London, England) (Robinson et al., 1972). Each preparation was assayed three times at multiple dilutions. The separate potency estimates were combined to yield the mean potency of each compound.

Results

[DHCH-Arg-20,DHCH-Arg-25]hPTH-(1-34) and Regenerated hPTH-(1-34). Table I compares the amino acid analyses obtained after acid hydrolysis of hPTH-(1-34), [DHCH-Arg-20,DHCH-Arg-25]hPTH-(1-34), and hPTH-(1-34) regenerated from arginine-modified material. Reaction with 1,2-dicyclohexanedione resulted in an 85% decline in unmodified arginine content. Content of all other residues was preserved. Chemical reversal of the arginine modification was demonstrated by nearly complete restoration of arginine content.

Thin-layer electrophoresis (pH 6.5, pyridine–acetic acid–water, 30:1:270, 100-μm cellulose-coated plates, Pauli stain) showed 32% decrease in electrophoretic mobility of the DHCH-Arg-containing peptide in the presence of a borate buffer. No unmodified hPTH-(1-34) was detected after treatment with 1,2-cyclohexanedione. Reversal of the arginine modification restored electrophoretic mobility to that of hPTH-(1-34), and no heterogeneity was detected.

Bioassay. Figure 2 depicts stimulation of the renal cortical membranes in the adenylate cyclase assay by hPTH-(1-34), [DHCH-Arg-20,DHCH-Arg-25]hPTH-(1-34), and hPTH-(1-34) regenerated from arginine-modified hormone. The mean potency of hPTH-(1-34) was 2700 (2150-3300) MRC units/mg. The arginine-modified analogue had an ac-

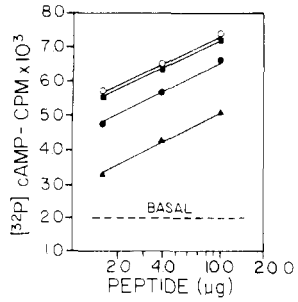

FIGURE 2: Representative bioassay of hPTH-(1-34) (○), [DHCH-Arg-20,DHCH-Arg-25]hPTH-(1-34) (▲), regenerated hPTH-(1-34) (■), and native bovine hormone standard, bPTH-(1-84), 2500 MRC units/mg (●). Each point is the mean of triplicate determinations. Each peptide was quantitated by amino acid analysis.

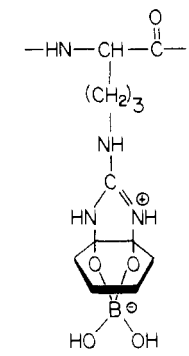

FIGURE 3: Structure of the complex formed by borate with DHCH-Arg, as previously suggested (Patthy and Smith, 1975a).

tivity of 430 (400-460) MRC units/mg. Regenerated hPTH-(1-34) was identical in activity to hPTH-(1-34) starting material, 2700 (2450-3000) MRC units/mg. Hence, modification of arginine results in an 84% decline in biological activity. Reversal of the arginine modification completely restores biopotency, demonstrating that the potency decline observed after 1,2-cyclohexanedione treatment is not caused by nonspecific alterations of structure.

Discussion

Patthy and Smith (1975a) reported a highly specific method for modifying the guanidino function of arginine. They demonstrated that conversion of arginine residues in proteins to DHCH-Arg could be used in the determination of primary structure by restricting the action of trypsin to hydrolysis of peptide bonds involving lysine. They also demonstrated the utility of this method for investigating the role of arginine in enzyme function (Patthy and Smith, 1975b).

Subsequently, the technique has been employed to identify potentially functionally significant arginines in other enzymes (Austen and Smith, 1976; Rogers and Weber, 1977), enzyme inhibitors (Menegatti et al., 1977; Roosdorp et al., 1977), and a protein hormone (Sairam, 1976).

We have extended these studies to determine the role of arginine in the biological activity of a peptide hormone whose active region can be chemically synthesized. Because the reaction conditions are mild, an arginine-modified analogue of the active fragment of PTH could be prepared specifically without causing undesired side reactions. Hormonal structure was not compromised, except as desired at positions 20 and 25, as demonstrated by restoration of arginine content and complete biological potency when the arginine modification was reversed.

Because arginine is the most basic of the naturally occurring amino acids, it often receives attention in structure-activity studies (Rudinger, 1971; Nicolaides et al., 1963; Fujino et al., 1972; Hugli and Erickson, 1977). As depicted in Figure 1, the DHCH modification preserves the positive charge of the amino acid side chain, although the electrophoretic mobility of DHCH-Arg is somewhat decreased compared to arginine (Patthy and Smith, 1975a,b). However, the side chain of DHCH-Arg differs considerably from that of arginine—it contains vicinal hydroxyls, and is larger in volume and different in shape.

The structure actually evaluated by bioassay may differ from the analogue prepared. The instability of the 1,2-cyclohexanedione adduct at neutral pH complicates assessment of biological activity in vivo. However, DHCH-Arg is stable at physiological pH in borate buffer. The renal adenylate cyclase system was successfully modified to permit in vitro bioassay—incubations were conducted in borate buffer. The stability of DHCH-Arg in borate buffer results from formation of a borate-DHCH-Arg complex (Patthy and Smith, 1975a). The structure of the arginines in the analogue during bioassay was probably that suggested by Patthy and Smith (1975a) depicted in Figure 3. The bulk of the side chain is further increased, and a negative charge is introduced by formation of a complex with borate. The large steric change or charge modification, or a combination of these factors may cause the dramatic decline in biopotency through adverse effects on hormone-receptor interaction or induction of conformational changes in the PTH molecule.

It was not possible to exclude the instability of DHCH-Arg as responsible for the small amount of biological activity observed (15% the activity of unmodified hormone). Complete modification of arginine may have been accomplished and not reflected in the amino acid analyses because up to 20% of DHCH-Arg reverts to arginine under the conditions of acid hydrolysis. Alternatively, regeneration of arginine during the bioassay may have occurred. The renal cortical membranes used in the assay are prepared in a Tris buffer, and the membranes themselves may contain amines. Small amounts of arginine may be regenerated in the presence of these nucleophiles during the 10-min incubation period of the bioassay. Hence, it is possible that the arginine-modified analogue is completely devoid of biological activity.

By using a mild, selective, and reversible reagent to explore the role of arginine in PTH bioactivity, we have shown that alteration of arginines in the active region of PTH produces a large decline in biopotency. These studies indicate that future synthesis of analogues containing structurally more subtle substitutions for one or both arginines of the active region may result in compounds of considerable biological interest, particularly hormone inhibitors (Rosenblatt et al., 1977).

Application of the arginine-modification technique should prove of further general interest as solid-phase peptide synthesis is applied to the larger peptide hormones, particularly those containing arginine near the carboxyl terminus.

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Effect of Membrane Phospholipid Compositional Changes on Adenylate Cyclase in LM Cells†

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ABSTRACT: Adenylate cyclase activities were examined in mouse LM cell membranes which had been supplemented with polar head groups and/or fatty acids. Basal, fluoride-, and PGE_1-stimulated activities varied systematically with changes in phospholipid composition, and PGE_1-stimulated activities correlated with the average degree of unsaturation of the phospholipid fatty acids or with the primary amino group character of the phospolipid polar head groups. In addition, the K_m for ATP of basal adenylate cyclase was systematically changed by both polar head group and fatty acid supplementation. Alteration of the membrane lipid composition also changed the temperature dependence of the enzyme and the lag time between addition of PGE_1 and the onset of a change in catalytic rate. However, none of the alterations in the enzyme activity could be correlated with the viscosities of supplemented membranes and, instead, seemed to be characteristic for a specific polar head group or fatty acid composition. The data suggest a specific interaction of the enzyme with phospholipids and indicate that structural features of phospholipids may play a role in regulating adenylate cyclase activity. It is proposed that adenylate cyclase can exist in several different conformations in the membrane depending upon the phospholipid composition.

Adenylate cyclase is associated with the plasma membranes of a number of animal cells (Butcher et al., 1965; Pohl et al., 1971a; Bilezikian & Aurbach, 1974; Engelhard et al., 1976a) and has also been reported to be associated with particulate fractions of many other cell types. Several studies have implicated the importance of membrane lipids for adenylate cyclase activity and hormone stimulation. For example, membranes have been treated with nonionic detergents (Sutherland et al., 1962), digitonin (Pohl et al., 1971b), phospholipases

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