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Molecular organisation of the ice nucleation protein InaV from *Pseudomonas syringae*

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Abstract A new ice nucleation gene from *Pseudomonas syringae* was isolated and overexpressed as a fully active protein in *Escherichia coli* in order to gain experimental data about the structure of ice nucleation proteins. No evidence of a signal sequence or secondary glycosylation was found. Differences in the extent of aggregation were shown to modulate the ice nucleation activity. The circular dichroism spectrum of the purified protein indicated the presence of β-sheet structure. This finding supports a recently proposed hypothetical model for the structure of ice nucleation proteins, which provides a plausible explanation for their aggregation tendency.

Key words: Bacterial ice nucleation; *inaV* sequence; Protein aggregation; β-strand; *Pseudomonas syringae*

1. Introduction

Pure water can be supercooled, i.e. cooled below its freezing point without freezing, to temperatures as low as −35°C until stable ice nuclei are formed on which ice can grow [1]. Several Gram-negative bacteria, including strains of *Pseudomonas syringae*, can catalyse the formation of ice in supercooled water at temperatures as high as −2°C. These bacteria have been shown to possess ice nucleation proteins, which are thought to act as templates for the assembly of seed crystals of ice [2,3]. Localisation studies in *P. syringae* showed that the ice nucleation protein is associated with the outer membrane where it forms large aggregates [4–6].

The ice nucleation active (INA) phenotype can be transferred to *Escherichia coli* by expressing the single *ina* gene in this host. Several *ina* genes have been sequenced and encode large proteins of 1200–1500 amino acid residues with a highly conserved sequence (77% identity) [2,3]. Ice nucleation proteins are composed of three distinct domains: an N-terminal domain (15% of the protein) containing three or four potential transmembrane spans but no clear signal peptide [7], a C-terminal domain (4% of the protein), and a central domain composed of repeats given by a 8-, 16- and 48-residue periodicity (consensus sequence of the 16-residue repeat: AGYGST-TA- - -S- - -), which is most probably the site acting as template for ice crystal formation [2,3]. The tertiary structure of ice nucleation proteins has so far not been determined. Computer modelling of the larger 48 amino acid repeat predicts three β-hairpins interacting with each other by side chains [8].

Ice nucleation is measured by the number of active ice nuclei at a given temperature [9]. A culture of INA bacteria always displays heterogeneous ice nucleators with threshold temperatures ranging from −2 to −10°C with only few ice nucleators (corresponding to about one nucleator per 10^6 cells) active at −2°C, and the majority being active at colder temperatures. Ice nucleators have been classified into three subpopulations with threshold temperatures of −4°C or warmer (type 1), −5 to −7°C (type 2), and −8 to −10°C (type 3) [10]. The theory of heterogeneous ice nucleation predicts that the larger the nucleating site, the higher the threshold temperature of its ice nucleation activity [11]. Therefore, the aggregation of individual ice nucleation proteins into different sizes of ice nucleators is believed to be the main biochemical basis for these subpopulations. An alternative hypothesis concerning the distribution of the ice nucleation activity is based on the different degrees of secondary modification of ice nucleation proteins: glycosylation, which may play a role in aggregation, and phosphatidylinositol for anchoring of the ice nucleation protein to the outer membrane [12,13].

In the present study, we analysed the InaV protein from different subcellular compartments and at different stages of the purification, which led us to the following conclusion. Protein aggregation appears to be the basis for activity and the extent and organisation of aggregation are the basis for the variation of the threshold temperatures.

2. Materials and methods

2.1. Bacterial strains and culture conditions

P. syringae strain INA5 was cultured in King's broth [14] at 30°C with constant shaking for 48 h and stored for 3 days at 4°C before the cells were collected and further processed. *E. coli* strain BZ234 [15] was cultured in double concentrated Luria-Bertani medium [20 g tryptone (Difco), 10 g yeast extract (Difco) and 5 g NaCl per litre] at 37°C with constant shaking. For InaV protein expression, a 1% inoculum of BZ234 containing plasmid pDP245 was incubated in the presence of 100 μg/ml ampicillin to A_{600} of 1.0, IPTG (isopropyl-thio-β-D-galactoside) added to a final concentration of 1 mM and incubated for a further 3 h.

2.2. Isolation of P. syringae strain INA5

15-g samples of a collection of apparently healthy leaves of raspberry (*Rubus idaeus*), hazel (*Corylus avellana*) and great bindweed (*Calystegia sepium*) were mixed and washed with 100 ml sterile peptone-phosphate buffer (0.1% peptone-50 mM phosphate, pH 7) in a shake flask. The buffer was centrifuged at 10000×*g* to pellet the bacteria and debris. The pellet was resuspended in 10 ml peptone-phosphate buffer and 0.05-ml samples were plated onto King's agar

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plates supplemented with 40 μg/ml cycloheximide and 0.1% cetramide. *P. syringae* strain INA5 was detected by screening of individual colonies for ice nucleation activity at −5°C.

2.3. DNA techniques

Total DNA was isolated from *P. syringae* INA5 as described for *E. coli* [16]. Single and double restriction digestions of INA5 genomic DNA were separated by agarose gel electrophoresis and transferred to a Zetaprobe nylon membrane (BioRad). The filter was hybridised according to Maniatis et al. [17] with the ^{32}P-labelled oligonucleotide 5′-GCNGGNTAYGGNAGYACNCARAC-3′ designed from the highly repeated peptide motif AGYGSTQT. The *Eco*RI+*Bgl*II double digestion gave a band of 4.5–5.5 kbp. This region of DNA was electro-eluted, ligated into pUC19 [18] and transformed into the *E. coli* strain BZ234. After screening the transformed colonies with the above-mentioned hybridisation probe, a positive clone with the plasmid pUC19-*inaV* was identified.

The complete DNA sequence of the genomic insert was determined from subclones using dideoxy-chain terminations with the T7-sequencing kit of Pharmacia. The sequence was compiled and analysed using the GCG suite of programs [19].

The plasmid pDP245 was constructed essentially as described by Wolber et al. [20]. The complete *inaV* open reading frame (ORF) was cut out as a *Dra*I-*Hin*dIII fragment and cloned into the vector pKK223-3 (Pharmacia) previously digested with *Sma*I and *Hin*dIII.

2.4. Antibodies and electron microscopy

A polyclonal antiserum against the purified InaV protein was prepared by Neosystem Laboratoire (Strasbourg, France) and the IgG fraction was isolated by passage over a protein A Superose FPLC column (Pharmacia). The InaV-specific antibodies were purified from the IgG fraction by passage over CNBr-activated Sepharose 4B (Pharmacia) coupled with purified InaV protein. For transmission electron microscopy, cultured bacteria were collected by centrifugation at 4000×*g* for 10 min at 4°C. The cells were fixed overnight at 4°C with 0.1% glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer, pH 7.4 and washed with phosphate buffer. The cells were mixed with 2% warm agar and after solidification small pieces were cut with a razor blade and dehydrated in a graded series (50–100%) of ethanol. The agar blocks were infiltrated with L.R. White resin (Hard), cut into ultra-thin sections (60–80 nm) with a Reichert ultramicrotome and floated on 1% bovine albumin, 0.2% polyethylene glycol (Carbowax 20-M, Union Carbide) in 50 mM Tris chloride, pH 8.0. The sections were incubated with the purified antibodies against the InaV protein at a 1:1000 dilution for 3 h at 4°C and washed in Tris chloride buffer. The labelling was performed with immunogold conjugates (goat anti-rabbit IgG gold conjugate, 10 nm, Bio Cell) and the sections were examined in a Philips CM 12 at an acceleration voltage of 60 kV.

2.5. Cell fractionation and InaV protein purification

Cultures (1 l) of either *P. syringae* or *E. coli* were collected by centrifugation at 18000×*g* for 20 min at 4°C and washed in 100 ml 50 mM Tris chloride, pH 7.5. The cells were suspended in 50 ml 50 mM Tris chloride, pH 8.0, containing 100 mM NaCl, 1 mM EDTA, 1 mM 1,4-dithio-DL-threitol, ribonuclease and deoxyribonuclease (3 mg of each) and the proteinase inhibitors 6-amino-*n*-hexanoic acid (100 mM), benzamidine (5 mM), phenylmethylsulphonyl fluoride (1 mM). The cells were then disrupted in a French press cell. Intact bacteria were removed by centrifugation at 20000×*g* for 10 min at 4°C and the supernatant was separated by ultracentrifugation at 144000×*g* for 1 h at 4°C into crude membranes (pellet) and cytoplasmic fractions (supernatant). From the cytoplasm InaV aggregates could be isolated by further ultracentrifugation at 144000×*g* for 15 h at 4°C. The InaV pellet contained approximately 10% contamination of cosedimenting proteins (result not shown).

For purification of InaV protein, the cytoplasmic fraction was diluted in an equal volume of distilled water and loaded onto an anion-exchange column (Q Sepharose Fast Flow, Pharmacia) equilibrated in buffer A (50 mM Tris chloride pH 8.0, 1 mM EDTA, 1 mM 1,4-dithio-DL-threitol). The run-through fraction was collected and ammonium sulphate was slowly added to a final concentration of 20% with constant stirring at 4°C. After 30 min incubation at 4°C the solution was centrifuged at 14000×*g* for 10 min at 4°C and the supernatant discarded. The pellet was resuspended at 100 mg wet weight/ml in 50 mM Tris chloride, pH 8.0, 10 mM EDTA, 10 mM 1,4-dithio-DL-threitol, 2% sodium *N*-lauroyl-sarcosinate (NLS), 10% glycerol, incubated for 2 h at 25°C and centrifuged at 10000×*g* for 5 min at 20°C. The supernatant was chromatographed by gel filtration (Sephacryl S-400 High Resolution, Pharmacia) in buffer B (50 mM Tris chloride, pH 8.0, 1 mM EDTA, 1 mM 1,4-dithio-DL-threitol, 0.1% NLS), the fractions were analysed by SDS-PAGE and those containing the InaV protein peak were pooled. Purification in guanidine hydrochloride was essentially the same except that after ammonium sulphate precipitation the pellet was solubilised and chromatographed in buffer A containing 6 M guanidine hydrochloride and that the InaV protein was renatured by dialysis against buffer A.

2.6. Solubilisation of crude membranes with different detergents

Crude membranes were solubilised (35 mg wet weight/ml) in buffer A with 1 mM phenylmethylsulphonyl fluoride and 1% of a detergent, either NLS, SDS, octyl-β-D-thioglucopyranoside (OSGP) or Triton X-100. After incubation for 3 h at 25°C the samples were centrifuged (144000×*g* for 1 h at 4°C), the supernatants were dialysed against buffer A and analysed by SDS-PAGE.

2.7. Characterisation of the InaV protein

NH_2-terminal sequence analysis was performed on a sequencer model 477A from Applied Biosystems. Ice nucleation frequencies were measured in a refrigerated waterbath by the method originally described by Vali [9], which was modified so that single freezing events could be measured in sample volumes of 100 μl in thin-walled glass tubes. Circular dichroism (CD) spectra were performed at 22°C on a Jasco J-720 spectropolarimeter using a cell of 0.2 mm light path length. The spectra were scanned 10 times at a scan rate of 20 nm/min, the signals averaged and the baseline contributions subtracted. The resulting signal was subjected to a noise reduction procedure (Jasco Standard Analysis Software).

2.8. Nucleotide sequence accession number

The DNA sequence of the *inaV* gene has been deposited under accession number AJ001086.

3. Results and discussion

The gene responsible for the INA phenotype of a locally isolated *P. syringae* strain was cloned, characterised and overexpressed in the heterologous host *E. coli*. The 3557-bp *Bgl*II-*Eco*RI fragment contained a single open reading frame encoding a protein of 1200 amino acids. *E. coli* cells, transformed with this gene, were active in ice nucleation (Fig. 1) and their lysates, analysed by SDS-PAGE, showed a band corresponding to 170 kDa which was not present in non-transformed

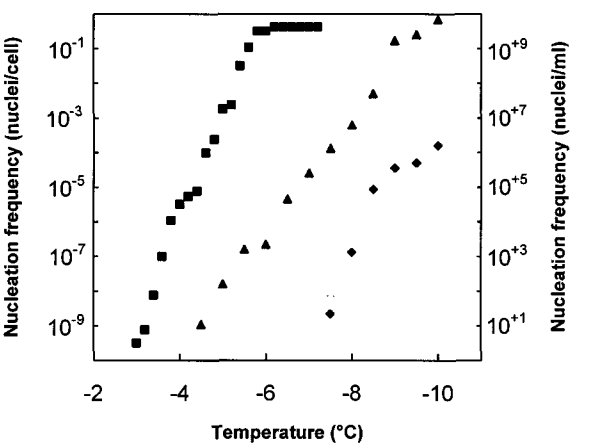

Fig. 1. Ice nucleation spectra of intact cells of *E. coli* transformed with pDP245/*inaV* (■, nuclei/cell), of their cytoplasm (▲, nuclei/ml) and of the purified InaV protein (◆, nuclei/ml). The cell number was determined by plating out serial dilutions of the culture.

cells. This protein was purified and the first nine amino acid residues of the purified InaV protein (determined by N-terminal Edman sequencing) corresponded to those predicted by the translation of the putative ORF. The gene *inaZ* from the *P. syringae* strain S203, described by Green and Warren [21], shares 92.4% identity at nucleotide level and 96.2% identity at amino acid level with *inaV*. In spite of the high homology, the apparent molecular mass of the InaV protein on SDS-PAGE of 170 kDa differed slightly from the 153 kDa reported for the InaZ protein [20].

In *P. syringae* the ice nucleation protein was observed to be located only in the outer membrane fraction [5] while in the heterologous host *E. coli* the localisation of the bulk of ice nucleation protein seemed to depend on the degree of overexpression. For low expression the ice nucleation protein was found in the outer membranes [5], for medium expression in a membrane fraction with an unusual density [20], whereas for high overexpression the product was found mainly in inclusion bodies [22]. We analysed different cellular fractions by immunoblotting with antibodies raised against the purified InaV protein to study subcellular localisation of the ice nucleation protein (Fig. 2). We found that the ice nucleation protein in *E. coli* as well as in *P. syringae* is present in both cytoplasmic and crude membrane fractions, and has in all cases the same molecular size on SDS-PAGE. The InaV protein is therefore probably not synthesised as precursor with a signal sequence. The minor bands represent degradation products of the InaV protein. By comparing the intensities of the bands of the total cell extracts of *P. syringae* and *E. coli* on SDS-PAGE (Fig. 2) and taking into account the dilution of the samples, we concluded that in *E. coli inaV* was approximately 1000-fold overexpressed compared to *P. syringae*. Whereas the latter showed an equal distribution of the InaV protein between the cytoplasm and the membrane fraction, in *E. coli* the InaV protein was mainly found in the cytoplasm (Fig. 2). This indicates that the *E. coli* membranes were probably saturated with InaV and the excess produced was deposited in the cytoplasm, which was readily visible, by immunoelectron microscopy of the InaV overexpressing *E. coli* cells. As control, non-transformed *E. coli* cells showed no staining (Fig. 3). In *P. syringae* the concentration of the ice nucleation protein was too low to be detected by immunoelectron microscopy.

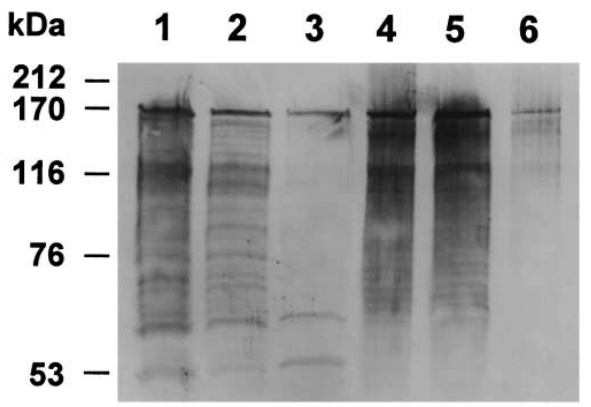

Fig. 2. Detection of InaV protein in different cell fractions of *P. syringae* and transformed *E. coli*. Total cell extract (after French press treatment), cytoplasm and crude membranes from *P. syringae* (lanes 1, 2 and 3) and *E. coli* (lanes 4, 5 and 6) were analysed by Western blot (7.5% polyacrylamide gel), probed with anti-InaV protein antibodies and alkaline phosphatase-labelled secondary antibodies.

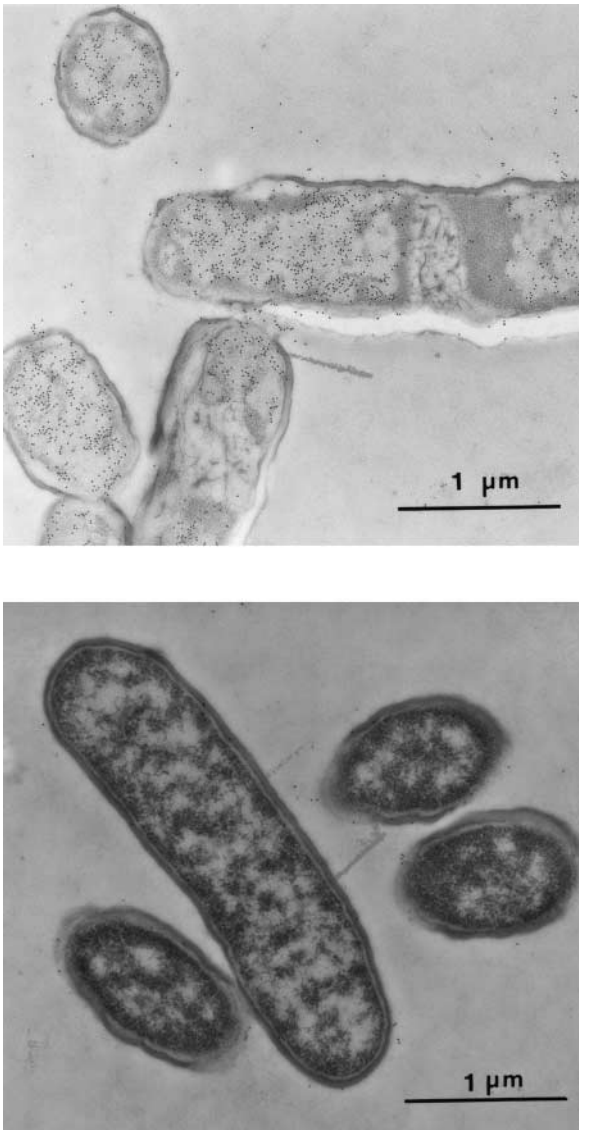

Fig. 3. Cellular localisation of InaV protein synthesised in *E. coli*. Electron micrographs of *E. coli* BZ234 transformed with pDP245/*inaV* (top panel) and *E. coli* BZ234 without plasmid (bottom panel). InaV proteins were detected by gold-labelled secondary antibodies.

Cultures of *P. syringae* and *inaV* expressing *E. coli* and the total cell lysates of both organisms after French press treatment displayed ice nucleation thresholds of about −3°C, indicating the presence of type 1 ice nuclei [10]. As shown for *E. coli*, this high threshold temperature was reduced after separation into membranes and cytoplasm by ultracentrifugation, implying that the fractionation process destroyed the nucleators with the highest threshold. The resuspended membrane fraction displayed an ice nucleation activity with a threshold temperature at −4°C while the cytoplasm was active at −4.5°C (for cumulative ice nucleation spectra, see Fig. 1). The anionic non-denaturing detergent NLS was found to solubilise the InaV protein from isolated membranes as efficiently as the denaturing, anionic detergent SDS. The mild, non-ionic detergents OSGP and Triton X-100 were less efficient in solubilising the ice nucleation protein. After solubili-

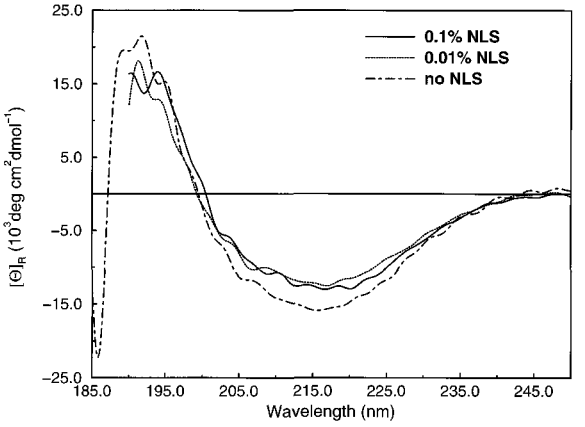

Fig. 4. CD spectra of purified InaV protein in various concentrations of the detergent NLS. The protein was in buffer A at concentrations of 0.24 mg/ml (0.1% NLS), 0.25 mg/ml (0.01% NLS) and 0.17 mg/ml (no NLS).

sation in NLS, OSGP or Triton X-100, centrifugation and dialysis of the soluble proteins against buffer B to remove the detergent, the samples showed an ice nucleation activity with a threshold temperature at −7.5°C. By contrast, SDS-solubilised InaV protein was inactive, apparently irreversibly denatured by the detergent.

As the cytoplasm contained the bulk of the protein product after expression of the *inaV* gene, this fraction was chosen for further purification. The ice nuclei from the cytoplasm which were active between −4.5°C (after ultracentrifugation to remove the membranes) and −6.0°C (after anion-exchange chromatography) are of type 2 [10]. The cytoplasmic InaV protein was highly aggregated as it could be pelleted by overnight ultracentrifugation. The aggregation is probably the reason for the unexpected behaviour of the ice nucleation protein on anion-exchange chromatography. Although the theoretical, calculated p*I* value of the highly homologous InaZ protein is 4.64, the InaV protein, assumed to be negatively charged at pH 8.0, did not bind to the anion-exchange column. After solubilisation of these aggregates in either glycerol/NLS or guanidine hydrochloride the InaV protein could be purified by gel filtration to a single band on SDS-PAGE. Both solubilisation procedures yielded about 10 mg pure protein from 1 l culture.

InaV protein, purified in NLS, displayed an ice nucleation activity with a threshold temperature of −7.5°C (Fig. 1), corresponding to type 3 activity. The solubilised InaV protein eluted in presence of 0.1% detergent near the void volume of the gel filtration column, implying a molecular mass of more than 2000 kDa. The pure protein in 0.1% NLS was therefore still aggregated, composed of more than 12 monomers. After extensive dialysis to remove the detergent completely, the ice nucleation activity remained unchanged, even though the InaV aggregates became larger as seen by the turbidity of the dialysed solution. Gel filtration in guanidine hydrochloride produced two pure InaV protein fractions with apparent molecular masses of 900 kDa and 380 kDa respectively in a ratio of 1 to 4 (result not shown). Reconstitution of ice nucleation activity was achieved by dialysis against buffer A containing the reducing agent dithiothreitol, which was found to be indispensable for recovery of activity. During this dialysis the protein solution became turbid as a consequence of protein reaggregation. The ice nucleation activity of this solution displayed a threshold temperature of −8.0°C. The finding that InaV is aggregated even in the presence of detergents or denaturing agents is in agreement with results from Wolber et al. [20]. On gel filtration chromatography in 2 M urea/12 mM OSGP most of InaZ showed an apparent molecular mass in excess of 400 kDa; the specific ice nucleation activity was even more concentrated in this fraction, indicating that a monomer of InaZ is not functioning as ice nucleator. We found that InaV protein synthesised in *E. coli*, isolated from the crude membranes as well as purified from the cytoplasm, gave no signal in a carbohydrate detection assay (results not shown). Apparently, the protein is not linked to an oligosaccharide and therefore not anchored to the outer membrane via phosphatidylinositol as proposed by Turner et al. [12] and Kozloff et al. [13].

The fact that we could not find indications for glycosylation as secondary modification and the strong tendency of InaV to self-aggregate imply that the different types of ice nucleators do not differ in the structure of the monomers composing them but in their aggregation/organisation state. After solubilisation, both membrane- and cytoplasm-derived ice nucleation proteins displayed just type 3 ice nucleation activity, indicating the necessity of a membrane environment or aggregation. Purified InaV protein isolated from cytoplasm was found to reaggregate after removal of the solubilising agent but without regaining type 2 activity, the process apparently being irreversible. The most active ice nucleators were in the membrane fraction. We considered them InaV with type 1 activity even though they were slightly less active than those from entire cells were. Type 1 activity requires membrane association, which may support and arrange the aggregation. All attempts to reconstitute purified InaV protein into a set of different phospholipid vesicles failed. The CD spectrum of the NLS-purified protein, which still represented an aggregated form of at least 10 InaV subunits and displayed type 3 activity, showed a well defined secondary structure (Fig. 4): the ratio of intensities between positive and negative bands and the point of intersection of the NLS-free spectrum at 187 nm are indicative of β-sheet structure [23]. The structure did not change upon variation of the detergent concentration. The overall shape of the CD spectra is compatible with the computer modelling structure of InaZ recently proposed by Kajava and Lindow [8]. This hypothetical structure of the 48-residue unit is composed of three β-hairpins, which resemble, in the arrangement of donors-acceptors of hydrogen bonds, that of ice and might promote aggregation of ice nucleation proteins by interdigitation with the 48-residue units of the neighbouring proteins.

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