Modulating furin activity with designed mini-PDX peptides: Synthesis and in vitro kinetic evaluation

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Received 16 May 2005; revised 4 July 2005; accepted 25 July 2005

Available online 8 August 2005

Edited by Judit Ovádi

Abstract **A peptide was designed from reactive site loop structure of α1 Antitrypsin Portland known as α1 PDX as a novel mini-PDX inhibitor of furin. The sequence was derived from (367–394) that contains the crucial furin cleavage motif RIPR^{382}. A P3 mutant replacing Ile^{380} by Leu was prepared as a first model peptide. A Cys residue was inserted at each terminal of the peptide for purpose of cyclisation which was accomplished by air or iodine-induced oxidation. This mini-PDX peptide both cyclic and acyclic form inhibited in vitro furin activity (IC_{50} in nM) when measured against either substrates Boc-RVRR⇓MCA or QVEGF-C [Abz-QVHSIIRR⇓SLP-Y(NO_2)-A-$CONH_2$, Abz = 2-amino benzoic acid and Y(NO_2) = 3-nitro tyrosine], latter being derived from vascular endothelial growth factor-C (VEGF-C) processing site. The geometrically constrained structure mimicking PDX reactive loop is crucial for enzyme inhibition. Our study further revealed that both mini-PDX peptides inactivate furin in a slow tight binding manner, with disulfide-bridged cyclic form being slightly more potent. Unlike PDX, these peptides inhibit furin via a different mechanistic pathway. The study provides an alternate strategy for development of efficient peptide-based inhibitors of Proprotein Convertases including furin.**

Keywords: Alpha 1 antitrypsin Portland; Proprotein convertases; Furin; Serpin; Miniserpin; Protease inhibitor; Disulfide bridge; Cyclic peptide; Reductive alkylation

1. Introduction

Furin is a Ca^{2+}-dependent cellular endoprotease that activates a large number of precursor proteins in secretory pathway compartments [1,2]. This endoprotease is a key member of subtilisin-kexin like mammalian proprotein convertases (PCs) that cleave peptide bonds at the general motif R/K/H–X–X/K/R–R↓ [3–5], where X = any amino acid usually not Cys. Proteolytic processing of larger inactive precursor proteins into functionally active smaller forms is an important cellular event implicated in both normal and abnormal pathophysiology. Over the years this hypothesis was well substantiated by numerous research publications [reviewed in 1,2]. Owing to this role, proteolytic enzymes remain in the forefront as important therapeutic targets for intervention of many diseases and metabolic disorders, even though several of these proteases are also linked to normal development and important physiological functions [2–4]. Thus design of potent and selective protease inhibitors became a valuable research subject in drug design and therapeutic applications. Several inhibitors directed against host or foreign invading proteases have been described in the literature for treatment of conditions such as viral infections like AIDS, inflammatory diseases such as chronic obstructive pulmonary disorder, prostate cancer and others [6–8]. Furin's critical role in many cellular events associated with infections of HIV [9], SARS [10], Ebola [11], anthrax [12], and influenza [13] as well as cancer [14], neurological dementia like Alzheimer's disease [15] has made this enzyme a therapeutic target. So far several furin inhibitors have been reported that are either macromolecular proteins or small molecule peptides to non-peptides [16]. Among them the most potent is the bioengineered serpin protein α1-Antitrypsin (AT) Portland (known as PDX) in which the reactive-centre Ala^{P4}–Ile–Pro–Met$^{382(P1)}$ sequence of α1 AT has been replaced by furin motif Arg–Ile–Pro–Arg^{382}↓ [17].

Despite several reports [16–19] not much success has been achieved in designing small compound inhibitors of furin, that are more attractive and useful as therapeutic agents because of their stability and ease of accessibility. In this study, we developed a small peptide with the constrained reactive site loop (RSL) structure of bioengineered serpin PDX as a modulator of furin activity. It was already demonstrated that RSL of all serpins are prime interactive domains responsible for their protease inhibitory function [20–23]. In this paper, we describe a linear and a corresponding disulfide-bridged cyclic peptide variant based on RSL structure of α1-PDX and demonstrated that both inhibit furin activity but with differential manner. To our understanding, this is the first report of a PC inhibitor based on PDX loop structure. Previously, we reported that chymotrypsin inhibitor 2 (CI-2) loop can be successfully used to develop inhibitors of PCs [24].

2. Materials and methods

All fluoren-9-yl methoxycarbonyl (Fmoc) amino acids (L-configuration), the coupling agents and organic solvents were purchased from PE Applied Biosystems (Framingham, MA, USA), Calbiochem–Novabiochem (San Diego, CA, USA), Chem-Impex International (Wood Dale, IL, USA) and Aldrich Chemical Company (Milwaukee, WI, USA). Recombinant furin enzyme and its substrate Boc-RVRR-MCA (MCA = 4-methyl coumaryl-7-amide) used for kinetic study

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0014-5793/$30.00
doi:10.1016/j.febslet.2005.07.062

were purchased from New England BioLabs Inc. (Ipswich, MA, USA) and Peptides International (Louisville, KY, USA), respectively. The intramolecularly quenched fluorogenic substrate, QVEGF-C [Abz-QVHSIIRR$^{227}\Downarrow$SLP-Y(NO_2)-A-$CONH_2$, MW 1703, Abz = 2-amino benzoic acid and Y(NO_2) = 3-nitro tyrosine] used in the present study as a furin substrate was prepared as previously reported by us [25]. This peptide was derived from the physiological processing site RR$^{227}\Downarrow$SL of VEGF-C. Acetonitrile (ACN), 4-hydroxycinnamic acid, HCl, triflouro ethanol (TFE), and all other reagents were purchased from Sigma–Aldrich Company, Milwaukee, MI, USA.

2.1. Peptide synthesis

All peptides were synthesized as C-terminal amides on an automated solid-phase peptide synthesizer (Pioneer, PE-Perseptive Biosytems Inc, Framingham, MA, USA), following *O*-hexafluoro-phospho-[7-aza-benzotriazol-1-yl]-*N*,*N*,*N*′,*N*′-tetramethyluronium/di-isopropylethyl-amine-mediated Fmoc chemistry using unloaded PAL-PEG (polyamino linker polyethylene glycol) resin. The following amino acid side chain protecting groups were used: *t*-butyloxycarbonyl (Boc) for Lys; 2,2,4,6,7-pentamethyl dihydrobenzo furan-5-sulfonyl for Arg; *t*-butyl for Ser, Thr, Asp and Tyr and trityl for His, Asn and Gln. A deprotection cocktail (Reagent B) containing TFA, phenol, water and triisopropylsilane was used and added to the peptide-bound resin (5 ml) [6,19]. After 3 h of treatment at room temperature, the peptide was recovered as described [6,19].

2.2. RP-HPLC protocol for peptide purification

All peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC, Rainin Dynamax) using a semi-preparative column (CSC Exsil, C_{18}, 25 cm × 1.0 cm, Chromatography Specialty Co., St-Laurent, Quebec, Canada). The buffer system consisted of an aqueous 0.1% (v/v) trifluoroacetic acid solution and an organic phase of ACN also containing 0.1% (v/v) trifluoroacetic acid. The elution was carried out by using a linear gradient from 15% to 60% organic phase in 105 min following a 10-min isocratic step at 100% aqueous phase (gradient A, semipreparative run) or from 0% to 60% organic phase in 60 min (gradient A, for analytical run). The flow rate was adjusted to 2.0 or 1.0 ml/min, respectively, and the separation was monitored by UV absorbance at 230 nm.

2.3. Amino acid analysis and N-terminal micro-sequencing

Quantitative amino acid analyses were performed following 24 h hydrolysis in 6 N HCl in a sealed tube at 110 °C in vacuo using the ion chromatography system (ICS-2500, Dionex, Oakville, ON, Canada) equipped with conductance detector [25]. The sequence of each peptide was confirmed by N-terminal microsequencing using Procise instrument (PE Biosystem Inc, Framingham, MA, USA) and phenylthio hydantoin chemistry [10].

2.4. Mass spectral analysis

The identity of each peptide was fully confirmed by matrix-assisted laser-desorption ionization-time of-flight (MALDI-TOF) mass spectrometry (Voyageur-DE Pro, PE-Biosystems Inc, Framingham, MA, USA) or surface enhanced laser desorption ionization time of flight (SELDI-TOF) (Ciphergen, CA, USA) on gold chips (http://www.ciphergen.com) using 4-hydroxycinnamic acid or 1,2-dihydroxy benzoic acid as energy absorbing matrix [10,19,26].

2.5. CD spectroscopy

All CD spectra were recorded in Jasco-810 spectropolarimeter (Easton, MD, USA) in 0.1-cm thick rectangular quartz cell in a total volume of 300 μL (100 μM final concentrations) at 0.1 nm intervals from 185 to 240 nm wavelength at 25 °C. Each spectrum was analyzed using CD Estima and CONTIN software for estimation of various α-helix, β-sheet, β-turn, and random structures (Softspec Co, NJ, USA). The final corrected CD spectra were obtained by subtracting the spectrum obtained with control solvent from those of samples [26].

2.6. Cyclic peptide formation and characterization

For preparation of disulfide-bridged cyclic peptides, oxidation by air or iodine solution was used. For air oxidation, air was continuously bubbled through a dilute solution (100 ml, 0.015 μmol/ml)) of the peptide (I) in aqueous ammonia, pH 8.0 at 25 °C for overnight. Aliquots were removed at various time intervals and analyzed by RP-HPLC. For iodine oxidation, a minor modification was made regarding the concentration of the reagent used [27]. Thus 1-ml solution of peptide (2.5 μmol/ml) in water:acetic acid (1:4) was treated at 25 °C with iodine solution (100 μL, 0.3 μmol/ml). The mixture was stirred for 3 h following which 1 ml of 1 M sodium thiosulfate solution was added to terminate the reaction. The mixture was concentrated by rotary evaporation under reduced pressure and analyzed by RP-HPLC as described above. All significant peaks were collected and subjected to analysis by MALDI-TOF mass spectrometry, amino acid analysis and N-terminal microsequencing. The formation of cyclic peptide was confirmed by co-mass spectrometry with acyclic peptide, RP-HPLC as well as N-terminal micro-sequencing and mass spectrometry following reduction with tri(2-carboxyethyl)phosphine (TCEP) [28] and alkylation with iodoacetamide as described in Focus™ protein Reduction–Alkylation kit (http://www.alphelys.com/site/fr/pKPEL_FOCUS_Prot_Alkyl.htm).

2.7. Enzyme assay and kinetic evaluation

All in vitro enzyme studies were performed using initial rate and/or end-time assays at room temperature in a final volume of 100 μl in 96-well flat-bottom black plates (Micro-fluor, Dynatec, Va, USA). The buffer consisted of 25 mM Mes, 25 mM Tris and 2.5 mM $CaCl_2$ (pH 7.4). Assays were performed using both Q-VEGF-C and Boc-RVRR-MCA as substrates at two different concentrations namely 10 and 30 μM of each. The release of highly fluorescent 7-amino 4-methyl coumarin (AMC) from Boc-RVRR-MCA and 2-amino benzoic acid (Abz) containing N-terminal fragment from QVEGF-C were monitored by a spectrofluorometer instrument (Perkin–Elmer model LS50B) at excitation and emission wavelengths of 370 nm/460 nm and 320 nm/420 nm, respectively. Recombinant furin (2 μL of 5-fold diluted stock sample) from New England BioLabs, representing 0.8 U (unit) of activity, one U = amount of furin that will release 1 pmol of free AMC from Boc-RVRR-MCA (50 μM) was pre-incubated for 30 min at 25 °C with various concentrations of the mini-PDX peptide. The fluorogenic substrate Boc-RVRR-MCA or QVEGF-C was then added and the released AMC or Abz-containing peptide was measured. K_i (inhibition constant) values were derived from Cornish–Bowden plots as described in [29]. For measurement of both K_i and IC_{50} (the concentration necessary to achieve 50% inhibition of enzymic activity) values, the inhibitor concentrations were varied over a range wide enough to yield residual activities of 25–75% of the control value [18,19,30].

2.8. Digestion of QVEGF-C peptide by furin

QVEGF-C (10 μg) was incubated at 37 °C with recombinant furin (2 μL, 5-fold diluted of commercial stock as described above) in 25 mM Tris + 25 mM Mes + 2.5 mM $CaCl_2$, pH 7.4 in a total volume of 100 μl in the absence and presence of various amounts of cyclic and acyclic mini-PDX peptides (I and II). Following 4 h digestion, the reaction was terminated by adding glacial acetic (1 μl) and product analyzed by RP-HPLC chromatography.

3. Results and discussion

3.1. Design of mini-PDX peptides

Sequence alignment of a number of known serpin inhibitors near their reactive site cleavage loops (Table 1), revealed a common resemblance of types of amino acids present at various *P* and *P*′ positions as indicated in the table. Of particular significance is the presence of near conserved alkyl side chain containing hydrophobic amino acids at P_6, P_5, P_4 and P'_2 positions. It is further noted that in general they all contain negatively charged Glu and/or Pro residues at positions between P'_3 and P'_6. Moreover, the physiological serpin Spn4a and the bioengineered serpin α1 PDX, the double mutant variant of α1 AT (not shown in Table 1), both of which inhibit furin activity also contain crucial P_1 and P_4 Arg residues. We hypothesize that the reactive site loop domain of either of these