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Review

Azurocidin — inactive serine proteinase homolog acting as a multifunctional inflammatory mediator★

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Azurocidin, also known as cationic antimicrobial protein 37 kDa (CAP37) or heparin-binding protein (HBP) is an inactive homolog of serine proteinases residing in granulocytes. The ability to cleave peptide bond was lost due to replacement of two of the three residues from the conserved catalytic triad characteristic for serine proteinases. Azurocidin has a broad spectrum of antimicrobial activity, mainly against Gram-negative bacteria. It is also recognized as a multifunctional inflammatory mediator for its contracting effects on endothelial cells causing an increase of vascular permeability, capacity to bind endotoxin and ability to attract monocytes to inflammation sites.

Trypsin-like serine proteinases play a key role in many physiological processes such as protein digestion and turnover, regulation of defense system protecting the organism from tissue damages and infection and proenzyme, prohormone and complement activation (Neurath, 1986). Their catalytic mechanism is based on the existence of a catalytic triad formed by histidine, aspartic acid and serine residues (Blow *et al.,* 1969).

During evolution a few members of the trypsin superfamily have lost proteolytic activity due to substitutions in the catalytic triad. They have preserved structural homology to serine proteinases but have different biological functions. Invertebrate examples of such "sterile enzymes" are antimicrobial horseshoe crab factor D (Kawabata *et al.,* 1996), adhesion molecule – masquerade protein (Muragasu-Oei *et al.,*1995), or the mos-

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Abbreviations: BPI, bactericidal permeability increasing protein; BPTI, bovine pancreatic trypsin inhibitor; CAP37, cationic antibacterial protein 37 kDa; DPP I, dipeptydyl peptidase I; HBP, heparin-binding protein; IL-1β, interleukin 1β, IL-6, interleukin 6; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α.

quito infection-responsive serine protease-like protein ispl5 involved in mosquito immune response (Dimopoulos *et al.,* 1997). Vertebrate inactive serine protease homologs include haptoglobin (Kurosky *et al.,* 1980), protein Z (Hojrup *et al.,* 1985), hepatocyte growth factor (Nakamura *et al.,* 1989) and human neutrophil azurocidin (Gabay *et al.,* 1989).

STRUCTURE OF HUMAN AZUROCIDIN

In 1984 Shafer *et al.* isolated and purified from human neutrophil granules a cationic antimicrobial protein of 37 kDa and named it CAP37. Azurocidin was purified from human neutrophil azurophilic granules by Wilde *et al.* (1990). Using heparin affinity chromatography Flodgaard *et al.* (1991) isolated from human and porcine neutrophils and sequenced protein, CAP37 and azurocidin are the same protein.

Azurocidin is a single polypeptide glycoprotein synthesized as a 251 amino-acid precursor processed by removal of 26 amino-acid residues from the N-terminus and three residues from the C-terminus (Almeida *et al.,* 1991; Morgan *et al.,* 1991) (Fig. 1). The mature polypeptide consists of 222 amino-acid residues with calculated molecular mass of 24 kDa (Pohl *et al.,* 1990). Sequence analysis shows 45% homology to human neutrophil elastase, 42% homology to proteinase 3, and 32% homology to cathepsin G from human neutrophils (Almeida *et al.,* 1991; Morgan *et al.,* 1991).

Histidine 41 and serine 175 from the catalytic triad have been replaced by serine and glycine residues, respectively, resulting in an inability to bind [^{3}H]diisopropylfluorophos-

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    Met Thr Arg Leu Thr Val Leu Ala Leu Leu Ala Gly Leu Leu Ala
    Ser Ser Arg Ala Gly Ser Ser Pro Leu Leu Asp Ile Val Gly Gly     4
  5 Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu Ala Ser Ile Gln    19
 20 Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu Ile His Ala Arg    34
 35 Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser Gln Asn Pro Gly    49
 50 Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu Arg Arg Arg Glu    64
 65 Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser Met Ser Glu Asn    79
 80 Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu Met Leu Leu Gln    94
 95 Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val Thr Ile Leu Pro   109
110 Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly Thr Arg Cys Gln   124
125 Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly Arg Leu Ser Arg   139
140 Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro Glu Asp Gln Cys   154
155 Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr Arg Arg Gly Gly   169
170 Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val Cys Glu Gly Leu   184
185 Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro Cys Gly Arg Gly   199
200 Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg Asp Trp Ile Asp   214
215 Gly Val Leu Asn Asn Pro Gly Pro Gly Pro Ala
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Figure 1. Amino-acid sequence of azurocidin (Almeida *et al.,* 1991).

The sequences of signal peptide, N-terminal (5+2) propeptide and C-terminal propeptide are shadowed. Amino-acid residues forming inactive catalytic triad are underlined, potential N-glycosylation sites are in italic.

two heparin-binding proteins (hHBP and pHBP, respectively) with chemotactic properties for monocytes and fibroblasts. The amino-acid sequence of CAP37 (Pohl *et al.,* 1990) and the nucleotide sequence of CAP37 (Morgan *et al.,* 1991) and azurocidin (Almeida *et al.,* 1991) cDNAs proved that heparin-binding phate and to cleave synthetic and natural proteinase substrates. The third member of the triad — aspartic acid 89 — is preserved (Campanelli *et al.,* 1990; Flodgaard *et al.,* 1991; Pohl *et al.,* 1990; Pereira *et al.,* 1990b). Despite the loss of catalytic activity, human azurocidin is still able to bind efficiently bo-

vine pancreatic trypsin inhibitor (BPTI) (K_d = 0.1 μM) (Petersen *et al.,* 1993), making BPTI-affinity chromatography a very efficient method for azurocidin purification (Wątorek *et al.,* 1996). Replacement of Gly 175 by the bulky Gln residue eliminates BPTI binding (Kastrup *et al.,* 2001). This confirms that BPTI binds to the inactive "catalytic" site pocket of azurocidin. High affinity towards BPTI is not preserved in porcine azurocidin (Petersen *et al.,* 1993), suggesting that Kunitz-type-inhibitor binding is probably without physiological significance for azurocidin function.

The three-dimensional structure of azurocidin was resolved from crystals of its recombinant form (Fig. 2). The protein has two domains typical for trypsin-like proteinases, each being a β-barrel of six antiparallel β-strands (Iversen *et al.,* 1997; Karlsen *et al.,* 1998). The overall structure of azurocidin is homologous to neutrophil elastase with some differences in the loop areas and different charge distribution on the surface. Basic and acidic amino-acid residues form separate patches on opposite surfaces of the molecule (Iversen *et al.,* 1997). The four disulphide bridges are conserved.

Three putative N-glycosylation sites (Asn 100, Asn 114 and Asn 145) are differentially glycosylated (Pohl *et al.,* 1990), which may account for the heterogeneity in molecular mass (28–37 kDa) found in different azurocidin preparations (Gabay *et al.,* 1989; Pohl *et al.,* 1990; Shafer *et al.,* 1984; Shafer *et al.,* 1986). A detailed analysis of human azurocidin carbohydrate moiety (Olczak & Wątorek, 2002) lists 21 different N-glycan structures, the majority of them being neutral (79.8%), the rest mono- (13.1%) and disialylated (1.2%). Glycans have no effect on protein targeting to azurophilic granules, as it has been shown for cathepsin G by Garwicz *et al.,* (1998), but have influence on azurocidin biological activity. Iversen *et al.* (1999) have found that nonglycosylated mutant of recombinant azurocidin has lower biological activity compared to glycosylated form.

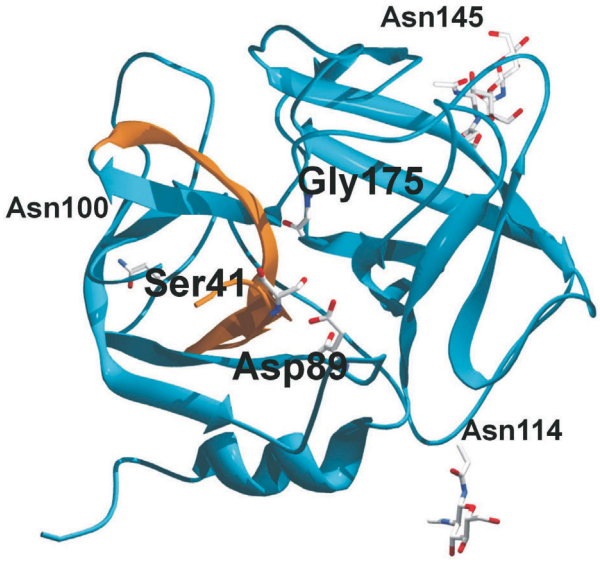

Figure 2. The tertiary structure of azurocidin.

Amino-acid residues forming inactive catalytic triad (Ser 41, Asp 89, Gly 175) and N-glycosylation sites (Asn 100, 114 and 145) are marked. The putative LPS-binding V-shaped substructure is shown in red. Figure was made with Swiss-PDB Viewer (Guex & Peitsch, 1997) and rendered in POV-Ray 3.5.

BIOSYNTHESIS AND PROCESSING

Phylogenetic studies show that azurocidin belongs to sixth class of serine proteases containing subfamily of proteinases of the immune defense system (Jenne, 1994). This family includes also neutrophil elastase, proteinase 3, cathepsin G, chymase and granzymes. All these proteins are localized inside granular structures of different immune effector cells and contribute to the destruction of ingested microorganisms. Genes for cathepsin G, granzymes B and H and chymase are grouped in one cluster on human chromosome 14 (Caughey *et al.*, 1993). A second cluster of genes for neutrophil elastase, proteinase 3 and azurocidin is located in the terminal region of the short arm of human chromosome 19 (Zimmer *et al.*, 1992).

The neutrophil elastase, proteinase 3 and azurocidin genes are constitutively expressed

in a cell-specific manner during the promyelocyte stage of neutrophil differentiation and, after processing, the proproteins are targeted to azurophilic granules (Gullberg *et al.*, 1999). The very low constitutive expression of these three proteins detected in monocytes and macrophages is probably the effect of residual mRNA translation early in myelomonocytic cell differentiation (Caughey, 1994). Small amounts of azurocidin in human and porcine platelets were observed by Flodgaard *et al.* (1991). However, azurocidin gene transcription has never been demonstrated in platelets, which suggests that neutrophil-derived azurocidin is presumably taken up by platelet precursors from human blood (Jenne, 1994). The majority of azurocidin and proteinase 3 is located in azurophilic granules (as a result of expression in early stages of neutrophil differentiation), but recent data show that there is also an easily secretable pool of molecules residing in secretory vesicles distinct from azurophilic granules (Witko-Sarsat *et al.*, 1999; Tapper *et al.*, 2002). These structures are formed at the last stages of neutrophil differentiation (Gullberg *et al.*, 1999), which suggests the possibility of late expression of azurocidin and proteinase 3.

Azurocidin and proteinase 3 expression can be induced also in nonmyeloid cell types. Human endothelial cells are able to express both proteinase 3 (Mayet *et al.*, 1993) and azurocidin (Lee *et al.*, 2002). Azurocidin expression was also found to be inducible in corneal epithelium by tumor necrosis factor α (TNF-α) and interleukin-1β (Il-1β) (Ruan *et al.*, 2002).

Azurocidin and other members of immune defense system serine proteinase subfamily (neutrophil elastase, proteinase 3, cathepsin G, granzymes) are processed according to one general scheme (Gullberg *et al.*, 1999). After cotranslational removal of the signal peptide, a short N-terminal propeptide is released yielding active protein. The N-terminal propeptides for cathepsin G, elastase and proteinase 3 consist of Gly-Glu, Ser-Glu and Ala-Glu, respectively. The enzyme responsible for the cleavage is dipeptydyl peptidase I (DPP I) (McGuire *et al.*, 1993). Azurocidin propeptide comprises seven amino-acid residues which are removed in two steps. The initial removal of five residues by an unknown enzyme (neither an aminopeptidase nor a serine or cysteine protease) is followed by cleavage of the Leu-Asp dipeptide (Lindmark *et al.*, 1999). The second cleavage is not inhibited by bestatin (a DPP I inhibitor), suggesting that the enzyme responsible for the dipeptide removal is different from DPP I. Both cleavages occur in the post-Golgi compartment. The N-terminal processing is followed by C-terminal peptide cleavage. Three amino-acid residues are removed from azurocidin, seven from proteinase 3 (Rao *et al.*, 1996), twenty and eleven from neutrophil elastase and cathepsin G C-termini, respectively (Salvesen & Enghild, 1990). Removal of the N-terminal propeptide converts the inactive zymogens of neutrophil elastase and cathepsin G to proteolytically active forms (Salvesen & Enghild, 1990; Gullberg *et al.*, 1994). In the case of azurocidin the relation between propeptide removal and the gain of biological activity is unclear and has to be investigated (Lindmark *et al.*, 1998). Site directed mutagenesis of cDNA for elastase, cathepsin G and azurocidin showed that the N- and C-terminal propeptides are dispensable for targeting to azurophilic granules (Gullberg *et al.*, 1995; Garwicz *et al.*, 1998; Lindmark *et al.*, 1999).

ANTIMICROBIAL ACTIVITY

Azurocidin was purified as an antimicrobial protein (Shafer *et al.*, 1984; Shafer *et al.*, 1986; Campanelli *et al.*, 1990) active in the range of 10^{-5}–10^{-6}M (Spitznagel, 1996). Its microbicidal activity is preferentially directed against Gram-negative bacteria and strongly depends on ionic strength and pH (Gabay *et al.*, 1989; Shafer *et al.*, 1986). At low ionic strength additional activity against Gram-positive bacteria and the pathogenic yeast *Can-*

dida albicans was demonstrated (Gabay *et al.,* 1989). The exact mechanism of the azurocidin microbicidal activity is unknown. Pereira *et al.* (1993) have undertaken a systematic approach in this direction, synthesizing a set of overlapping peptides based on azurocidin sequence. The highest bactericidal activity was found for peptide 20-44, however, it was 10-fold lower than that of native whole molecule of azurocidin (Pereira *et al.,* 1993). This peptide has maximum activity at pH between 5 and 6 (pH conditions characteristic for phagolysosome). The presence of disulphide bridge between Cys 26 and Cys 42, is necessary for antibacterial action. The activity of peptide 20-44 is abolished in a dose-dependent manner by LPS fragment (lipid A) (Karlsen *et al.,* 1998). Azurocidin binds with high affinity ($K_{ass} = 0.8 \times 10^9 M^{-1}$) lipid A of LPS endotoxin (Linde *et al.,* 2000), and the antibacterial activity is probably initiated by binding of azurocidin to LPS lipid A. The putative site for lipid A was initially suggested to reside within peptide 20-44 (Iversen *et al.,* 1997). Residues 20-44 form a V-shaped substructure stretched through the entire molecule, constructed mainly from two antiparallel β-strands (residues 26–30 and 35–39) (Fig. 2). The exposed Phe 25, Cys 26, Cys 42 and Phe 43 from the top of the V substructure form a hydrophobic pocket probably involved in lipid A fatty acid chain or glycosaminyl ring binding. The adjacent hydrophilic residues Asn 20, Gln 21 and Arg 23 could be responsible for lipid A phosphate group binding. Recently, the same laboratory has constructed an azurocidin mutant with Arg 23 replaced by Ser and Phe 25 replaced by Glu (Kastrup *et al.,* 2001). Such mutations extensively change the charge distribution within the putative LPS binding site which should result in inhibition of LPS binding. Surprisingly, there was no change in the affinity to lipid A, which suggests that another, not yet identified domain may be involved in lipid A binding. The bactericidal activity of azurocidin is not limited to peptide 20-44. Directed mutagenesis of eight basic residues (Arg5Gln, Lys6Gln, Arg8Gln, Arg10Gln, Arg61Gln, Arg62Gln, Arg63Gln and Arg65Gln) markedly diminished the antimicrobial activity of azurocidin against *Escherichia coli* and *C. albicans* (McCabe *et al.,* 2002). At the same time there was no change in the antimicrobial activity against the Gram-positive *Streptococcus faecalis*, suggesting that different azurocidin domains participate in the antibacterial activity against Gram-negative and Gram-positive bacteria. Heinzelman *et al.* (1998b) suggested that the antibiotic effect of azurocidin on Gram-positive bacteria is a result of binding to bacterial cells and subsequent phagocytosis by monocytes. According to their hypothesis, azurocidin would be an opsonin for Gram-positive microorganisms. This hypothesis is supported by the fact that almost 90% of neutrophil-derived azurocidin is released during phagocytosis of *Streptococcus aureus* (Pereira *et al.,* 1990a).

CHEMOTACTIC ACTIVITY

In addition to antibiotic activity, azurocidin was found to be a potent (at 10^{-8}–10^{-10}M) chemoattractant to monocytes (Pereira *et al.,* 1990), it also attract fibroblasts (Flodgaard *et al.,* 1991), lymphocytes T and, to some extent, neutrophils (Chertov *et al.,* 1996; 1997).

Upon activation, neutrophils are able to secrete almost 90% of its azurocidin content (Pereira *et al.,* 1990a). Under similar conditions only 5% of neutrophil-derived bactericidal permeability increasing protein (BPI) is released. Such massive efflux of azurocidin is possible as a result of the existence of a secretable azurocidin pool in secretory vesicles (Witko-Sarsat *et al.,* 1999; Tapper *et al.,* 2002). The secreted azurocidin binds to a scavenger type, Ca^{2+} dependent receptor expressed on attracted monocytes (Heinzelmann *et al.,*1998c) and is subsequently internalized, probably *via* the clathrin coated pathway (Heinzelmann *et al.,*1999). Internalized azuro-

cidin prolongs monocytes survival (Østergaard & Flodgaard, 1992) and enhances TNF-α and IL-6 production by LPS-stimulated monocytes (Heinzelmann *et al.,* 1998a; Heinzelmann *et al.,* 1999; Rasmussen *et al.,* 1996).

Recently, Pereira *et al.* (2003) reported that azurocidin is also a chemoattractant and an activator of microglia – mononuclear phagocytic cells of the central nervous system. Microglial cells activated by azurocidin show upregulated expression of TNF-α, IL-1β and such chemokines as fraktalkine and RANTES.

INTERACTION WITH ENDOTHELIUM

Adhesion of neutrophils to endothelium releases a factor modifying endothelial cells and in consequence alters vascular permeability (Wedmore & Williams, 1981). The nature of this factor remained elusive until a recent report by Gautam *et al.* (2001) identifying it as azurocidin. First suggestion that azurocidin may be responsible for neutrophil penetration through endothelium came from the same laboratory much earlier (Østergaard & Flodgaard, 1992). Their recent data (Gautam *et al.,* 2001) show that the adhesion of neutrophils to endothelial lining causes the engagement of leukocytic β_2 integrin in triggering of azurocidin exocytosis and release. The released azurocidin binds to endothelial cell membrane proteoglycans (Olofsson *et al.,* 1999). The interaction with proteoglycans is electrostatic in nature: a cluster of basic amino-acid residues localized on one side of the protein (Iversen *et al.,* 1997) binds to negatively charged proteoglycan molecules. The inactive "catalytic site" of azurocidin is not involved in this interaction. Native protein and its recombinant form mutated in this site exert an identical effect on endothelium. BPTI strongly diminishes the endothelium-directed activity, probably by neutralization of the dipolar nature of azurocidin. This would suggest that the anti-inflammatory effect of BPTI administered to patients after surgery (Peters & Noble, 1999) is based not only on its antiproteolytic activity, but is also a consequence of BPTI-azurocidin interaction (Gautam *et al.,* 2001). Binding of azurocidin induces a Ca^{2+}-dependent rearrangement of the cytoskeleton resulting in cell contraction and increased permeability of the endothelium. The exact mechanism of the azurocidin mediated cytoskeleton structure rearrangement is unknown. Pereira *et al.* (1996b) have noticed that azurocidin activates endothelial cell protein kinase C, an enzyme involved in signaling. Part of endothelium-bound azurocidin molecules is internalized and protects endothelial cells from apoptosis (Olofsson *et al.,* 1999).

There are reports that azurocidin is expressed in endothelial cells in response to inflammatory mediators. Expression in cerebral microvasculature was found only in Alzheimer's disease and it was induced *in vitro* by cytokines and β-amyloid (Pereira *et al.,* 1996a). This would be consistent with the inflammatory effect of azurocidin and the concept that chronic inflammation could play a significant role in Alzheimer's disease pathogenesis (McGeer & McGeer, 1995). Lee *et al.* (2002) described inducible expression of azurocidin in vascular endothelium and localized this protein in atherosclerotic plaques. These findings support the hypothesis that azurocidin is involved in inflammatory-mediated diseases.

CONCLUSIONS

Azurocidin is an example of a protein which lost in evolution its primary proteolytic function, but gained another activity – it became an important mediator of inflammatory response. As a component of neutrophil azurophilic granules it participates in oxygen-independent killing mechanisms functioning in phagocytosing neutrophils. Released extracellularly, azurocidin causes contraction of endothelial cells. Gaps formed in

this way between the cells of endothelial lining enable neutrophil extravasation. Secreted azurocidin attracts monocytes and is responsible for their influx into inflammation sites. These properties make azurocidin a plausible target in new therapies directed against the harmful effects of inflammatory response.

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