Translocation of a Thioether-Bridged Azurin Peptide Fragment via the Sec Pathway in *Lactococcus lactis*[▽]

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This study demonstrates for the first time that a thioether-containing peptide, an azurin fragment, can be translocated via the Sec pathway. This methyl-lanthionine was introduced by the nisin modification enzymes. The Sec pathway can therefore be a successful alternative for those cyclized peptides that are inefficiently transported via NisT.

Azurin, a cupredoxin produced by *Pseudomonas aeruginosa*, can selectively enter human cancer cells and induce apoptosis (24) via binding to the tumor suppressor protein p53 (1). The azurin peptide fragment p28, containing amino acids 50 to 77 (LSTAADMQGVVTDGMASGLDKDYLPDD), still enters human cancer cells and inhibits tumor proliferation (20). Importantly, novel cancer treatments can be based on azurin peptide fragments and derivatives thereof (T. Das Gupta and A. Chakrabarty, 20 March 2008, patent application WO2008033820). Although the pharmacokinetic property of therapeutic peptides is promising, lack of biostability is the major hurdle for their successful application. Consequently, it is very relevant to explore the possibilities for enhancing biostability of these peptides.

In our group, we developed a technology to improve the stability of therapeutic peptides by exploiting the nisin synthetase enzymes NisB and NisC for the introduction of thioether bridges. We applied a two-plasmid expression system (7, 8, 12), in which the NisBTC-encoding plasmid is compatible with the substrate-peptide-encoding plasmid. *Lactococcus lac-*

tis containing this expression system can secrete nonantibiotic peptides which are dehydrated or stabilized by a thioether ring (8, 16). NisB dehydrates serines and threonines in substrate peptides, NisC couples dehydrated residues stereo- and regio-selectively to cysteines, and NisT, the ABC transporter, translocates the modified peptides out of the cell (10, 11, 13, 15). The leader peptide is essential for targeting and modification of the propeptides (23).

When transport via NisT is impaired or is less efficient, the Sec pathway of *L. lactis* is a successful alternative in translocation of dehydrated peptides. When the nisin leader is preceded by a Sec signal peptide or a Tat signal peptide 27 or 44 amino acids long, respectively, modification by NisB and NisC still occurs (12; G. N. Moll, A. Kuipers, R. Rink, A. J. M. Driessen, and O. P. Kuipers, 15 June 2006, patent application WO 2006062398). However, NisC-cyclized prenisin was not translocated via the Sec system (12). This is likely due to the dimensions of fully modified nisin (3), which is too large to fit in the SecY pore (12, 21). Here, we report for the first time that the Sec pathway of *L. lactis* can translocate a p28 azurin fragment analog with a thioether ring.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics ^a	Reference
Strain		
NZ9000	MG1363 derivative; <i>pepN::nisRK</i> ⁺	5
Plasmids		
pIL253 derived		19
pIL3BTC	<i>nisBTC</i> cloned behind <i>P_{nis}</i> promoter; Cm ^r	17
pILBC	<i>nisBC</i> cloned behind <i>P_{nis}</i> promoter; Em ^r	12
pILB	<i>nisB</i> cloned behind <i>P_{nis}</i> promoter; Em ^r	12
pNZ8048 derived		14
pNZazu	<i>P_{nis}</i> + sequence encoding nisin leader fused to azurin(50–77) peptide fragment; Em ^r	This study
pNG41azu	<i>P_{nis}</i> + sequence encoding SP _{Usp45} fused to nisin leader fused to azurin(50–77) peptide fragment; Cm ^r	This study
pNG51azu	<i>P_{nis}</i> + sequence encoding SP _{YwbN} fused to nisin leader fused to azurin(50–77) peptide fragment; Cm ^r	This study

^a *P_{nis}* is the nisin-inducible *nisA* promoter; Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance. Boldface indicates mutations: S51A, M56C, and S66A in the azurin(50–77) peptide fragment (LATAADCQGVVADGMAAGLDKDYLPDD).

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We previously demonstrated that under culturing conditions, the highly reactive dehydroalanines can spontaneously couple to cysteines, either intra- or extracellularly, whereas the less reactive dehydrobutyrines do not (16). To exclude spontaneous thioether ring formation by dehydroalanines, we mutated serines in positions 51 and 66 to alanines, whereas a single dehydratable threonine was kept in position 52 of the azurin(50-77) peptide fragment. Position 56 was mutated to a cysteine to allow posttranslational introduction of a thioether bridge (Table 1; pNZ8048-derived plasmids).

When the azurin peptide fragment fused behind the nisin leader was coexpressed with the enzymes NisB, NisC, and NisT in *L. lactis*, no secreted (un)modified peptides in the supernatant were detected. Hence, we made use of the Sec pathway of *L. lactis* for export of the azurin peptide fragment. The nisin leader of the substrate peptide was preceded by the Sec signal peptide of Usp45, SP_{Usp45}. This fusion peptide was coexpressed with NisB and NisC in *L. lactis* in the absence of NisT. The strains and plasmids used are listed in Table 1. The culture of *L. lactis* NZ9000(pNG41azurin)(pILBC) was grown in minimal medium. Peptides from 4-ml induced cultures were isolated and purified with bond elute C₁₈ cartridges from Varian. Dissolved peptides were analyzed by mass spectrometry directly, as well as after incubation with TCEP [Tris(2-carboxyethyl)phosphine] and, subsequently, CDAP (1-cyano-4-dimethylamino-pyridinium tetrafluoroborate) (16). Mass spectra were recorded with a Voyager DE PRO matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometer in the linear mode.

We were able to detect Sec-secreted azurin peptides in the supernatant by mass spectrometry. Some of the dehydrated peptides in the supernatant contained disulfide-bonded cysteine adducts, meaning that not all of the formed dehydrobutyrines were coupled to the peptide's cysteines (data not shown). This observation of partial ring formation was confirmed by mass spectrometry. Peptides were first reduced with the phosphine TCEP and then alkylated with CDAP; formation of the thiocyanate results in a mass shift of +25 Da. (Fig. 1A). These data indicate that transport of an azurin peptide fragment with a thioether ring via the Sec pathway is possible, when preceded by SP_{Usp45} and the nisin leader.

As demonstrated before, replacement of SP_{Usp45} with a Tat signal peptide of YwbN from *Bacillus subtilis*, SP_{YwbN}, resulted in reduced transport efficiency and simultaneously enhanced the extent of dehydration of the substrate peptide (12). To examine the effect of reduced transport on the extent of NisC-mediated cyclization, SP_{YwbN} was fused N terminally to the nisin leader and azurin peptide fragment (Table 1) and coexpressed with NisB and NisC. Peptides in the supernatant of induced cultures were analyzed by mass spectrometry as described above and analyzed on a Tricine gel (18) by silver staining (Invitrogen). As a control, the substrate peptide was also coexpressed with only NisB. In the case of coexpression of NisB and NisC, the secreted peptides were almost fully dehydrated and no cysteinylations were seen (data not shown), suggesting that the peptides were fully ring closed. TCEP treatment and CDAP incubation (Fig. 1B) of the purified isolated peptides confirmed this observation. As expected, in the supernatant of the control which had only coexpression of NisB (pILB), fully dehydrated peptides were seen. No cyclization

FIG. 1. Transport of cyclized and dehydrated azurin peptide fragments via the Sec pathway. Culture supernatant was analyzed by matrix-assisted laser desorption/ionization–time of flight mass spectrometry. The expected mass of the processed, protonated, and fully dehydrated fusion peptide (STKDFNLDLVSVSKKDSGASPR-LAT AADCQGVVADGMAAGLDKDYLPD) is 5,141 Da. (A) Supernatant of *L. lactis* NZ9000(pILBC)(pNG41azu), with the fusion peptide preceded by SP_{Usp45}. (B) Supernatant of *L. lactis* NZ9000(pILBC)(pNG51azu), with the fusion peptide preceded by SP_{Usp45}. (C) Supernatant of control *L. lactis* NZ9000(pILB)(pNG51azu).

took place, and therefore all of the free cysteines reacted with CDAP (Fig. 1C). This control experiment clearly demonstrated that no spontaneous cyclization had occurred in the absence of NisC. Hence, the thioether bridge formation observed in the experiments presented in Fig. 1A and B should result from intracellular NisC-mediated cyclization. The data therefore convincingly prove that indeed this intrinsically stable thioether-bridged peptide is transported via the Sec system.

Analyses on silver-stained gel showed that no transport at all

FIG. 2. Amount of secreted peptides in the supernatant. Peptides from 2 ml of supernatant of induced cultures were applied to a gel. Lanes: M, kaleidoscopic marker (Bio-Rad); 1, NZ9000(pIL3BTC)(pNZazu); 2, NZ9000(pIL3BC)(pNG41azu); 3, NZ9000(pIL3BC)(pNG51azu).

via the transporter NisT had occurred when the azurin peptide fragment was preceded by only the nisin leader (Fig. 2, lane 1). In full contrast, transport of the modified azurin peptide fragment via the Sec pathway was successful. The transport of the peptide fragment was less efficient when SP_{YwbN} was used instead of SP_{Usp45} (Fig. 2), which is in full agreement with previous data (12).

These data demonstrate for the first time that a peptide with an intramolecular thioether bridge can be translocated in vivo via the Sec pathway of *L. lactis*. A Sec system that has been studied in more detail is that of *Escherichia coli*. The Sec translocase in the membrane is composed of a highly conserved protein-conducting channel, SecYEG (6). The Sec translocase transports unfolded proteins, which is driven by the ATPase SecA. Homologues of SecYEG and SecA are also found in *L. lactis* (2, 9). In vitro studies demonstrated that the translocon SecYEG of *E. coli* can also translocate the polypeptide proOmpA with a disulfide-bridge, which can have a loop of 18 amino acids or smaller (22). Other in vitro data demonstrated that proOmpA labeled with bulky fluorescent probes, assessing up to 16 Å, was also transported (4). These in vitro data with the Sec system of *E. coli* suggested that the SecY translocon is not that rigid and could be used for other purposes, such as the in vivo translocation in *L. lactis* of peptides with thioether bridges. Interestingly, the efficiency of translocation in *E. coli* can be drastically enhanced by *priA* (*secY*) mutations (6). Likely, in the near future such mutations in the SecY translocon of *L. lactis* can contribute to an even more successful application of the Sec pathway for transport of therapeutic peptides with thioether bridges.

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