Biochemical and Biophysical Research Communications 374 (2008) 635–640

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

The functional importance of the N-terminal region of human prolylcarboxypeptidase

J. Mallela [a,b], R. Perkins [a], J. Yang [a], S. Pedigo [c], J.M. Rimoldi [d], Z. Shariat-Madar [a,b,*]

[a] Department of Pharmacology, University of Mississippi, 317 Faser Hall, University, MS 38677-1848, USA
[b] Research Institute of Pharmaceutical Sciences University of Mississippi, University, MS 38677-1848, USA
[c] Department of Chemistry & Biochemistry University of Mississippi, University, MS 38677-1848, USA
[d] Department of Medicinal Chemistry, University of Mississippi, University, MS 38677-1848, USA

ARTICLE INFO

Article history:
Received 10 July 2008
Available online 24 July 2008

Keywords:
Prolylcarboxypeptidase
Renin–angiotensin system
Kallikrein-kinin system
Prekallikrein
High molecular weight kininogen
Angiotensin converting enzyme 2

ABSTRACT

The renin–angiotensin-system cascade pathway generates the vasopressor and prothrombotic hormones, angiotensin II (Ang II) and angiotensin III (Ang III) from angiotensinogen. One of the key enzymes for the generation of angiotensin 1–7 (Ang 1–7) and angiotensin 2–7 (Ang 2–7) from Ang II and III, respectively, is prolylcarboxypeptidase (PRCP). To understand the contribution of the N-terminal region to catalysis, an N-terminal truncated form, lacking 179 N-terminal residues of PRCP ($rPRCP_{40}$) was constructed. The circular dichroism (CD) spectrum of $rPRCP_{40}$ illustrated that it was structured with significant helical content as indicated by local minima at ~220 and 208 nm. The main products of Ang III metabolized by $rPRCP_{40}$ were Ang 2–7 plus phenylalanine as determined by LC–MS. Angiotensin I (Ang I) blocked the metabolism of Ang III by $rPRCP_{40}$. These investigations showed that the C-terminal region of the $rPRCP_{40}$ contributes to PRCP's catalytic function, and provided additional experimental evidence for this suggestion.

Published by Elsevier Inc.

The renin–angiotensin system (RAS) regulates blood pressure, water, and electrolyte balance [1,2]. The RAS has been implicated as a major effector of hypertension and other cardiovascular diseases arising from its production of angiotensin II (Ang II) and angiotensin III (Ang III) [3]. Angiotensin II can be metabolized by at least 13 proteases, among which are angiotensin converting enzyme 2 (ACE2) and prolylcarboxypeptidase (PRCP) which convert Ang II to angiotensin 1–7 (Ang 1–7) [4]. ACE2 and PRCP are switches that transform this peptide from a vasoconstrictor to a vasodilator. Ang (1–7) may act in a synergistic manner to modulate bradykinin-induced vasodilation at the local level. In doing so, ACE2 and PRCP regulate the blood flow through active tissues in order to preserve the internal environment [5]. Thus, ACE2 and PRCP have cardiovascular protective roles [6].

Recently, we found that when the complex of high molecular weight kininogen (HK) and prekallikrein (PK) binds to endothelial cell membranes, PK is rapidly converted to kallikrein by PRCP. Kallikrein then cleaves HK to liberate bradykinin (BK) which is a potent vasodilator by activating constitutive bradykinin B2- and inducible bradykinin B1 receptors-mediating nitric oxide and prostacyclin formation [7]. The PRCP-dependent BK generating pathway might be considered as an additional negative regulator of the pressor actions of the RAS.

In humans, PRCP has been recognized to have 2 mRNAs, termed as PRCP1 (Gene Bank NP_005131) and PRCP2 (NP_955450). The N-terminus of PRCP is where these isoforms diverge. There is no evidence suggesting whether PRCP2 mRNA encodes a functional protein.

PRCP is a polypeptide that folds back on itself to form a three-dimensional functional structure. The monomeric PRCP has the ability to undergo dimerization yet by an uncharacterized intramolecular mechanism of reaction. The residues involved in protein dimerization differ from protein to protein. Generally, N-terminal, C-terminal, and transmembrane domains of a variety of proteins are found to be implicated in dimeric interaction [8–10]. The first 539 base pairs on the 5′-end of PRCP encode the putative transmembrane domain and the non-catalytic region [6]. PRCP1 had less than 30% similarity with the 3D structures of molecules found in the Protein Data Bank. Hence, it was difficult to guess the critical side chains. Therefore we focused on the functional importance of the N-terminal region of PRCP1 and test its role by site-directed mutagenesis, protein expression, and kinetic assessment.

The present investigation was designed to describe the expression and purification of human PRCP which does not contain the first 539 base pairs on the 5′-end ($rPRCP_{539-1601}$). The $rPRCP_{539-1601}$ contains the catalytic region and is highly conserved with human PRCP family of enzymes and with PRCP in other

* Corresponding author. Address: Department of Pharmacology, University of Mississippi, 317 Faser Hall, University, MS 38677-1848, USA. Fax: +1 662 915 5148.
E-mail address: madar@olemiss.edu (Z. Shariat-Madar).

0006-291X/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.bbrc.2008.07.069

species. The $rPRCP_{539-1601}$ is a N-terminal truncated form (ΔN179-PRCP), lacking 179 N-terminal residues of PRCP with an apparent molecular mass of 40 kDa. The 40 kDa recombinant human PRCP ($rPRCP_{40}$) was characterized with respect to its biochemical and physical properties. Collectively, the metabolism of angiotensin molecules by $rPRCP_{40}$ brings forth a new understanding into the mechanism of actions of PRCP, therefore allowing better insight into structure–function relationships.

Materials and methods

Materials. Schneider insect (S2) cells were purchased from Invitrogen (Carlsbad, CA). Serum free insect cell growth medium was purchased from HyClone (Logan, UT). Diethylaminoethyl (DEAE) cellulose was purchased from Whatman (Fairfield, NJ). SP-Sephadex and all reagents were purchased from Sigma–Aldrich (St. Louis, MO). Angiotensin III, angiotensin 1–7, angiotensin I, H-Ala-Pro-pNA, and z-Pro-Pro-aldehyde-dimethyl acetate (Z-Pro-Prolinal) were purchased from Bachem (King of Prussia, PA). Angiotensin 2–7 was obtained from New England Peptide LLC (Gardner, MA). Phenylmethylsulfonyl fluoride (PMSF) was obtained from Calbiochem (San Diego, CA).

Expression and purification of wild-type and the mutant PRCP. In the past, the wild-type PRCP ($rPRCP_{52}$) cDNAs was incorporated into pMT/BIP/V5-HisC inducible/secreted expression vector and expressed in Schneider (S2) insect cells [11]. Therefore, we used the same strategy to clone and express a variant cDNA that deleted a sequence of 539 base pairs on 5′-end $PRCP_{539-1607}$ cDNA into pMT/BiP/V5. The sense primer contained a 5′-encoding sequence (5′-GAA.TTT.CCT.GAC.ATC.AGA.ACA.AGC.TC-3′) and an EcoRI restriction site at the 5′-end. The antisense primer (3′-CCG.GAA.TTC.TCA.GTG.CTG.CTT.TCC.TGC.ACT-5′) had an EcoRI restriction site at the 3′-end. The EcoRI and EcoRI restriction sites were for direct cloning into the expression vector pMT/BiP/V5-His C (Invitrogen, Carlsbad, CA).

pMT/BiP-PRCP plasmid was expressed according to a previously described method [11]. The fidelity of the cDNA for PRCP was confirmed by DNA sequence analysis. The expressed wild-type (with a predicted molecular mass of about 52 kDa) and mutant PRCP (with a predicted molecular mass of 40 kDa) were separated by 10% reduced SDS–polyacrylamide gel electrophoresis (data not shown). The concentration of rPRCP was calculated by measuring its absorbance at 280 nm in a 1 cm pathlength cuvette using an UV–Vis spectrophotometer. The molar extinction coefficient was calculated to be 90,000 M^{-1} cm^{-1}. Using the Beer–Lambert Law, the concentration of protein was determined [12].

Circular dichroism (CD) measurements. Far UV CD spectra and thermal-denaturation experiments were recorded on an AVIV 62D CD spectropolarimeter. Spectra in a 0.05 cm pathlength cuvette were at room temperature (22 °C) with 9.6 μM $rPRCP_{40}$ in reaction buffer (0.01 M sodium acetate, 0.07 M potassium phosphate, and 1 mM EDTA, pH 4.8). Spectra in a 1 cm pathlength cell were at 25 °C with 1 μM recombinant PRCP mutant protein in reaction buffer. Angiotensin III was added at 1 μM when present. Spectra were not background corrected. The thermal-denaturation experiments were performed in a 1 cm pathlength cell with a sealed top through which a temperature probe was inserted. Temperature was scanned from 15 to 85 °C while the CD signal was monitored at 225 nm. The concentration of recombinant PRCP mutant was at 1 μM in reaction buffer. Data are plotted against the probe temperature.

Data were analyzed using IGOR Pro (ver.4.0; Wavemetrics), with procedure files written in-house. Temperature induced unfolding transitions monitored by CD were fit to a 2-state unfolding model represented as follows:

$$Native \leftrightarrow Unfolded \tag{1}$$

The equilibrium constant, K, is given by

$$K = \frac{[U]}{[N]} \tag{2}$$

The mole fraction of the unfolded species is given by

$$f_U = \frac{K}{1+K}; \quad K = e^{\frac{-\Delta G}{RT}} \tag{3}$$

The relationship between free energy change and temperature is given by the equation shown below

$$\Delta G = \Delta H_m\left(1 - \frac{T}{T_m}\right) + \Delta C_p\left(T - T_m - T \cdot \ln\left(\frac{T}{T_m}\right)\right) \tag{4}$$

where ΔH_m is the enthalpy of unfolding at the melting temperature, T_m is the melting temperature and ΔC_p is the heat capacity at the melting temperature [13]. Unfolding data from the CD were fit to Eq. (3) and (4) with baseline slopes and intercepts as fitted parameters.

Kinetic studies. The rate constants for PRCP substrate were determined for both the wild-type and purified mutant PRCPs in reaction buffer containing 0.01 M Na-acetate, 0.07 M potassium phosphate, pH 5.8, at 37 °C. The slope of the initial linear increase in absorbance at 304 nm/min was used to determine PRCP activity. Blanks with substrate but no enzyme were run to verify that PRCP activity was measured as opposed to substrate auto-hydrolysis. The blank values were subtracted from those in the presence of enzyme to obtain enzyme catalyzed hydrolysis of the substrate. The Henri–Michaelis–Menten constant (K_m, V_{max}) were calculated from Lineweaver–Burke ($1/S$ versus $1/V$) plots and verified by Hanes–Woolf (S versus S/V) analysis.

LC–MS studies. LC–MS experiments for the analysis of angiotensin III and its metabolites were conducted based on the method of Cui [14], with modification. The instrumentation platform used consisted of a Waters Micromass ZQ single quadrupole mass spectrometer coupled to a Waters 2695 HPLC, controlled by Mass Lynx 4.0 Software. Peptide incubation mixtures were separated on a reverse-phase C18 column (Phenomenex Prodigy, 5 μm, ODS3, 100 × 4.6 mm) using a mobile phase consisting of water (spiked with 0.1% formic acid) and CH_3CN at ambient temperature using a constant flow rate of 0.2 mL/min. The mobile phase gradient of 5% CH_3CN in water was increased linearly to 20% CH_3CN from 0–10 min, to 50% CH_3CN from 10–25 min, and decreased linearly to 5% CH_3CN from 25–40 min. Electrospray ionization (ESI) parameters: capillary voltage, 3.2 kV; cone voltage, 15 V; extractor, 3 V; desolvation temperature, 250 °C, desolvation gas flow, 400 L/h, source temperature, 120 °C. LC–MS was performed using both TIC and SIR (single ion recording) mode. Angiotensin III (m/z 466.4 $[MH]^{+2}$; angiotensin III-Phe (m/z 392.9 $[MH]^{+2}$). 10 μL of the incubation mixture containing Ang III and PRCP was diluted with water (240 μL) and 5 μL of this solution was used for analysis.

Results

Structure and stability of recombinant PRCP

The CD spectrum of $rPRCP_{40}$ (Fig. 1A) illustrates that it is structured with significant helical content as indicated by local minima at ~220 and 208 nm. Addition of equimolar levels of Ang III did not change the spectrum. Thermal denaturation of $rPRCP_{40}$ is shown in (Fig. 1B). The transition appears to be biphasic illustrating that unfolding occurs in a multistep process. Data were modeled as a simple 2-state transition (Eq. (1)) to provide estimates of the enthalpy of unfolding and melting temperature. Fits to Eq. (3) and (4) resolved an enthalpy change upon unfolding of 33 ± 1 kcal/