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 $\Delta H_m$ is the enthalpy of unfolding at the melting temperature, $T_m$ is the melting temperature and $\Delta C_p$ 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/

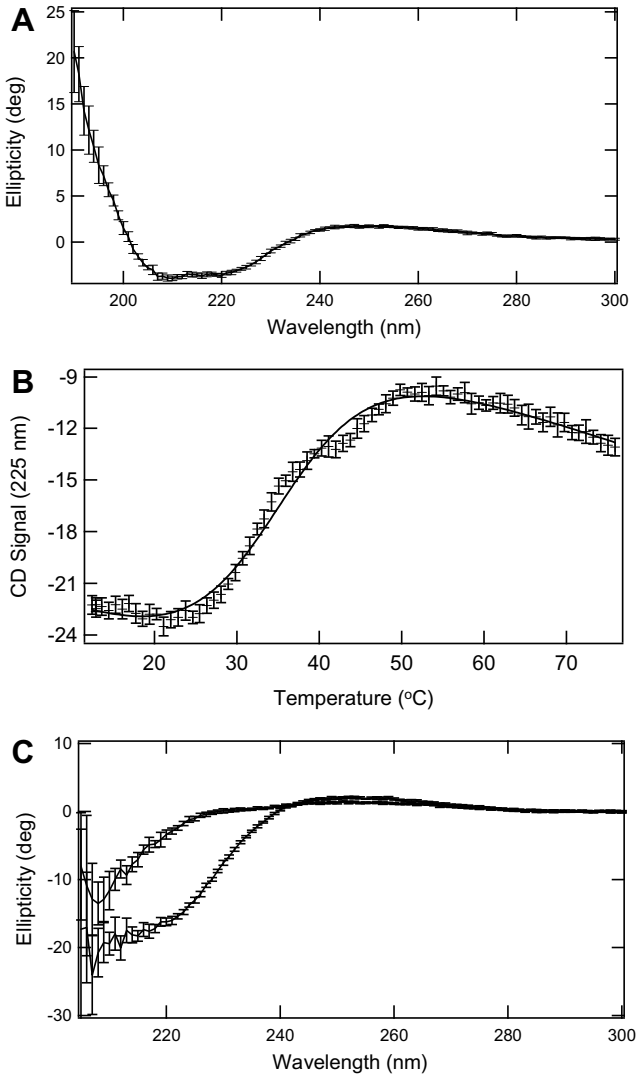


**Fig. 1.** CD spectroscopy. (A) CD spectrum of $rPRCP_{40}$ at 25 °C (0.05 cm quartz cuvette, 9.6 μM protein). (B) Temperature-dependence of molar ellipticity of $rPRCP_{40}$ at 225 nm. Protein concentration is 1 μM. Solid line through the data is simulated based on the resolved values to fits to Eqs. (3) and (4). The native baseline was fixed to the value resolved for the unfolded baseline. (C) CD spectra of $rPRCP_{40}$ in the presence of 1 μM Ang III at 25 °C before (bottom spectrum) and after (top spectrum) heating to 60 °C. Protein concentration is also 1 μM in a 1 cm quartz cuvette. (Error bars are one standard deviation of the average value.)

mol and $T_m$ 35.3 ± 0.2 °C ($\Delta C_p$ was fixed to 0 cal/mol K). The enthalpy change resolved from a fit to a 2-state model less than the enthalpy for the individual fits to a 3-state transition. There was significant change in the CD spectrum after heating. Fig. 1C shows a comparison of the spectra of PRCP with Ang III added to before and after a thermal-denaturation experiment. These data suggest that the unfolding of $rPRCP_{40}$ was irreversible even in the presence of Ang III.

To determine whether $rPRCP_{40}$ behaved like native PRCP under steady-state kinetic conditions, we monitored the percent liberation of *p*-nitroanilide from Ala-Pro-*para*-nitroanilide over a range of temperature with a fixed time and enzyme concentration. The stability of $rPRCP_{40}$ was tested by measuring Ala-Pro-pNA hydrolysis at various temperatures for 1 h. The activity of the $rPRCP_{40}$ increased with assay temperature until the temperature reached 37 °C. $rPRCP_{40}$ activity gradually decreased at higher temperature, and became completely inactive, presumably by becoming denatured at temperatures above 60 °C. These experiments along with thermal-denaturation experiments (Fig. 1C) support the ideas that the N-terminal of PRCP may be involved in its structural stabilization.

*Kinetic characterization of $rPRCP_{40}$.*

We next determined the kinetic parameters of $rPRCP_{40}$. The specificity of $rPRCP_{40}$ was studied by determining the $k_{cat}/K_m$ values for the hydrolysis of Ala-Pro-pNA and angiotensin molecules substrates. Linearity of the reaction with time and amount of enzyme was determined in all experiments performed in this study. The rate of hydrolysis of Ala-Pro-pNA by $rPRCP_{40}$ was plotted as a function of substrate concentration and is shown in (Fig. 2). A hyperbolic dependence was observed for $rPRCP_{40}$, consistent with Henri-Michaelis–Menten kinetics, using Ala-Pro-pNA. $rPRCP_{40}$ like $rPRCP_{52}$ as shown previously metabolized Ala-Pro-pNA in a dose- and time-dependent fashion (Fig. 2A) [11]. The ratio of enzyme-to-chromogenic substrate was adjusted so that the rate of *para*-nitroanilide release was linear over the course of the assay. The $K_m$ of $rPRCP_{40}$ and of wild-type rPRCP ($rPRCP_{52}$) for Ala-Pro-pNA was 1.5 ± 0.4 mM and 3.1 ± 0.3 mM, respectively. The catalytic efficiency ($k_{cat}/K_m$) of the $rPRCP_{40}$ protein towards Ala-Pro-pNA is 18.6 ± 3.4/sec. To demonstrate that Ala-Pro-pNA hydrolysis was not due to the activities of aminopeptidases and cathepsins, $CdCl_2$ (3 mM), 1,10-phenanthroline (10 mM) and cystatin (1 μM), inhibitors of aminopeptidases A and N, cathepsin B, H and L were added to the incubation cocktail. Our results suggested that the ability of $rPRCP_{40}$ to hydrolyze the substrate was significantly improved in comparison to $rPRCP_{52}$ ($p \leqslant 0.05$) [11].

Recently, we showed that Ang II causes substrate inhibition of native endothelial PRCP and $rPRCP_{52}$ [11,15]. To further characterize $rPRCP_{40}$, substrate inhibition studies were performed. In the substrate inhibition studies, Ang III (a PRCP substrate), but not Ang 1–7 (as control) inhibited $rPRCP_{40}$ in a dose-dependent manner suggesting the functional similarity of $rPRCP_{40}$ with the native endothelial PRCP (Fig. 2B) [15]]. The effect of Ang II precursor, angiotensin I (Ang I) on $rPRCP_{40}$ was determined. Ang I inhibited PRCP in a dose-dependent manner with an $IC_{50}$ of 1 mM in the presence of 3 mM chromogenic substrate (Fig. 2C).

For the first time, we developed a new and an accurate assay to determine PRCP catalytic activity. The reaction products from the conversion of Ang III, Ang 2–7, and Ang I by $rPRCP_{40}$ were determined by LCMS. Reaction mixtures lacking enzyme served as negative controls, while $rPRCP_{40}$ in the presence of the substrate was used as a positive control. As shown in (Fig. 3A), $rPRCP_{40}$ has the ability to convert Ang III ($Arg^2$-$Val^3$-$^{125}I$-$Tyr^4$-$Ile^5$-$His^6$-$Pro^7$-$Phe^8$) to Ang 2–7 ($Arg^2$-$Val^3$-$^{125}I$-$Tyr^4$-$Ile^5$-$His^6$-$Pro^7$) and phenylalanine. Metabolism of Ang III (1 mM) by $rPRCP_{40}$ (100 ng) resulted in three major peaks in comparison to the control (no enzyme) (Fig. 3A). The unmetabolized amount of Ang III appeared at 31.58 min, whereas Ang 2–7 and phenylalanine appeared at 28.33 and 26.11 min, respectively. Unmetabolized fraction of Ang III by $rPRCP_{40}$ had a similar retention time as the control (Ang III alone) (Fig. 3A, inset). This is a novel finding because N-terminally truncated PRCP like that of endothelial PRCP, which is known for its ability to cleave Pro-X bonds to release the C-terminal amino acid [16], can generate identical metabolites (Ang 2–7 and phenylalanine). To determine the generation of potential secondary metabolites, Ang 2–7 or Ang 1–7 were incubated with $rPRCP_{40}$. No secondary metabolites were formed by $rPRCP_{40}$ (Fig. 3B) in comparison to the control (Fig. 3B, inset). Similarly, no secondary metabolites were formed when $rPRCP_{40}$ was incubated with Ang1–7 (Fig. 3C). Ang 1–7 in the absence of $rPRCP_{40}$ was used as a negative control (Fig. 3C, inset). The incubation of $rPRCP_{40}$ with Ang I (an ACE 2 substrate) resulted in no metabolite generation (Fig. 3D). Ang III was used as a standard and shown in (Fig. 3E).

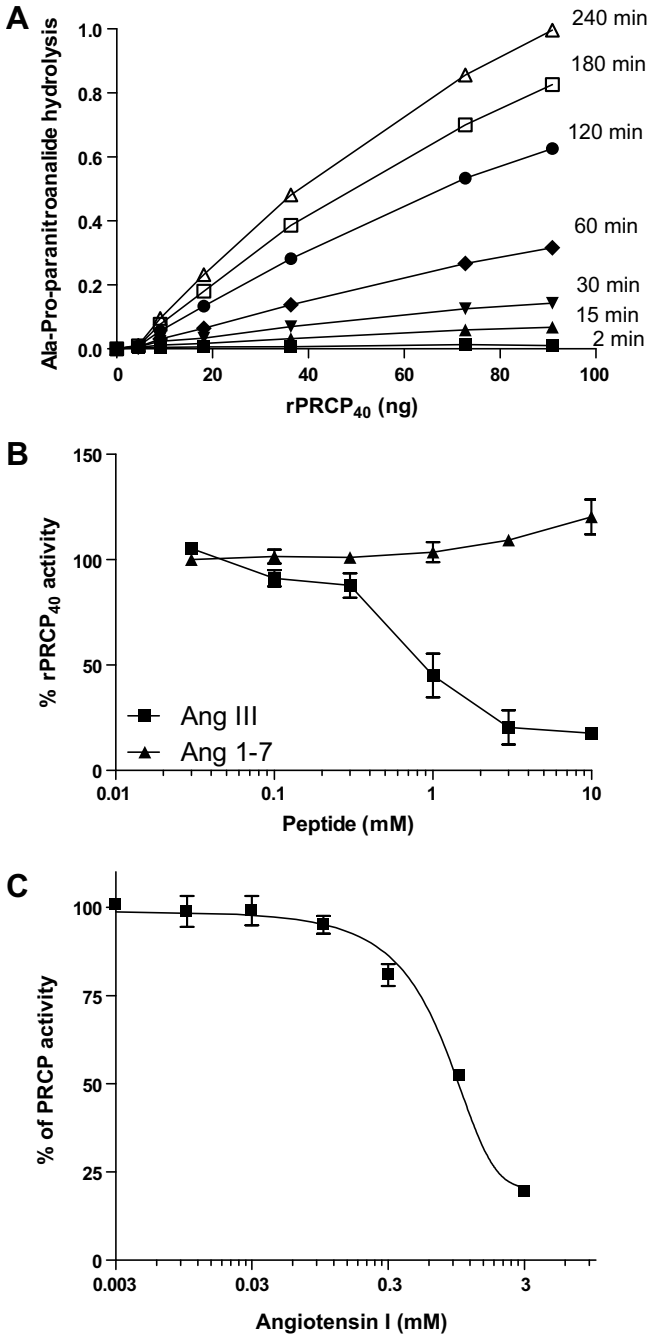


**Fig. 2.** Enzymatic analysis of $rPRCP_{40}$. (A) Time course and enzyme dependency of $rPRCP_{40}$ on *para*-nitroanalide generation. The indicated concentration of $rPRCP_{40}$ was incubated with 1.8 mM Ala-Pro-*para*-nitroanalide (APpNA) in 0.01 M Na-acetate, 0.07 M $KH_2PO_4/K_2HPO_4$ buffer pH 5.8 at 37 °C for 2 (■), 15 (▲), 30 (▼), 60 (•), 120 (●), 180 (□), and 240 (△) minutes and the amount of generated *para*-nitroanalide expressed as OD was assessed for each $rPRCP_{40}$ concentration. (B) Substrate inhibition of $rPRCP_{40}$. The amount of *para*-nitroanalide formed in the presence of $rPRCP_{40}$ was determined by incubating each well with 1 mM Ala-Pro-*para*-nitroanalide in the absence or presence of increasing concentrations of angiotensin III (•) or angiotensin 1–7 (■). (C) Effect of angiotensin I (Ang I) on $rPRCP_{40}$. $rPRCP_{40}$ was incubated with various concentrations of Ang I in the presence of $rPRCP_{40}$ substrate, Ala-Pro-*para*-nitroanalide (APpNA). Hydrolysis of APpNA was monitored at 405 nm for 3 h. The results presented are means ± SEM of three separate experiments and are expressed as % $rPRCP_{40}$ activity.

When a combination of Ang I (1 mM) and Ang III (1 mM) were incubated with $rPRCP_{40}$, it resulted in the generation of Ang 2–7. However, the level of Ang 2–7 generated by $rPRCP_{40}$ in the presence of Ang I was significantly reduced, suggesting that Ang I might be the endogenous inhibitor of PRCP (Fig. 3F). These data suggested that the main products of Ang III or Ang II metabolized by $rPRCP_{40}$ are Ang 2–7 and phenylalanine or Ang 1–7 and phenylalanine, respectively.

## Discussion

In view of the role of PRCP in RAS and KKS pathways, investigations were performed to characterize molecular structure and function analysis of recombinant PRCP on the metabolism of Ang III and Ang II. To our surprise, the enzymatic properties of $rPRCP_{40}$ and mature $rPRCP_{52}$ characterized in the present investigation indicate that the two proteins have similar functional activities on Ang II and Ang III.

$rPRCP_{40}$ was tested for its ability to metabolize Ala-Pro-pNA, as well as Ang II and Ang III. The affinity for Ala-Pro-pNA for $rPRCP_{40}$ was higher than for the wild-type enzyme. The hydrolysis of substrate by $rPRCP_{40}$ followed Henri–Michaelis–Menten kinetics with the $K_M$ value decreased by a factor of two when compared with the wild-type. The catalytic efficiency of $rPRCP_{40}$ was approximately twofold higher than the wild-type. In substrate inhibition studies, Ang III but not Ang 1–7 in increasing concentration blocked Ala-Pro-pNA hydrolysis. Angiotensin converting enzyme 2 (ACE 2) forms angiotensin 1–9 from angiotensin 1 (Ang 1). Ang I blocked both Ala-Pro-pNA metabolism (Fig. 2C) and Ang III metabolism (Fig. 3F), suggesting that it may be an endogenous inhibitor of PRCP.

The most prominent effect caused by extended incubation of Ang III with PRCP is a near total inactivation of Ang III which eliminates its ability to cause $AT_1$ receptor stimulation (Fig. 3A). PRCP converted Ang III to Ang 2–7 with no other metabolites being observed.

The temperature inactivation and inhibition studies confirmed the uniqueness of the $rPRCP_{40}$'s function. $rPRCP_{40}$ was found to be much less stable upon incubation at temperatures higher than 50 °C than endothelial PRCP as was observed earlier, [16,17]. This finding could only be due to folding nature of recombinant PRCP.

Efficient conversion of Ang II to Ang 1–7 by ACE2 as well as PRCP and Ang III to Ang 2–7 by PRCP are critical on endothelium that are continuously exposed to a variety of vasopressor molecules. Therefore, vasodilation rather than vasoconstriction pathways are favored, sustaining function, and preparing endothelium to receive the next wave of vasoconstrictor stimuli. PRCP activity provides a measure of endothelium relaxation capacity due to its ability to initiate nitric oxide and prostaglandin generation via kallikrein generation [7,15]. ACE2 and PRCP are gauges of endothelium relaxation processes.

PRCP is a homodimer and the biochemical and physical nature of this dimerization is not known. It is tempting to speculate that the first 179 residues of N-terminal region of PRCP, which is lacking in $rPRCP_{40}$ could play an important role in the folding and the dimerization of the polypeptides. Our observation that β-mercaptoethanol does not reduce substrate hydrolysis indicates that cysteine oxidation may not be involved in $rPRCP_{40}$-induced substrate hydrolysis. The inability of β-mercaptoethanol or DTT to inactivate $rPRCP_{40}$ has several implications. First, the PRCP monomer containing the catalytic domain retains the ability to metabolize Ang III and Ala-Pro-pNA. Secondly, dimers of PRCP could be the inactive form. Identification and characterization of yet another unidentified modulator that might regulate the equilibrium between the active monomer and the inactive dimers of PRCP will require further investigations.

In conclusion, the structural basis of how PRCP can bind and selectively metabolize a variety of different peptides and protein

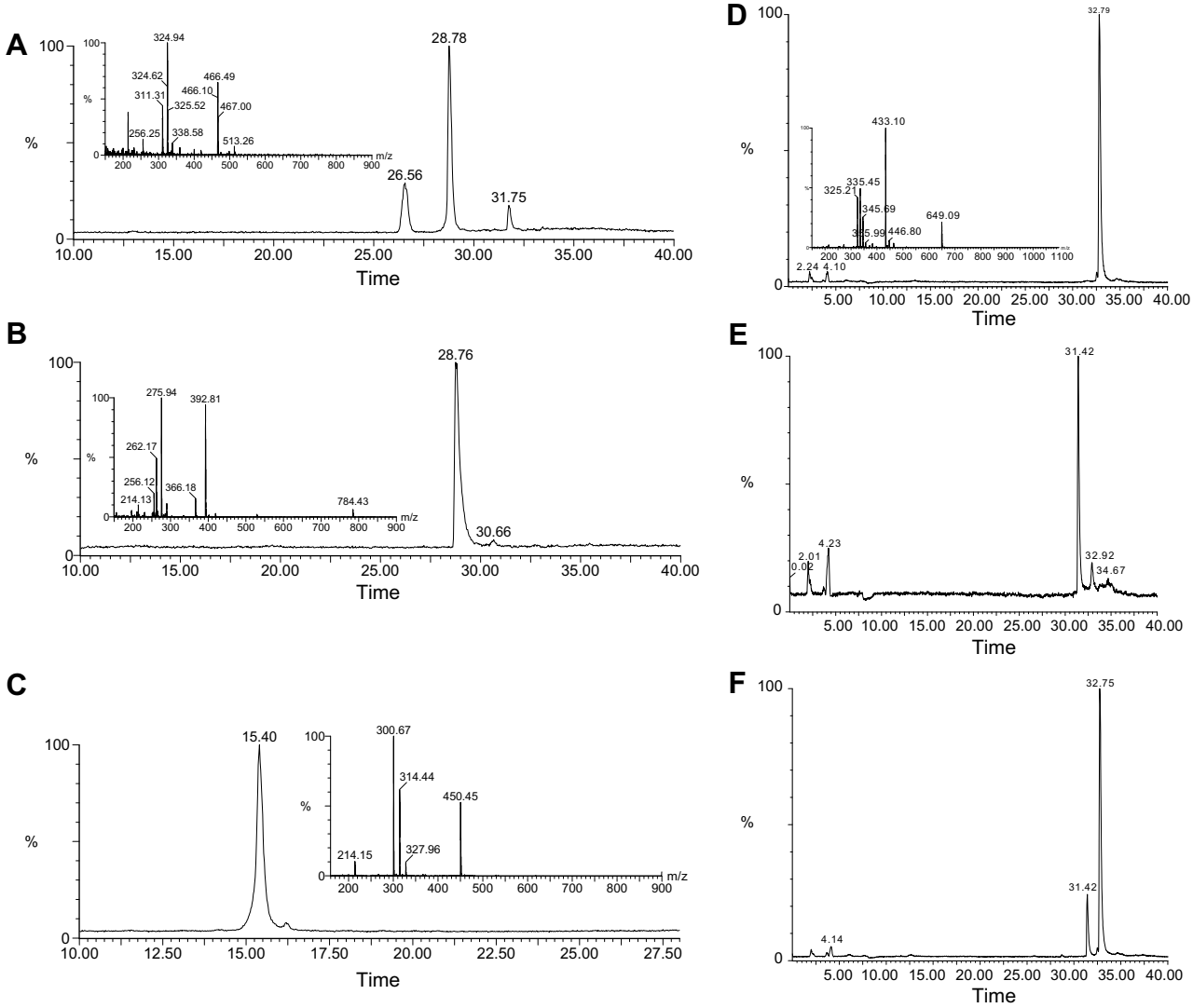


**Fig. 3.** LC–MS chromatograms of angiotensin peptides. The metabolism of the angiotensin peptides in the absence or presence of $rPRCP_{40}$ was determined by LC–MS. (A) 1 mM angiotensin III (Ang III) was incubated with 100 ng $rPRCP_{40}$ for 3 h at 37 °C and the metabolites of angiotensin III were determined and the inset is the Ang III standard mass spectra. (B) 1 mM angiotensin 2–7 was incubated with 100 ng $rPRCP_{40}$ for 3 h at 37 °C and the metabolites of angiotensin 2–7 were determined. The inset is the mass spectra of Ang 2–7. (C) 1 mM angiotensin 1–7 was incubated with 100 ng $rPRCP_{40}$ for 3 h at 37 °C and the metabolites of angiotensin 1–7 were determined. The inset is the mass spectra of Ang 1–7. (D) 1 mM angiotensin I (Ang I) was incubated with 100 ng $rPRCP_{40}$ for 3 h at 37 °C and the metabolites of Ang I were determined. (E) Ang III alone was used as standard. (F) 1 mM Ang I in combination with 1 mM Ang III was incubated with 100 ng $rPRCP_{40}$ for 3 h at 37 °C and the metabolites of Ang molecules were determined.

is far from clear. The work presented here is the first description of the role of N-terminal region of PRCP in the production of Ang 1–7 and Ang 2–7 using LC–MS. It is also the first demonstration of an instance in which Ang I blocks the conversion of Ang III to Ang 2–7 by PRCP. The data thus suggest that a selective increase in Ang I in plasma may be helpful to prevent hypotensive effects of Ang 1–7, Ang 2–7, and bradykinin through PRCP-mediated PK activation. Despite the incomplete characterization of PRCP, we showed that the C-terminal region of the PRCP contributes to PRCP's catalytic function, and provided additional experimental evidence for this suggestion.

## Acknowledgements

The authors thank Robert Speth and Vardan Karamyan for discussion. This study was supported by American Heart Association 0330193N and P20RR021929 from the National Center for Research Resources (NCRR/NIH) to ZSM.

## References

[1] J.E. Hall, Control of blood pressure by the renin–angiotensin–aldosterone system, Clin. Cardiol. 14 (1991) IV6–IV21.
[2] M.J. McKinley, A.L. Albiston, A.M. Allen, M.L. Mathai, C.N. May, R.M. McAllen, B.J. Oldfield, F.A. Mendelsohn, S.Y. Chai, The brain renin–angiotensin system: location and physiological roles, Int. J. Biochem. Cell Biol. 35 (2003) 901–918.
[3] E.K. Jackson, Renin and angiotensin, in: L.L. Brunton, J.S. Lazo, K.L. Parker (Eds.), Goodman & Gilman's the Pharmacological Basis of Therapeutics, McGraw-Hill, New York, 2006, pp. 789–821.
[4] V.T. Karamyan, R.C. Speth, Enzymatic pathways of the brain renin–angiotensin system: unsolved problems and continuing challenges, Regul. Pept. 143 (2007) 15–27.
[5] C.S. Flordellis, D. Goumenos, G. Kourounis, S.A. Tsementzis, H. Paris, J. Vlachojiannis, The shift in the "paradigm" of the pharmacology of hypertension, Curr. Top. Med. Chem. 4 (2004) 487–498.
[6] J. Mallela, J. Yang, Z. Shariat-Madar, Prolylcarboxypeptidase: a cardioprotective enzyme, Int. J. Biochem. Cell Biol., in press, doi:10.1016/j.biocel.2008.02.022
[7] Y. Zhao, Q. Qiu, F. Mahdi, Z. Shariat-Madar, R. Rojkjaer, A.H. Schmaier, Assembly and activation of HK–PK complex on endothelial cells results in bradykinin liberation and NO formation, Am. J. Physiol. Heart Circ. Physiol. 280 (2001) H1821–H1829.

[8] T. Okazaki, Y. Nagaoka, K. Asami, Ion channels of N-terminally linked alamethicin dimers: enhancement of cation-selectivity by substitution of Glu for Gln at position 7, Bioelectrochemistry 70 (2007) 380–386.
[9] J. Rodriguez-Zavala, H. Weiner, Role of the C-terminal tail on the quaternary structure of aldehyde dehydrogenases, Chem. Biol. Interact. 130–132 (2001) 151–160.
[10] P.T. Zanna, B.L. Sanchez-Laorden, A.B. Perez-Oliva, M.C. Turpin, C. Herraiz, C. Jimenez-Cervantes, J.C. Garcia-Borron, Mechanism of dimerization of the human melanocortin 1 receptor, Biochem. Biophys. Res. Commun. 368 (2008) 211–216.
[11] Z. Shariat-Madar, F. Mahdi, A.H. Schmaier, Recombinant prolylcarboxypeptidase activates plasma prekallikrein, Blood 103 (2004) 4554–4561.
[12] C.N. Pace, F. Vajdos, L. Fee, G. Grimsley, T. Gray, How to measure and predict the molar absorption coefficient of a protein, Protein Sci. 4 (1995) 2411–2423.
[13] P.L. Privalov, Stability of proteins: small globular proteins, Adv. Protein Chem. 33 (1979) 167–241.
[14] L. Cui, K. Nithipatikom, W.B. Campbell, Simultaneous analysis of angiotensin peptides by LC–MS and LC–MS/MS: metabolism by bovine adrenal endothelial cells, Anal. Biochem. 369 (2007) 27–33.
[15] Z. Shariat-Madar, F. Mahdi, A.H. Schmaier, Identification and characterization of prolylcarboxypeptidase as an endothelial cell prekallikrein activator, J. Biol. Chem. 277 (2002) 17962–17969.
[16] C.E. Odya, D.V. Marinkovic, K.J. Hammon, T.A. Stewart, E.G. Erdos, Purification and properties of prolylcarboxypeptidase (angiotensinase C) from human kidney, J. Biol. Chem. 253 (1978) 5927–5931.
[17] C.E. Odya, E.G. Erdos, Human prolylcarboxypeptidase, Methods Enzymol. 80 (Pt. C) (1981) 460–466.