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REVIEW

Antibacterial Proline-Rich Oligopeptides and Their Target Proteins

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Abstract—This review presents findings on a new family of antibacterial proline-rich oligopeptides—pyrrhocoricin, drosocin, apidaecin, and formaecin—isolated from insects. The functional and physicochemical properties of proline-rich oligopeptides are considered, a role of proline in their antibacterial activity is discussed, and experimental evidence is given in favor of the ability of these oligopeptides to suppress metabolism of bacteria by means of stereospecific interaction with heat shock protein DnaK and inhibition of DnaK-dependent protein folding. Binding of the peptides under investigation with DnaK correlates with their antibacterial activity. Evidence that pyrrhocoricin, drosocin, apidaecin, and formaecin are nontoxic for human and animal cells serves as a prerequisite for their use as novel antibiotic drugs.

Key words: proline-rich oligopeptides, heat shock proteins, folding, antibacterial activity, EROP-Moscow database

Natural antibacterial oligopeptides have been discovered in practically all living organisms. Whereas in prokaryotes these oligopeptides regulate competition between separate species occupying ecological niches and function as the signaling molecules in processes of intercellular communication [1], in eukaryotes they are localized in every organ and tissue and play a key role in innate immunity [2].

The functional class of antimicrobial oligopeptides involves structures, which display an unusually broad spectrum of antibacterial, antifungal, antiviral, and antitumor activities. These peptides are able to suppress or kill not only Gram-negative and Gram-positive bacteria, but also fungi, parasites, cancer cells, as well as HIV virus and herpes simplex virus. Antimicrobial oligopeptides that are known today, produced in response to infection or injury, are quite selective for microbes over eukaryotic cells. In animals, antimicrobial peptides are found in different body parts and organs most likely to come into contact with pathogenic microbes. They have been detected in the skin, ears, and eyes, on epithelial surfaces of the tongue, tracheas, lungs, and gut, and in the bone marrow and testes; in blood they are most prevalent in neutrophils [3].

Interest in antimicrobial, especially antibacterial oligopeptides is determined by their medicinal potential. This is particularly important because of the continuous emergence of novel strains of bacteria that are resistant to natural and synthetic antibiotics, which prompts the search for new efficacious remedies against pathogenic bacteria. The functional properties of antibacterial oligopeptides are realized in different ways depending on the peculiarity of their structure. These peptides are characterized by compact, completely or partially helical structures formed with cysteine bridges as well as by disordered structures. An important role in formation of disordered oligopeptide structures belongs to proline residues, which constitute a large part of the total number of amino acid residues in relatively short oligopeptide molecules. Because of a significant number of proline-rich natural antibacterial oligopeptides, a question arises as to how unique is the mechanism of action of these peptides.

This review systematizes the data on structure and functions of some structural-homologous members of families of proline-rich oligopeptides (PRO) possessing antibacterial activity, and it contains a discussion of the mechanism of their action.

OLIGOPEPTIDES WITH HIGHER CONTENT OF PARTICULAR AMINO ACID RESIDUES

The ANTIMIC database [4] contains information on ~1700 natural antimicrobial peptides that differ in their chemical structures and functions. The EROP-

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Moscow (**E**ndogenous **R**egulatory **O**ligo**P**eptides) database [5], involving data on peptide molecules with 2-50 amino acid residues, usually called oligopeptides [6-8], contains about 1000 antimicrobial peptides. Currently, about 100 novel natural antimicrobial peptides containing no more than 50 amino acid residues are discovered every year (Fig. 1). These include molecules with higher proline content [9-26]. Twenty-eight oligopeptides with high proline content are selected and presented in Fig. 2.

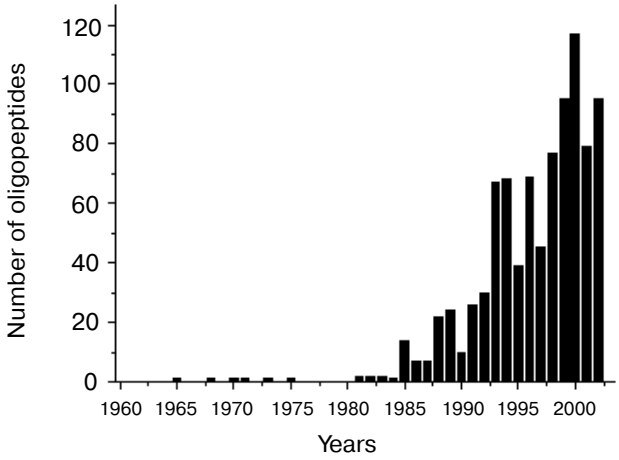

Fig. 1. Decoding chronology of primary structures of natural antimicrobial peptides.

FUNCTIONAL PROPERTIES

Proline-rich antimicrobial peptides are a group of linear peptides related to innate immunity, isolated from mammals and insects and characterized by high content (up to 50%) of proline residues [27-29]. Members of this group are predominantly active against Gram-negative bacteria, which they kill without bacterial membrane lysis, at variance with the majority of the known oligopeptides of this functional class.

One of the most extensively studied among antibacterial peptides from mammals is the proline-arginine rich peptide PR-39 related to the antibacterial peptide apidaecin obtained from hemolymph (the functional equivalent of blood) of the honeybee *Apis mellifera*. PR-39 has been isolated from pig intestine and consists of 39 amino acid residues, of which 19 are proline residues [30, 31]. This oligopeptide is predominantly active against Gram-negative bacteria, killing those by inhibition of DNA and protein synthesis without lysis of the bacterial membrane [32]. PR-39 can also inhibit NADPH oxidase activity in neutrophils, which suggests a role of this oligopeptide in inhibition of inflammation [27]. Besides, PR-39 prevents the post-ischemic production of toxic oxygen radicals and can induce angiogenesis both *in vitro* and *in vivo* [33]. Whereas stimulation of angiogenesis by PR-39 results from specific inhibition of proteasomal degradation of the transcription factor HIF-1α, its antiinflammatory activity is associated with inhibition of IκBα (inhibitory subunit of NFκB, nuclear factor κB) degradation, that, in turn, prevents the activation of NFκB-dependent gene expression [34]. Gaczynska et al. [35] established that in both cases the mechanism of action of PR-39 involves allosteric inhibition of the proteasome activity due to the interaction of PR-39 with the proteasomal noncatalytic subunit R7.

One of the shortest oligopeptides is indolicidin, an antimicrobial oligopeptide isolated from cytoplasmic granules of bovine neutrophils, composed of only 13 amino acid residues, three of which are proline residues [9]. This peptide exhibits activity against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria; like PR-39, it does not lyse the bacterial membrane, and it also possesses antifungal activity. Indolicidin has been shown to inhibit DNA synthesis selectively in *E. coli* cells without affecting RNA and protein synthesis [36].

Antibacterial activity is also found in the oligopeptide +AGAPEPAEPAQPGVYz, the 152-166 fragment of the high-molecular-weight vasopressin precursor (vasopressin-neurophysin-2-glycopeptide) isolated from bovine neurohypophysis secretory granules [37, 38]. This oligopeptide, amidated on the C-terminus, comprises 15 amino acid residues, four of which are proline residues, and it also reveals various biological activities [39-42]. In particular, it induces antibody production in human lymphocyte culture to *Yersinia enterocolitica*, *Salmonella typhimurium*, *Shigella flexner*, *Klebsiella pneumoniae*, and *S. aureus* [43]. *In vitro* investigations have shown this peptide to inhibit phytohemagglutinin P- and interleukin-2-induced activation of peripheral blood T lymphocytes, and spontaneous proliferation of Jurkat cells (human T cell leucosis) [44], and to stimulate myelopoiesis and activate neutrophils [45]. *In vitro* experiments have also demonstrated that this neurohypophyseal oligopeptide, depending on its concentration, shows stimulatory or inhibitory effects on the rate of tyrosine hydroxylation catalyzed by tyrosine-3 monooxygenase from rat hypothalamus [46].

The most promising among antimicrobial peptides are members of a new family of antibacterial oligopeptides isolated from insects [10, 12, 13, 17, 47, 48]. The amino acid sequence diversity of the PRO of insects, on one hand, is caused by errors in amino acid sequences of the target proteins in the pathogens induced by multiple mutations [48]. On the other hand, due to evolution of the insects' immune system, various species of insects have developed specific antibacterial peptides adapted to the environment where they reside and to pathogens that threaten their existence [49]. Even the same insect species produces different antimicrobial peptides depending

upon the immunizing microorganisms [50, 51]. Induction of the expression of various peptides was found to depend on the type of challenging infectious agent [52]. Seven distinct antimicrobial peptides (plus isoforms) can be detected in insect hemolymph as early as 2-4 h after a septic injury. Five of them represent one of the major antibacterial peptide families. This family includes cecropin, defensin, drosocin, diptericin, and attacin. Two additional peptides, drosomycin and metchnikowin, possess antifungal activity.

Of particular interest are some relatively short PRO—pyrrhocoricin, drosocin, apidaecin, and formaecin. These peptides, isolated from insects, consist of 18-20 amino acid residues [53, 54]. The names of the antibacterial PRO reflect their origin rather than subdivision among the individual amino acid sequences.

Pyrrhocoricin, drosocin, apidaecin, and formaecin, mostly killing Gram-negative bacteria, show structural similarities with longer, distantly related insect- and mammal-derived antimicrobial peptides discovered earlier [12, 17, 47, 55, 56]. The latter appear to carry multiple functional domains, do not have a specific macromolecular target, and are toxic to eukaryotic cells. For example, cecropin, defensin, and magainin interact with and disrupt the cell membrane [57-60], while buforin 2 binds nonspecifically to bacterial DNA [61]. Some of the anti-

E01472	Indolicidin (bovine)	+ILPWKWPWWPWRRz	[9]
E00841	Apidaecin Ia (honey bee)	+GNNRPVYIPQPRPPHPRI-	[10]
E00842	Apidaecin Ib (honey bee)	+GNNRPVYIPQPRPPHPRL-	[10]
E00843	Apidaecin II (honey bee)	+GNNRPIYIPQPRPPHPRL-	[10]
E02190	Apidaecin (European bumblbee)	+GN RPVYIPPPRPPHPRL-	[16]
E00939	Abaecin (honeybee)	+YVPLPNVPQPGRRPFPTFPGQGPFNPKIKWPQGY-	[11]
E02191	Abaecin (European bumblebee)	+FVPYNPPRPGQSKPFPSFPGHGPFNPKIQWPYPLPNPGH-	[16]
E01498	Lebocin 1 (silkworm)	+DLRFLYPRGKLPVPTPPPFNPKPIYIDMGNRY-	[14]
E01499	Lebocin 2 (silkworm)	+DLRFLYPRGKLPVPTPPPFNPKPIYIDMGNRY-	[14]
E01500	Lebocin 3 (silkworm)	+DLRFLYPRGKLPVPTLPPFNPKPIYIDMGNRY-	[14]
E02356	Drosocin (fruit fly)	+GKPRPYSPRPTSHPRPIRV-	[12]
E02357	Formaecin 1 (red bulldog ant)	+GRPNPVNNKPTPHPRL-	[17]
E02358	Formaecin 2 (red bulldog ant)	+GRPNPVNTKPTPYPRL-	[17]
E03135	Metchnikowin A1 (fruit fly)	+HRHQGPIFDTRPSPFNPNQPRPGPIY-	[15]
E03136	Metchnikowin A2 (fruit fly)	+HRRQGPIFDTRPSPFNPNQPRPGPIY-	[15]
E04594	Heliocin (tobacco budworm)	JRFIHPTYRPPPQPRRPVIMRA-	[18]
E04500	Bombesin-related peptide (belly toad)	JKKPPRPPQWAVGHFMz	[19]
E03041	Pyrrhocoricin (firebug)	+VDKGSYLPRPTPPRPIYNRN-	[13]
E03137	Metalnikowin I (green shield bug)	+VDKPDYRPRPRPPNM-	[15]
E03140	Metalnikowin IIA (green shield bug)	+VDKPDYRPRPWPRPN-	[23]
E03141	Metalnikowin IIB (green shield bug)	+VDKPDYRPRPWPRNMI-	[23]
E03142	Metalnikowin III (green shield bug)	+VDKPDYRPRPWPRPNM-	[23]
E01015	Bactenectin, Bac 5 (bovine)	+RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGPP-	[20]
E03657	Bactenectin, Bac5 (bovine)	+RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGPFPz	[22]
E03659	Bactenectin, Bac5 (sheep)	+RFRPPIRRPPIRPPFRPPFRPPVRPPIRPPFRPPFRPPIGPFPz	[24]
E03658	Bactenectin, Bac5 (goat)	+RFRPPIRRPPIRPPFNPPFRPPVRPPFRPPFRPPFRPPIGPFPz	[25]
E01260	PR-39 (pig)	+RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFPz	[21]
E03665	Propherin-18 (pig)	+PPPPPFRPPPFGPPRFPz	[26]

Fig. 2. Natural antimicrobial peptides with high proline content contained in the EROP-Moscow database [5]. As a result of alignment they are grouped in structural-homologous families. Oligopeptide identification number in the EROP-Moscow database [5], name of the oligopeptide, isolation source, the amino acid sequence, and the reference are consecutively indicated in each line. Standard single-letter symbols have been used for designation of amino acid residues; "+" and "–" are N- and C-open termini bearing positive and negative charges, respectively; "z" indicates an amide on the C-terminus.

bacterial peptides, such as histatin or NAP-2, act as inhibitors of bacterial enzymes either by serving as a pseudosubstrate or by binding tightly to the enzyme active site, thus eliminating the accessibility of the native substrate [62]. Unlike those, pyrrhocoricin, drosocin, apidaecin, and formaecin consist of only minimal determinants needed to penetrate through the cell membrane and bind to the target biopolymer. Pyrrhocoricin, drosocin, apidaecin, and formaecin inactivate bacterial intracellular biopolymers without associating with and disrupting the cell membrane, bind to the target biopolymer in a stereospecific manner, and are nontoxic to mammals [54, 63, 64].

PHYSICOCHEMICAL PROPERTIES

Despite the variety of their primary structures, antibacterial oligopeptides have a general property (for those not containing cysteine residues)—they all possess a higher conformational mobility when not bound by crosscovalent bridges. However, PRO are of particular interest because of the unique properties of proline itself and of its residue. Proline, as the amino acid, has extremely high solubility in comparison with all other amino acids. The solubility of proline is 162 g per 100 ml H_2O at 25°C, whereas that of glycine, the next amino acid by scale of solubility, is only 25 g per 100 ml H_2O [65]. The proline residue is also unique as the only element of the natural peptide chain which, when integrated in regular structure of its backbone, fixes forcedly the configuration of the site devoid of torsion angles Ω and ϕ [66, 67], and constrains possibilities of intramolecular rotations.

Thus, proline residues along with cysteine are the critical amino acid residues determining the spatial structure of oligopeptide molecules and their activity [68, 69]. Both residues, although differently, modify configuration of the linear peptide chain, and as a result the amino acid residues distant in primary structure get closer [68]. Higher content of the abovementioned residues is evidence of a high potential of the oligopeptide molecule to fold [70]. In prolyl-containing molecules, the approach occurs due to the turn of the oligopeptide backbone in sites containing proline residues that constrains oligopeptide intramolecular mobility [71].

In PRO, proline residues disposed consecutively in the linear peptide chain usually have a disordered left-handed polyproline II helix structure [72, 73]. Nuclear magnetic resonance (NMR) investigations reveal unordered structures in mucin [72] and drosocin [55] typical for molecules with polyproline II helix conformation. Drosocin circular dichroism (CD) spectra also demonstrate polyproline II helix structure for this peptide [53, 74]. The secondary structure of bioactive drosocin comprises two reverse turns, which constitute the binding sites to the target molecule [55]. The general fold of native pyrrhocoricin, as determined by NMR and CD investigations, is similar to the above [63]. Reverse turns in pyrrhocoricin structure, localized at the oligopeptide termini and bridged by an extended peptide domain, have been identified as pharmacologically important elements [63].

Study of the effect of disaccharides on the conformation of glycosylated PRO, especially on drosocin conformation, reveals subtle differences in the small populations of folded conformers between the glycosylated and non-glycosylated peptides [55]. In particular, the turn at residues 10-13 in the drosocin molecule tends toward a more extended structure upon glycosylation, while there is some tightening of the downstream turn at residues 17 and 18 [64]. On the basis of structural studies and determining of the biological activity of drosocin fragments, a model has been built according to which the interaction of drosocin with its target protein may involve the locally structured regions 4-7 and 17-19, and glycosylation may aid in correctly orienting these separate binding regions. Like drosocin, the structure of pyrrhocoricin appears to be random coil, and there is little change in the backbone conformation upon glycosylation [48]. As has been shown for mucin 1, carbohydrates stabilize polyproline II helix structure [75]. Glycosylation is suggested to promote folding and stability of glycopeptides [76].

The role of proline in the cell-penetrating ability of oligopeptides into bacterial cells was studied by confocal fluorescence microscopy of buforin 2 [77]. This histone H2A-derived oligopeptide kills bacteria by binding to nucleic acids [61]. The sequence of buforin 2 consists of 21 amino acid residues with a single proline in mid-chain position [78]. Buforin 2 and its truncated analogs penetrate the cell membrane of *E. coli*, but like the PRO do not bind to it [77]. In contrast with buforin 2, which penetrates the bacterial cell membrane and accumulates in the cytoplasm, proline-free magainin remains associated with the inner leaflet of the lipid bilayer after translocation of an artificial membrane, disrupting the membrane [79]. When the buforin single proline residue is replaced with an alanine, the mode of action of this oligopeptide is changed from one featuring an intracellular target to magainin-type membrane destruction [48]. From these data, it was postulated that a proline hinge is the key structural element for the bacterial cell penetration by PRO, particularly of buforin 2, without membrane association [64]. Since the type of functional activity of buforin 2 depends on a single proline residue, the multiple proline residues in the short PRO should influence activities of these peptides much more efficiently.

To study the ability of the peptides to permeate the outer and cytoplasmic membranes of *E. coli*, to bind to liposomes, and to form channels in planar lipid bilayer membranes, oligopeptide analogs were synthesized that were either free of proline or contained one or two proline residues [80]. All peptides bound to the bacterial

lipopolysaccharide and permeated the outer membrane of the bacteria to a similar extent. However, their ability to permeate the cytoplasmic membrane decreased substantially as the number of proline residues increased. Though increase in proline residues in oligopeptides decreased the ability of the peptides to form channels in planar lipid bilayers, proline-free molecules tended to cause rapid breakage of planar membranes. It has been established that when the key proline is replaced with leucine in antibacterial oligopeptides with amphiphilic α-helical structure [81], the ability of the oligopeptide to accumulate intracellularly decreases, and therefore the antibacterial activity is reduced. The positive charged amino acid residues in the short antimicrobial PRO are suggested to enhance bacterial cell entry, and the interspersed proline residues may prevent helical structure formation and toxicity to the host [82]. A proline-rich structural module, promoting permeability of PRO into bacterial cells, may be common for all natural antibacterial oligopeptides [83].

MECHANISM OF ACTION OF PROLINE-RICH ANTIBACTERIAL PEPTIDES

Penetration of proline-rich oligopeptides into the cell. There is evidence available that PRO enter the bacterial cell, penetrate into the cytoplasm, bind to the specific molecular targets essential to growth of bacteria, and thus kill them. Similar to many antibacterial oligopeptides [84, 85], pyrrhocoricin, drosocin, apidaecin, and formaecin are rich in positively charged residues of such amino acids, as arginine, lysine, and histidine [83], because of which the oligopeptides interact electrostatically with the negatively charged bacterial phospholipids of the cell membrane [86, 87]. The interaction of these oligopeptides with the outer membrane of the bacterial cell is followed by their invasion of the periplasmic space and by a specific engagement with a receptor that may be bound to the inner membrane or, most likely, associated with a component of a permease-type transporter system [88]. Finally, the oligopeptide is translocated into the interior of the cell where it meets its ultimate target, a component of the protein synthesis machinery [89].

Interaction with intracellular target proteins. Biopolymers in the bacterial cell, which are targets for PRO, have been identified by mass spectrometry, Western blotting, and fluorescence polarization [88, 90]. Investigation results have established that pyrrhocoricin, drosocin, and apidaecin, after penetration into the bacterial cell with participation of lipopolysaccharide 1, interact with the heat shock proteins (in a specific manner with the 70-kD DnaK chaperone, and in a nonspecific manner with the 60-kD chaperonin GroEL) [90].

Functions of DnaK and GroEL chaperones. Heat shock proteins (Hsps) arise in prokaryote and eukaryote cells in response to stress (heat, oxidative, or toxic) and protect the cells from stress-induced damage, performing chaperone functions [91, 92]. In stress conditions a disturbance of protein native structure may occur, which is accompanied by protein aggregation. Chaperones DnaK and GroEL (with corresponding co-chaperones DnaJ and GrpE for DnaK and GroES for GroEL) in prokaryotes, as well as Hsp70 (with co-chaperone Hsp40) and Hsp60 in eukaryotes are able to bind with hydrophobic sites on surfaces of non-native protein substrates, participating in their refolding and preventing protein aggregation [91-93]. DnaK and Hsp70 interact with nascent, unfolded polypeptide chains that are still connected with ribosomes and stabilize them by promoting polypeptide folding [94, 95]. GroEL interacts with newly synthesized polypeptide chains after their removal from ribosomes and is responsible for mechanisms of multimeric protein folding/unfolding [96-98].

Structure of DnaK chaperone. DnaK and other 70-kD heat shock proteins are monomeric proteins composed of three domains: N-terminal domain, C-terminal domain, and lid (amino acid residues 1-388, 389-508, and 509-638, respectively) [99-101]. Hendrickson and coauthors [99] obtained crystals of N- and C-terminal domains of *E. coli* DnaK and determined the three-dimensional structures of these domains. A scheme of DnaK structure is represented in Fig. 3.

The N-terminal domain of *E. coli* DnaK with ATPase activity (~44 kD) is a bilobed structure that contains a deep channel between the two lobes, where nucleotides (ATP and ADP) bind [102-104]. The C-terminal peptide-binding domain (~27 kD) [99] consists of a uniquely folded β-sandwich subdomain and is followed by a domain that consists of five antiparallel α-helices (αA-αE) [99, 105]. The α-helical domain is like a lid over the β-sandwich subdomain [97]. The oligopeptide in the C-domain pocket restricted by β-sandwich loops is encapsulated due to α-helical structure [106]. The α-helical domain is followed by disordered, flexible subdomain (amino acid residues 609-638). By sequentially deleting the flexible C-terminal subdomain and the individual lid helices, Slepenkov et al. [101] established the importance of each structural unit for stabilization of the DnaK–peptide complex. Thus, αE and αD helices were found to be the key helices for lid stability. αD helix, stabilizing the lid, plays the key role in creation of long-lived DnaK–peptide complex. The presence of the αB helix impacts the rate of the ATP-induced high-to-low affinity conformational change of the C-domain. The deletion analysis revealed that residues 596-638, which comprise the αE helix and flexible C-terminal subdomain, affect ATP binding.

Mechanism of action of DnaK. DnaK binds mainly with unfolded proteins or with newly synthesized polypeptide chains [107]. Because association of DnaK with polypeptides is sensitive to the inhibitor of protein

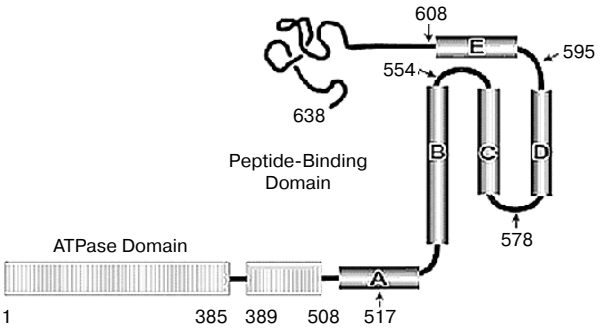

Fig. 3. Schematic representation of DnaK domains. The lid region is not present in the scheme [101].

synthesis puromycin [108], it is suggested that DnaK interacts with nascent unfolded polypeptides associated with ribosomes [94, 95]. However, the intracellular concentration of DnaK (~50 μM) [109] exceeds that of ribosomes (~30 μM) [91], assuming a cytosolic distribution of DnaK non-bound to ribosomes.

Interactions contributing to DnaK–substrate binding include hydrophobic forces, hydrogen bonds, van der Waals forces, and electrostatic interactions [99, 110-112]. Polypeptide sites to which DnaK binds are ~7 residues long and typically contain hydrophobic leucine and isoleucine residues in their central region [91]. These binding sites occur every ~40 residues. However, the key role in the DnaK–peptide complex formation and stabilization belongs to electrostatic interactions between charged amino acid residues located on the chaperone and peptide surfaces [112].

Energy released on ATP hydrolysis by DnaK is coupled to the repeated cycles of polypeptide binding and release [106, 113]. On ATP binding, the α-helical lid over the peptide-binding pocket of DnaK opens and the chaperone interacts with the polypeptide substrate. Polypeptide binding is accompanied by closing of the peptide-binding pocket. This DnaK conformation is induced by ATP hydrolysis. The generated ADP–DnaK–polypeptide complex is relatively stable. Fluorescence studies reveal two conformations of DnaK that differ with regard to the polypeptide substrate [114, 115]. Whereas in complex with ADP DnaK is in high-affinity state and tightly binds to target polypeptide, ADP release and ATP binding induce conversion of the chaperone in low-affinity state characterized by weak binding to the substrate. ATP binding to the N-terminal domain of DnaK induces allosteric shifts in the C-terminal domain of the chaperone and release of the polypeptide [116, 117].

The cycling of two DnaK conformations associated with ATP hydrolysis and binding of the polypeptide substrate is regulated by the co-chaperones DnaJ (41 kD) and GrpE (a two-subunit homodimer), which accelerate certain steps in the DnaK activity cycle [106, 109]. The N-terminal domain of DnaJ (J) binds to DnaK and accelerates hydrolysis of ATP, thus facilitating peptide capture [118]. The C-terminal domain of DnaJ functions as a chaperone in recognizing hydrophobic peptides and can thus recruit DnaK to nascent chains [119, 120]. It is suggested that DnaJ plays a pivotal role *in vivo* by increasing the affinity of the polypeptide substrate for DnaK [121]. GrpE, also termed nucleotide-exchange factor, induces the release of ADP from DnaK complex [104]. As a result of rebinding of ATP the DnaK–peptide complex dissociates, completing the reaction cycle. The binding of newly synthesized polypeptide to DnaK results in stabilization of the polypeptide substrate in conformation that prevents its aggregation.

Structure and mechanism of action of GroEL chaperonin. GroEL is a protein complex composed of 14 identical 57-kD subunits, forming two heptameric rings [122, 123]. Each GroEL subunit consists of two separate domains (equatorial and apical) connected through an intermediate, hinge-like domain [122]. The GroEL equatorial domain contains the ATP binding site. The apical domain contains residues of hydrophobic amino acids exposed toward the cylinder cavity for binding by hydrophobic interactions with unfolded polypeptide substrates [124]. The substrate-binding amino acid residues in the apical domains of GroEL are also responsible for GroEL interaction with GroES co-chaperone in a manner regulating the GroEL ATPase activity required for GroEL-mediated protein folding [91, 96, 106].

The mechanism of GroEL action differs from that of DnaK, although in both cases protein binding and release are regulated by ATP. GroEL does not bind to nascent polypeptides and is thus likely to interact with protein substrates after their release from DnaK. Non-native protein substrate folds after transfer to the cylinder central cavity of GroEL, which prevents its aggregation with other non-native proteins [108].

Interaction of proline-rich peptides with DnaK. Interaction of PRO with DnaK has been studied on the basis of effects of these oligopeptides on two major functions of DnaK, the ATPase activity and refolding of proteins [88]. The ATPase activity was determined on *E. coli* recombinant DnaK by measurements of inorganic phosphate release from ATP. The protein folding ability was assessed by measuring activity of expressed enzymes in cultures of live bacteria upon incubation with PRO.

Effect of proline-rich peptides on the ATPase activity of DnaK. *In vitro* experiments show that simultaneous addition of the biologically active L-pyrrhocoricin and DnaK to the ATPase activity assay mixture is accompanied by decrease of the ATPase activity of DnaK [88]. Comparative analysis of inhibition of the recombinant DnaK ATPase activity by native pyrrhocoricin and by its fragments demonstrates that the N-terminal 1-9 fragment of pyrrhocoricin is as effective as the full-size oligopeptide molecule [88]. The inhibiting ability of the C-terminal 10-20 fragment was not as significant as the inhibiting

activity of the N-terminal fragment 1-9. It has been established that pyrrhocoricin inhibits ATPase activity of DnaK due to interaction of the N-terminus of the oligopeptide with the allosteric site of DnaK located between the peptide binding cavity and the C-terminus of the chaperone [88]. Use of modified, fluorescein-labeled versions of the native pyrrhocoricin has demonstrated that deletion of the C-terminal half of the oligopeptide eliminates the ability of the peptide to enter bacterial or mammalian cells. Whereas the N-terminus is a necessary fragment for the inactivation of bacteria, the C-terminal fragment is responsible for the delivery of the molecule into the interior of the bacterial cell.

Because the ATPase activity of DnaK is inhibited by biologically active oligopeptides made of L-amino acids, in particular by pyrrhocoricin, it can be concluded that antibacterial activity of PRO and their binding with DnaK correlate [63]. The antibacterial oligopeptides cecropin A and magainin 2, associating with and destroying the bacterial membrane, failed to inhibit the DnaK-mediated phosphate release from ATP [48]. Pyrrhocoricin does not influence the ATPase activity of recombinant Hsp70, the human equivalent of DnaK, which is evidence suggesting that pyrrhocoricin would not be toxic to humans.

Effect of proline-rich oligopeptides on DnaK-dependent protein folding. The effect of PRO on protein folding was estimated by assaying the β-galactosidase and alkaline phosphatase activities upon incubation of an *E. coli* strain TG-1 culture, expressing β-galactosidase and alkaline phosphatase, with L-pyrrhocoricin or drosocin. The results showed a reduction in activities of these enzymes under the effect of L-pyrrhocoricin or drosocin [88]. Inhibition of β-galactosidase was more appreciable in comparison with inhibition of alkaline phosphatase and depended on L-pyrrhocoricin concentration. The inhibitory ability of L-pyrrhocoricin was found to be higher than that of drosocin. While the effect of L-pyrrhocoricin on the β-galactosidase activity could be detected as early as 1 h after introduction of the peptide, the effect of drosocin developed only after 6 h. D-Pyrrhocoricin, magainin 2, or buforin 2, an antimicrobial oligopeptide involved in binding to bacterial nucleic acids, had but negligible effect on the activity of β-galactosidase. It was concluded on the basis of these results that pyrrhocoricin and drosocin inhibit DnaK-assisted protein folding [88].

These findings suggested that the mechanism of action of pyrrhocoricin and, possibly, of drosocin and apidaecin, includes binding of the peptides to the multihelical lid located over the peptide-binding pocket. The binding of PRO with DnaK results in permanently closing the peptide-binding pocket and inhibition of the DnaK-assisted protein folding [88].

Identification of crucial residues for the antibacterial activity of proline-rich oligopeptides. Essential sites of pyrrhocoricin for binding to *E. coli* DnaK were identified by analysis of the results on connection of the synthetic DnaK fragments or the mutated derivatives to fluorescein-labeled pyrrhocoricin or its analogs [83]. According to fluorescence polarization and dot blot analysis of synthetic DnaK fragments and labeled pyrrhocoricin analogs, it was established that the oligopeptide binds with a K_{dis} of 50.8 μM to the hinge region around the C-terminal helices D and E. As pyrrhocoricin diminishes the ATPase activity of recombinant DnaK, the D-E helix region is likely to be one of those C-terminal domains that allosterically influence the ATPase activity of the chaperone [88]. In line with the lack of ATPase inhibition, the drosocin binding site with DnaK appears to be slightly shifted toward the D helix. Nevertheless, both peptides inhibited DnaK-mediated protein folding as demonstrated by a significant reduction in β-galactosidase and by the less prominent, but still observable reduction of the alkaline phosphatase activities. Pyrrhocoricin binding was not observed to the homologous DnaK fragment of *S. aureus*, a pyrrhocoricin non-responsive strain.

Molecular modeling of DnaK–pyrrhocoricin interactions. The biochemical results were supported by molecular modeling of DnaK–pyrrhocoricin interactions (Fig.

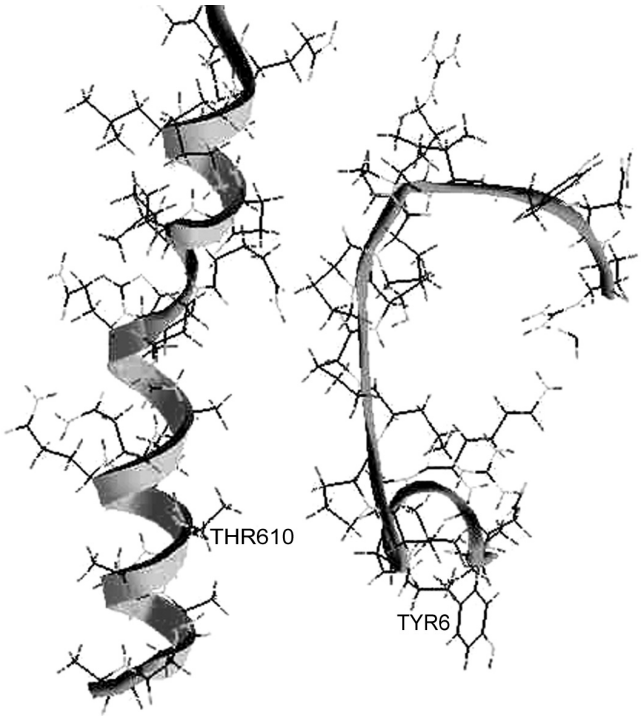

Fig. 4. Model structures of pyrrhocoricin and the D-E helix region of *E. coli* DnaK as they were generated by the flexible docking process [64]. The helical domain on the left corresponds to D and E helices (at the top and bottom, respectively). The right structure corresponds to pyrrhocoricin (N-terminus is at the bottom and C-terminus is at the top). Reprinted with permission from [64], ©2002 Springer-Verlag.

4) [88]. The structure of pyrrhocoricin was docked into DnaK for molecular modeling using the FlexiDock module of SYBYL. The structure of DnaK was fixed in space, only side chains of residues 397-439 (peptide-binding pocket) and residues 587-615 (helices D and E) were flexible. All bonds, except for the peptide bonds, were flexible in the structure of pyrrhocoricin. According to the molecular modeling results, the N-terminal region of pyrrhocoricin (amino acid residues 1-9) binds to the D-E helix region of DnaK, and the binding surface probably extends further down to residues 11-12. Apparently, the strong binding of pyrrhocoricin to the D-E helix hinge region permanently closes the lid over the peptide-binding cavity and prevents DnaK-dependent protein folding.

It is evident from molecular modeling that the pyrrhocoricin fragment that is most intimately associated with helix E of *E. coli* DnaK [64] is the experimentally identified Tyr6-Pro10 hexapeptide segment [88]. This pyrrhocoricin fragment is by itself unable to penetrate into cells, but is an absolute requirement for antibacterial activity.

Identification of amino acid residues crucial for the antibacterial activity of the proline-rich oligopeptides. Study of the antibacterial activity of pyrrhocoricin analogs, in which each residue was replaced with alanine (Ala-scan), has been performed to identify amino acid residues whose replacement may lead to a significant reduction in antibacterial activity of the oligopeptide [83]. According to the results, an identical fragment in pyrrhocoricin is active against *E. coli* and *Agrobacterium tumefaciens* used as targets. No activity was detected against either bacterium when Asp2, Lys3, Tyr6, Leu7, Pro8, Arg9, or Pro10 were replaced with alanine, indicating that the most crucial residue for antibacterial activity is the Asp2-Pro10 peptide fragment. The importance of Arg19 residue for antibacterial activity of pyrrhocoricin is also suggested, because the deletion of the C-terminal Arg19-Asn20 dipeptide results in a reduction of the pyrrhocoricin activity against *A. tumefaciens* [54]. The partial loss of the pyrrhocoricin activity after replacing the two C-terminal arginine residues suggests that this region is responsible for the initial interaction of pyrrhocoricin with the negatively charged bacterial surface and the ensuing entry of the oligopeptide into bacterial cells.

Because drosocin, pyrrhocoricin, and formaecin molecules contain O-glycosylated threonine residues [88], it was of interest to study the role of carbohydrates in the antibacterial activity of PRO. Synthetic O-glycosylated pyrrhocoricin was found to be less active than the non-glycosylated peptide [54]. However, synthetic analog of formaecin without carbohydrate is less active than the O-glycosylated analog carrying sugar on Thr11 [17]. These data suggest that the presence of carbohydrates in drosocin and pyrrhocoricin have no effect on the antibacterial activity of the oligopeptides [83]. Glycosylated residue of threonine, possibly, participates in binding of the oligopeptides to target protein, and may modify either the interaction of oligopeptides with the cellular surface or their ability to penetrate cells [83].

Thus, native PRO are able to inhibit metabolism of bacteria. The mechanism of action of pyrrhocoricin and drosocin has been established, their binding sites to recombinant DnaK of *E. coli* have been identified, and the correlation between antibacterial activity of these peptides and two main functions of DnaK, the ATPase activity and protein folding, has been shown. Pyrrhocoricin is the most active oligopeptide among members of this family; it is nontoxic for humans and mammals and is able to protect them against bacterial infection. The species-specific inhibition of DnaK-assisted protein folding in the presence of PRO includes the control of bacteria, but also of fungi, parasites, and insects. The studies have shown that analogs of natural antibacterial proline-rich oligopeptides can be used as medicines against bacteria with resistance to existing antibiotics.

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