Bioorganic Chemistry 69 (2016) 159–166

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Paramagnetic bradykinin analogues as substrates for angiotensin I-converting enzyme: Pharmacological and conformation studies

Luis Gustavo Deus Teixeira [a], Luciana Malavolta [b], Patrícia Alessandra Bersanetti [c], Shirley Schreier [d], Adriana K. Carmona [a], Clovis R. Nakaie [a,*]

[a] Department of Biophysics, Escola Paulista de Medicina, Universidade Federal de Sao Paulo, 04044-020 Sao Paulo, SP, Brazil
[b] Department of Physiological Sciences, Santa Casa de Sao Paulo, School of Medical Sciences, 01221-020 Sao Paulo, SP, Brazil
[c] Department of Health Informatics, Universidade Federal de Sao Paulo, 04023-062 Sao Paulo, SP, Brazil
[d] Department of Biochemistry, Institute of Chemistry, Universidade de Sao Paulo, 05513-970 Sao Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 5 July 2016
Revised 10 October 2016
Accepted 26 October 2016
Available online 27 October 2016

Keywords:
Bradykinin
Angiotensin I-converting enzyme
ACE
Toac
Spin label
Biological activity

ABSTRACT

This study uses EPR, CD, and fluorescence spectroscopy to examine the structure of bradykinin (BK) analogues attaching the paramagnetic amino acid-type Toac (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid) at positions 0, 3, 7, and 9. The data were correlated with the potencies in muscle contractile experiments and the substrate properties towards the angiotensin I-converting enzyme (ACE). A study of the biological activities in guinea pig ileum and rat uterus indicated that only Toac^{0}-BK partially maintained its native biological potency among the tested peptides. This and its counterpart, Toac^{3}-BK, maintained the ability to act as ACE substrates. These results indicate that peptides bearing Toac probe far from the ACE cleavage sites were more susceptible to hydrolysis by ACE. The results also emphasize the existence of a finer control for BK-receptor interaction than for BK binding at the catalytic site of this metallodipetidase. The kinetic *kcat/Km* values decreased from 202.7 to 38.9 μM^{-1} min^{-1} for BK and Toac^{3}-BK, respectively. EPR, CD, and fluorescence experiments reveal a direct relationship between the structure and activity of these paramagnetic peptides. In contrast to the turn-folded structures of the Toac-internally labeled peptides, more extended conformations were displayed by N- or C-terminally Toac-labeled analogues. Lastly, this work supports the feasibility of monitoring the progress of the ACE-hydrolytic process of Toac-attached peptides by examining time-dependent EPR spectral variations.

1. Introduction

Many applications have been reported since the inception of the non-coded paramagnetic amino acid-type and fluorescent quenching probe Toac (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid) in the chemistry of peptides, firstly at the N-terminal end [1,2] and later at any internal position of the sequence [3]. These applications mainly involve electron paramagnetic resonance (EPR) spectroscopy. This cyclic spin label presents great sensibility to detect the motion and orientation of coupled macromolecules. This unique property is based on the fact that Toac has a constrained $C^{\alpha,\alpha}$-tetrasubstituted cyclic structure in which the rotation about side chain bonds is hampered by the presence of nitroxide nitrogen and C^{α}, C^{β}, and C^{γ} atoms in its heterocyclic moiety.

Different types of Toac applications in biology and chemistry have been described [4,5]. This unnatural spin label amino acid has been used to investigate the structure of different peptide segments [6–8], for their correlation with biological activities [9–14], or to examine G-protein coupled receptor fragments [15–18]. Toac has also been used to evaluate membrane-mimetic environments [19–21], measure intermolecular distances [22,23], and optimize peptide synthesis in a polymeric matrix [24–29].

In structure-function studies of Toac-attached peptides, we have mainly focused on investigating important vasoactive peptides [2,30–33], such as angiotensin II (AngII, DRVYIHPF) [34–36] and bradykinin (BK, RPPGFSPFR) [37,38]. More recently, studies testing for novel uses of Toac-peptide investigated the catalytic effect of the metallopeptidase angiotensin I-converting enzyme (EC 3.4.15.1 or ACE) upon angiotensin I (AngI, DRVYIHPFHL) bearing Toac group at several positions [39,40]. This enzyme releases the strong vasoconstrictor AngII from AngI and inactivates BK, hydrolyzing its structure sequentially at scissile bonds 7–8 and 5–6 [41,42].

* Corresponding author.
E-mail address: cnakaie@unifesp.br (C.R. Nakaie).

http://dx.doi.org/10.1016/j.bioorg.2016.10.006
0045-2068/

Following this approach, a multidisciplinary strategy was designed based on the insertion of the Toac probe in the BK sequence at positions 0, 3, 7, and 9. These labeled peptides were first evaluated in terms of conformational features using EPR, circular dichroism (CD), and fluorescence spectroscopy. The pharmacological activity of the synthesized peptides was examined in muscle contractile experiments (guinea pig ileum and rat uterus) according to a previous report [30], and the susceptibility to ACE's peptidase activity was evaluated. The progressive release of ACE's Toac-BK catalytic fragments was examined in a comparative time-course experiment for the possibility of using the EPR method to monitor this enzymatic hydrolysis process.

2. Abbreviations

ACE: angiotensin I-converting enzyme; Ang I: angiotensin I; Ang II, angiotensin II; BK, bradykinin; Boc: *tert*-butyloxycarbonyl; EPR, electron paramagnetic resonance; Fmoc: 9-(fluorenylmethy loxycarbonyl); 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-tetra methylpiperidine-1-oxyl-4-amino-4-carboxylic acid.

3. Materials and methods

3.1. Materials

Boc and Fmoc amino acids were purchased from Bachem (Torrance, CA, USA), and purified rabbit lung ACE was obtained from Sigma (St. Louis, MO, USA). Dimethylformamide (DMF) 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. The molar concentration of rabbit lung ACE was determined by active site titration with lysinopril, as previously described [43].

3.2. Methods

3.2.1. Peptide synthesis

BK derivatives bearing the Toac probe were synthesized manually according to previously reported combined Boc/Fmoc strategies [3,30]. All synthetic steps were performed through Fmoc chemistry [44], and anhydrous HF (Boc chemistry) [40,45] was used to remove the peptide from the solid support. The C-terminally coupled $Toac^9$-BK synthesis began with a Toac-copolystyrene-1% divinylbenzene support that was previously obtained by attaching Fmoc-Toac to a chloromethyl resin according to a standard protocol [45]. The crude spin-labeled peptides were subjected to alkaline treatment (pH 10 for 1 h at 50 °C) to completely reverse the N—O protonation that occurs during the HF reaction. 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 solvents 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, mass spectrometry, and amino acid analysis.

3.2.2. Analytical RP-HPLC

RP-HPLC analyses were carried out in a TFA/acetonitrile gradient using a Waters Associates HPLC system consisting of two 510 HPLC pumps, an automated gradient controller, a Rheodyne manual injector, a 486 UV detector, and a 746 data module. The column was a Vydac C18 column (0.46 × 15 cm; 5-μm particle size; 300-Å pore size) with detection at λ = 210 nm and the following solvent systems: solvent A: 0.1% TFA/H_2O; solvent B: 60% acetonitrile/0.1% TFA/H_2O. A gradient of 5–95% B in 30 min at a flow rate of 1.5 mL/min was used.

3.2.3. Mass spectrometry

Liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS) experiments were performed on a system consisting of a Waters Alliance model 2690 separation module and model 996 photodiode array detector (Waters, Eschborn, Germany), which were controlled with a Compaq AP200 workstation coupled to a Micromass model ZMD mass detector (Micromass, Altrincham, UK). The samples were automatically injected into 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.

3.2.4. Amino acid analysis

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

3.2.5. 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 of less than one-fifth of the line widths, and the microwave power was 5 mW to avoid saturation effects. The rotational correlation time (τ_C) of paramagnetic compounds were calculated from spectral line heights and line widths [46,47]. The concentration of peptides was 10^{-4} M in 0.02 M phosphate buffer at pH 7.

3.2.6. 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 an emission path length of 10 mm. the excitation and emission slits were 5 nm, and the concentration of peptides was 10^{-4} M in 0.02 M phosphate buffer at pH 7. The excitation wavelength was 255 nm for BK peptides.

3.2.7. CD studies

Spectra were obtained on a Jasco J-810 spectropolarimeter at room temperature that was continually flushed with ultra-pure nitrogen. The peptide concentration was 10^{-4} M in 0.02 M phosphate buffer at pH 7.0 or with the addition of TFE. Equivalent results were obtained in triplicate for the EPR, fluorescence, and CD experiments.

3.2.8. Bioassays

The biological potencies of BK and their Toac-attached derivatives were examined in rat uterus and guinea-pig ileum according to a previous report [30]. Briefly, uterine horns were removed from female rats (200–240 g) that had received 100 mg of diethylstilbestrol per 100 g of weight 24 h before the experiments. The horns were mounted in 5-mL organ baths containing De Jalon's solution, which was bubbled with a gas mixture of 95% O_2 and 5% CO_2, and the temperature was kept at 30 °C in order to avoid the spontaneous contractions observed at higher temperatures. For