DESIGN, SYNTHESIS, AND BIOLOGIC EVALUATION OF HORMONE ANALOGUES*

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Two analogues of bovine parathyroid hormone (bPTH), [Nle-8, Nle-18, Tyr-34]bPTH-(3-34)amide and [Nle-8, Nle-18, o-NPS-Trp-23, Tyr-34]bPTH-(3-34)amide, were synthesized by the solid phase method. The analogues represent the NH₂-terminal fragment bPTH-(3-34), previously shown to be a hormone antagonist, but with the addition of structural features associated with enhancement and stabilization of biological activity of the full agonist bPTH-(1-34). When tested in the in vitro renal adenylyl cyclase assay, these analogues, containing potency-enhancing modifications imposed on an inhibitory core of bPTH, proved to be strong hormonal inhibitors completely lacking agonist activity. Inhibition was in each case proportional to the dose of inhibitor and inversely related to the quantity of native hormone: the analogues in high dose completely suppressed bPTH-stimulated adenylyl cyclase activity. When present in a ratio equimolar to native bPTH-(1-84), [Nle-8, Nle-18, Tyr-34]bPTH-(3-34)amide and [Nle-8, Nle-18, o-NPS-Trp-23, Tyr-34]bPTH-(3-34)amide resulted in 44 and 57% inhibition, respectively, of native bPTH-(1-84) action. Detailed study of the kinetics of inhibition of the analogue [Nle-8, Nle-18, Tyr-34]bPTH-(3-34)amide revealed parameters characteristic of competitive inhibition. For this inhibitor, Kᵢ, derived by Eadie-Hofstee plots was (1.24 ± 0.13) x 10⁻⁴ M. The affinity of this inhibitor for the physiological receptor in vitro is of the same order of magnitude as that of the native hormone, Kᵢ = 0.45 x 10⁻⁴ M. These analogues represent an improvement, greater than 30-fold, in inhibitory potency over the unsubstituted fragment bPTH-(3-34), which has an approximate Kᵢ = 5.3 x 10⁻⁴ M obtained from the inhibitor concentration resulting in 50% inhibition of native bPTH action. These analogues should prove valuable in further analysis of the dichotomy between receptor binding and adenylyl cyclase activation for parathyroid hormone, and as models for the synthesis of other inhibitory analogues of possible physiologic and medical application.

Although parathyroid hormone (PTH) is a single-chain 84-amino acid peptide in several species (1-4), the structural requirements necessary for full biologic activity are virtually satisfied by the NH₂-terminal 34-amino acid fragment bPTH-(1-34) (5, 6). Progressive amino acid deletions from either the NH₂ or COOH terminus of the active fragment result in a progressive decline in biologic activity and have led to the determination that the minimum sequence necessary for biologic activity is a continuous sequence region 2-27 (6). However, the structural requirements for binding to receptors are somewhat separable from those for activation. A synthetic fragment shortened at the NH₂ terminus by only two amino acids, bPTH-(3-34), lacks agonist activity in vitro and in vivo, yet appears able to bind to hormone receptor sites in target tissue, as evidenced by its inhibition of the action of native bPTH-(1-84) and synthetic bPTH-(1-34) (7). The two NH₂-terminal amino acids, however, are not only critical for stimulation of adenylyl cyclase, but must also have an effect on avidity of receptor binding, inasmuch as bPTH-(3-34) does not inhibit bPTH-(1-84) on an equimolar basis, but requires addition of a several hundred-fold excess of inhibitor to agonist to effect 50% inhibition.

Structure-activity studies of synthetic analogues of the fully active bPTH-(1-34) revealed certain modifications to be activity-enhancing or to confer resistance to oxidation (8, 9). Substitution of tyrosine for phenylalanine at the COOH terminus and conversion of the COOH-terminal carboxyl group to a carboxamide (CONH₂) are activity-enhancing; substitution of norleucines for the methionines of positions 8 and 18 affords resistance to oxidation. In an attempt to enhance the inhibitory activity of the fragment bPTH-(3-34), synthesis of analogues of bPTH-(3-34) containing activity-enhancing or stabilizing features was undertaken. The analogues [Nle-8, Nle-18, o-NPS, Tyr-34]bPTH-(3-34)amide and [Nle-8, Nle-18, o-NPS, Tyr-34]bPTH-(3-34)amide were synthesized by the solid phase method. The analogues represent the NH₂-terminal 34-amino acid fragment bPTH-(3-34), which has an approximate Kᵢ = 5.3 x 10⁻⁴ M obtained from the inhibitor concentration resulting in 50% inhibition of native bPTH action. These analogues should prove valuable in further analysis of the dichotomy between receptor binding and adenylyl cyclase activation for parathyroid hormone, and as models for the synthesis of other inhibitory analogues of possible physiologic and medical application.

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1 The abbreviations used are: PTH, parathyroid hormone; bPTH, bovine parathyroid hormone; o-NPS, orthonitrophenylsulfonyl; Boc, tertiary butyloxycarbonyl.
2 Specialized preparations of canine renal membranes have revealed some biological activity present in sequence regions shorter than 2-27; however, such activity has always been less than 1% of that of native PTH.
Tyr-34)bPTH-(3-34)amide and [Nle-8, Nle-18, o-NPS-Trp-23, Tyr-34)bPTH-(3-34)amide that incorporate these modifications proved to be extremely potent inhibitors. The unexpected finding that these analogues of bPTH-(3-34) are more potent as antagonists than are the corresponding bPTH-(1-34) analogues as agonists provides further impetus to systematic analysis of structural features important for activation as distinct from receptor binding.

**EXPERIMENTAL PROCEDURES**

**Preparation of Hormone Analogues**—Synthetic peptide hormone analogues were prepared by a modification of the Merrifield solid phase technique (8, 10). The analogues [Nle-8, Nle-18, Tyr-34)bPTH-(3-34)amide and [Nle-8, Nle-18, o-NPS-Trp-23, Tyr-34)bPTH-(3-34)amide are depicted in Fig. 1. Synthesis was performed with a Beckman model 990 automated synthesizer. Benzhydrylamidine hydrochloride resin (polystyrene/1% divinylbenzene) was employed as the solid support in order to effect the carboxamide (CONH2) COOH-terminal modification. All Boc-α-amino acids were obtained from Beckman, with the exception of Boc-norleucine and Boc-O-2,6-dichlorobenzyl tyrosine, which were obtained from Bachem. Each amino acid incorporating step was qualitatively monitored for completeness of reaction by the fluorescamine test (11). The details of the synthetic procedure and purification methods have been reported previously (8). All synthetic peptides were shown to correspond to theoretical composition by acid and enzymic hydrolysis followed by automated amino acid analysis (12) and were shown to be homogeneous by sequence analysis employing repetitive Edman degradation (13).

The analogue [Nle-8, Nle-18, o-NPS-Trp-23, Tyr-34)bPTH-(3-34)amide was prepared by treating 30 mg of synthetic [Nle-8, Nle-18, Tyr-34)bPTH-(3-34)amide with 30 mg of o-nitrophenoxybenzyl chloride (o-NPS-Cl) (Pierce) as previously reported (14). Conversion of tryptophan to the o-NPS derivative was accompanied by the expected appearance of an additional absorption peak in the UV spectrum at 365 nm (15), as well as the complete absence of unmodified tryptophan content by amino acid analysis after enzymic hydrolysis.

Native bPTH-(1-84) used as a standard in the adenylyl cyclase assay for potency determinations was Medical Research Council (MRC) Research Standard, Lot MRC 72/286 (National Institute for Medical Research, London, England). For all other studies, purified bovine parathyroid hormone (approximately 3000 MRC units/mg) was used as a standard in the adenylyl cyclase assay. Approximations of the inhibitory constant, K, of native bPTH-(1-84) and synthetic bPTH-(1-34) have activities, respectively, of 3000 (2500 to 4000) MRC units/mg and 6800 (5800 to 7500) MRC units/mg (expressed as mean potency with 95% confidence limits). The synthetic analogues were assessed for inhibition of bPTH-(1-84)-stimulated adenylyl cyclase activity over a concentration range of 3.7 x 10^-9 to 5.9 x 10^-8 M.

**Protein**—Protein was determined by the method of Lowry et al. (21) using bovine serum albumin as a standard. Actual peptide content of the doses of native hormone and synthetic bPTH analogues was obtained by amino acid analysis of a separate aliquot of the peptide-containing delivery solution.

**RESULTS**

The two analogues reported herein, [Nle-8, Nle-18, Tyr-34)bPTH-(3-34)amide and [Nle-8, Nle-18, o-NPS-Trp-23, Tyr-34)bPTH-(3 34)amide, were both devoid of agonist activity at multiple doses up to a maximum of 1 x 10^-4 M. Native bPTH-(1-84) and synthetic bPTH-(1-34) have activities, respectively, of 3000 (2500 to 4000) MRC units/mg and 6800 (5800 to 7500) MRC units/mg (expressed as mean potency with 95% confidence limits). The synthetic analogues were assessed for inhibition of bPTH-(1-84)-stimulated adenylyl cyclase activity over a concentration range of 3.7 x 10^-9 to 5.9 x 10^-8 M. Each analogue was demonstrated to be a complete hormone antagonist that caused total suppression of bPTH-stimulated adenylyl cyclase activity. Fig. 2 illustrates the reverse-sigmoid dose-response curves of inhibition of bPTH-stimulated adenylyl cyclase activity by the synthetic analogues. This figure illustrates that the substituted synthetic analogues are markedly more potent hormone inhibitors than the fragment bPTH-(3-34). Approximations of the inhibitory constant, K, obtained from the dose at which 50% inhibition of native bPTH action occurred (at the single, near maximum stimulatory concentration of 1.2 x 10^-7 M of bPTH-(1-84) were 1.6 x 10^-7 M for [Nle-8, Nle-18, Tyr-34)bPTH-(3-34)amide, and 9.0 x 10^-6 M for [Nle-8, Nle-18, o-NPS-Trp-23, Tyr-34)bPTH-(3-34)amide. Hence these compounds demonstrate 44 and 57% antagonism of hormone expression, respectively, when present at concent-
K, was used to derive Ki independently at each of three
molar for receptor binding. NH,-terminal deletions cause a simi-
lar some portion thereof) at the COOH terminus appears essen-
tial for receptor binding and cyclase activation; thus the sequence region 27-34 (or
adenylyl cyclase activity; thus the sequence region 27-34 (or
bPTH-(1-26) neither stimulates nor inhibits bPTH-stimulated
residues at the COOH-
derived from the slope of the Eadie-Hofstee
plot in the absence of inhibitor, was 0.45 × 10^-8 M. In the
presence of inhibitor, the expression, slope = -q × (1 + ([Z/K,])), where
used to derive K, independently at each of three
concentrations of inhibitor (I)). The mean K, derived in this
manner was (1.24 ± 0.07) × 10^-4 M. In these plots, the y
intercept is equivalent to the V[max] of the hormone agonist. Fig.
4 illustrates that V[max] remains constant at 537 pmol of CAMP/
mg of protein × 10 min over a full range of concentrations of
inhibitors, thus demonstrating the kinetics characteristic of
competitive inhibition.

DISCUSSION

Investigations directed at determining the minimum se-
quence necessary for hormonal activity in bPTH revealed that
stepwise deletion of amino acids from either the NH2 or COOH
terminus of the fully active hormone fragment, bPTH-(1-34),
results in a progressive decline in biological activity (9). How-
ever, there is a striking difference in the apparent role of
residues at the COOH- versus the NH2-terminal region of
bPTH-(1-34) on receptor binding and cyclase activation.
bPTH-(1-26) neither stimulates nor inhibits bPTH-stimulated
adenylyl cyclase activity; thus the sequence region 27-94 (or
some portion thereof) at the COOH terminus appears essen-
tial for receptor binding. NH-terminal deletions cause a simi-
lar decline in biologic potency but the hormone fragment
bPTH-(3-34), although not a hormone agonist, is still a hor-
mone antagonist and, hence, must be able to occupy receptor
sites without stimulating adenylly cyclase (7). A similar di-
chotomy of receptor binding and adenylyl cyclase stimulation
has been observed in many peptide hormone systems (25-27).

If amino acids at positions 1 and 2 are truly essential for the
expression of hormonal activity, then one approach to the
design of hormonal antagonists would be to incorporate modi-
fications known to enhance biological activity in the agonist
bPTH-(1-34) into analogues lacking the two most NH2-terminal
residues. In this manner, receptor binding and, hence,
inhibitory properties might be expected to increase without
restoration of any hormonal activity. Structure-activity stud-
ies of synthetic analogues of the fully active hormonal frag-
ment, bPTH-(1-34), reveal two independent COOH-terminal

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**Fig. 2.** Inhibition of bPTH-(1-84)-stimulated generation of cAMP
by synthetic analogues. A single near maximum dose of bPTH-(1-84)
(1.2 × 10^-7 M) was assayed in the presence of variable doses of
inhibitors as noted: ○, bPTH-(3-34); ●, [Nle-8, Nle-18, Tyr-34]bPTH-
(3-34)amide; x, [Nle-8, Nle-18, o-NPS-Trp-23, Tyr-34]bPTH-(3-34)
amide. The 50% inhibition level is marked by --. Each point
represents the mean of triplicate determinations. The limits noted
represent standard error of the mean.

![Graph](https://via.placeholder.com/150)

**Fig. 3.** Dose-response curves of bPTH-(1-84) at concentrations of
4.55 × 10^-9 M, 1.35 × 10^-8 M, 4.06 × 10^-8 M, and 1.22 × 10^-7 M, each
in the presence of variable doses of [Nle-8, Nle-18, Tyr-34]bPTH-(3-34)
amide as follows: ○, no inhibitor; ●, 4.18 × 10^-9 M; △, 1.24 × 10^-8 M;
●, 3.73 × 10^-9 M; ■, 1.13 × 10^-8 M. Basal activity is indicated by ---.
All points represent the mean of triplicate determinations. The
limits noted represent standard error of the mean.

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**Fig. 4.** Michaelis-Menten analysis of adenylly cyclase inhibition
by Eadie-Hofstee Plots of V versus (V/S). Each line is derived from
the data depicted in Fig. 3. Agonist (native bPTH) concentration is
varied in the presence of a constant dose of inhibitor (t[Nle-8, Nle-18,
Tyr-34]bPTH-(3-34)amide). The concentrations of inhibitor are rep-
resented by: ○, no inhibitor; □, 4.18 × 10^-9 M inhibitor; △, 1.24 × 10^-9 M;
●, 3.73 × 10^-9 M; ■, 1.13 × 10^-8 M. V is
corrected for basal CAMP activity. The y intercept yields V[max] K, =
(=) slope of agonist activity in the absence of inhibitor, i.e. the slope
of ○---○. In the presence of inhibitor, the K, can be calculated from
the relationship: slope = -q × K, where q = (1 + [(I/K,)]).
The points noted are the mean of triplicate determinations. The
limits noted represent standard error of the mean.
modifications that enhance biologic activity. Substitution of tyrosine for phenylalanine at the COOH terminus, position 34, results in an analogue 139% as potent as bPTH-(1-34) (8). Conversion of the COOH-terminal carboxylic acid group to a carboxamide (CONH₂) is accompanied by nearly a 3-fold increase in biologic activity (9). Both of these structural modifications were incorporated into the two analogues presented here. In addition, a modification of the central region of the active fragment was selected as a stabilizing feature. The nearly isosteric substitution of norleucines for the two methionines at positions 8 and 18 had been demonstrated previously to be well tolerated in terms of biopotency, while at the same time providing analogues that withstand vigorous oxidizing conditions with full preservation of biologic activity (8). Without norleucine substitution, oxidation of methionine results in a complete loss of both agonist and antagonist activity (7, 28, 31). An additional structural modification was undertaken based on studies of ACTH (14, 32, 33). PTH, like ACTH, contains only 1 tryptophan residue, and it lies within the active core. For ACTH, treatment with o-nitrophenylsulfenyl chloride (o-NPS-Cl) to yield the o-NPS-tryptophan derivative yielded a potent inhibitor of ACTH-mediated cyclic AMP production and lipolysis. However, preparation of the o-NPS derivative involves exposure of the peptide to oxidizing conditions. Hence, an analogue in which norleucine is substituted for methionine is necessary to provide a suitable substrate for formation of the o-NPS derivative of PTH.

The analogues [Nle-8, Nle-18, Tyr-34]bPTH-(3-34)amide and [Nle-8, Nle-18, o-NPS-Trp-23, Tyr-34]bPTH-(3-34)amide lacked agonist properties and were demonstrated to be very effective hormone inhibitors, causing 44 and 57% inhibition of native hormone activity, respectively, at concentrations equivalent to native bPTH. They also were shown to be complete antagonists; that is, total suppression of bPTH-stimulated adenyl cyclase activity was accomplished by doses of the analogues of approximately 6 × 10^-8 M (Fig. 2). Detailed studies of the analogue [Nle-8, Nle-18, Tyr-34]bPTH-(3-34)amide (Figs. 3 and 4) confirmed true competitive inhibition, a feature that could not be examined with bPTH-(3-34) in previously reported studies (7) because of the relative weakness of the inhibitory properties of this unsubstituted fragment.

It must be emphasized, of course, that the cyclase assay system cannot yield a test of inhibition as direct as that which can be obtained in formal studies of the interaction of substrate and inhibitor with an enzyme in which Kᵣ and Kᵢ are directly measured. Although receptor-site occupancy by hormone is thought to be the initial event in a series of interactions that culminates in cyclic AMP production, binding is obviously not directly determined by this assay system. Rather, the cyclase assay only reflects receptor-site occupancy of a membrane-associated receptor-enzyme complex through the measurement of substrate ([³²P]ATP)-to-product ([³²P]) cyclic AMP) conversion (34), assuming no nonsystematic alteration of product recovery; thus only apparent Kᵣ and Kᵢ for agonist and antagonist are derived. However, the kinetics of the inhibition seen, i.e. the agreement in derived Kᵣ independent of relative dose of inhibitor or agonist (Fig. 4) and the unaltered V_max of the enzyme (Fig. 4) are consistent with truly competitive inhibition at the receptor locus.

The high potency as antagonists of substituted peptides, such as those reported here, should now help in more direct tests of receptor binding per se, as well as in direct elucidation of the initial steps in hormone action. Because the two norleucine-containing analogues are resistant to oxidation, they can be radioactively labeled with ¹²⁵I by conventional techniques without alteration of biologic properties, as has been done successfully with the previously reported norleucine-substituted agonist analogues of bPTH-(1-34) (8). Because inhibitors may differ from agonists in their rates of association with and dissociation from receptors, a radioactively labeled hormone inhibitor may prove to be a valuable tracer compound in direct studies of binding to physiologically relevant receptors (34), an area that has not yet yielded fully satisfactory results.

In addition, certain other features of potential interest concerning structure-activity relations are evident from these studies. The ability to impose activity-enhancing structural modifications upon an inhibitory core molecule without concomitant conversion to partial agonism further emphasizes the dichotomy between binding and subsequent activation for this hormone in renal tissues. Previous analysis (8, 9) of the effect of the structural modifications employed in the present studies had indicated an enhancement of the agonist properties of bPTH-(1-34) analogues of, at most, only 3-fold greater than unmodified bPTH-(1-34); the increase in antagonist potency of the inhibitory analogues containing the same structural modifications is at least 30-fold to several hundred-fold or more than unmodified bPTH-(3-34) (Figs. 2 and 4). Further analysis of structure-activity relations based on design and testing of related analogues may help define structural features important for binding versus activation.

Additional information concerning the structural requirements for receptor binding was derived by assessment of the o-NPS analogue, [Nle-8, Nle-18, o-NPS-Trp-23, Tyr-34]bPTH-(3-34)amide. Attachment of the bulky o-NPS group to the indole nitrogen of tryptophan is tolerated without significant change in inhibitory potency. Hence, an unmodified tryptophan at position 23 is not essential for hormone binding.

It may be possible now to further examine the physiologic role of guanylnucleotides such as GTP on hormonal action through the use of these inhibitors (34-36), particularly to address the issue of receptor versus postreceptor locus of action for GTP.

The marked increase in inhibitory effectiveness of these analogues makes practical for the first time the synthesis of sufficient quantities of these compounds for evaluation in vivo of hormonal inhibition in animal models, a study that we now plan to undertake. Design and synthesis of PTH antagonists active in vivo would provide direction toward the preparation of hormone analogues of potential therapeutic application in disorders of parathyroid hormone excess.

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