Epimerases

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A Lanthipeptide-like N-Terminal Leader Region Guides Peptide Epimerization by Radical SAM Epimerases: Implications for RiPP Evolution

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Dedicated to Prof. Dr. Gerhard Bringmann on the occasion of his 65th birthday

Abstract: *Ribosomally synthesized and posttranslationally modified peptide natural products (RiPPs) exhibit diverse structures and bioactivities and are classified into distinct biosynthetic families. A recently reported family is the proteusins, with the prototype members polytheonamides being generated by almost 50 maturation steps, including introduction of* D*-residues at multiple positions by an unusual radical SAM epimerase. A region in the protein-like N-terminal leader of proteusin precursors is identified that is crucial for epimerization. It resembles a precursor motif previously shown to mediate interaction in thioether bridge-formation in class I lanthipeptide biosynthesis. Beyond this region, similarities were identified between proteusin and further RiPP families, including class I lanthipeptides. The data suggest that common leader features guide distinct maturation types and that nitrile hydratase-like enzymes are ancestors of several RiPP classes.*

Ribosomally synthesized and posttranslationally modified peptides (RiPPs) are a natural product class of remarkable structural and functional diversity.[1] Most known RiPPs derive from precursors that are organized into N-terminal leader and C-terminal core segments.[1a,2] The core region is posttranslationally altered by maturation enzymes that can introduce a great variety of modifications. Leaders vary in length and amino acid composition and are often used for RiPP classification.[3] In several studies, leaders were shown to facilitate the interaction with their cognate modifying enzymes, resulting in more efficient core maturation.[2,3] Well-studied examples are class I lanthipeptides, such as nisin, for which an FD/NLN/D motif (termed FNLDmotif) and the surrounding residues in the NisA leader mediate introduction of lanthionine bridges.[4] For this and other cases, interactions are mediated by PqqDlike domains in the modifying enzymes.[4a,5] Such domains are present in more than 50% of all RiPP families, including class I lanthipeptides, proteusins, cyanobactins, and linear azol(in)econtaining peptides (LAPs), where they are supposed to act as structurally related RiPP precursor peptide recognition elements (RREs).[6]

Proteusins have recently emerged as a new RiPP family that computational analyses predict to be widespread in bacteria.[1c,7] The prototype members polytheonamides are highly cytotoxic pore formers isolated from the sponge *Theonella swinhoei*,[8] but recently attributed by us to the metabolically versatile uncultivated bacterium *Candidatus* Entotheonella factor.[7] Polytheonamides are the most extensively modified peptides reported to date, featuring 48 modifications that include 18 D-amino acids. The latter are generated by the radical *S*-adenosyl methionine (rSAM) epimerase PoyD.[9] Related epimerases were found in several other proteusin gene clusters and shown to irreversibly and regioselectively introduce diverse D-amino acid patterns into peptides.[9] The polytheonamide precursor (PoyA) and precursors of other proteusin biosynthetic gene clusters containing epimerase genes, such as OspA[9] from *Oscillatoria* sp. PCC 6506, feature a large (up to 96 aa) leader region resembling the α-subunit of nitrile hydratase (NHαs) proteins.[1c,7,10] Nothing is known about the function of such NHα-like leader peptides (NHLPs) and the significance of their large size and enzyme-like features. Such knowledge is, however, crucial to utilize epimerization for rational peptide engineering and to integrate the epimerase function into pathways of other RiPP families.

To interrogate a possible leader role in proteusin epimerization, we selected the OspA/OspD precursor–epimerase pair as model. An advantage of this system over the homologues PoyA/PoyD is that OspD introduces only two epimerizations (18 in polytheonamides). Furthermore, non-epimerized OspA is soluble, whereas non-epimerized PoyA, which is either due to absence of PoyD or to effects of leader mutations, was not solubly produced in *E. coli*. OspD epimerizes a Val and an Ile residue on the 16-aa core of OspA (Figure 1a), resulting in different HPLC retention times of unepimerized and epimerized peptide cores.[9]

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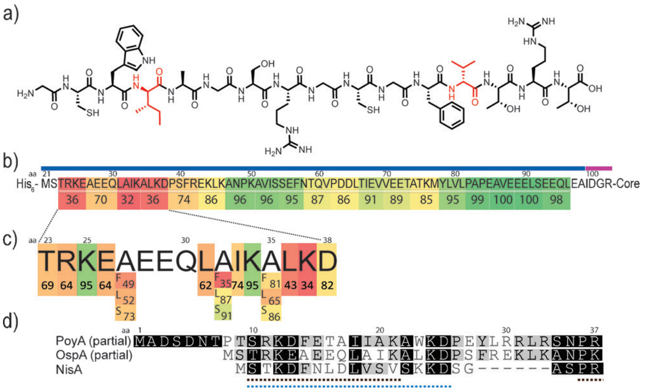

Figure 1. a) OspA core peptide with D configured residues shown in red. b) OspA leader (blue line) with an N-terminal His_6-thrombin cleavage site fusion (His_6), as well as a factor Xa protease site (pink line) between leader and core. Letters in colored boxes indicate four-residue blocks that were replaced with Ala residues in individual mutants. The degree of epimerization [%] and a colored heat map is provided for each mutant. The average degree of epimerization for the wild-type was 94%. c) Effect of single-site *x* to Ala mutants as well as mutations of natural Ala residues in important areas of the leader (to Phe (F), Leu (L), and Ser (S). d) N-terminal leader alignment of PoyA and OspA showing pronounced sequence similarities to NisA regions that are important for interaction with NisB and C (dotted black line). The OspA leader region with a strong influence on core epimerization is indicated by the blue dotted line.

To investigate the relevance of the leader for core epimerization, we constructed 19 His_6OspA-leader mutants (Figure 1b). These block mutants featured individual stretches of four consecutive Ala residues as replacements of the original four residues and covered the entire leader up to the leader-core interface, where a factor Xa site was added (Figure 1b).[9] Naturally occurring Ala residues in the OspA leader were not replaced. Genes of the mutated precursors as well as of the control were individually co-expressed in *E. coli* together with *ospD*. After purification of the precursors and cleavage with factor Xa, product mixtures were analyzed by LC-MS for epimerization efficiencies. All His_6-OspA precursors were solubly produced (Supporting Information, Figure S1) and the wildtype showed an average degree of 94% core epimerization (Figure 1b; Supporting Information, Figure S2; Table S1). The degree of epimerization was calculated based on peak areas of extracted ion chromatograms, using a weighted formula for appropriate representation (see the Supporting Information). In most experiments, leader mutations did not drastically impair core epimerization or even improved it. However, for a small set of mutants, we detected a strongly reduced epimerization efficiency. The greatest effects were observed for Ala mutants located in a confined N-terminal region of the OspA leader. Here, replacement of the regions TRKE, LAIK, or ALKD by Ala series decreased the epimerization efficiency from 94% to below 40% (Figures 1b; Supporting Information, Figure S2, Table S1). The reproducibility of these effects was tested for mutants as well as the wildtype in triplicates, resulting in little variation (Supporting Information, Table S2). 19 point mutants within the N-terminal leader region were tested (Figure 1c). Some did not affect epimerization, even though the properties of the side chains were strongly altered. For example, while mutants K25A and K34A showed no effect, K37A strongly decreased epimerization (Figures 1c; Supporting Information, Figures S3,S4, Table S3). Replacements of natural Ala residues in the N-terminal region with Phe, Leu, and Ser revealed that even minor changes at residue A27 (A27L) have a relatively strong impact on epimerization (Figures 1c; Supporting Information, Figures S5,S6, Table S4).

Thus, even though the OspA leader is much larger as compared to, for example, class I lanthipeptide leaders, our results suggest important functions during core maturation. Similar interactions of maturases and leader peptides have been shown for NisA and subclass II lanthipeptides.[4a,11]

These parallels prompted us to investigate if proteusin leaders share similarities with leaders of other RiPP classes. An initial alignment of the OspA, PoyA, and NisA leaders indeed revealed pronounced N-terminal sequence similarities despite their affiliation to different RiPP classes (Figure 1d). The similar regions include the OspA and NisA leader portions that are most important for interactions with OspD and NisB, respectively. As mentioned, interactions of NisB with the extended FNLD motif are mediated by a widespread maturase RRE.[4a,6] Burkhart et al. also identified an RRE at the C-terminus of the epimerase PoyD.[6] In agreement, a Phyre2 analysis[12] revealed an RRE at the C-terminus of OspD as well (Supporting Information, Figure S7). A homology model of this RRE in OspD using the GROMOS force field 54A7[13] and the MODELLER[14] web server suggested a stable winged helix-turn-helix motif with adjacent β-strands as it has been found for other RREs (Figure 2; Supporting Information, Figure S8).

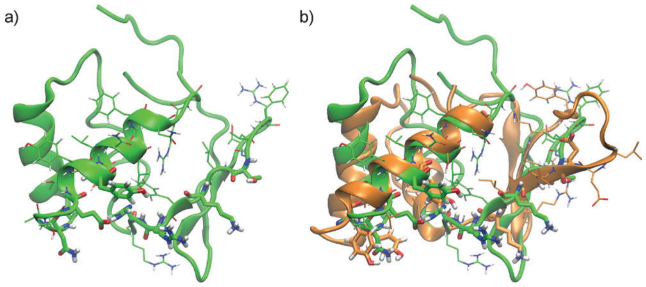

Figure 2. a) Homology model of the OspD RRE motif with the known winged helix-turn-helix-motif based on NisB (PDB: 4WD9).[4a,15] b) Overlayed models of the RREs of OspD (green) and NisB (orange).

A larger-scale analysis that also included other leader types as well as the NHα enzymes revealed unexpected further sequence similarities (Supporting Information, Figures S9,S10, Table S5). Within NHLP proteusins, the motif is conserved in a large number of gene clusters lacking epimerase genes. It might therefore also govern interactions with additional maturases. Along with the homology between a large number of NHLP and class I lanthipeptide leaders, similarities became apparent to several class II lanthipeptide leaders (Supporting Information, Figure S9; for a more detailed description see the Supporting Information, text I). Remarkably, these regions not only included the N-terminal motif but spanned the entire length of leaders. In case of LAP leaders, which also interact with enzymes containing RREs,[6] we detected only faint similarities to the N-terminal leader areas of NHLPs and class I lanthipeptides (Supporting Information, Figure S11). However, it should be noted that residues F14 and L18 of the LAP microcin B17 leader (residue numbering, see the Supporting Information, Figure S11) are essential for core maturation,[16] and both residues are also widespread in NHLP proteusins and class I lanthipeptide leaders such as NisA and PoyA (Supporting Information, Figure S11). As an exception of the consensus between NisA, microcin B17, and PoyA-like NHLP leaders, F14 is replaced by Ala in OspA (Figure 1 d). This Ala, however, turned out to be highly important for epimerization of OspA. To investigate the role of F14 in PoyA, we analyzed its influence on epimerization of a PoyA variant with the truncated core residues 1–25 (PoyA25), which has improved solubility (Supporting Information, Figure S12).[7] Ala mutants of the FETA block and F14 strongly decreased core epimerization efficiencies. These findings corroborate the importance of the alignment position and point towards an evolutionarily conserved leader-maturase recognition region in OspA, PoyA, and NisA.

The general conservation of this N-terminal region in NHLP leaders (Figure 3 a) and similarities to other RiPP leaders that interact with RREs suggest a shared RREbinding sequence (REBS) in NHLP leaders. However, some more variable residues were found to be important for OspA epimerization (for example, TRKE), while less important residues exhibit strong conservation (for example, D16). One reason could be that less conserved residues might guarantee specificity of single leader-maturase pairs, thus preventing cross reactivity between different RiPP pathways in the same organism. On the other hand, conserved but less-important residues might be involved in interactions with other maturases or contribute to the overall structure and stability of the leader. No similarity of NHLP leaders to bottromycin, cyanobactin, thiopeptide, sactipeptide, and lasso peptide leaders was identified.[1a] Previous studies have shown that PatDtype heterocyclases involved in cyanobactin biosynthesis, which also harbor RREs, interact with a motif termed recognition sequence 1.[17] This motif is unrelated to the proposed REBS identified here.

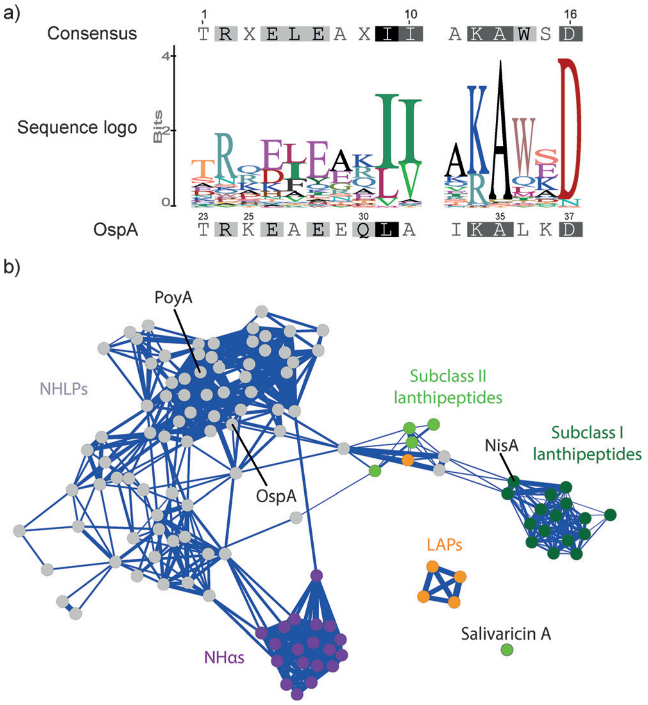

Figure 3. a) 79 NHLP leader sequences were analyzed to display the most highly conserved residues in the area that shows strongest influence on OspA core epimerization. A consensus sequence is given and gray to dark shading indicates an increasing degree of conservation. The corresponding OspA sequence is shown below, using the same numeration as in Figure 1 b. b) REBS area sequence identity network. Cutoff: 30% identity; $k_{max} = 17$. The network is based REBS of NHLPs as well as similar region in subclass I and II lanthipeptides, and NHαs (Supporting Information, Figure S8). Edge thickness represents the percentage of identity of the connected nodes.

To further evaluate leader similarities, we constructed a sequence identity network from N-terminal leader regions, which also comprised the corresponding NHα sequences (Figure 3 b). We allowed each node of the network to interact with ≤ 17 other nodes and set a minimal sequence identity of 30% for node interactions. As a result, NHLP proteusins clustered with the homologous regions in NHαs, their probable ancestors. Subclass I and II lanthipeptides formed connected subclusters, thus underlining sequence similarities among leaders of different RiPP classes (Figure 3 b).

Based on our mutagenesis data, similarities among leaders of different RiPP classes (NHLP, class I and II lanthipeptides) might result from an interaction with structurally similar RREs. These similar leader regions could be a consequence of convergent evolution, recombinatorial exchange, or shared evolutionary origin of leaders. Bayesian phylogenetic analysis including NHαs (Supporting Information, Figure S13) lacks resolution near the root because of the shortness of the alignments, and neighbor-net analysis[18] (Supporting Information, Figure S14) reveals a certain amount of reticulate events (for example, convergence, recombination) among branches. However, a general common ancestry is supported by the existence of further similarity regions beyond the N-terminal region as well as the overall alignment topology (Supporting Information, Figure S9), which suggests that major differences between NHαs and NHLP and lanthipeptide leaders are due to block deletions of larger gene sections. A putative evolutionary scenario might therefore be that NHLPs, class I, and class II lanthipeptides would be descendants of NHα-like enzymes. NHLP leaders lost the region containing the metal-chelating residues of NHαs, while more extensive deletions gave rise to the much shorter lanthipeptide leaders (for more discussion on NHαs as possible origin of several RiPP classes, see the Supporting Information, Text II). Conservation of a preserved REBS motif during this process might have fostered co-evolution of RREs in unrelated RiPP-modifying enzymes, such as NisB and OspD.

In summary, this study revealed common features shared by RiPP precursor families that were previously considered unrelated. We identified an NHLP proteusin leader region, here termed REBS, that is important for core epimerization, showing interesting parallels to the NisA/NisB recognition pair. Further studies are required to test whether cognate REBS-RRE pairs are a more widespread feature governing maturation in diverse RiPP classes, as the presence of both motifs in unrelated precursor-maturase pairs suggests. Our study also identified larger similarities between proteusin and type I and II lanthipeptide leaders beyond the N-terminal region. This finding suggests a unifying evolutive scenario that involves NHα-type enzymes as ancestors of these distinct RiPP precursors. Understanding general principles underlying the remarkable biosynthetic diversity of RiPPs are an important basis to use epimerases and other maturation enzymes for rational metabolic engineering independent of pathway families.

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