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COMMUNICATIONS

Published on Web 12/06/2006

Peptide Macrocyclization: The Reductase of the Nostocyclopeptide Synthetase Triggers the Self-Assembly of a Macrocyclic Imine

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Nonribosomal peptides (NRPs) have attracted considerable attention from scientific and medical communities and gave rise to a wide assortment of therapeutic agents.[1] Specifically, the biological activity of many of these complex natural products, including the immunosuppressant cyclosporin and the last resort antibiotic daptomycin, relies on the macrocyclization of their peptide backbone.[2] The correlation between macrocyclization and biological activity is underlined by the discovery of ribosomally produced proteins and peptides with circular topology. This family of cyclic peptides encompasses, for instance, the antimicrobials Microcin J25, produced by certain *E. coli* strains[3] and the mammalian rhesus θ defensins (RTD).[4] For NRPs macrocyclization can be achieved via two different strategies hitherto known: The formation of macrolactams or macrolactones. During biosynthesis, the ring closing reaction is performed by enzymatic catalysts called thioesterase domains (TE domain), the most downstream domains in multidomain modular assembly lines of nonribosomal peptide synthetases (NRPSs).[5] Recently, a unique and so far unknown type of peptide cyclization was reported for the nostocyclopeptide (ncp) (Figure 1), a cytotoxin produced by the terrestrial cyanobacterium *Nostoc* sp. ATCC53789.[6] In this heptapeptide the C- and N-terminal amino acids are fused together via a stable imino bond, a novel type of head-to-tail linkage that had not been reported before in a naturally derived cyclic peptide. In principle, different scenarios for imine macrocyclization are possible, including the existence of a so far unknown macrocyclization catalyst. As we show in this work, a C-terminal reductase domain of the respective ncp NRPS catalyzes the reductive release of the mature peptide chain from the synthetase, and thereby triggers the spontaneous formation of the imino head-to-tail linkage. Our experiments evidenced that the ncp molecular self-assembly is based on the intrinsic conformation of the linear heptapeptide aldehyde and strongly depends on the nature of the C- and N-terminal amino acids.

The sequencing of the ncp biosynthetic gene cluster revealed that the assembly of the linear heptapeptide chain is achieved by a linear NRPS, consisting of two subunits ncpA and ncpB (Figure S1 in Supporting Information).[6] Instead of the commonly found TE domain, a relatively rare reductase domain (R domain), containing a putative NAD(P)H binding motif, is found at the C-terminal end of ncpB. Although other C-terminal reductase domains in NRPSs are known, such as in the biosynthesis of lysine in yeast,[7] myxobacterial natural products,[8−10] and linear gramicidin,[11] the associated compounds are unexceptionally primary amines and alcohols that do not possess a macrocyclic structure. In order to characterize the role of the ncp R domain in imine macrocyclization, we overexpressed the ncp R domain, and ncp R together with its adjacent peptidyl-carrier protein (PCP), as recombinant proteins (Figure S2). The reductase activity of ncp PCP−R was tested in vitro with peptidyl-thioester substrates that were generated

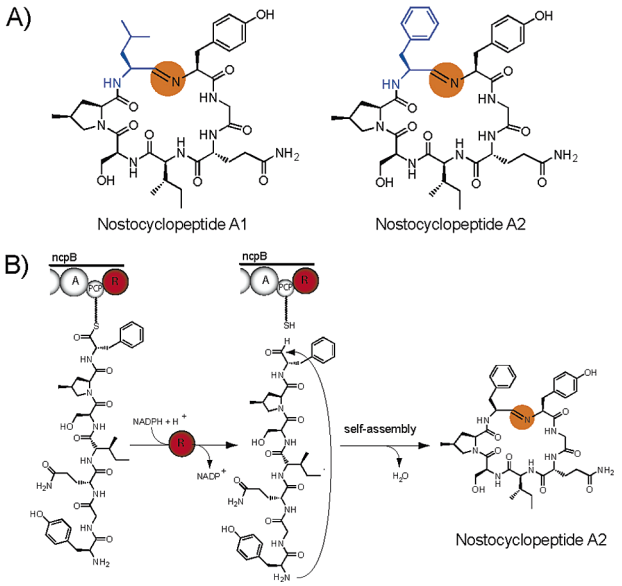

Figure 1. (A) The nostocyclopeptides A1 and A2 are macrocyclic imines differing at the C-terminal amino acid position (highlighted in blue); (B) mechanism of imine macrocyclization via molecular self-assembly.

via solid-phase peptide synthesis (SPPS) (Supporting Information). For synthetic reasons we replaced the naturally occurring methylproline with proline. The side-chain protected linear peptides were C-terminally activated with four different leaving groups, such as thiophenol, SNAC, ppan, and CoA, to mimic the natural situation, in which the fully assembled peptide chain is thioester-bound to the PCP of the last module (Figure 1). The assay was carried out as reported in Supporting Information. Subsequent HPLC−MS analysis revealed product formation for peptidyl-CoA substrates in the presence of NADPH (Figure 2). NADH was not accepted as electron donor. Mass analysis confirmed that the two linear substrate mimics ncpA1 and ncpA2 (Figure S3) were converted mainly into the corresponding cyclic imines and only traces of ncpA1 and ncpA2 aldehydes were detected. In contrast to CoA- and ppan-activated substrates, thiophenol and SNAC were not suited as leaving groups (data not shown). The alone standing R domain displayed significantly lower catalytic activity. We believe that the adjacent PCP has stabilizing influence on the excised R domain. Therefore subsequent experiments were performed with recombinant ncp PCP−R. The in vitro results clearly evidenced that ncp R is a strictly NADPH dependent enzyme responsible for the reductive release of the linear peptide aldehyde intermediate. Thus, we could exclude the possible existence of an additional protein involved within imine macrocyclization. However, the role of the R domain in imino bond formation remained unclear at this stage.

To further investigate this issue, we chemically synthesized peptide aldehydes with sequences identical to ncpA1 and ncpA2 (Figure S4). These compounds were incubated in aqueous HEPES-

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10.1021/ja0667458 CCC: $33.50

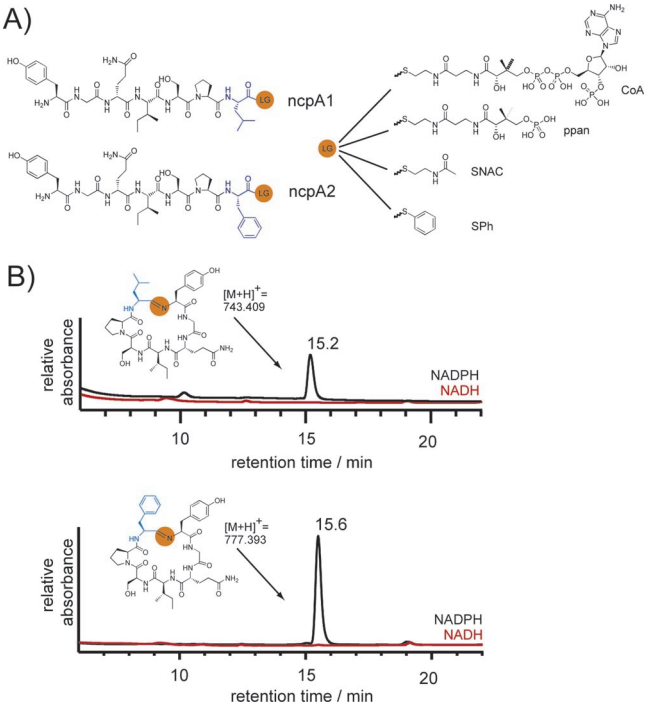

Figure 2. (A) Peptidyl-thioester substrates used in this study. The C-terminus is activated with a thioester leaving group; (B) HPLC-MS of the ncp PCP−R activity assay in vitro.

buffer at pH 6 with and without the recombinant ncp PCP−R under otherwise identical conditions (Supporting Information). In both cases, the formation of the macrocyclic imine was detected in identical amounts (Figure S5). These results were significant as they demonstrated that the macrocyclization of the linear heptapeptide aldehydes was not influenced by recombinant ncp PCP−R. With this experiment we uncoupled the imine formation from the ncp R-mediated reductive release of the ncp aldehydes and showed that the cyclization reaction is completely enzyme independent. This led us to the conclusion that the offloading of the reactive peptide aldehyde by ncp R triggers the self-assembly of the macrocyclic imine. A prerequisite for this process would be that the free linear heptapeptide aldehyde can adopt a "productlike" conformation without the help of ncp PCP−R, so that the distant ends of the peptide aldehyde are in close proximity for cyclization. This hypothesis was further supported by an alanine scanning mutagenesis of the peptidyl substrates. We generated CoA-derivatives, in which one single amino acid at positions 2−6 of the ncp peptide sequence was substituted by alanine. Assaying revealed that Pro_6, Ser_5, and Ile_4 side chains were not crucial for imine self-assembly, whereas the replacement of $DGln_3$ and Gly_2 abolished macrocyclization almost completely (Table S3). This strongly suggests that Gly_2 may serve as a flexible hinge that allows the peptide to achieve a precyclic conformation. The amino acid with the highest polarity, $DGln_3$, within the ncp sequence is likely to be involved in hydrogen bonding with the peptide backbone, and thereby critically contributes to the preorganisation of the linear peptidyl substrate. According to previous studies on TE domain mediated peptide cyclization, we anticipated an important role for the C- and the N-terminal amino acids. However, ncp occurs in nature as a mixture of two compounds ncpA1 and ncpA2 that differ in the C-terminal position, which can be occupied by either phenylalanine or valine.[5] Despite these aromatic and aliphatic amino acids we incorporated threonine, lysine, and aspartate at this position. In additional peptidyl-CoA substrates phenylalanine was conserved at the C-terminus, while the N-terminal tyrosine was substituted with leucine, threonine, lysine, and aspartate. Remarkably, ncp R reduced all modified peptidyl-CoA substrates, although those carrying aspartate or lysine either at the C- or N-terminus were not capable of imine-cycle formation (Table S4). These findings give rise to speculations, whether electronic effects caused by these charged amino acids may influence the proper conformation of the linear heptapeptide, required for the molecular self-assembly.

The experimental data were accomplished by determining the kinetic rates for ncp PCP−R reductase activity spectrophotometrically (Table S5). Remarkably, ncp PCP−R was the first recombinant R domain excised from NRPSs that exhibited multiple turnover reaction in vitro.

In conclusion, we have demonstrated that ncp R triggers the self-assembly of a macrocyclic imine by the reductive release of a reactive peptide aldehyde. This distinguishes ncp R from all other known NRPS reductase domains that are engaged in the formation of linear peptide alcohols or amines.[7−11] Additionally, it became obvious that the intrinsic folding of the linear ncp is the basis for imine macrocyclization. NMR studies are currently on the way to further investigate the intriguing conformation of the linear aldehyde precursor.

Our results are significant as they elucidate this unique and novel type of peptide macrocyclization strategy. In general, the molecular self-assembly of complex NRPs may be more commonplace than originally thought. The antitumor antibiotic saframycin A is assumed to undergo a speculated intramolecular Schiff base reaction after the formation of a peptide aldehyde precursor.[12,13] It will be of interest to see whether this cyclization process is analogously triggered by a putative reductase domain.

Acknowledgment. We thank Dr. Uwe Linne for help with MS-experiments, as well as Katja Kräling and Johannes Ahrens for excellent experimental assistance. Funding was provided by DFG (M.A.M.) and the Fonds der Chemischen Industrie (F.K.).

Supporting Information Available: Experimental details and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Fischbach, M. A.; Walsh, C. T. *Chem. Rev.* **2006**, *106*, 3468−3496.
(2) Grunewald, J.; Marahiel, M. A. *Microbiol Mol. Biol. Rev.* **2006**, *70*, 121−146.
(3) Martinez-Bueno, M.; Maqueda, M.; Galvez, A.; Samyn, B.; Van Beeumen, J.; Coyette, J.; Valdivia, E. *J. Bacteriol.* **1994**, *176*, 6334−6339.
(4) Tang, Y. Q.; Yuan, J.; Osapay, G.; Osapay, K.; Tran, D.; Miller, C. J.; Ouellette, A. J.; Selsted, M. E. *Science* **1999**, *286*, 498−502.
(5) Kohli, R. M.; Walsh, C. T. *Chem. Commun.* **2003**, 297−307.
(6) Becker, J. E.; Moore, R. E.; Moore, B. S. *Gene* **2004**, *325*, 35−42.
(7) Ehmann, D. E.; Gehring, A. M.; Walsh, C. T. *Biochemistry* **1999**, *38*, 6171−6177.
(8) Gaitatzis, N.; Kunze, B.; Muller, R. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 11136−11141.
(9) Silakowski, B.; Kunze, B.; Nordsiek, G.; Blocker, H.; Hofle, G.; Muller, R. *Eur. J. Biochem.* **2000**, *267*, 6476−6485.
(10) Silakowski, B.; Nordsiek, G.; Kunze, B.; Blocker, H.; Muller, R. *Chem. Biol.* **2001**, *8*, 59−69.
(11) Schracke, N.; Linne, U.; Mahlert, C.; Marahiel, M. A. *Biochemistry* **2005**, *44*, 8507−8513.
(12) Pospiech, A.; Cluzel, B.; Bietenhader, J.; Schupp, T. *Microbiol.* **1995**, *141* (8), 1793−1803.
(13) Pospiech, A.; Bietenhader, J.; Schupp, T. *Microbiol.* **1996**, *142* (4), 741−746.

JA0667458