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NMR and circular dichroism studies of the lantibiotic nisin in non-aqueous environments

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The lantibiotic, nisin, which is known to interact with membranes of certain Gram-positive bacteria, was studied in three model systems which mimic a membrane-like environment, i.e. a mixture of trifluoroethanol and water, or micelles of sodium dodecyl sulfate or dodecylphosphocholine. The ^{1}H NMR spectra of nisin in the non-aqueous environments, at 40°C and pH 3.5, have been assigned completely. The CD and NMR results indicate that the conformation of nisin in the three non-aqueous environments differs from that in aqueous solution, and that the conformation in the two micellar systems is similar. The major conformational changes, relative to nisin in aqueous solution, occur in the N-terminus.

Bacteriocin; Lantibiotic; Nisin; NMR, Two-dimensional; Circular dichroism; Micelle

1. INTRODUCTION

The lantibiotics constitute a group of polycyclic polypeptides containing unusual amino acids such as lanthionine [1]. One of the most prominent lantibiotics is nisin, produced by a number of *Lactococcus lactis* strains (Fig. 1). Because of its antimicrobial activity against a wide spectrum of Gram-positive organisms and its inhibitory effect on the sporulation of *Bacillus* and *Clostridium* species, it is exploited as a food preservative in the canning and dairy industry [2]. Nisin is capable of inducing pore formation in membranes of sensitive bacteria in a voltage-dependent fashion, leading to an efflux of small molecules and a depolarization of the membrane potential [3–5], which results in cell death.

To gain insight into the biological activity of nisin we have initiated a systematic NMR study to elucidate the 3D structure of this lantibiotic in various media. Previously, we reported the ^{1}H NMR assignments and the 3D structure of nisin in aqueous solution [6,7]. The conformation of nisin at its target site, the membrane, may differ from the one in water. Therefore we set out to study the molecule in a membrane-like environment. Micelles, or a solvent less polar than water, can be used as membrane mimetics, since native membranes or phospholipid vesicles are not suited for high resolution NMR due to particle size. We used trifluoroethanol (TFE)/water, sodium dodecyl sulfate (SDS) and dodecylphosphocholine (DPC). The results show that nisin forms complexes with SDS and DPC micelles. Sample conditions were optimized for recording of ^{1}H NMR spectra of these systems and complete resonance assignments are reported. The chemical shifts of several protons, mainly from the N-terminal region, change upon going from water to the membrane-mimicking conditions. Also the CD spectrum of nisin is strongly affected; hence it is concluded that nisin undergoes a structural transition when brought to a membrane-like environment.

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Abbreviations: Dha, dehydroalanine; Dhb, dehydrobutyrine (3-methyldehydroalanine); TFE, trifluoroethanol; SDS, sodium dodecyl sulfate; DPC, dodecylphosphocholine; CMC, critical micelle concentration; RP-HPLC, reversed phase-high performance liquid chromatography; CD, circular dichroism; 2D NMR, two-dimensional nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlated spectroscopy; P.COSY, purged correlated spectroscopy.

2. MATERIALS AND METHODS

Nisin was purchased from NBS Biologicals (lot no. 03232G) and was purified by RP-HPLC [8]. [$^2H_{25}$]SDS and [$^2H_{38}$]DPC were obtained from MSD Isotopes and [$^2H_3$]TFE from Cambridge Isotope Laboratories.

CD measurements were performed at room temperature on a Jasco-600 spectropolarimeter, using 0.2 mm path length cells with a scan speed of 50 nm/min, a time constant of 0.125 s and a bandwidth of

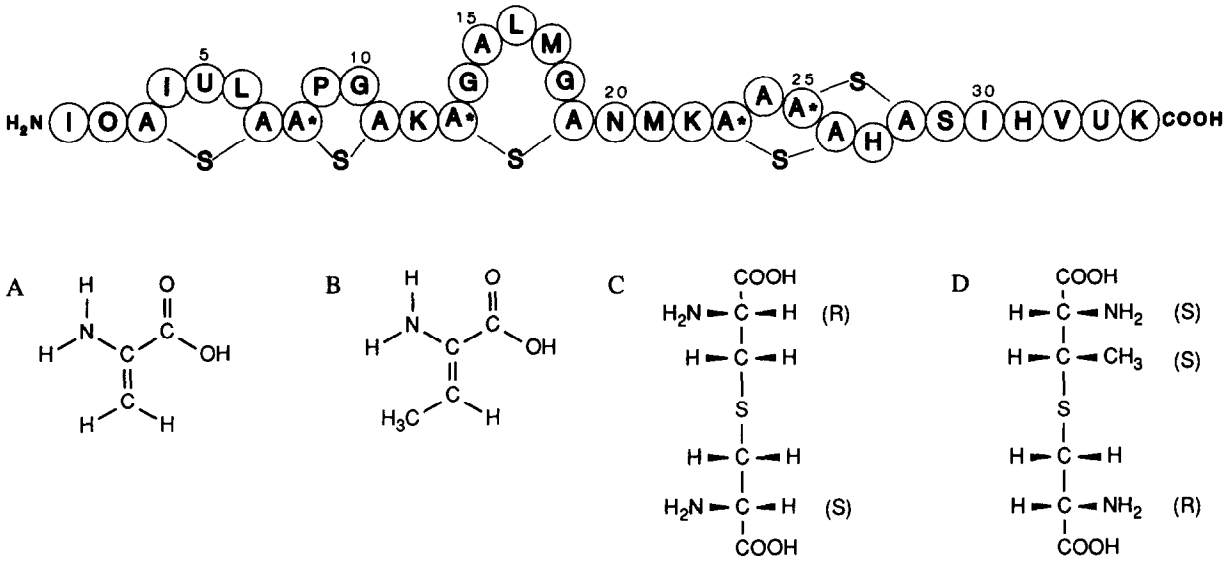

Fig. 1. The primary structure of nisin and structures of the unusual amino acids (A) U = dehydroalanine, (B) O = dehydrobutyrine, (C) $\overset{A}{\underset{S}{\lfloor\rfloor}}\overset{A}{}$ = lanthionine and (D) $\overset{A^*}{\underset{S}{\lfloor\rfloor}}\overset{A}{}$ = 3-methyllanthionine.

1 nm. The data processing consisted of a subtraction of a spectrum of a protein-free control sample and smoothing of the resulting spectrum.

The ^{1}H NMR experiments were carried out with samples containing 3–5 mM of nisin. The polypeptide was directly dissolved in 75% [$^2H_3$]TFE/25% H_2O (in % by volume) or in 75% [$^2H_3$]TFE/25% ^2H_2O. For the micellar samples nisin was first dissolved in $H_2O/^2H_2O$ (9:1) or ^2H_2O and subsequently a 30–35-fold excess of [$^2H_{25}$]SDS or a 45-fold excess of [$^2H_{38}$]DPC was added. The pH of all samples was adjusted to 3.5 (pH meter reading). 1D spectra, TOCSY and P.COSY spectra were recorded at 400 MHz on a Bruker AM400, interfaced to an Aspect 3000 computer. NOESY spectra were recorded at 600 MHz on a Bruker AM600, interfaced to an Aspect 3000 computer. Acquisition and processing parameters were essentially the same as those described in [6,7]. The spectra were referenced to sodium 3-(trimethylsilyl)-1-propanesulphonate (DSS).

3. RESULTS AND DISCUSSION

The influence of TFE, SDS and DPC on the conformation of nisin was examined by ^{1}H NMR and CD spectroscopy. [$^2H_3$]TFE, [$^2H_{25}$]SDS and [$^2H_{38}$]DPC were added to an aqueous solution of nisin at room temperature and pH 3.5 and the effect was monitored by ^{1}H NMR. The spectrum of nisin changed gradually upon addition of TFE. The position of isolated resonances, as a function of TFE concentration, suggested a conformational transition, which was complete at about 70% TFE. Consequently, 2D NMR experiments were performed at 75% TFE. Supportive evidence for a conformational change was obtained by CD spectroscopy (vide infra). In Fig. 2A and B the spectral region containing the amide and vinyl proton resonances of nisin in aqueous solution and in 75% TFE/25% H_2O is presented.

In the case of SDS a titration is not possible, since more than an 18–20-fold excess had to be added to obtain a clear solution. The resonances of nisin were broadened upon the addition of more than a 20-fold excess of SDS (cf. Fig. 2A and C). This line broadening may be due to chemical exchange, or complexation with micelles. The 1D NMR spectrum showed no significant changes when the nisin-to-SDS ratio was changed from 1:20 to 1:60 (data not shown), which makes exchange unlikely. The observed line broadening is indicative of an interaction between nisin and SDS micelles.

The titration with DPC can be analyzed in terms of line width and chemical shift. An example is shown in Fig. 3 for the $H^{\beta 1}$ proton of Dha5, a resonance for which the effect is most pronounced and the shifting could be readily followed in the 1D spectra. For DPC concentrations below the critical micelle concentration (CMC = 1.1 mM and 22°C [9]) no spectral changes were observed, which suggests that there is no interaction between nisin and monomeric DPC. Just above the CMC of DPC shifting and broadening of resonances was observed. The resonances sharpen again with increasing amounts of DPC and shift continuously until a nisin-to-DPC ratio of approximately 1:30 is reached. Above that ratio the spectrum is no longer affected by the addition of DPC, although the nisin resonances remain twice-to-three times as broad as those of nisin in aqueous solution. These results were interpreted as evidence for the formation of micelles containing 30 DPC molecules per nisin molecule. The aforementioned line broadening and subsequent sharpening of the resonances of nisin with DPC concentrations above the

CMC indicate that there is an intermediate-fast exchange between free and micelle-bound nisin.

The resonances of nisin in the three membrane-like environments, at 40°C and pH 3.5, have been assigned completely using the standard strategy [6,10,11]. Tables of all resonance assignments and an overview of sequential NOEs are available upon request. The assignments will be deposited in the BioMagResBank, a NMR structure database [12].

The spectra of nisin in TFE/water and of nisin complexed to DPC micelles revealed a single set of resonances, and all NOEs could be interpreted as intramolecular contacts, which strongly suggests a monomeric structure of nisin in these environments. The spectra of nisin complexed to SDS micelles revealed the presence of a minor component (≤10%). The resonances of the major component have been assigned. In Fig. 2C the two components can be seen for the Dhb^{NH}_2 and Dha^{β}_5 protons at 10.11 and 5.60 ppm, respectively.

The chemical shifts of backbone NH and H^{α} protons of nisin in the three membrane mimetics were compared to those observed in water (see Fig. 4). The global pattern of the differences in the chemical shifts is similar for the three model systems. The resonances of protons in the N-terminus exhibit the largest differences in chemical shift, suggesting a different conformation for the

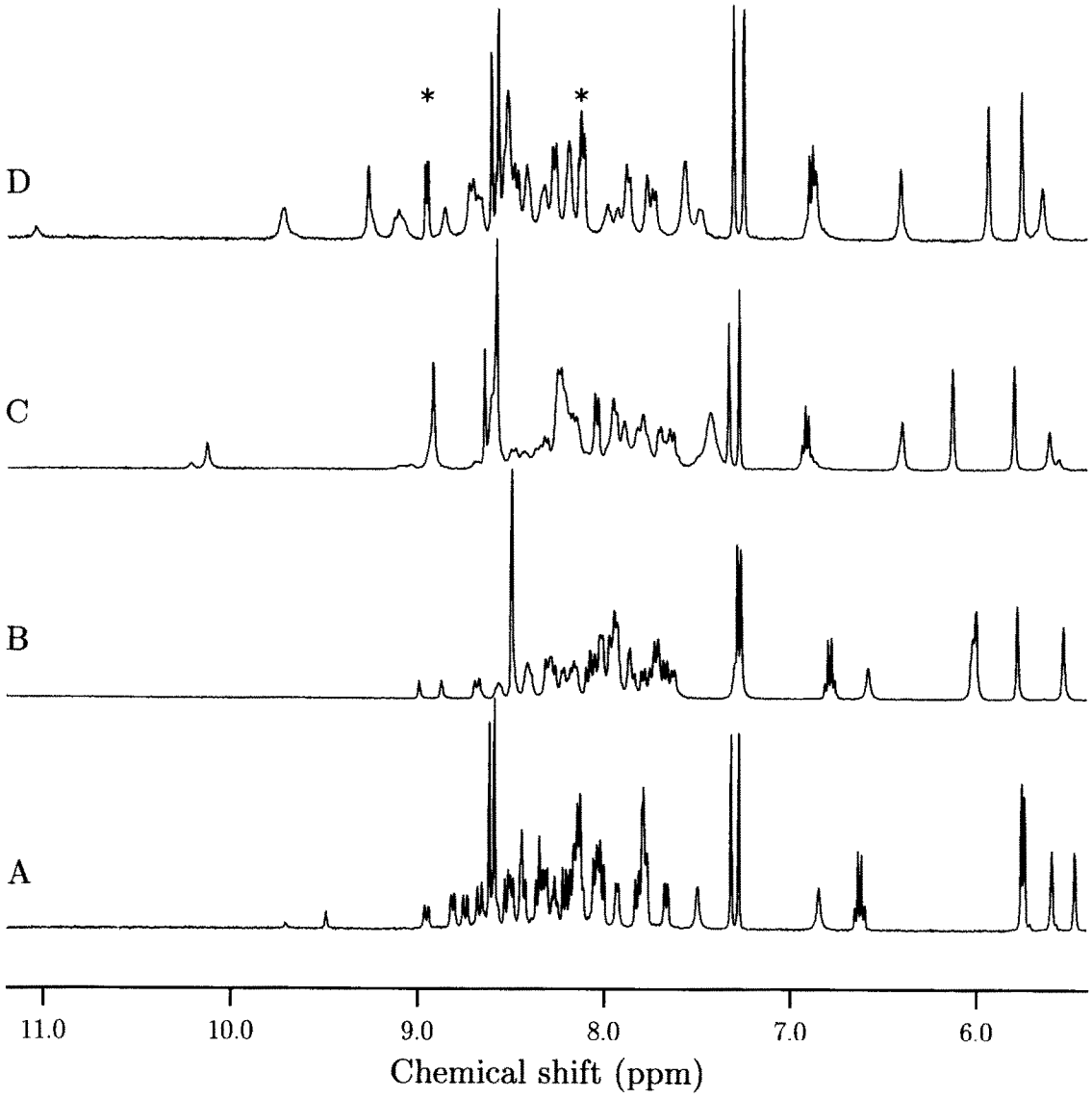

Fig. 2. The amide and vinyl proton region of the ^1H NMR spectrum of nisin, at 40°C and pH 3.5, (A) in aqueous solution, (B) in 75% TFE/25% H_2O, (C) complexed to SDS micelles (nisin/SDS = 1:35) and (D) complexed to DPC micelles (nisin/DPC = 1:45). Pyridine is present as an impurity in DPC and two of its resonances at 8.95 and 8.12 ppm are indicated by asterisks; the third resonance at 8.58 ppm overlaps with resonances of nisin and is not indicated.

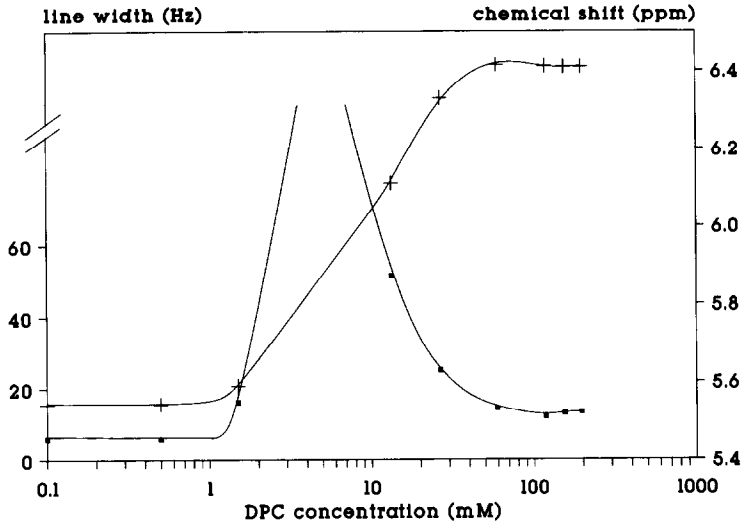

Fig. 3. Line width and chemical shift vs. DPC concentration. An aqueous solution of 3.3 mM of nisin at 25°C and pH 3.5 was titrated with [^{2}H]DPC. The line width (■) and chemical shift (+) were determined in the measured spectra for the $H^{\beta 1}$ proton of Dha5. This resonance was broadened beyond detection for DPC concentrations between 3 and 7 mM.

N-terminus in the non-aqueous environments compared to nisin in aqueous solution. In principle, chemical shift data can be interpreted in terms of α or β secondary (protein) structure [13]. However, since 80% of the residues of nisin are present in lanthionine ring structures, regular secondary structure is not to be expected here. Moreover, no data are available to correlate chemical shift to structure for lanthionines and α,β unsaturated amino acids.

To supplement the findings based on chemical shift data CD measurements were performed. The CD spectra of nisin in the three model systems differ significantly from the CD spectrum of nisin in aqueous solution (see Fig. 5), suggesting that the conformation of nisin in the three membrane mimetics is different from that in aqueous solution. The CD spectra of nisin complexed to SDS and DPC micelles are similar, indicating a similar conformation. The CD spectrum of nisin in TFE/water showed global features, which were also observed in the micellar spectra. The conformation of nisin in TFE/water might resemble the micellar conformation. Again, in view of the presence of lanthionines and α,β unsaturated amino acids, no attempt was made to deduce average secondary structure from the CD spectra.

Another striking difference between nisin in aqueous and non-aqueous environments is the pH dependence of its solubility. In water nisin is soluble at low pH; the solubility decreases strongly with increasing pH, when the two histidines are deprotonated (both have a pK_a of about 6.5 [6]). In the non-aqueous environments nisin is soluble both at low and high pH. In all three systems, TFE/water, SDS and DPC micelles, both histidines have nearly identical pK_a's of 6.0, 7.5 and 6.5, respectively, as determined from chemical shift vs. pH curves. Apparently, the presence of two charged histidines is no longer needed for the solubility of nisin in non-aqueous environments.

Qualitative analysis of NOE data (not shown) does not reveal substantial differences in comparison with the results obtained for aqueous solution. Especially the 'long range' NOEs are few in number, and are rather weak. Hence, we expect no dramatic change in overall tertiary structure upon binding of nisin to the micelles. Elucidation of more subtle structural changes requires careful analysis of NOE intensities in terms of interproton distances, taking spin diffusion into account [14], followed by distance geometry calculations and energy minimization. This work is currently in progress.

In conclusion, nisin has been studied in membrane-mimicking model systems, for which the ^{1}H NMR spectra were assigned completely. CD spectra and chemical shift data indicate that nisin adopts a different conformation in a membrane environment as compared to nisin in aqueous solution. According to the NOE data this conformational change may be rather limited and does not appear to affect the overall fold of the molecule. The main differences are indicated for the N-terminus. In this respect, it is interesting to note that degradation at Dha5, and not at Dha33, causes inactivation of nisin [8,11].

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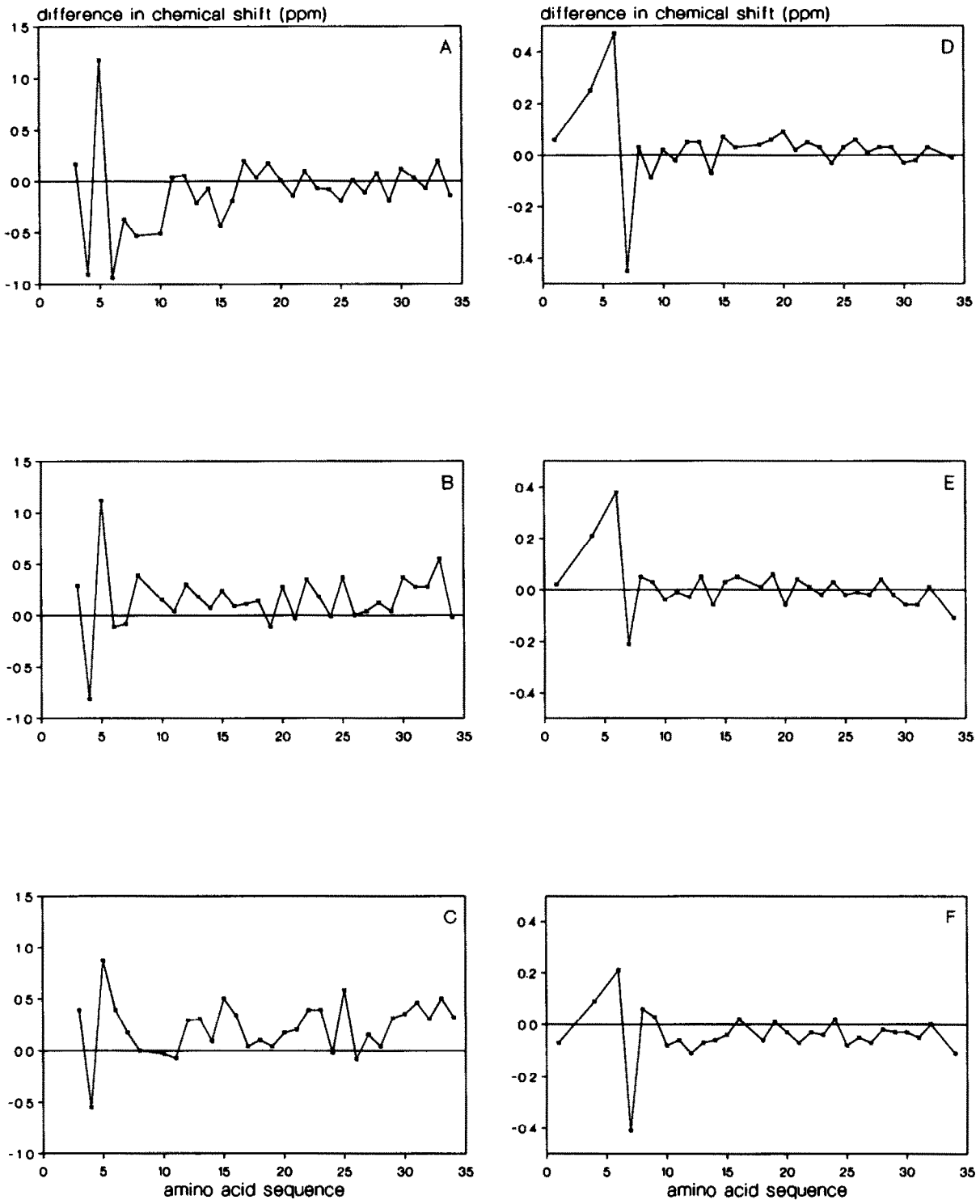

Fig. 4. Difference in chemical shifts of nisin in aqueous solution with respect to nisin in a non-aqueous environment for (A) amide NH protons of nisin complexed to DPC micelles, (B) NH protons of nisin complexed to SDS micelles, (C) NH protons of nisin in TFE/water, (D) H^{α} protons of nisin complexed to DPC micelles, (E) H^{α} protons of nisin complexed to SDS micelles, and (F) H^{α} protons of nisin in TFE/water.

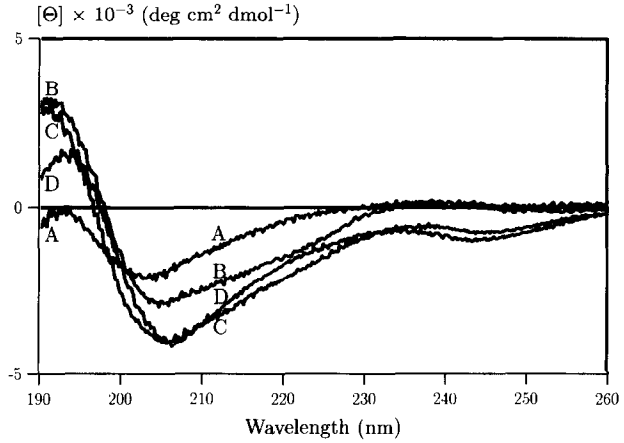

Fig. 5. CD spectra of 0.3 mM nisin (A) in aqueous solution, (B) in 75% TFE/25% water, (C) in the presence of 8 mM SDS and (D) in the presence of 12 mM DPC.

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