Biochimie 93 (2011) 331–338

Contents lists available at ScienceDirect

Biochimie

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

Research paper

Mapping the eosinophil cationic protein antimicrobial activity by chemical and enzymatic cleavage

Daniel Sánchez, Mohammed Moussaoui, Esther Carreras, Marc Torrent, Victòria Nogués, Ester Boix*

Dpt. Bioquímica i Biologia Molecular, Fac. Biociències, Universitat Autònoma de Barcelona, 08193-Cerdanyola del Vallès, Spain

ARTICLE INFO

Article history:
Received 30 July 2010
Accepted 8 October 2010
Available online 15 October 2010

Keywords:
Antimicrobial peptides
Host defense
Proteolysis
Chemical cleavage
Eosinophil cationic protein

ABSTRACT

The eosinophil cationic protein (ECP) is a human antimicrobial protein involved in the host immune defense that belongs to the pancreatic RNase A family. ECP displays a wide range of antipathogen activities. The protein is highly cationic and its bactericidal activity is dependant on both cationic and hydrophobic surface exposed residues. Previous studies on ECP by site-directed mutagenesis indicated that the RNase activity is not essential for its bactericidal activity. To further understand the ECP bactericidal mechanism, we have applied enzymatic and chemical limited cleavage to search for active sequence determinants.

Following a search for potential peptidases we selected the Lys-endoproteinase, which cleaves the ECP polypeptide at the carboxyl side of its unique Lys residue, releasing the N-terminal fragment (0–38).

Chemical digestion using cyanogen bromide released several complementary peptides at the protein N-terminus. Interestingly, ECP treatment with cyanogen bromide represents a new example of selective chemical cleavage at the carboxyl side of not only Met but also Trp residues. Recombinant ECP was denatured and carboxyamidomethylated prior to enzymatic and chemical cleavage. Irreversible denaturation abolishes the protein bactericidal activity.

The characterization of the digestion products by both enzymatic and chemical approaches identifies a region at the protein N-terminus, from residues 11 to 35, that retains the bactericidal activity. The most active fragment, ECP(0–38), is further compared to ECP derived synthetic peptides. The region includes previously identified stretches related to lipopolysaccharide binding and bacteria agglutination. The results contribute to define the shortest ECP minimized version that would retain its antimicrobial properties. The data suggest that the antimicrobial RNase can provide a scaffold for the selective release of cytotoxic peptides.

1. Introduction

The eosinophil cationic protein (ECP) is an host defense protein with antipathogen capacities [1]. Its bactericidal activity is not dependant on its enzymatic RNase activity [2] and the protein antibacterial activity correlates with its membrane disruption capacity [3]. To further understand ECP mechanism of action we have characterized the protein effect at the membrane and bacteria wall level [4–6]. The antimicrobial protein binds to bacteria cells and triggers the cell death, with a particular agglutination activity for *Escherichia coli* [7].

Antimicrobial peptides in living organisms serve as a defense system in addition to, or complementary to, the highly specific cell-mediated immune response. As antibiotic resistance develops rapidly as soon as new agents are introduced, there is an increased interest to develop antimicrobial compounds with alternative mechanisms of action [8].

Antimicrobial peptides can act both at the bacteria envelope and at a specific intracellular target [9–12]. While intracellular vital components could be targeted by specific enzymatic activities, such as RNases, DNases or proteases, many antimicrobial polypeptides trigger the pathogen death by a direct action at the cell surface. Many antimicrobial peptides bind specifically to bacteria wall components, as peptidoglycans or lipopolysaccharides (LPS), and have a membrane lytic activity [13–15]. We also find several cases where a well characterized host defense enzyme can simultaneously disrupt the cellular membrane by an unspecific lysis mechanism. As an example, lysozyme, not only attacks the peptidoglycan layer of Gram-positive cells but can also promote the cell aggregation and lead to the Gram-negative cell lysis [16]. Lysozyme specifically binds to LPS and can disrupt the permeability barrier that represents the outer membrane of Gram-negative strains [17].

Abbreviations: CFU, colony forming units; CNBr, cyanogen bromide; ECP, eosinophil cationic protein; LB, Luria Broth; LPS, lipopolysaccharides; poly(U), polyuridylic acid; TFA, trifluoroacetic.

* Corresponding author. Tel.: +34 93 5814147; fax: +34 93 5811264.
E-mail address: Ester.Boix@uab.es (E. Boix).

0300-9084/$ – see front matter © 2010 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2010.10.005

Interestingly, denatured lysozyme looses its muramidase activity but retains its membrane disruption ability [9]. Aprotinin, a protease inhibitor, denatured and devoid of protease inhibiting activity still retains its bactericidal activity [18]. Another example is cathepsin, a neutrophil proteolytic enzyme, which retains its bactericidal properties after ablation of its catalytic site [19]. These examples may represent a more generalized strategy that combines a multilevel mechanism of action, with a first mechanical step and a specialized intracellular function. Multifunctional host defense agents that can target cells simultaneously at diverse cellular levels are good candidates to reduce the development of bacteria resistance mechanisms [12,20].

There are also many examples in the literature of antimicrobial proteins which suffer post-translational proteolysis modifications. In fact, the proteolytic processing is used extensively in immunology cascade events. A local cleavage can release the active peptides in the area of infection or inflammation [21]. As an example, a specific cleavage of cathelicidins is a critical step carried out by several proteases dependant on the polypeptide sequence and post-tranlational modifications, which provides a mechanism for *in situ* activation [22]. Limited proteolysis of an inactive protein precursor can release *in vivo* an active fragment, which often corresponds to the N- or C-terminus [23]. The research pharmacology industry has often taken as templates these proteolysis products for drug design. A recombinant fragment corresponding to the N-terminal end of the bactericidal permeability- increasing protein (BPI), expressed in neutrophils, is currently under clinical assay in phase III, as an alternative antibiotic. Lactoferricin is a naturally occurring microbicidal peptide derived from the N-terminus of lactoferrin, by action of pepsin in the stomach [24], and is very effective against some antibiotic resistant strains[25]. Cathelicidins, expressed in neutrophils [26,27] or cryptidins, secreted by Paneth cells [28], are active peptides liberated from inactive proprotein precursors, by a specific proteolytic activation.

Interestingly, several members of the RNase A superfamily with antimicrobial activity, have conserved their active site architecture but do not require their enzymatic RNase activity for bacteria cytotoxicity [29]. Moreover, derived peptides from the chicken leukocyte RNase A-2 reproduce most of the protein original bactericidal activity [30]. Some cytotoxic RNases may then serve as a scaffold of active peptides [31].

We describe here the analysis of ECP digestion products released both by chemical cleavage and enzymatic proteolysis.

2. Methods

2.1. Materials

Endoproteinase Lys-C (E.C. 3.4.24.20) from *Lysobacter enzymogenes* was purchased from Sigma–Aldrich. pET11 expression vector and *E. coli* BL21 (DE3) cells were from Novagen, Madison, WI. Iodoacetamide was from Sigma–Aldrich. Symmetry C18 chromatography column was from Waters Corporation, Milford, MA. *Staphylococcus aureus* 502 A strain was from ATCC, Rockville, MD. BacTiter-Glo™ assay was from Promega. Microcon and Amicon filtering devices were from Millipore. Synthetic peptides ECP (1–19), ECP(24–45) and ECP(1–45) were synthesized and purified at the Proteomics and Protein Chemistry Unit, Pompeu Fabra University, Spain, as previously described [32].

2.2. ECP expression and purification

Recombinant ECP was expressed using a synthetic gene for human ECP coding sequence and the pET11c expression plasmid [33]. Protein expression in the *E. coli* BL21 (DE3) strain, recovery of the protein from inclusion bodies, and the purification steps were carried out as previously described [33]. The protein was then desalted and lyophilized.

2.3. ECP reduction and carboxyamidomethylation

Reduced and carboxyamidomethylated ECP was prepared by a modification of the protocol previously described [34]. Lyophilized ECP was dissolved to a concentration of 10 mg/ml in 1 M Tris–HCl, pH 8, buffer containing 2 mM EDTA and 6 M Guanidinium hydrochloride. After an incubation of 1 h at 37 °C, reduction of disulfide bridges was performed by addition of 25 mM dithiotreitol final concentration (10-fold molar excess over the total cysteine content). The mixture was incubated for 90 min at 37 °C, and then iodoacetamide was added at a 250 mM final concentration. The sample was further incubated for 1 h at 37 °C in the dark. The alkylated protein was filtrated and buffer exchanged in 50 mM acetic acid in Amicon filtrating tubes (3000 Da cut-off), and lyophilized.

2.4. Enzymatic proteolysis by Lys-C proteinase cleavage

Limited proteolysis of ECP was performed using the Lys-C-endoproteinase, which cleaves polypeptides at the carboxyl side of lysyl residues [35]. Proteolysis was achieved by incubating, for 3 h at 25 °C, 1 mg of the reduced and carboxyamidomethylated ECP in 50 μl of 0.1 M Tris/HCl pH 7.8 buffer with 0.05 U/ml final concentration of Lys-endoproteinase. The two fragments resulting from the process were separated using a Microcon filtrating tubes with the appropriate molecular exclusion ranges (10000 and 3000 Da cut-off). The cleavage efficiency was checked by SDS-PAGE, and confirmed by N-terminal sequencing and the molecular mass was determined by MALDI-TOF. The N-terminal sequencing of the largest cleaved fragment identified the sequence NQNTFLR, thus confirming that a unique cleavage has taken place at Lys38 residue, releasing the N-terminus 0–38 peptide. The presence of a Met0 residue, from the recombinant expression in a prokaryote system should also be taken into account.

2.5. Chemical cleavage of ECP by cyanogen bromide

Cyanogen bromide cleavage was performed by a modification of the protocol previously described [36], as detailed [37]. Briefly, the reduced and carboxyamidomethylated lyophilized ECP was dissolved in 0.1 N HCl at 10 mg/ml and added to a CNBr solution at 45 mg/ml in 0.1 N HCl, assuring a 100 M fold excess. The mixture was stirred for 24 h at room temperature and lyophilized.

The CNBr cleavage products of the chemical proteolysis of ECP were analysed by SDS-PAGE. Purification of the peptides was performed by HPLC reverse phase chromatography, using a Vydac C4 column and a Symmetry C18 column, by a modification of the previously described protocol [38]. A sequential gradient from solvent A (water-0.05% TFA) to solvent B (100% acetonitrile-0.05% TFA) was applied, up to 25% of solvent B in 10 min, then to 35% of solvent B for 65 min and finally to 100% solvent B for 20 min. Fractions of 2 ml were collected at a flow rate of 1 ml/mim. Peptide elution was followed at 214 and 280 nm. The main representative peaks were analysed by N-terminal sequencing and molecular mass determination was carried out by MALDI-TOF methodology.

2.6. RNase activity

Zymograms or activity-staining gels were performed as described [39] to confirm the ECP denaturation process. Samples were mixed with the loading buffer (60 mM Tris–HCl, 10% glycerol, 0.015% bromophenol blue, 3% SDS, pH 6.8) and analysed for RNase