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Engineering Dehydrated Amino Acid Residues in the Antimicrobial Peptide Nisin*

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The small antimicrobial peptide nisin, produced by *Lactococcus lactis*, contains the uncommon amino acid residues dehydroalanine and dehydrobutyrine and five thio ether bridges. Since these structures are post-translationally formed from Ser, Thr, and Cys residues, it is feasible to study their role in nisin function and biosynthesis by protein engineering. Here we report the development of an expression system for mutated nisin Z (*nisZ*) genes, using nisin A producing *L. lactis* as a host. Replacement by site-directed mutagenesis of the Ser-5 codon in *nisZ* by a Thr codon, led to a mutant with a dehydrobutyrine instead of a dehydroalanine residue at position 5, as shown by NMR. Its antimicrobial activity was 2–10-fold lower relative to wild-type nisin Z, depending on the indicator strain used. In another mutagenesis study a double mutation was introduced in the *nisZ* gene by replacing the codons for Met-17 and Gly-18 by codons for Gln and Thr, respectively, as in the third lanthionine ring of the related antimicrobial peptide subtilin from *Bacillus subtilis*. This resulted in the simultaneous production of two mutant species, one containing a Thr residue and the other containing a dehydrobutyrine residue at position 18, both having different bacteriocidal properties.

Nisin (Gross and Morell, 1971; Hurst, 1981), produced by several *Lactococcus lactis* strains, is a prominent member of the group of lanthionine and/or β-methyllanthionine containing peptides, called lantibiotics (Schnell *et al.*, 1988). Other members of this group include subtilin (Gross *et al.*, 1973), epidermin (Allgaier *et al.*, 1986), pep5 (Sahl and Brandis, 1981), lacticin 481 (Piard *et al.*, 1992), ancovenin (Wakamiya *et al.*, 1985), and duramycin (Gross and Brown, 1976; Fredenhagen *et al.*, 1991). Lantibiotics are small-sized (<4 kDa) antimicrobial peptides which are active against a broad range of Gram-positive bacteria. Nisin is used as a natural preservative in the food industry (Delves-Broughton, 1990). Two natural variants of nisin have been found, *i.e.* nisin A and nisin Z (Fig. 1*A*), harboring at position 27 a His and an Asn residue, respectively. The structural genes encoding nisin A and nisin Z have been cloned and named *nisA* (Buchman *et al.*, 1988; Kaletta and Entian, 1989; Dodd *et al.*, 1990; Rauch and de Vos, 1992) and *nisZ* (Mulders *et al.*, 1991; Kuipers *et al.*, 1991b).

In lantibiotics the uncommon amino acid residues dehydroalanine (Dha)[1] and dehydrobutyrine (Dhb) (Fig. 1*B*) are formed post-translationally from Ser and Thr residues, respectively. Presumably, these dehydration reactions are enzyme-catalyzed. Subsequent intramolecular stereospecific addition of cysteine thiol groups to the double bonds of Dha or Dhb results in the formation of (2*S*,6*R*)-lanthionine (lanthionine) or (2*S*,3*S*,6*R*)-3-methyllanthionine (β-methyllanthionine), respectively (Fig. 1*B*). The three-dimensional structure of nisin A in aqueous solution has been studied by NMR techniques, showing that the nisin A molecule is quite flexible and that the fragment from residues 3 to 19 has a strongly amphiphilic character (van de Ven, *et al.*, 1991; Lian *et al.*, 1992). The structure, function, and biosynthesis of lantibiotics have recently been reviewed (Jung, 1991).

The mode of action of lantibiotics is still not fully elucidated. However, several studies on different lantibiotics including nisin have indicated that these compounds can form voltage-dependent channels in membranes, thereby dissipating the membrane potential and causing the efflux of cellular constituents (Sahl, 1991; Benz *et al.*, 1991; Gao *et al.*, 1991). The function of lanthionines and dehydrated amino acid residues in lantibiotics is not clear yet. The lanthionines might be important for the preservation of a local rigid structure, which could be a prerequisite for efficient pore formation in membranes. It has been suggested that lanthionines also contribute to the thermostability of nisin (Hurst, 1981). Dehydrated amino acid residues in lantibiotics could contribute to the antimicrobial activity by their ability to react with free sulfhydryl groups of bacterial cell-wall proteins (Morris *et al.*, 1984; Liu and Hansen, 1990). Another hypothesis is that dehydrated residues induce specific conformations in the backbone of the precursor peptide during biosynthesis, thus allowing subsequent thio ether bridge formation.

In this paper a protein engineering strategy is presented which enables the production of mutant nisin Z species by site-directed mutagenesis. For this purpose, a plasmid-free *L. lactis* strain which harbored the nisin-sucrose transposon Tn*5276* (Rauch and de Vos, 1992) and produced nisin A was used as a host for expression plasmids containing the *nisZ* gene. This strain was chosen to ensure both nisin immunity and the presence of all proteins involved in the biosynthesis of nisin, *i.e.* dehydration, thio ether bridge formation, proc-

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[1] The abbreviations used are: Dha, dehydroalanine; Dhb, dehydrobutyrine; NOESY, nuclear Overhauser effect spectroscopy; RP-HPLC, reversed phase-high performance liquid chromatography; PCR, polymerase chain reaction.

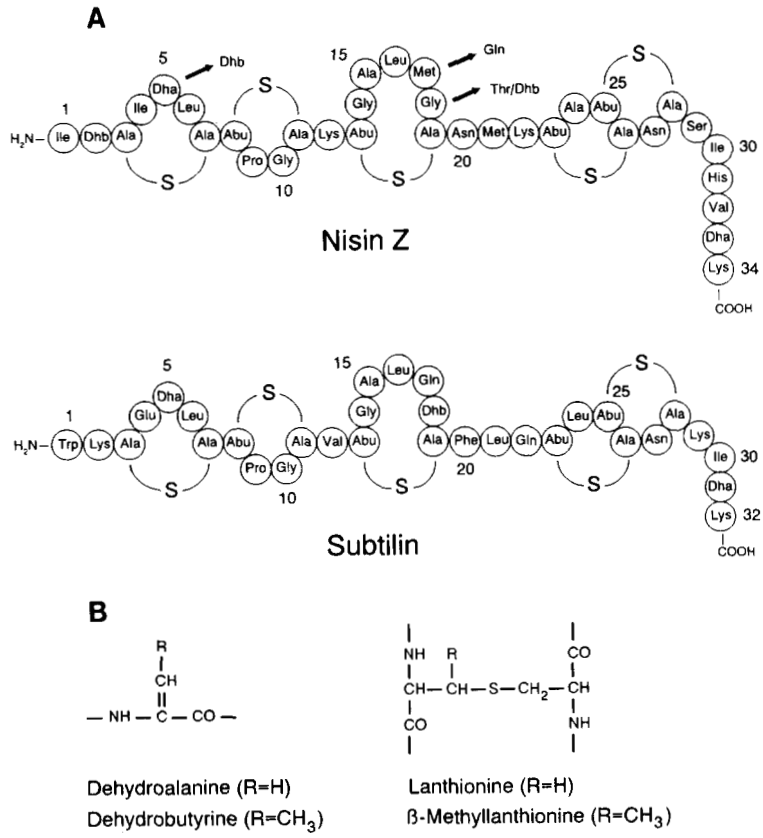

FIG. 1. *Panel A*, primary structures of nisin Z and subtilin. The *arrows* indicate the mutations which are described in this paper. *Panel B*, structures of dehydrated amino acids and lanthionine residues.

essing, and secretion. The presence of the expression plasmid harboring *nisZ* in the *L. lactis* strain described resulted in the simultaneous production of nisin A and nisin Z, which were fully separated by reversed phase HPLC.

Since it has been reported that residue Dha-5 is of great importance for biological activity in both nisin and subtilin (Morris *et al.*, 1984; Chan *et al.*, 1989a; Rollema *et al.*, 1991), we decided to investigate the role of Dha-5 in nisin in more detail. For this purpose a mutant *nisZ* gene was produced by site-directed mutagenesis, in which the codon for Ser-5 was changed to a codon for Thr. In an attempt to introduce a fourth dehydrated residue in nisin Z, the third ring of the homologous lantibiotic subtilin (Fig. 1*A*), which contains a Dhb at position 18, was mimicked in nisin Z, by simultaneous replacement of the codons for Met-17 and Gly-18 by the codons for Gln and Thr, respectively. The secreted mutant nisin Z species were purified to homogeneity, studied by two-dimensional NMR techniques to investigate their primary structures, and further characterized by determining their antimicrobial activities.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Growth Conditions—*Escherichia coli* MC1061 (Casadaban *et al.*, 1980) was used as intermediate host for cloning and handled as described (Sambrook *et al.*, 1989). *L. lactis* T165.5 containing a single copy of the nisin-sucrose transposon Tn*5276* encoding nisin A production (Rauch and de Vos, 1992) was used to express mutated *nisZ* genes. Nisin Z producing *L. lactis* NIZO 22186 (Mulders *et al.*, 1991) was used as source for the *nisZ* gene. Plasmid pNZ123 is a polylinker-containing derivative of pNZ12 based on the heterogrametic replicon of pSH71 (de Vos, 1987). *L. lactis* strains were grown in M17 broth (Difco) supplemented with 0.5% of glucose. If appropiate, media contained 10 μg/ml chloramphenicol. For isolation of wild-type or mutant nisin, *L. lactis* strains were grown without aeration at 30 °C in a medium containing 1% sucrose, 1% peptone (Difco), 1% yeast extract (Difco), 0.2% NaCl, 0.002% $MgSO_4 \cdot 7H_2O$, and 1% KH_2PO_4. During 10-liter fermentations the pH was maintained at 6.0 by pH-stat control using a 10 M solution of NaOH.

Site-directed Mutagenesis, DNA Manipulation, and DNA Sequence Analysis—Sequencing of purified DNA fragments was performed by the dideoxy chain termination method (Sanger *et al.*, 1977). Site-directed mutagenesis and PCR were performed using the following primers: 1) 5′-GATTAAATTCTGCAGTTTGTTAG-3′ (*Pst*I); 2) 5′-CCCTAAAAAGCTTATAAAAATAGG-3′ (*Hin*dIII); 3) 5′-CAACCCATCAGAGCTCCTGTT-3′ (*Sst*I); 4) 5′-AACAGGAGCTCTGCAGACTTGTAACATGAA-3′ (*Sst*I, M17Q/G18T); and 5) 5′-GCATTACAAGTATTACACTATGTACACCCGG-3′ (S5T). Sites of mutation are in *italics* and restriction enzyme cleavage sites used in cloning as well as codon changes are indicated in parentheses. All mutations were made in the *nisZ* gene (Mulders *et al.*, 1991). For PCR reactions approximately 100 ng of bacterial chromosomal DNA was used in a total volume of 50 μl, containing 1 unit of Taq polymerase (Bethesda Research Laboratories), 50 mM NaCl, 10 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$, 10 μg of gelatin, 200 μM of each dNTP, 10 pmol of each primer, 2.5 μl of stabilizer (1% W-1, Bethesda Research Laboratories), and covered with 100 μl of light mineral oil. PCR was performed in 30 cycles, each cycle consisting of a denaturing step at 93 °C for 1 min, a primer annealing step at 54 °C for 1.5 min, and an extension step at 72 °C for 2.5 min. The DNA fragments were obtained by agarose gel electrophoresis followed by excision of the DNA fragments and purification by the Gene-Clean procedure (Bio 101, La Jolla, CA). The 280-base pair PCR fragment, containing the *nisZ* gene, was obtained using primer 1 and 2 with total DNA from *L. lactis* NIZO 22186 as a template. Other PCR fragments were made with primers 1 and 3 and primers 2 and 4, respectively; after digestion of the PCR fragments with the enzymes indicated, 0.5 μg of each fragment was ligated with 0.2 μg of pNZ123, digested with *Eco*RI and *Hin*dIII, and 0.5 μg of an *Eco*RI-*Pst*I fragment containing the *lac*-promoter of the lactose-operon of *L. lactis*, derived from pNZ3003 (Van Rooijen *et al.*, 1992). This ligation mixture was used to transform *E. coli* MC1061. Mutation of residue 5 was performed with the method described previously (Kuipers *et al.*, 1991a). Chloramphenical-resistant transformants were sequenced to confirm the mutations and the integrity of the *nisZ* gene. The desired plasmids were isolated and introduced by electroporation into *L. lactis* T165.5 (Rauch and de Vos, 1992). Transformants were analyzed again for sequence integrity of the mutant *nisZ* gene.

Purification of (Mutant) Nisin Species—Nisin species were purified from 10 liters of culture supernatants of producing strains, which were grown for 7 h. Purification of the nisin species using Fractogel TSK butyl 650-S (Merck) column chromatography followed by preparative RP-HPLC was performed essentially as described previously (Mulders *et al.*, 1991). Preparative RP-HPLC was performed at 30 °C using a 250 × 21.5-mm Hi-Pore RP-318 column (Bio-Rad). Nisin components were eluted by a linear gradient from 23 to 28% buffer B (90% aqueous acetonitril, 0.07% trifluoroacetic acid) in buffer A (10% aqueous acetonitril, 0.1% trifluoroacetic acid) during 50 min using a flow rate of 10 ml/min. The absorbance of the effluent was monitored at 220 nm. Absolute amounts of nisin were estimated using the specific absorbance at 220 nm of 21 mg^{-1} ml cm^{-1}, obtained for nisin A by relating the absorbance at 220 nm to the nisin concentration determined by ^1H NMR spectroscopy.

NMR Experiments—^1H NMR spectra were taken at 400.13 MHz on a Bruker AM400 spectrometer interfaced to an Aspect 3000 computer. The nisin species were dissolved in 10% D_2O·90% H_2O, pH 3.5, at concentrations of 2–5 mM and all spectra were recorded at 25 °C. The solvent resonance was suppressed by low power preirradiation. The spectra were referenced to external 3-(trimethylsilyl)-propionic 2,2,3,3-d_4 acid. Total correlation spectroscopy spectra were taken using a MLEV-17 mixing sequence with durations of 20 and 70 ms. NOESY spectra were recorded with a mixing time of 400 ms. In the latter experiments the solvent resonance was also irradiated during the mixing time in order to maintain saturation. All two-dimensional spectra were recorded in phase-sensitive mode using time proportional phase incrementation. In all experiments 512 T_1 increments, each with 2048 data points, were used. Data processing was performed either on a Bruker X32 or a SUN workstation using UXNMR and NMR2 software, respectively. Cosine-bell squared windows were used in f_2; in f_1 cosine-bell windows were used in combination with zero filling. The final digital resolution was approximately 5 Hz/point in f_2 and 10 Hz/point in f_1.

Determination of Antimicrobial Activities—Antimicrobial activities were determined using a bioassay with *Micrococcus flavus* DSM 1790 (obtained from the German Collection of Microorganisms, Braunschweig, Germany), *Bacillus cereus* P7 (NIZO collection), or *Streptococcus thermophilus* R_s (NIZO collection) as indicator strains. Determination of minimal inhibitory concentration values was performed in the following way. Overnight cultures of indicator organisms were diluted to an $A_{600\,nm}$ of 0.05 (*M. flavus*) or 0.025 (*S. thermophilus* and *B. cereus*), and were divided into 10 aliquots of 5 ml. These tubes were inoculated with different amounts of (mutant) nisin Z and were incubated under the following conditions: *M. flavus*, aerobic with shaking at 30 °C for 8 h; *S. thermophilus*, aerobic without shaking at 37 °C for 3 h; *B. cereus*, aerobic with shaking at 30 °C for 6 h. Subsequently, the $A_{600\,nm}$ of the contents of each tube were measured. Without nisin addition, the final $A_{600\,nm}$ values were about 1.2 for *M. flavus*, 0.5 for *S. thermophilus*, and 0.8 for *B. cereus*. All experiments were performed in triplicate. Inhibition curves were made by plotting $A_{600\,nm}$ at the end of incubation *versus* nisin concentration. The minimal inhibitory concentration values were determined from the inhibition curves by interpolation. The lowest concentration of nisin at which less than 1% of the total increase in the $A_{600\,nm}$, measured in the absence of nisin, had occurred, was taken as the minimal inhibitory concentration value.

RESULTS AND DISCUSSION

Development of an Expression System for (Mutated) nisZ Genes—A plasmid was constructed by ligation of a 280-base pair PCR fragment containing the *nisZ* gene, together with a fragment containing the *lac*-promoter of the lactose-operon of *L. lactis* (Van Rooijen *et al.*, 1992) in the vector pNZ123. The resulting plasmid, pNZ9013 (Fig. 2), can be used to transform both *E. coli* and *L. lactis* strains and was used to transform the nisin A producing strain *L. lactis* T165.5 (Rauch and de Vos, 1992). The resulting transformant secreted both nisin A and nisin Z, which can be fully separated by RP-HPLC (Mulders *et al.*, 1991). The total nisin yield from 10-liter cultures with strain T165.5, harboring pNZ9013, was about 30 mg/liter, whereas strain T165.5 without this plasmid produced about 10 mg of nisin A/liter. This implies that the expression system already gives a 3-fold higher production of nisin. This system was used for the expression of site-directed mutants of nisin Z.

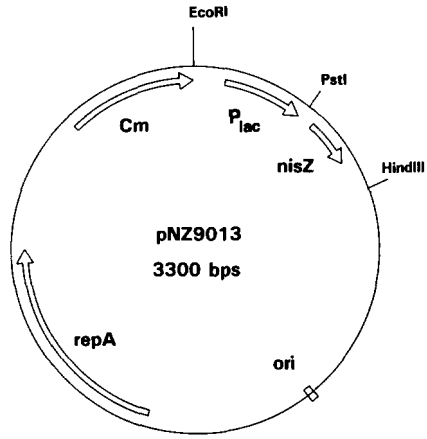

FIG. 2. **Schematic map of plasmid pNZ9013, showing the restriction sites used for construction, the *lac*-promoter region, and the *nisZ* structural gene.** *bps*, base pairs.

Production and Structural Characterization of the Dha5Dhb, M17Q/G18T, and M17Q/G18Dhb Nisin Z Species—Mutant nisin Z species were produced by recombinant strains in a 10-liter fermentation. Yields of mutant nisin Z species, relative to the nisin A produced by the same strain, varied greatly, ranging from 15% for Dha5Dhb nisin Z and M17Q/G18T nisin Z to 150% for M17Q/G18Dhb nisin Z. The low yield of two of the mutants could be due to several phenomena, such as a lower biosynthesis rate, a less efficient processing, or reduced secretion of the mutant peptide. Final purification of the mutant nisins was achieved by RP-HPLC. All mutant nisin Z species showed shorter retention times than nisin A, whereas native nisin Z eluted after nisin A.

To verify the mutation(s) structurally and to establish the degree of post-translational processing, the mutant nisin species were examined by two-dimensional NMR techniques and compared to nisin Z. For that purpose total correlation spectroscopy and NOESY spectra of the mutants were recorded. The assignment of the spectra was essentially as described for other nisin species (Chan *et al.*, 1989b; Slijper *et al.*, 1989; Rollema *et al.*, 1991): the TOCSY data were used for amino acid pattern recognition; sequential assignment was based on short range cross-peaks observed in the NOESY spectra. Finally the lanthionine residues were characterized by their β-β and β-γ connectivities in the NOESY spectrum.

The assignment of the spectrum of nisin Z is given in Table I. Comparison of the assignment of nisin Z with that of nisin A (Chan *et al.*, 1989b; Slijper *et al.*, 1989; Lian *et al.*, 1992) shows that, apart from the residues close to position 27, the spectra of both nisin species are very similar. This suggests that there are no major differences in the global structures of nisin A and nisin Z. The chemical shifts of residues of the mutants, differing appreciably from those of nisin Z, are presented in Table II. For all mutants a correct and complete formation of thio ether bridges and unsaturated amino acids was observed, indicating that the post-translational modification process was not affected by the mutations. For the mutant in which the codon for Ser-5 was changed to a codon for Thr, complete dehydration of the Thr residue at position 5 had occurred; the codon replacements M17Q and G18T resulted in the simultaneous production of a minor amount (~10%) of a nisin Z species with an unprocessed Thr-18, and a major fraction (~90%) containing a Dhb at position 18 (Fig. 3). The engineered Dhb residues at position 5 and 18 appeared to have the same stereochemistry as Dhb-2 in native nisin. In

TABLE I

Assignment of the (400 MHz) ^1H NMR spectrum of nisin Z

Experimental conditions were 10% D_2O, 90% H_2O, pH 3.5, 25 °C. Amino acid nomenclature is according to IUPAC-IUB recommendations (*Eur. J. Biochem* (1984) 138, 9–37). Ala* and Abu* represent the alanyl- and β-methylalanyl- moieties of lanthionines. Dehydroalanine and dehydrobutyrine are denoted by Dha and Dhb, respectively.

Residue	Chemical shift			
	$N^{\alpha}H$	$C^{\alpha}H$	$C^{\beta}H$	Other
			ppm	
Ile-1		4.13	2.13	$C^{\gamma}H_2$ 1.33, 1.57 $C^{\beta'}H_3$ 1.11 $C^{\delta}H_3$ 1.01
Dhb-2			6.63	$C^{\gamma}H_3$ 1.84
Ala*-3	8.24	4.63	3.14, 3.29	
Ile-4	7.85	4.35	2.10	$C^{\gamma}H_2$ 1.19, 1.38 $C^{\beta'}H_3$ 0.96 $C^{\delta}H_3$ 0.87
Dha-5	9.88		5.46, 5.57	
Leu-6	8.95	4.43	1.75	$C^{\gamma}H$ 1.68 $C^{\delta}H_3$, $C^{\delta'}H_3$ 0.91, 0.97
Ala*-7	8.24	4.54	2.95, 3.06	
Abu*-8	8.88	5.12	3.61	$C^{\gamma}H_3$ 1.33
Pro-9		4.44	1.83, 2.47	C^4H 1.96, 2.18 C^5H 3.46
Gly-10	8.66	3.59, 4.43		
Ala*-11	7.93	4.02	3.04, 3.67	
Lys-12	8.63	4.35	1.82	$C^{\gamma}H_2$ 1.40, 1.52 $C^{\delta}H_2$ 1.71 $C^{\epsilon}H_2$ 3.01 $N^{\zeta}H_3^+$ 7.56
Abu*-13	8.33	4.65	3.63	$C^{\gamma}H_3$ 1.34
Gly-14	8.32	4.08, 4.15		
Ala-15	8.58	4.24	1.48	
Leu-16	8.48	4.32	1.76	$C^{\gamma}H$ 1.67 $C^{\delta}H_3$, $C^{\delta'}H_3$ 0.92
Met-17	7.82	4.66	2.13, 2.29	$C^{\gamma}H_2$ 2.47, 2.64 $C^{\epsilon}H_3$ 2.10/2.12[a]
Gly-18	8.11	3.85, 4.12		
Ala*-19	7.70	4.52	2.99	
Asn-20	8.56	4.70	2.83	$N^{\gamma}H_2$ 6.94, 7.61
Met-21	8.28	4.52	2.02, 2.14	$C^{\gamma}H_2$ 2.53, 2.62 $C^{\epsilon}H_3$ 2.10/2.12[a]
Lys-22	8.42	4.32	1.86	$C^{\gamma}H_2$ 1.45, 1.51 $C^{\delta}H_2$ 1.73 $C^{\epsilon}H_3$ 3.03 $N^{\zeta}H_3^+$ 7.56
Abu*-23	8.81	4.99	3.59	$C^{\gamma}H_3$ 1.36
Ala-24	8.22	4.68	1.47	
Abu*-25	9.15	4.84	3.56	$C^{\gamma}H_3$ 1.46
Ala*-26	7.89	3.98	2.82, 3.73	
Asn-27	8.66	4.98	2.73,2.91	$N^{\gamma}H_2$ 6.91, 7.62
Ala*-28	7.80	4.36	2.80, 3.66	
Ser-29	8.44	4.49	3.87	
Ile-30	8.06	4.18	1.84	$C^{\gamma}H_2$ 1.16, 1.38 $C^{\beta'}H_3$ 0.86 $C^{\delta}H_3$ 0.86
His-31	8.63	4.78	3.19, 3.26	C^2H 8.62 C^5H 7.29
Val-32	8.31	4.19	2.10	$C^{\gamma}H_3$, $C^{\gamma'}H_3$ 0.98
Dha-33	9.68		5.74	
Lys-34	8.22	4.33	1.82, 1.92	$C^{\gamma}H_2$ 1.42 $C^{\delta}H_2$ 1.72 $C^{\epsilon}H_2$ 3.03 $N^{\zeta}H_3^+$ 7.56

[a] The sequential assignment of the $C^{\epsilon}H_3$ resonances was not feasible because of their small difference in chemical shift.

all cases strong $N^{\alpha}H - C^{\gamma}H_3$ and weak $N^{\alpha}H - C^{\beta}H$ intraresidue NOESY cross-peaks were observed. In the case of Dhb-5 the amide proton of Leu-6 showed a strong connectivity to the β-proton and a weak connectivity to the γ-protons of Dhb-5. These observations are characteristic of a Z-conformation of the dehydrobutyrine residue, *i.e.* the $-C^{\gamma}H_3$ group is directed towards the N-terminal side of the residue. Apparently the dehydration of Thr residues occurs in a stereospecific way. Table II shows that the Dha5Dhb substitution has an effect on the chemical shifts of only Leu-6. In this mutant there are apparently little changes in the overall structure of the molecule, which is not surprising considering the nature of this substitution. In contrast, the mutations at positions 17 and 18 considerably affect chemical shifts of residues in a relatively large segment of the molecule, comprising residues 10–21. In the case of M17Q/G18T nisin Z, effects on residues 3, 7, and 9 are also observed. Obviously, these substitutions at positions 17 and 18 have a distinct effect on the overall structure of nisin Z. Surprisingly, this effect is confined to a smaller segment of the molecule when a Dhb residue is present at position 18, although the planar configuration of the C^{α} atom of this residue would be expected to cause more alterations in the polypeptide backbone of the third ring of nisin than the presence of a Thr residue.

For nisin Z and the mutants described here, relatively few long range NOESY connectivities were observed. Although some differences between the various species were observed in this respect, no indications for major structural differences between the molecules were found. However, it should be realized that a complete structural characterization of the mutants would require more experimental data and a quantitative evaluation of the NOESY data. Finally, it is interesting to note that for residues 13–19 in M17Q/G18Dhb nisin Z, chemical shifts very similar to those of the corresponding residues in subtilin are observed (Chan *et al.*, 1992). Apparently, in this mutant nisin Z the conformation of the third ring is very similar to that in subtilin.

Functional Properties of Dha5Dhb Nisin Z—The fact that the change of the Ser-5 codon to a Thr codon resulted in a nisin Z mutant harboring a Dhb instead of a Dha residue (Fig. 3) demonstrates that either Ser or Thr at position 5 can be dehydrated. Moreover, it indicates that the nisin A producer is also immune to Dha5Dhb nisin Z. Dehydration of the residue at position 5 in nisin might be a prerequisite for subsequent biosynthetic processes. The planar geometry of the C^{α} atom of residue 5 induces a particular conformation of the peptide backbone and may thereby facilitate the specific thio ether bridge formation between residues Dha-3 and Cys-7. The latter hypothesis is supported by our observation that a change of the Ser-5 codon into an Ala codon in the *nisZ*

TABLE II

Assignment of the ^1H NMR spectra of the mutants of nisin Z

Only those residues are shown which exhibit chemical shift values differing more than 0.05 ppm from those of the native nisin Z species. Experimental conditions: 10% D_2O, 90% H_2O, pH 3.5, 25 °C. Amino acid nomenclature as in Table I.

Residue	Chemical shift			
	N^nH	C^nH	$C^\beta H$	Other
			ppm	
Dha5Dhb nisin Z				
Dhb-5	9.90		6.25	$C^\gamma H_3$ 1.78
Leu-6	8.59	4.47	1.72	$C^\gamma H$ 1.70 $C^\delta H_3/C^{\delta'} H_3$ 0.90, 0.96
M17Q/G18T nisin Z				
Ala*-3	8.23	4.70	3.13, 3.29	
Ala*-7	8.26	4.63	2.95, 3.04	
Pro-9		4.42	1.82, 2.46	C^4H 1.96, 2.13 C^5H 3.45
Gly-10	8.78	3.67, 4.37		
Ala*-11	8.07	3.97	3.03, 3.64	
Lys-12	8.61	4.43	1.80, 1.86	$C^\gamma H_2$ 1.46 $C^\delta H_2$ 1.71 $C^\epsilon H_2$ 3.02 $N^\epsilon H_3^+$ 7.66
Abu*-13	8.22	4.72	3.54	$C^\gamma H_3$ 1.31
Gly-14	8.41	3.99, 4.12		
Ala-15	8.43	4.29	1.45	
Leu-16	8.35	4.33	1.81	$C^\gamma H$ 1.68 $C^\delta H_3$, $C^{\delta'} H_3$ 0.91, 0.96
Gln-17	8.16	4.24	2.18	$C^\gamma H_2$ 2.40 $N^\delta H_2$ 6.89, 7.55
Thr-18	7.82	4.37	4.37	$C^\gamma H_3$ 1.23
Ala*-19	8.27	4.65	3.04	
Asn-20	8.35	4.60	2.80, 2.88	$N^\gamma H_2$ 6.90, 7.58
Met-21	8.19	4.51	2.04, 2.14	$C^\gamma H_2$ 2.55, 2.61 $C^\epsilon H_3$ 2.11
M17Q/G18Dhb nisin Z				
Gly-10	8.74	3.65, 4.38		
Ala*-11	7.99	4.00	3.05, 3.66	
Lys-12	8.55	4.47	1.80	$C^\gamma H_2$ 1.40, 1.49 $C^\delta H_2$ 1.68 $C^\epsilon H_2$ 3.02 $N^\epsilon H_3^+$ 7.57
Gly-14	8.36	4.05, 4.27		
Ala-15	8.65	4.20	1.46	
Leu-16	8.57	4.27	1.79	$C^\gamma H$ 1.68 $C^\delta H_3$, $C^{\delta'} H_3$ 0.94
Gln-17	7.72	4.46	2.24	$C^\gamma H_2$ 2.42 $N^\delta H_2$ 6.82, 7.52
Dhb-18	8.69		6.96	$C^\gamma H_3$ 1.78
Ala*-19	7.60	4.51	2.95	
Met-21	8.22	4.54	2.03, 2.14	$C^\gamma H_2$ 2.52, 2.61 $C^\epsilon H_3$ 2.11

gene did not yield any detectable amount of secreted mutant nisin Z.[2]

The Dhb-5 mutant displayed 2–10-fold lower bacteriocidal activities than nisin Z towards three different indicator strains (Table III), confirming the important function of Dha-5 in nisin. It is very likely that the replacement of Dha-5 by Dhb will not result in a large conformational change of the first ring, which is also confirmed by the NMR data. It might be speculated that the additional methyl group of the Dhb residue affects the interaction of the nisin molecules with the bacterial cell membrane during pore formation. However, the most likely explanation for the reduced activity is that the reactive double bond of Dha-5 is crucial for the mechanism of nisin action, since the less reactive double bond of Dhb would then indeed be expected to yield a lower bacteriocidal capacity. This hypothesis is supported by the results of studies on nisin degradation products, which showed that saturation of the double bond in Dha-5 leads to a dramatic reduction in bacteriocidal activity of nisin (Chan *et al.*, 1989a; Rollema *et al.*, 1991; Lian *et al.*, 1992).

Functional Properties of M17Q/G18T and M17Q/G18Dhb Nisin Z—The replacement of the codons for Met-17 and Gly-18 in *nisZ* by codons for Gln and Thr, respectively, resulted in the production of two mutant species both displaying antimicrobial activities. Characterization of these two species by ^1H NMR spectroscopy (see above) showed that the main mutant produced contained a Dhb residue at position 18, as in subtilin, whereas a smaller amount of mutant nisin Z contained an unmodified Thr residue at this position (Fig. 3). This result demonstrates first that although some of the mutant nisin molecules escape dehydration at position 18, this does not prevent their further maturation and secretion. Second, although nisin A producing *L. lactis* strains are sensitive to subtilin, the introduction of the third ring of subtilin into nisin Z obviously does not make this nisin-subtilin hybrid lethal to *L. lactis*. The antimicrobial activity of subtilin against nisin-producing *L. lactis* must therefore reside in some other part of the subtilin molecule. The Dhb-18 (and Gln-17) containing species showed similar activities as nisin Z towards three different indicator strains (Table III), indicating that an additional dehydrated residue at position 18 in nisin Z does not enhance or reduce antimicrobial activity. On the other hand the nisin mutant containing Thr-18 (and Gln-17) was about 4-fold less active against two of the indicator strains but twice as active against *M. flavus* than wild-type nisin Z. This result clearly shows that by genetic engineering the antimicrobial activity of nisin can be modulated, depending on the choice of target organism. The change in relative sensitivities of these strains to the Thr-18 containing mutant could be due to either differences in membrane phospholipid composition of the three indicator strains, or to different membrane potentials. The substitutions at positions 17 and 18 appear to influence the global structure of nisin appreciably, which may also contribute to the altered activity spectrum.

Conclusion—Our results show that it is possible (i) to overproduce wild-type and mutant nisins in *L. lactis*, (ii) to introduce or exchange dehydrated residues in nisin by protein

[2] O. P. Kuipers and H. J. Boot, unpublished results.

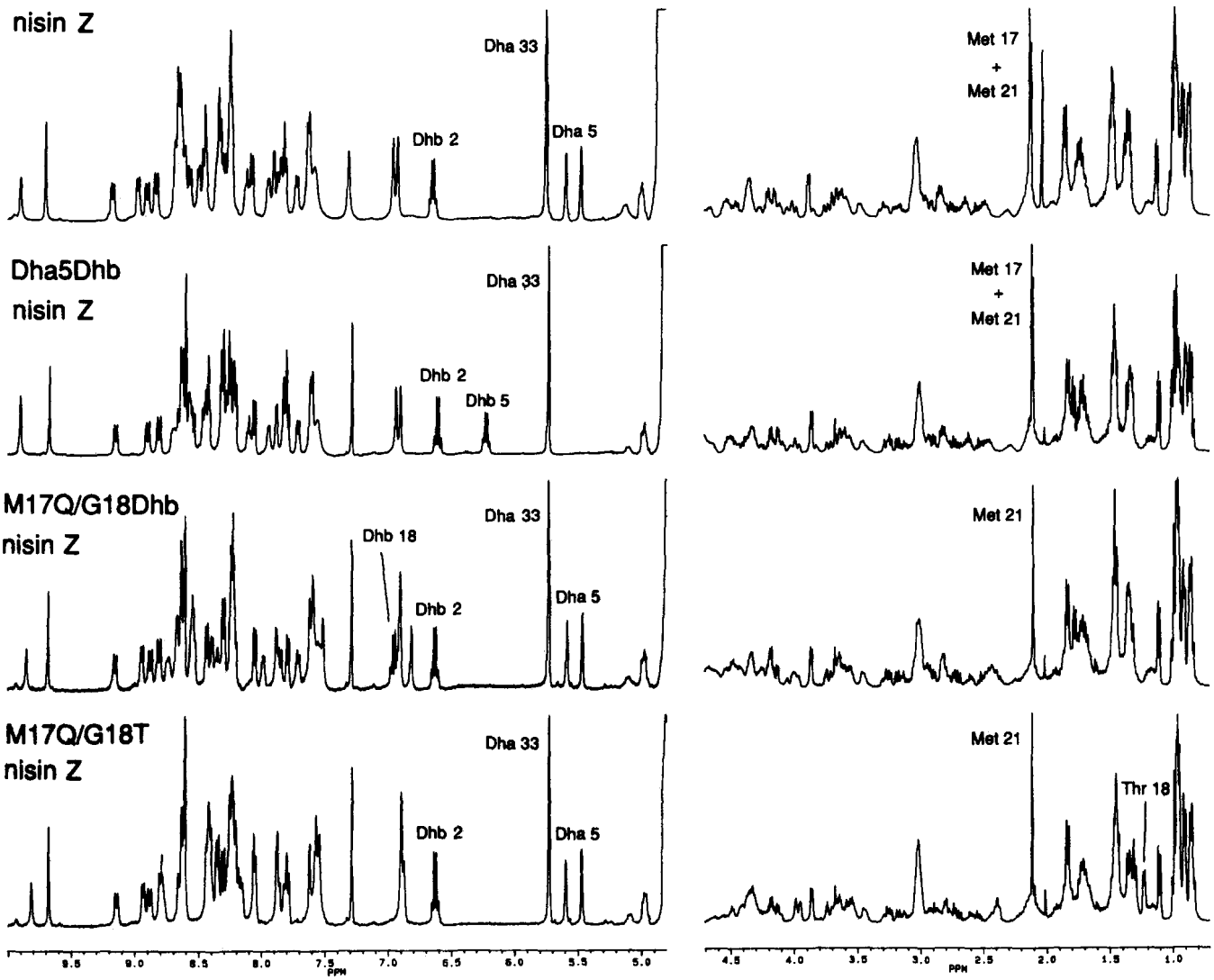

FIG. 3. **^{1}H NMR spectra of wild-type nisin Z, Dha5Dhb nisin Z, M17Q/G18Dhb nisin Z, and M17Q/G18T nisin Z.** Resonances of relevant residues are indicated. Experimental conditions: 10% D_2O·90% H_2O, pH 3.5, 25 °C.

TABLE III

Antimicrobial activities of nisin Z, Dha5Dhb nisin Z, M17Q/G18T nisin Z, and M17Q/G18Dhb nisin Z

Standard errors were less than 15% of the values shown.

Nisin Z	Minimal inhibitory concentration		
	M. flavus	*S. thermophilus*	*B. cereus*
		μg/liter	
Wild-type	11	6	130
Dha5Dhb	21	65	640
M17Q/G18T	6	24	570
M17Q/G18Dhb	13	8	130

engineering, and (iii) to construct modified nisins with altered antimicrobial activity and specificity. The engineering of dehydrated residues in lantibiotics offers the perspective of *de novo* design and production of biologically important substances and enlarges the variety of amino acid residues that can be used in protein engineering.

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