Synthesis of [α-methyltyrosine-4]angiotensin II: Studies of its conformation, pressor activity, and mode of enzymatic degradation

(angiotensin II analog/resistance to α-chymotrypsin degradation/full pressor activity/NMR and circular dichroism spectra similar to those of angiotensin II)

M. C. Khosla*, K. Stachowiak*, R. R. Smeyb, F. M. Bumpus*, F. Priou§, K. Lintner§, and S. Fernandjian‡

*Research Division, The Cleveland Clinic Foundation, Cleveland, Ohio 44106; and §Service de Biochimie, Department de Biologie, Centre d’Etudes Nucleaires de Saclay, P. B. No. 2, F-91190 Gif-sur-Yvette, France

Communicated by Irvine H. Page, October 15, 1980

ABSTRACT Modifications in angiotensin II and its antagonistic peptides that should have increased in vivo half-lives but not reduced biological activity were studied by determining the effect of α-methylation of the tyrosine in position 4. [α-Methyltyrosine-4]angiotensin II, synthesized by the solid-phase procedure, showed 92.6 ± 5.3% pressor activity of angiotensin II. Incubation with α-chymotrypsin for 1 hr indicated absence of degradation although, under the same conditions, angiotensin II was completely degraded to two components. Comparison of the 1H NMR spectra in aqueous solution and the circular dichroism spectra in trifluoroethanol of angiotensin II and [α-methyltyrosine-4]angiotensin II suggested that α-methylation of the tyrosine residue in angiotensin II does not lead to major changes in the overall solution conformation. These results are in contrast to those obtained with N-methylation in position 4, which drastically reduced the biological activity and produced remarkable changes in the peptide backbone and a severe limitation in rotational freedom of the side chains in tyrosine. Thus, it may be possible to synthesize potent angiotensin II analogs that have greater resistance to enzymatic degradation by α-methylation in position 4 (or 5) and simultaneous suitable modification at the NH2 and COOH termini.

The antagonists for the pressor hormone angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe; All) have proved useful in the study of experimental hypertension and as possible new clinical diagnostic tools (1). However, their long-term application has been limited because they have a short in vivo half-life and, due to degradation by peptidases, are not orally active. We therefore attempted to make these peptides resistant to this enzymatic degradation by replacing the natural amino acid residues with N-methylamino (2) or β-homoamino (3) acid residues. These modifications drastically reduced the biological activity of these analogs, and conformation studies suggest that N-methylation in positions 4 or 5 results in remarkable changes in the peptide backbone and a severe limitation in the rotational freedom of the side chains in tyrosine, isoleucine, and histidine residues (4). The replacement of an α proton by an α methyl group, however, is thought to produce minimal changes in backbone and side-chain structures. The analogs thus obtained might mimic the parent hormone in recognizing and binding with the receptor on the cell membrane and, in addition, be stable to enzymatic degradation (4, 5). Based on this hypothesis, we have synthesized [α-methyltyrosine-4]angiotensin II (α-MeTyr4)All. And, indeed, this peptide is resistant to chymotrypsin degradation and yet retains almost the full pressor activity of All. Conformation studies suggest minimum changes in backbone and side-chain structures. These results again suggest that, for full biological activity (agonist or antagonist), the backbone and side-chain structure of the analog should resemble that of the hormone, All (2, 4). These observations may be helpful in the design of new potent analogs.

EXPERIMENTAL

O-Benzyl-α-Methyl-L-Tyrosine. The procedure used for the synthesis of this compound is a modification of the one used by Khosla et al. (2) for the synthesis of O-(2,6-dichlorobenzyl)-L-tyrosine.

α-Methyl-L-tyrosine (3.904 g; 20 mmol) and 1.6 g of NaOH (20 mmol) in 10 ml of H2O were treated with 2.5 g of CuSO4·5H2O (10 mmol) in 10 ml of H2O. The mixture was shaken on a wrist shaker for 10 min, and 60 ml of MeOH was added, followed by 2.38 ml of C6H5CH2Br (20 mmol). The flask was stoppered, and the mixture was shaken for 20 hr at room temperature. The copper complex was removed by filtration, washed with three 10-ml portions of H2O, two 10-ml portions of MeOH, and two 10-ml portions of Et2O. The semi-air-dried product (6.2 g) was suspended in 40 ml of 1 M HCl, pulverized, and filtered out. The precipitate was repeatedly macerated with 1 M HCl and filtered out until the filtrate was almost colorless. The residue was then stirred with 20 ml of 16.4% AcONa; filtered out, washed with hot water, and dried at reduced pressure over P2O5. O-Benzyl-α-methyl-L-tyrosine thus obtained was homogeneous: thin layer chromatography (TLC) on silica gel, Rf 0.43 in n-BuOH/AcOH/H2O (4:1:5); Rf 0.57 in n-PrOH/ H2O (1:1).

t-Butyloxy carbonyl-O-Benzyl-α-Methyl-L-Tyrosine. A suspension of 1.42 g of O-benzyl-α-methyl-L-tyrosine (5 mmol) in 30 ml of Me2SO was treated with 0.72 ml of NEt3 (5 mmol) and 0.3 g of t-butyloxy carbonyl azide (0.3 g), and the mixture was stirred at 40°C under anhydrous conditions for 24 hr. The addition of Boc-N3 (0.3 g) and NEt3 (0.72 ml) was repeated every 24 hr until the solid went into solution (1 week or more). The solution was diluted with 40 ml of H2O, treated with NaOH to pH 9, and extracted with three 25-ml portions of Et2O. The aqueous layer was evaporated to dryness at 20°C at reduced pressure on a rotary evaporator. The residue was dissolved in 7 ml of H2O, and the solution was cooled to 0°C and treated with ice-cold citric acid solution to pH 4.5. The solution was then saturated with solid NaCl and extracted with AcOEt; the organic phase was washed with H2O and then with saturated NaCl solution, dried with Na2SO4, and evaporated to yield 2.36 g of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.
product: TLC on silica gel, Rf 0.67 in EtOH, Rf 0.81 in CHCl₃/ AcOH (95:5); Rf 0.73 in CHCl₃/MeOH/AcOH (85:10:5).

[α-MeTyr⁴]AII. COO-H Terminal tert-butyloxycarbonyl-L-phenylalanine was attached to 2% crosslinked chloromethyl polymer as described, and chain elongation was performed on a manual nitrogen-stirred apparatus (6) by using the protocol described (7). In general, the coupling reaction was carried out twice for 6–12 hr each time. 1-Hydroxybenzotriazole (2 mmol per mmol of tert-butyloxycarbonylamino acid) was used as an additive during coupling with N,N-dicyclohexylcarbodiimide. Apart from facilitating the coupling reaction, this procedure also avoids racemization of the histidine residue during coupling with tert-butyloxycarbonyl-L-α-benzyllhistidine (8). Coupling with tert-butyloxycarbonylvaline was difficult and was repeated three times by using a 3-fold excess of this derivative and a 6-fold excess of 1-hydroxybenzotriazole each time. At the end of the synthesis, the peptide was removed from the polymer with HBr/CF₃COOH and hydrogenated over 5% palladium/BaSO₄ in MeOH/AcOH/H₂O (5:1:1). The crude product was purified on a column of Bio-Rad anion exchange resin (AG 1 × 2, 200–400 mesh, acetate form) by eluting with ammonium acetate buffer (pH 8.5). Fractions containing the major component were pooled, lyophilized, and rechromatographed on successive columns of Sephadex G-25 by using n-BuOH/pyridine/H₂O (85:35:35, upper phase) and n-BuOH/AcOH/H₂O (4:1:5, upper phase) as the solvent systems. TLC on cellulose (E. Merck), Rf 0.73 in n-BuOH/AcOH/H₂O (4:1:5); Rf 0.55 in n-BuOH/pyridine/H₂O (10:2:5); Rf 0.68 in n-BuOH/pyridine/H₂O (85:35:65); Rf 0.71 in n-PrOH/H₂O (2:1); Rf 0.76 in n-BuOH/AcOH/H₂O/pyridine (30:6:24:20). Ionophoresis was carried out on Schleicher & Schuell 2403A filter paper strips in a model R, series D, Beckman electromorphosis cell at 400 V in 0.5 N H₂CO₃/AcOH buffer (pH 1.95) prepared by diluting 60 ml of HCO₃⁻ and 240 ml of AcOH to 10 liters with distilled H₂O and Beckman barbiturate buffer B-2 (pH 8.6). Histidine was run simultaneously as a reference compound; E(His) indicates electrophoretic mobility relative to His = 1.00; E(His) 0.92 (pH 1.95); E(His) 1.25 (pH 8.6). The peptide was hydrolyzed in a sealed tube in 12 M HCl/proionic acid (1:1) at 155°C for 2 hr in the presence of 0.1 ml of 90% aqueous phenol. Amino acid analysis, performed on a model MM-100 Glenco amino acid analyzer, gave the ratio: Asp 1.0, Arg 0.99, Val 0.89, Tyr(α-Me) 1.14, Ile 0.95, His 0.98, Pro 0.98, Phe 0.98.

Pressor Activity. Determination of the pressor activity (9) was carried out on vagotomized ganglion-blocked rats; [α-MeTyr⁴]AII showed 92.62 ± 5.32% (n = 24, 8 rats) pressor activity of AII.

Enzymatic Degradation with α-Chymotrypsin. Incubation of the peptide solution (0.1 M ammonium acetate buffer, pH 8.5) with chymotrypsin (beef pancreas, triple crystallized) (3) for 1 hr followed by TLC indicated the absence of degradation products when the chromatogram was sprayed with ninhydrin. Under the same conditions, angiotensin II was completely degraded into two components (presumably tetrapeptides).

³H NMR. The peptides AII and [α-MeTyr⁴]AII were studied under identical conditions—0.01 M in the cationic state in deuterated dimethyl sulfoxide. Tetramethylsilane was used as internal reference. ¹H NMR spectra were obtained on 250-MHz and 400-MHz instruments. Signal assignment was carried out on the basis of double resonance experiments and by comparison with previous work (10).

Circular Dichroism (CD). CD spectra were recorded on a Dichrograph model III (Jobin Yvon) using fused quartz cells of 1.0-cm and 0.01-cm path length. The results are expressed in molar ellipticity, and pH titrations were carried out as described (11).
group) from pH 1 to pH 4 increases the intensity of the tyrosine signal, and titration of the histidine imidazole group causes a total inversion of the CD signal. The amplitude of the titration—i.e., the difference of ellipticity, \( \theta_{275} \) between pH 4 and pH 8—is a function of the respective orientations of the tyrosine and histidine side chains, in other words, of the time-averaged distance between them (4, 10). The increased amplitude of titration in \([\alpha\text{-MeTyr}^4]\text{AII}\) with respect to that of AII hints at a slightly reduced distance, perhaps due to a sterically more hindered rotation of the tyrosine side chain (modified rotamer distribution). At higher pH values, the tyrosine chromophore titrates, going from the phenol to phenolate form; the CD spectrum at pH 12 consists of a strong positive band at 293 nm, just as in AII.

**DISCUSSION**

The conformational properties of \(\alpha\)-methylated amino acids, such as \(\alpha\text{-aminoisobutyric acid}\), have been investigated by theoretical and experimental methods. Whereas early calculations suggested preference for helical-type conformations around this residue (14), IR and NMR spectroscopy studies of peptides containing this amino acid in inert solvents showed that, in solution, no such preference exists (15, 16). \(\alpha\)-Aminoisobutyric acid has been found in the C\(_7\) conformation both in solution (15) and in the crystal state (\(\phi = 70, \psi = 64^\circ\)) (17) and in the corner positions 2 and 3 of \(\beta\)-turns (15, 16); this residue and, by extrapolation, any \(\alpha\)-methylated amino acid residue, displays nearly the same conformational properties as other usual residues. It is therefore not surprising that \(\alpha\)-methylolation of the tyrosine residue in AII does not lead to any major change in the overall solution conformation of the peptide. Small localized effects that can be detected by the sensitive spectroscopic methods CD and NMR

**FIG. 1.** \(^1\text{H NMR spectra at 400 MHz of AII and [}\alpha\text{-MeTyr}^4]\text{AII}.**

**FIG. 2.** Ellipticity at 275 nm as a function of pH for AII and [\alpha\text{-MeTyr}^4]AII.
do not extend far beyond the site of the chemical modification. This corroborates the results of Marshall et al. (18), who suggested that α-methylation introduces only minor NMR modifications.

Considering the ever stronger correlation between the conformation and the biological activity of all peptides, it is logical to predict full biological activity for [α-MeTyr]4AII. As α-methylation of the tyrosine residue practically eliminates the susceptibility of the peptide to chymotryptic hydrolysis, the synthesis of further analogs of this type (which conserve the essential conformation while being resistant to enzymatic attack) can be expected. In addition to α-methylation in position 4 (or 5), suitable modifications at the NH2 and COOH termini are necessary to stabilize these peptides against enzymatic attack by endopeptidases and exopeptidases.

We are grateful to Drs. R. Hirschmann and D. F. Veber (Merck Sharp & Dohme) for a generous supply of L-α-methyltyrosine and to Messrs. E. Bachynsky, S. Forgac, and J. Blum and Miss C. Lakios for their excellent technical assistance. This work was supported in part by National Institutes of Health Grant HL-6835.
