DOI: 10.1002/cbic.200800272

Hydrophobic Interactions as Substitutes for a Conserved Disulfide Linkage in the Type IIa Bacteriocins, Leucocin A and Pediocin PA-1

Darren J. Derksen, Marc A. Boudreau, and John C. Vederas*[a]

Dedicated to Prof. Christoph Tamm (University of Basel) on the occasion of his 85th birthday.

Bacteriocins are antimicrobial peptides that are ribosomally synthesized and exported by bacteria to destroy competing microorganisms.[1] A variety of lactic acid bacteria produce unmodified type IIa bacteriocins (typically 37–48 residues) that have a relatively narrow spectrum of activity but show high potency against certain important food pathogens, including *Listeria monocytogenes*.[2] Although leucocin A (LeuA; **1**) was the first of these to have its sequence reported,[3] this group of several dozen peptides is now called the "pediocin class" after its most popular member, pediocin PA-1 (Ped, **2**; Figure 1). All

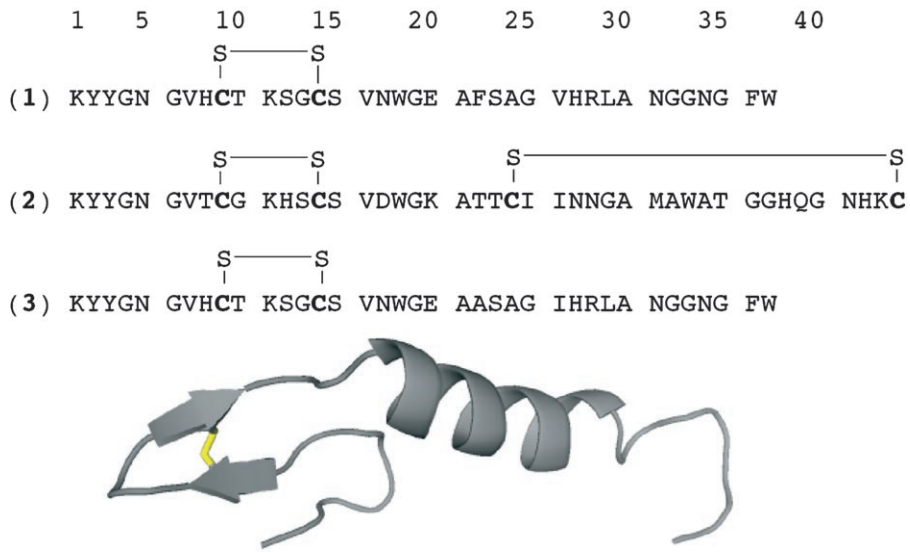

Figure 1. Amino acid sequences of leucocin A (**1**), pediocin PA-1 (**2**), and mesentericin Y105 (**3**) with the solution backbone structure of **1** showing the N-terminal β sheet and C-terminal α helix.

type IIa bacteriocins are believed to bind to a chiral receptor in bacterial cell membranes and create a pore that depolarizes the target cell. As expected with such a mechanism, enantiomeric D-LeuA—synthesized from all D-amino acids—does not show antimicrobial or antagonistic activity at physiologically relevant concentrations.[4] Investigations suggest that the target for LeuA and Ped is the mannose phosphotransferase (mpt) system.[5–7] The exact nature of the bacteriocin–receptor interaction is not yet understood, but it appears to be mediated by the membrane-bound proteins mptC and/or mptD. Synthesis of bacteriocin mutants and analogues provides valuable structure–activity relationships and tools to obtain further information on the peptide–receptor complex.[4,8,9]

The C-terminal portions (residue 20 onwards) of type IIa bacteriocins show little homology, but their sequences allow formation of an amphipathic α helix that appears critical for antimicrobial specificity and temperature-dependent activity of these peptides.[10–14] In contrast, the N-terminal sections are highly homologous, and LeuA and Ped differ by only six residues in the first 21 amino acids. All type IIa bacteriocins have a conserved YGNGVXC sequence in the N terminus, and the cysteine is part of a disulfide bond with another cysteine five residues away.[2] Structurally, the N terminus of LeuA consists of a three-strand antiparallel β sheet (residues 2–16) that is rigidified by this [9–14]-disulfide moiety.[10,15] Substitution of the cysteines with serines in LeuA or in its relative mesentericin Y105 (**3**) abolishes all activity.[8,16] The corresponding residues in Ped have also been reported to be essential based on NNK scanning.[17] Hence, the conserved disulfide bond would be expected to be required for antimicrobial action for all type IIa bacteriocins. However, recent results from our laboratory indicate that the disulfide bond in LeuA could be replaced by a noncyclic diallyl moiety without significant loss in activity.[8] Evidently, hydrophobic or π-stacking interactions can compensate for the absence of the disulfide in this molecule and assist receptor binding. In the present study, we report the synthesis and testing of a series of LeuA and Ped analogues to explore the cause and generality of this unexpected phenomenon.

In order to assess the relative contributions of π-stacking and hydrophobic interactions in the acyclic bis-L-allylglycine derivative, (Cys9AllylGly, Cys14AllylGly)-LeuA (**4a**), its saturated counterpart **4b**, which has two norvalines (Nva), was synthesized by solid phase peptide synthesis (SPPS) by using TGA resin and standard Fmoc methodology (Figure 2). Similarly, the L-allylglycine (AllylGly) residues were substituted with L-phenylalanines to give **4c**, in which π-stacking interactions could po-

[a] Dr. D. J. Derksen, Dr. M. A. Boudreau, Prof. J. C. Vederas
Department of Chemistry, University of Alberta
Edmonton, Alberta, T6G 2G2 (Canada)
Fax: (+1) 780-492-2134
E-mail: john.vederas@ualberta.ca

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

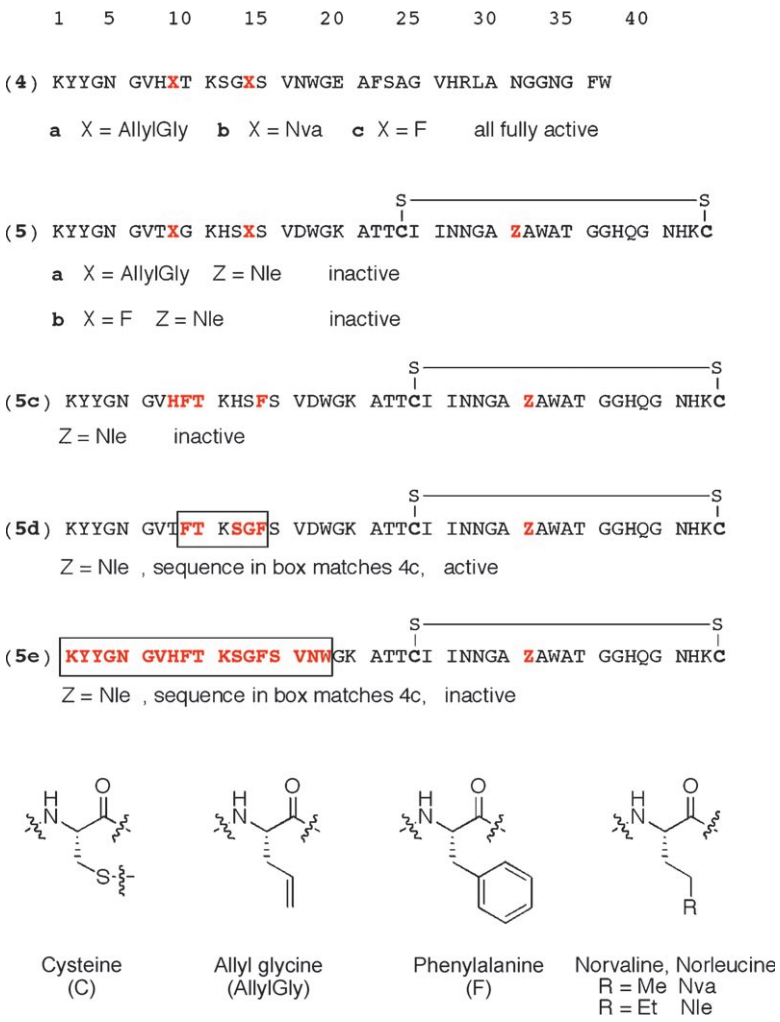

Figure 2. Synthetic analogues **4a–c** of LeuA and **5a–e** of Ped. The Ped analogue **5**, in which Z = Nle, has both disulfides (9–14 and 24–44) and is as active as natural Ped. Structures of the key amino acid residues used in disulfide bond mimics are shown.

tentially be enhanced. Within experimental error, peptides **4a–c** showed the same antimicrobial potency as the parent unmodified leucocin A (**1**) in spot-on-lawn tests with three standard test organisms (*Carnobacterium maltaromaticum* UAL26, *C. divergens* LV13, and *Listeria monocytogenes* EGDe; Table 1). The activity of the norvaline analogue **4b**, which lacks a double bond, demonstrates that hydrophobic interactions, as opposed to π stacking, can replace the disulfide bond in LeuA and promote the correct conformation required for receptor binding.

The concept of cysteine substitution with hydrophobic residues was then examined with analogues of pediocin (**2**). As **2** is prone to aerobic oxidation of the sulfur of Met31 to a sulfoxide and the corresponding Met31Nle mutant **5** is fully active,[12] all synthetic Ped analogues were made with this substitution. 2-Chlorotrityl resin was employed with Fmoc SPPS to produce (C9AllylGly, C14AllylGly, M31Nle)-Ped (**5a**) and (C9F, C14F, M31Nle)-Ped (**5b**). To examine the potential generality of such replacements, the corresponding (C24AllylGly, C44AllylGly, M31Nle)-Ped, which contains the natural 9–14 disulfide, was also synthesized. Surprisingly, in contrast to the LeuA analogues, all of these Ped derivatives, in which a disulfide moiety had been replaced, lacked any significant antimicrobial activity. If LeuA and Ped both interact with the mpt receptor system as suggested, it appears that either: 1) specific recognition of the sulfurs in the disulfide moiety by the protein is essential for Ped but not for LeuA, or 2) modest differences in the N-terminal sequence of Ped prevent attainment of the correct β-turn conformation when the 9–14 disulfide is missing.

To assess these two possibilities, the sequence between residues 8 and 14 of leucocin A and pediocin PA-1 was varied as this region is composed of the same constituent amino acids in both peptides, and differs only in their relative arrangement. Analysis of the NMR structure of LeuA[10] suggested that Tyr2, His8, and Thr10 might interact to form a cluster;[11] therefore, a pediocin analogue **5c**, (T8H, C9F, G10T, C14F, M31Nle)-Ped, was prepared that incorporated these substitutions in conjunction with replacement of residues 9 and 14 with the hydrophobic amino acid phenylalanine. The possibility that the intraring residues might be responsible for the activity of the acyclic analogue was explored by synthesis of a pediocin analogue **5d**, (C9F, G10T, H12S, S13G, C14F, M31Nle)-Ped, that substituted intraloop residues 10–13 from LeuA into the Ped sequence as well as the disulfide bond replacement by phenylalanines. To complete the structure–activity relationship study, a hybrid peptide **5e**, LeuA(1-18)(C9F,C14F)-Ped(19-44), in which the 9–14 disulfide was replaced by two phenylalanine residues, was also synthesized to examine the role of the entire N terminus on biological activity.

Antimicrobial testing as before showed that these Ped analogues, which lack the 9–14 disulfide, are

Table 1. Biological testing results of peptides against three indicator organisms.

	Residues 9–14[b]	Remaining sequence	*C. div*[d] LV13 [μM]	*C. malt*[e] UAL26 [μM]	*L. mono*[f] EGDe [μM]
1[a]	C**TKSG**C	LeuA	3	3	3
4a	a**TKSG**a[8]	LeuA	6	3	6
4b	n**TKSG**n	LeuA	3	3	3
4c	F**TKSG**F	LeuA	6	3	6
2[a]	CGKHSC	Ped	6	6	6
5a	aGKHSa	Ped	n.a.[g]	n.t.[h]	n.a.[g]
5b	FGKHSF	Ped	n.a.[g]	n.a.[g]	n.a.[g]
5c	F**TKHS**F	Ped	n.a.[g]	n.a.[g]	n.a.[g]
5d	F**TKSG**F	Ped	100	50	n.a.[g]
5e	F**TKSG**F	hybrid[c]	n.a.[g]	n.a.[g]	n.a.[g]

[a] Natural sequence; [b] a: allyl glycine; n: norvaline; [c] Leu(1–18)-Ped-(19–44); [d] *Carnobacterium divergens*; [e] *C. maltaromaticum*; [f] *Listeria monocytogenes*; [g] n.a.: no activity detected up to 100 μM. [h] n.t.: not tested. Note: All pediocin analogues contain the oxidatively stable norleucine in place of methionine.[12]

devoid of activity except for peptide **5d**, which has the intraring loop sequence of LeuA. This compound regains significant, but not full, activity against two of the three test organisms. This indicates that, as with LeuA, direct interaction of the receptor protein with the sulfurs in the 9–14 disulfide of Ped is not required, at least for *C. divergens* and *C. maltaromaticum*. Apparently the propensity of the intraloop sequence of LeuA to induce β turns[18] in combination with the hydrophobic interaction of the two Phe residues is sufficient to achieve the appropriate conformation for bioactivity. Receptor binding might occur on the surface of a three-strand antiparallel β sheet at the N terminus of the peptide as well as by recognition of the hydrophobic face of the amphipathic C-terminal α helix, which is known to be required[4,19] and determines specificity for particular organisms.[13] Surprisingly, when the first 18 residues of pediocin are replaced by (C9F, C14F)-LeuA(1–18), biological activity is not observed despite the presence of the intraloop residues from LeuA. These results indicate that although the N-terminal loop has a vital influence on the activity of the peptide, additional interactions at the C terminus with the receptor must match and also contribute to the overall activity. This is in accord with previous observations that the entire bacteriocin is essential for activity,[4,20] and that very small structural changes, such as removal of a single tryptophan residue from the C terminus of **3**[16] or mutation of the conserved Tyr3 to phenylalanine in closely-related carnobacteriocin B2[19] near the N terminus, have severely detrimental effects on activity.

It is clear that the β-turn structure seen in the NMR spectroscopy studies of type IIa bacteriocins[10–15] is critical for antimicrobial activity. Whether this structure arises from a conformation enforced by a disulfide bond or based on the inherent peptide sequence is more case dependent, and largely controlled by modest differences in the intraloop sequence. In the example of the pediocin (**2**) intraloop sequence, the disulfide is a prerequisite for biological activity, but with the leucocin A (**1**) loop the disulfide appears to be an assisting feature that serves to reinforce the tendency to form the required secondary structure of the peptide. It is intriguing that only the exchange of the positions of the four amino acids between residues 8–14 from pediocin PA-1 to leucocin A allows substitutions of cysteine for hydrophobic residues, such as phenylalanine or allylglycine.

Our results are consistent with the idea that the selectivity and activity of type IIa bacteriocins is dependent on their entire sequence and the overall three-dimensional structure preferred in membranes. It is important to note that all of these peptides (including the natural bacteriocins **1** and **2**) have random-coil structures in water and assume a defined conformation only in hydrophobic environments (e.g., micelles) or in solvents such as trifluoroethanol.[10–14] Limited solubility and difficulty in universal isotopic labeling of the synthetic peptides precludes detailed NMR analysis for complete determination of the three-dimensional structures of the unnatural analogues. However, CD studies and physical properties indicate that they resemble their parents in propensity to form α helices in the C-terminal section. More generally, our structure–activity results demonstrate that for peptides in which the propensity of a particular amino acid sequence to form a β turn is sufficiently great, substitution of cysteine disulfide bridges with hydrophobic interactions by using a variety of lipophilic side chains can very effectively assist attainment of the correct conformation. This effect is especially useful with phenylalanine substitution because it permits facile production of active bacteriocins from natural amino acids through genetic engineering. The possibility of such substitutions in other types of bioactive peptides is being explored.

Experimental Section

Synthesis of peptides was completed by using standard solid phase peptide synthesis (SPPS) methods with the incorporation of pseudoprolines[21] to disrupt aggregation in long sequences. Peptides were synthesized manually to identify problematic sequences, and then optimized methods were incorporated for synthesis by using an ABI 433A peptide synthesizer equipped with UV monitoring feedback control. The peptides were prepared on preloaded Fmoc-Trp(Boc)-TGA, Fmoc-Cys(Trt)-TGT, or H-Cys(Trt)-2-ClTrt resin by using standard side-chain protection. Oxidation to form the necessary disulfide bond(s) was completed by suspending the peptide in a 1:1 solution of 2,2,2-trifluoroethanol and ammonium bicarbonate buffer (1 mM; pH 8.0). The solutions were then saturated with oxygen and allowed to stir for 16 h. After oxidation, the nonaqueous portion of the solution was removed in vacuo and the aqueous portion was lyophilized to obtain the crude peptide. The crude peptide was then dissolved in a solution of acetonitrile (20%)/water and passed through a syringe filter before purification by HPLC. Fractions showing the desired mass by MADLI-TOF MS were pooled and the sample was repurified to homogeneity. Purified samples were lyophilized to yield a white powder and were tested by using the spot-on-lawn method as previously reported.[8] All peptide concentrations were obtained by measuring the A_{280} according to standard procedures.[22] Indicator organisms used for testing were *Carnobacterium maltaromaticum* UAL26 and *Carnobacterium divergens* LV13 (grown at 25 °C), and *Listeria monocytogenes* EGDe (grown at 37 °C). See the Supporting Information for further details.

Acknowledgements

Financial support by the Natural Sciences and Engineering Research Council of Canada (NSERC), the Advanced Foods and Materials Network (AFMNet), the Canada Research Chair in Bioorganic and Medicinal Chemistry, the Alberta Heritage Foundation for Medical Research (AHFMR), and the Alberta Ingenuity Fund is gratefully acknowledged. We thank Randy Whittal and Jing Zheng (University of Alberta) for help with mass spectral analyses.

Keywords: antimicrobial agents · leucocin A · pediocin PA-1 · peptides · structure–activity relationships

[1] J. Cleveland, T. J. Montville, I. F. Nes, M. L. Chikindas, *Int. J. Food Microbiol.* **2001**, *71*, 1–20.
[2] D. Drider, G. Fimland, Y. Héchard, L. M. McMullen, H. Prévost, *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 564–582.

[3] J. W. Hastings, M. Sailer, K. Johnson, K. L. Roy, J. C. Vederas, M. E. Stiles, *J. Bacteriol.* **1991**, *173*, 7491–7500.
[4] L. Z. Yan, A. C. Gibbs, M. E. Stiles, D. S. Wishart, J. C. Vederas, *J. Med. Chem.* **2000**, *43*, 4579–4581.
[5] D. B. Diep, M. Skaugen, Z. Salehian, H. Holo, I. F. Nes, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 2384–2389.
[6] M. Ramnath, S. Arous, A. Gravesen, J. W. Hastings, Y. Héchard, *Microbiology* **2004**, *150*, 2663–2668.
[7] B. Erni, *J. Bacteriol.* **2006**, *188*, 7036–7038.
[8] D. J. Derksen, J. L. Stymiest, J. C. Vederas, *J. Am. Chem. Soc.* **2006**, *128*, 14252–14253.
[9] a) N. Ghalit, J. F. Reichwein, H. W. Hilbers, E. Breukink, D. T. S. Rijkers, R. M. J. Liskamp, *ChemBioChem* **2007**, *8*, 1540–1554; b) N. Ghalit, J. Kemmink, H. W. Hilbers, C. Versluis, D. T. S. Rijkers, R. M. J. Liskamp, *Org. Biomol. Chem.* **2007**, *5*, 924–936.
[10] N. L. Fregeau Gallagher, M. Sailer, W. P. Niemczura, T. T. Nakashima, M. E. Stiles, J. C. Vederas, *Biochemistry* **1997**, *36*, 15062–15072.
[11] Y. J. Wang, M. E. Henz, N. L. Fregeau-Gallagher, S. Y. Chai, A. C. Gibbs, L. Z. Yan, M. E. Stiles, D. S. Wishart, J. C. Vederas, *Biochemistry* **1999**, *38*, 15438–15447.
[12] K. Kaur, L. C. Andrew, D. S. Wishart, J. C. Vederas, *Biochemistry* **2004**, *43*, 9009–9020.
[13] G. Fimland, O. R. Blingsmo, K. Sletten, G. Jung, I. F. Nes, J. Nissen-Meyer, *Appl. Environ. Microbiol.* **1996**, *62*, 3313–3318.
[14] G. Fimland, L. Johnsen, L. Axelsson, M. B. Brurberg, I. F. Nes, V. G. H. Eijsink, J. Nissen-Meyer, *J. Bacteriol.* **2000**, *182*, 2643–2648.
[15] T. Sprules, K. E. Kawulka, A. C. Gibbs, D. S. Wishart, J. C. Vederas, *Eur. J. Biochem.* **2004**, *271*, 1748–1756.
[16] Y. Fleury, M. A. Dayem, J. J. Montagne, E. Chaboisseau, J. P. Le Caer, P. Nicolas, A. Delfour, *J. Biol. Chem.* **1996**, *271*, 14421–14429.
[17] T. Tominaga, Y. Hatakeyama, *Appl. Environ. Microbiol.* **2006**, *72*, 1141–1147.
[18] a) K. W. Miller, R. Schamber, O. Osmanagaoglu, B. Ray, *Appl. Environ. Microbiol.* **1998**, *64*, 1997–2005; b) E. G. Hutchinson, J. M. Thornton, *Protein Sci.* **1994**, *3*, 2207–2216.
[19] L. E. N. Quadri, L. Z. Yan, M. E. Stiles, J. C. Vederas, *J. Biol. Chem.* **1997**, *272*, 3384–3388.
[20] G. Fimland, R. Jack, G. Jung, I. F. Nes, J. Nissen-Meyer, *Appl. Environ. Microbiol.* **1998**, *64*, 5057–5060.
[21] T. Wohr, F. Wahl, A. Nefzi, B. Rohwedder, T. Sato, X. C. Sun, M. Mutter, *J. Am. Chem. Soc.* **1996**, *118*, 9218–9227.
[22] S. C. Gill, P. H. Von Hippel, *Anal. Biochem.* **1989**, *182*, 319–326.

Received: April 22, 2008
Published online on July 18, 2008

