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Structural Variations in Nisin Associated with Different Membrane Mimicking and pH Environments

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Nisin is a membrane active antimicrobial peptide containing unusual dehydrated amino acid residues. The secondary structure of nisin in aqueous solution, membrane mimicking solvents and at various pH values was investigated using circular dichroism. In aqueous solution nisin is largely randomly coiled. In liposomes and at pH 6 and above, however, the presence of a maximum at 195 nm and a minimum at 190 nm was notable and indicative of β-turn formation in these environments. This change in structure was speculated to result in an increasing unavailability of the site for initial reaction of peptide and membrane at higher pH.

Nisin is a 34 amino acid residue cationic peptide produced by strains of the Gram-positive bacterium *Lactococcus lactis* (1, 2). This peptide demonstrates antimicrobial activity against a broad range of Gram-positive bacteria, including important foodborne pathogens such as *Listeria monocytogenes*, and is thus widely exploited as a preservative in the food industry (3–5). Nisin contains a number of unusual amino acids such as lanthionine and β-methyllanthionine, placing it in the class of antimicrobial peptides known as lantibiotics (2). The formation of these unusual amino acids is the result of post-translational dehydration of serines and threonines and the subsequent reaction of these modified residues with the thiol groups of cysteine residues (6).

Nisin acts by forming pores in the cytoplasmic membranes of target cells in a manner which is dependent on both trans-membrane potential and pH as well as the presence of acidic phospholipids (7–10). The net result of pore formation is an efflux of cellular components and subsequent depletion of the energy transducing ability of the cell (9, 11). The 3 dimensional structure of nisin in aqueous solution and in a number of membrane mimicking environments has been well studied using various techniques (12–20). Nisin consists of 2 amphipathic domains: a N-terminal domain (residues 3–19) containing 3 lanthionine rings and a C-terminal domain (residues 22–28) containing 2 lanthionine rings (17). These domains are separated by a flexible "hinge" region (residues 20–21) and flanked by regions of structural variability (15). In aqueous solution, nisin has a similar global structure to that in membrane mimicking environments, but varies with respect to the structure and orientation of the individual lanthionine rings (12–15).

The influence of pH on the antimicrobial activity of nisin is well documented with nisin being generally more effective in low pH environments (21–24). This feature has been attributed to the fact that both the solubility and stability of nisin increase as the pH decreases (21), with maximal activity seen around pH 5.5 (23, 24). At higher pH values (above 8.0), on the other hand, the molecule undergoes pH-induced physical modifications which are not well characterized but may include the formation of multimers (21, 24). Furthermore, low pH conditions may themselves often inhibit microbial growth and the combined effect of low pH and nisin may act synergistically (22). The secondary structure of nisin is important in its effect on the cell membrane since the lanthionine rings of the C-terminus appear to be responsible for initial interaction with the cell membrane (18, 20). The secondary structure of the molecule may be influenced by the pH of the environment and these changes may, in part, be responsible for the observed effects that pH has on nisin activity.

In this study circular dichroism (CD) was used to investigate the structure of nisin in various membrane mimicking environments and the possible induction of secondary structure changes in nisin in aqueous solution at pH 3 to 8.

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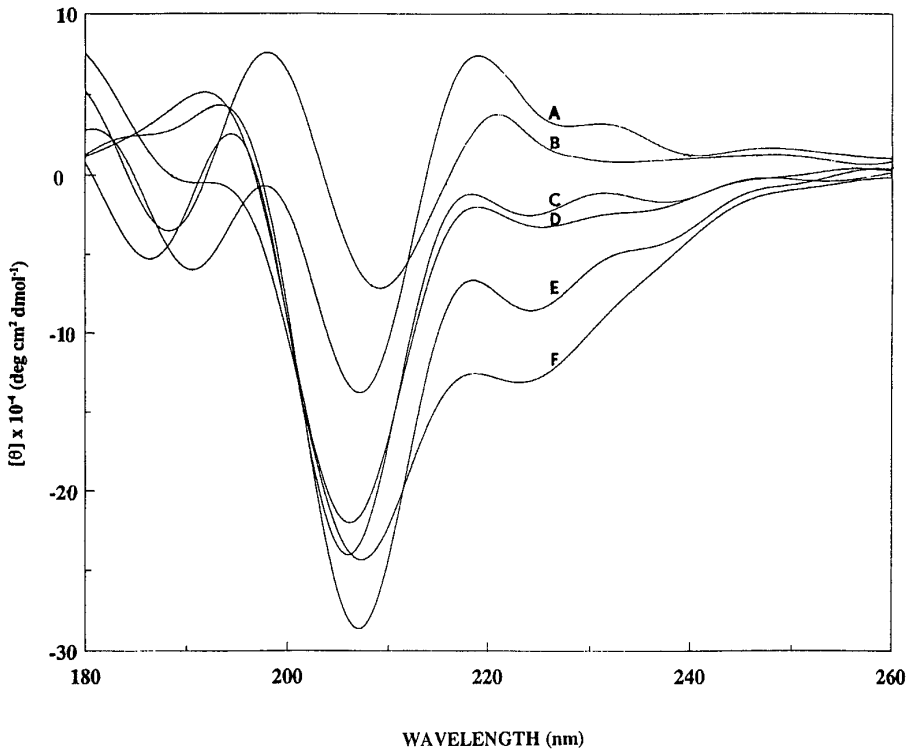

FIG. 1. Circular dichroism spectra of 0.3 mM nisin in aqueous solution and different membrane mimicking environments: (A) listerial lipid liposomes (30 mM), (B) POPC/POPG liposomes (30 mM), (C) 10 mM sodium acetate buffer (pH 5.0), (D) non-micellar SDS (2 mM), (E) micellar (10 mM), 50% trifluoroethanol.

MATERIALS AND METHODS

Chemicals. Nisin was purchased from the Sigma Chemical Co. (St. Louis, MO, USA) and further purified as previously described (25). The phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were obtained from Northern Lipids Inc. (Vancouver, BC, Canada). The method of Winkowski *et al.* (26) was used to extract lipids from mid-log phase cultures of a nisin sensitive strain of *Listeria monocytogenes* grown at 30°C in Brain Heart Infusion Broth (Acumedia, Baltimore, MY, USA). Phospholipid concentration was determined by phosphorus analysis (27).

Liposome preparation. Unilamellar liposomes (0.1 μm) were prepared from 10 mg ml^{-1} solution of POPC/POPG (7:3) or *Listeria monocytogenes.* This was achieved by forming multilamellar vesicles by the freeze thaw method (28) followed by extrusion through 0.1 μm double stacked Nucleopore filters using an extruding device (Lipex Biomembranes, Vancouver, BC, Canada) which promoted the formation of uniform sized liposomes.

Circular dichroism. Purified nisin was added to a final concentration 0.3 mM sodium acetate buffer (pH 5.0), 50% trifluoroethanol (TFE), non-micellar SDS (2mM), micellar. SDS (10mM), POPC/POPG (7:3) and *Listeria monocytogenes* phospholipid liposomes, respectively. In addition purified nisin was added to a final concentration of 0.3 mM to sodium phosphate buffer (pH 6.0 to pH 8.0) or sodium acetate buffer (pH 3.0 to pH 5.0) and to micellar SDS (10 mM) in each of the same buffers. Circular dichroism spectra were recorded between 180 nm and 260 nm for each of the above solutions at room temperature in a quartz cell (path length 1 mm) on a J-720 spectropolarimeter (JASCO, Tokyo, Japan). Data were digitally collected every 0.1 nm and the resulting spectra smoothed using the JASCO program. In all cases the CD spectra of the solutions alone were recorded and subtracted from experimental readings to compensate for background scattering. All spectra presented were the average of five scans. Results are expressed in terms of molar ellipticity.

RESULTS AND DISCUSSION

Circular dichroism is widely used to examine the secondary structure of peptides, often in conjunction with various deconvoluting techniques designed to estimate the degree of secondary structure (29, 30). The effects of unusual dehydrated amino acids and thio-ether bridges on the CD spectra of peptides is not well characterized and it has been suggested that attempts to estimate secondary structure from such spectra could be highly inaccurate. The presence of modified amino acids has, however, little effect on the overall pattern of the spectrum (14). For this reasons we have made no attempt to derive quantitative estimates of secondary structure in this study but have relied instead on data derived from the overall spectrum signatures.

The corrected CD spectra of nisin in different membrane mimicking environments are presented in Fig. 1. It is clear from these spectra that substantial variations in the secondary structure of nisin were apparent in liposomes as compared to different membrane mimicking environments which were themselves more simi-

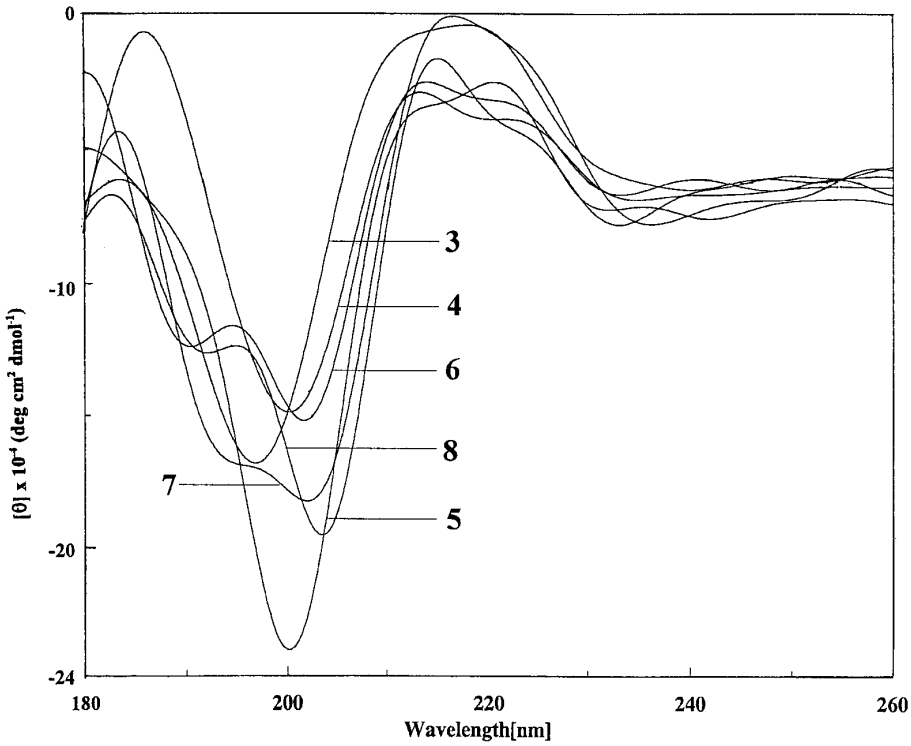

FIG. 2. Circular dichroism spectra of 0.3 mM nisin in aqueous solution at different pH values between 3 and 8 (indicated by numbers).

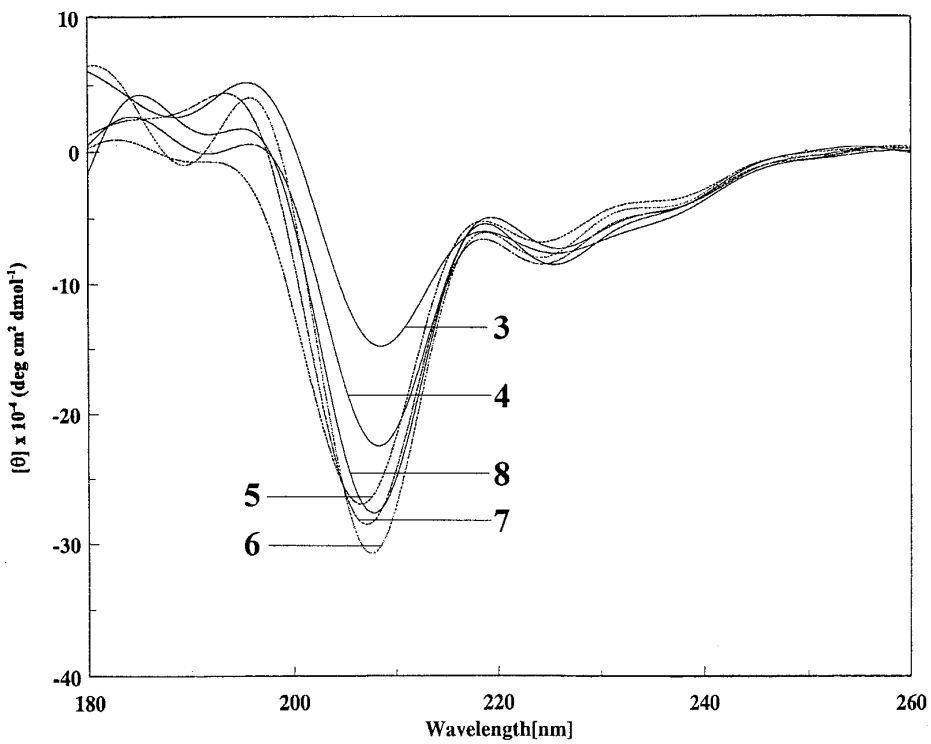

FIG. 3. Circular dichroism spectra of 0.3 mM nisin in micellar SDS (10 mM) at different pH values between 3 and 8 (indicated by numbers).

lar to, but different from, that in aqueous solution. In aqueous solution at pH 5 nisin displayed a signature fairly typical of random coil (maximum 220 nm, minimum 198 nm, maximum 180 nm) (30), although small differences in presence and position of peaks indicated some slight structure. This structure may be attributed to the presence of stabilizing thio-ether bridges in the lanthionine rings of the molecule. Non-micellar SDS appeared to alter the CD spectrum of nisin relatively little from that in aqueous solution, while micellar SDS and 50% TFE, on the other hand, promoted similar structural changes in the peptide. These changes were characterized by the strengthening of negative bands centred at 222 nm and 208 nm and of a positive band centred near 190 nm, all of which are typical indicators of α-helix formation in the molecule (30). In the presence of liposomes there were, however, strong positive bands centered at around 218 nm and 198 nm and a weakening of the 208 nm band observed in other environments. Bands centred at around 218 nm and 198 nm are typical of those observed for molecules displaying β-structure and in particular the formation of β-turn (30). The 218 nm positive band positive and 208 nm negative band observed for nisin incorporated into liposomes made from listerial lipids were stronger than the same bands in the POPC/POPG liposomes, while the positive band centred at 198 nm was weaker. In addition, there was a negative band centred at around 190 nm in the presence of listerial lipids as opposed to one at 185 nm in artificial POPC/POPG liposomes indicating slight differences in conformation in the different liposome environments.

It is a well established feature that the conformation of proteins and peptides are profoundly influenced by interaction with different solvent environments (31–33). In the case of membrane active peptides, these interactions have been shown to result in secondary structure which is similar to that seen upon interaction with membranes (31, 32). Among the solvents commonly used in studies of this type are alcohols such as trifluoroethanol and surfactants such SDS (31). The exact mechanisms by which these solvents act to induce secondary structure in peptides is not fully understood and varies between solvents (32). These solvents are supposed to present the peptides with an environment that mimic the hydrophilic/hydrophobic interfaces of the cell membrane. Not all solvents, however, induce the same degree or type of secondary, for example, TFE may promote α-helix formation in some peptides while these same peptides may show total or partial β-structure formation in the presence of non-micellar SDS (31).

The currently reported structure of nisin is based on NMR studies of the interaction of this peptide with membrane mimicking environments and have been used to devise a model of its interaction with the cell membrane itself (20). This model proposes that nisin forms a angle (β-turn) with the axis in the central hinge region when actively forming pores in the cell membrane. We show that the secondary structure of nisin as indicated by CD spectra is similar in a number of different solvent environments including SDS and TFE but differs from the structure proposed for nisin interaction with the cell membrane (Fig. 1). We also show, however, that the secondary structure of nisin in liposomes, which may be regarded as better models for the cell membrane, differ substantially from that in other solvent environments and contain a strong β-turn component, adding further support to the proposed membrane model (20).

TABLE 1

Position (nm) of Maxima and Minima Exhibited by Circular Dichroism Spectra of 0.3 mM Nisin in Aqueous Solution at Different pH Values

pH	Maxima (nm)				Minima (nm)	
3	180	—	210	220	—	198
4	186	—	212	223	—	200
5	185	—	212	220	—	200
6	180	195	212	220	190	202
7	182	195	215	222	192	202
8	182	196	215	223	192	203

The corrected CD spectra of nisin in aqueous solution and micellar SDS at different pH values between 3 and 8 are presented in Figs. 2 and 3 respectively. A greater degree of variation in secondary structure of nisin was apparent at the different pH values in aqueous solution than in micellar SDS. This result was not unanticipated since strong interactions between the cationic nisin and membrane mimicking solvents such as micellar SDS were expected (34, 35). Interaction with membrane mimicking solvents has previously been shown to result in secondary structure changes to nisin and this feature was confirmed in this study (18, 19, 34, 35). In particular the overall weakening of the maxima at around 220 nm and strengthening of the maxima centred between 180 and 190 nm in SDS as compared to buffer was similar to that noted in a previous CD study on nisin (14). Since the interaction of nisin with micellar SDS was likely to impose constraints on any structural effects caused by pH differences in the environment (34, 35), these spectra were therefore not examined in any further detail.

The spectra observed at different pH values in aqueous solution varied in both the exact position and number of maxima and minima as well as their relative intensities (Fig. 2). Table 1 gives the relative positions of the various maxima and minima of nisin for each of the pH values in aqueous solution. Maxima occurred at around 182, 212 and 220 nm and a minimum at 200 nm in all samples. The signature produced by nisin at pH 3 (Fig. 2, Table 1) was most typical of a random coiled structure while a successive shift away from this

signature was observed as pH became higher. This feature seems to indicate that random coiling is associated with high solubility and stability of the molecule. No consistent pattern of change was apparent in band intensity, however, when compared to variation in pH although at pH 5 very strong negative 220 nm minimum was noticeable.

In addition to the above features a weak but clear maximum and minimum occurred at about 195 nm and 190 nm, respectively, in samples with pH values of 6 and above (Fig. 2, Table 1). The intensity of these bands, as with those discussed previously, did not follow any clearly definable pattern. The occurrence of bands in these positions, as previously discussed, probably indicates an increase in β-turn structure in the molecule (30). This observed change in secondary structure at higher pH is clearly related to biological activity of the molecule since a sharp decline in inhibitory activity against bacteria is noticed above pH 6 (21–24). An increased β-turn content in the molecule may be due to low levels of multimer formation as previously suggested (21), but may also be due to constraints on the flexibility of the molecule around the central "hinge" resulting in a structure similar to that encountered in liposomes. In both scenarios the C-terminus of the molecule which is necessary for initial membrane binding may be less available resulting in the observed reduced activity (18, 20).

The highest inhibitory activity of nisin against microorganisms is observed at about pH 5.5 (23, 24), a point which corresponds to a watershed between the development of β-turn structure in the molecule at higher pH and random coiling of the molecule at lower pH. It seems likely that secondary structure of nisin at this pH, typified by the strongest 200 nm minimum, allows optimal initial of interaction of the molecule with the cell membrane. Thus, although solubility and stability of nisin increase as pH decreases, we show for the first time that specific pH-induced secondary structure features are probably also involved in pH-dependent activity changes in nisin. Furthermore, we speculate that the pre-induction of the membrane-active peptide structure by higher pH is detrimental to nisin activity.

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