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Analogues containing the paramagnetic amino acid TOAC as substrates for angiotensin I-converting enzyme

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Dedicated to the memory of Professor Antonio Cechelli de Mattos Paiva

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Abstract **The angiotensin I-converting enzyme (ACE) converts the decapeptide angiotensin I (Ang I) into angiotensin II by releasing the C-terminal dipeptide. A novel approach combining enzymatic and electron paramagnetic resonance (EPR) studies was developed to determine the enzyme effect on Ang I containing the paramagnetic 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) at positions 1, 3, 8, and 9. Biological assays indicated that TOAC^{1}-Ang I maintained partly the Ang I activity, and that only this derivative and the TOAC^{3}-Ang I were cleaved by ACE. Quenching of Tyr^{4} fluorescence by TOAC decreased with increasing distance between both residues, suggesting an overall partially extended structure. However, the local bend known to be imposed by the substituted diglycine TOAC is probably responsible for steric hindrance, not allowing the analogues containing TOAC at positions 8 and 9 to act as substrates. In some cases, although substrates and products differ by only two residues, the difference between their EPR spectral lineshapes allows monitoring the enzymatic reaction as a function of time.**

Keywords: Peptides; Angiotensins; Angiotensin I-converting enzyme; TOAC; Electron spin resonance spectroscopy; Enzymes

1. Introduction

After its introduction in the early 1980s [1,2] for applications in solid peptide chemistry method [3,4], the use of the cyclic and achiral 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) spin probe [5] increased significantly, mainly after the introduction of an unconventional approach which allows TOAC to be inserted at any position of the peptide sequence [6]. Thereafter, a great variety of TOAC applications using mainly electron paramagnetic resonance (EPR) spectroscopy were described in the literature.

Due to the fact that TOAC belongs to the same class of $C^{\alpha,\alpha}$-disubstituted glycines as does aminoisobutyric acid (Aib), its introduction into any peptide sequence will certainly produce a unique structure as a consequence of its bend-forming capacity. In this context, various authors have investigated in depth the characteristics of the TOAC probe as well as its insertion (single or double) into many types of model peptide sequences [7–11] some of them of biological relevance [12–16]. Many alternative experimental applications have been designed for the use of this spin label, always taking into account its principal and more advantageous characteristics, i.e., of being rigidly bound to a molecule or system and thus monitoring with more sensitivity the dynamics of the site to which it is attached [17]. Some approaches are aimed to evaluate membranes [18,19] or even the topology of integral membrane peptides [20], thus providing examples of the broad range of applications for this probe. In addition, its use has been also extended to the labeling of other systems such as those in which complex solvation effects of polymeric materials were investigated in order to improve the peptide synthesis methodology [21–24].

In keeping with this trend toward developing novel uses for the TOAC probe, we have initiated studies of an approach where a peptide substrate labeled at different positions with the TOAC residue has been examined with regard to its specificity for an enzyme. The system chosen as our model comprises the angiotensin I-converting enzyme (EC 3.4.15.1 or ACE) and its substrate angiotensin I (Ang I, DRVYIHPFHL), with the TOAC residue attached at positions 1, 3, 8, and 9.

Although the subject of many investigations and detailed more thoroughly in a variety of reviews [25–28], some aspects of the ACE functions in the organism have not as yet been fully clarified. This enzyme is a zinc-containing dipeptidyl carboxypeptidase responsible for the conversion of the decapeptide Ang I to the potent vasoconstrictor octapeptide angiotensin II (Ang II) by hydrolyzing the Phe^{8}-His^{9} peptide bond [29], and for the degradation of the non-apeptide hypotensive agent bradykinin (BK) [30], both important modulators of

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Abbreviations: ACE, angiotensin I-converting enzyme; Aib, α-aminoisobutyric acid; Ang I, angiotensin I; Ang II, angiotensin II; BK, bradykinin; Boc, *tert*-butyloxycarbonyl; EPR, electron paramagnetic resonance; Fmoc, 9-(fluorenylmethyloxycarbonyl); PBC, phosphate-borate-citrate buffer; LC/ESI-MS, liquid chromatography/electrospray ionization mass spectrometry; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TOAC, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid

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blood pressure. Innumerable studies have been conducted in attempts to increase the understanding of the enzyme mechanism, as well as the specificities of the substrate and inhibitor [31–34].

In this context, the possibility of conjugating the investigation of ACE specificity for unusual substrates, i.e., Ang I analogues containing TOAC at different positions seems to be of interest in order to simultaneously increase the knowledge of this physiologically essential exopeptidase and determine the effect of the non-natural, cyclic TOAC residue on substrate reactivity. Therefore, we synthesized these TOAC-bearing AI analogues and developed the following experimental approaches: (i) determination of biological potency in contractile tissues; (ii) conformational investigation by means of EPR and fluorescence spectroscopies; (iii) enzymatic assays of ACE and determination of kinetic parameters; (iv) determination of structure–activity relationships for the analogues in terms of biological potency and substrate specificity; (v) time course monitoring of ACE hydrolysis of labeled substrate analogues.

2. Materials and methods

2.1. Material

The Boc and Fmoc amino acids were purchased from Bachem (Torrance, CA, USA). Solvents and reagents were from Aldrich or Sigma (St. Louis, MO, USA). Dimethylformamide was distilled over P_2O_5 and ninhydrin was distilled under reduced pressure before use. All solvents were HPLC grade and all chemicals met ACS standards. Purified rabbit lung ACE and lisinopril were purchased from Sigma. The molar concentration of the enzyme was determined by active site titration with lisinopril, as previously described [35].

2.2. Methods

2.2.1. Peptide synthesis. All peptides bearing the TOAC probe were synthesized manually according to the combined Boc/Fmoc strategies as previously reported [6]. All synthetic steps were performed through Fmoc chemistry, and anhydrous HF (Boc chemistry) was used for removal of the peptide from the solid support. The crude spin-labeled peptides were submitted to alkaline treatment (pH 10, 1 h at 50 °C) for complete reversal of the N–O protonation that occurs during the HF reaction [6,13]. Unlabeled peptides were synthesized using the Boc strategy. The peptides were purified by preparative HPLC (C_{18}-column) using aqueous 0.02 M ammonium acetate (pH 5) and 60% acetonitrile solutions as solvent A and B, respectively (linear gradient of 30–70% B for 2 h, flow rate of 10 mL/min). Peptide homogeneity was determined through analytical HPLC, amino acid analysis and LC/ESI-MS. The synthesized peptides were Ang I and its analogues containing TOAC at positions 1, 3, 8, and 9. Ang II and its analogues TOAC^1-AII and TOAC^3-AII were synthesized previously in our laboratory [2,13].

2.2.2. EPR studies. Spectra were obtained at 9.5 GHz in a Bruker ER 200 spectrometer at room temperature (22 ± 2 °C) using flat quartz cells from Wilmad Glass Co., Buena, NJ, USA. The magnetic field was modulated with amplitudes less than one-fifth the line widths, and the microwave power was 5 mW to avoid saturation effects. Rotational correlation times, τ_B and τ_C, for pure reagents and products were calculated from spectral line heights and line widths making use of the equations given by Kivelson [36]. Only the latter parameter will be reported here.

2.2.3. Fluorescence studies. Static fluorescence spectra were obtained at room temperature (22 ± 2 °C) in a Hitachi F 2500 spectrofluorimeter (Hitachi, Tokyo), using cuvettes with excitation path lengths of 2 mm or 5 mm and emission path lengths of 10 mm. Excitation and emission slits were 5 nm. The excitation wavelength was 280 nm and peptides concentrations were 10^{-4} M.

2.2.4. Mass spectrometry. The LC/ESI-MS experiments were performed on a system consisting of a Waters Alliance model 2690 separations module and model 996 photodiode array detector (Waters, Eschborn, Germany) controlled with a Compaq AP200 workstation coupled to a Micromass model ZMD mass detector (Micromass, Altrincham, Cheshire, UK). The samples were automatically injected on a Waters narrow bore Nova-Pak column C_{18} (2.1 × 150 mm, 60 Å pore size, 3.5 μm particle size). The elution was carried out with solvents A (0.1% TFA/H_2O) and B (60% acetonitrile/0.1% TFA/H_2O) at a flow rate of 0.4 mL/min using a linear gradient from 5% to 95% B in 30 min. The condition used for mass spectrometry measurements was a positive ESI. Specifically for the case of monitoring enzymatic reactions, a different gradient of 35–50% (v/v) of solution B in 15 min, with flow rate of 1.5 mL/min was used.

2.2.5. Amino acid analysis. Peptide composition was monitored using amino acid analysis performed on a Biochrom 20 Plus amino acid analyzer (Pharmacia LKB Biochrom Ltd., Cambridge, England) equipped with an analytical cation-exchange column.

2.2.6. Determination of kinetic parameters for Ang I and TOAC-containing peptides. The hydrolysis of peptides by purified rabbit lung ACE was performed at 37 °C in 0.1 M Tris–HCl buffer containing 50 mM NaCl and 10 μM $ZnCl_2$ at pH 7.0 (1.0 mL final volume). The hydrolysis reaction was monitored by LC/ESI-MS as a function of the time as already described, and the peaks areas were used for determination of kinetic parameters. The enzyme concentration (0.44 nM) was chosen so as to hydrolyze less than 5% of the substrate present in order to obtain the initial rate. The peptide concentrations ($\times 10^{-5}$ M) were 1, 5, 10, 15 and 20. The hydrolysis reaction was interrupted at different times by adding TFA aquous solution until pH 3.5. The k_{cat}, K_m and k_{cat}/K_m values were obtained by analysis of the non-linear regression data using the GraFit program. The standard deviation of these parameters was less than 5%.

3. Results and discussion

Following a previously described experimental protocol [6], the four paramagnetic AI analogues (labeling at positions 1, 3, 8, and 9) were obtained in satisfactory yield. The synthesis scale for each sample was 0.2 mmol/g, and the general synthesis and purification protocol are detailed in Section 2.

3.1. Biological assays

To determine the biological properties of the paramagnetic Ang I analogues, guinea pig ileum and rat uterus were chosen as in vivo model systems for use in contraction experiments [13]. It is well known that the biological potency of Ang I is much lower than that of Ang II in tissues lacking sufficient quantities of ACE needed to transform Ang I into Ang II. This is the case of the rat uterus [37] where much lower activity is expected for Ang I or its TOAC analogues in this type of muscle preparation than in guinea pig ileum which contains ACE. Accordingly, the biological potencies of Ang I in these two muscle preparations were 1% and 11%, respectively, in comparison with those of Ang II, taken as 100%.

With regard to the paramagnetic analogues, only TOAC^1-Ang I maintained activity, presenting potency nearly two-times in the uterus and 18% in guinea pig ileum that of Ang I. These data represent relative activities of about 2% that of Ang II in both muscle preparations, thus not in accordance with previous investigation of TOAC^1-Ang II analogue where values nearly 20% were determined in the two in vivo systems [13]. These findings reveal unknown factors affecting the ACE cleavage of Ang I to produce Ang II in these muscle contractile assays, thus leaving the perspective of further more elaborated investigations correlating in vitro and in vivo experiments. Otherwise, the results are clearly in agreement with the previous observation that the N-terminal portion of Ang II (which is responsible for muscle contraction) is less important for maintaining its potency [38]. In particular, the lack of effect observed for TOAC^3-AI is in agreement with previous studies