Modeling *Pseudomonas syringae* Ice-Nucleation Protein as a β-Helical Protein

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ABSTRACT Antifreeze proteins (AFPs) inhibit the growth of ice, whereas ice-nucleation proteins (INPs) promote its formation. Although the structures of several AFPs are known, the structure of INP has been modeled thus far because of the difficulty in determining membrane protein structures. Here, we present a novel model of an INP structure from *Pseudomonas syringae* based on comparison with two newly determined insect AFP structures. The results suggest that both this class of AFPs and INPs may have a similar β-helical fold and that they could interact with water through the repetitive TXT motif. By theoretical arguments, we show that the distinguishing feature between an ice inhibitor and an ice nucleator lies in the size of the ice-interacting surface. For INPs, the larger surface area acts as a template that is larger than the critical ice embryo surface area required for growth. In contrast, AFPs are small enough so that they bind to ice and inhibit further growth without acting as a nucleator.

INTRODUCTION

Essentially all proteins interact with water, but two classes of proteins, antifreeze proteins (AFPs) and ice-nucleation proteins (INPs), have a function that specifically relates to ice. Despite this similarity, they have opposite functions in that AFPs are able to inhibit the growth of ice crystals, whereas INPs are able to promote their formation. The structures of several AFPs have been determined (Davies and Sykes, 1997), including two recent insect AFP structures (Graether et al., 2000; Liou et al., 2000). To date, the structural characterization of INPs has involved sequencing and truncation experiments, activity measurement, peptide studies, and theoretical modeling (for reviews see Gurian-sherman and Lindow, 1993; Warren and Wolber, 1991; Hew and Yang, 1992). In addition to the fundamental interests in understanding how INPs work, practical applications include fusing of other proteins with INPs for presentation on a bacterial cell surface (Beguin, 1999) and the production of artificial snow (Margaritis and Bassi, 1991).

Although several organisms have been identified as having ice-nucleation activity, the best characterized by biochemical methods are the bacterial INPs. Of these INPs, that of *Pseudomonas syringae* is often used as a representative protein. Analysis of the cloned *P. syringae* sequence shows that the protein can be divided into nonrepetitive N- and C-terminal domains and a highly repetitive central domain (Fig. 1). In the central region, the largest repeat element is 48 residues long, which itself consists of three repeats of 16 residues (AGYGSTXTAXXXSXLX, where X is a residue that is not repetitive). The N- and C-terminal regions consist of 175 and 49 residues, respectively, and are believed to anchor the protein to the outer membrane. Mutations of residues in the central domain showed that activity was lost only if the periodicity of the 48-residue repeats were disrupted (Green et al., 1988). This result suggests that the structure of this region is repetitive and modular.

In the absence of a heteronucleus, water can be supercooled to −40°C (Hobbs, 1974). INPs promote the formation of ice by raising the nucleation temperature. In in vitro assays, this temperature ranges from −14 to −2°C. The variability in threshold temperature is believed to come from the number of proteins that can cluster together to form a larger ice nucleus (Govindarajan and Lindow, 1988). To overcome ambiguities in activity measurements, INP assays are typically measured as a frequency of nucleation events over the number of cells present at various temperatures (Southworth et al., 1988).

Several research groups have previously presented theoretical structures of the highly repetitive region (Mizuno, 1989; Warren and Wolber, 1991) and one has described a model of 8, 16, and 24 residue peptides based on NMR data (Tsuda et al., 1997). Given the high degree of sequence repetition, it is commonly accepted that the structure of the central region of INP would also have a high degree of repetition. The simplest repetitive structure for a polymer is a helix, which has led several researchers to suggest some type of helical repeat for INP (Mizuno, 1989; Warren and Wolber, 1991).

Here, we present a new model of the central repeat region of INP as a β-helical fold based on the recently determined insect AFP structures. The structure is derived from modeling, energy minimization, and solvated molecular dynamic (MD) simulations. The result suggests that the insect AFPs and bacterial INPs may have a similar β-helical structure, despite having opposite effects on water molecules, and that the difference in function can be attributed to the difference in size of the ice-interacting surface.

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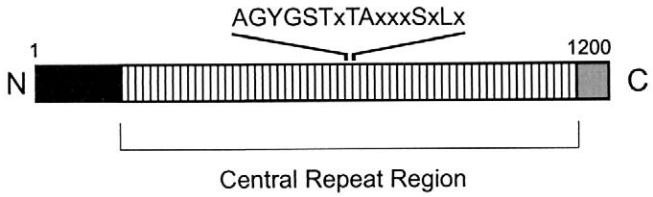

FIGURE 1 Domain structure and sequence repeats in *P. syringae* INP. The boxes show the domain arrangement of the protein with the N-terminal region shown in black, the C-terminal region in gray, and the 61 16-residue repeats as white boxes. Adapted from Wolber and Warren (1989).

MATERIALS AND METHODS

A peptide of the sequence EGSNLTAGYG STGTAGADSS LIAGYG-STQT SGSESSLTAG YGSTQT was synthesized by solid-state methods. The sample was dissolved in 90% H_2O/10% D_2O and the pH was adjusted to a value of 6.6. Circular dichroism (CD) spectra were collected on a Jasco CD-500 at both room temperature and 4°C from 180 to 260 nm. A two-dimensional nuclear Overhauser effect spectroscopy (NOESY) was performed with the sample on a Bruker 500-MHz Avance spectrometer at ambient temperature.

Modeling and MD simulation of INP was performed in Sybyl, version 6.6 (Sybyl, 1999). To start, a single loop of residues 36–53 of the β-helical protein UDP-acetylglucosamine acyltransferase (PDB code 1LXA) (Raetz and Roderick, 1995) was excised from the structure. Residues 52 and 53 were removed to reduce the 18-residue loop to a 16-residue loop and the sequence was modified according to a corresponding INP repeat (AGYG-STQTAGGDSALT). This substitution was subsequently repeated three times and the C- and N-termini of adjacent loops were linked by a peptide bond. To form a geometrically accepted peptide bond and to ensure that the side chains of the modified structure were positioned so as to avoid steric clashes, a 100-step energy minimization was performed using Sybyl. Subsequently, the 48-residue structure was repeated twice, the three repeats were linked together, and the sequence was modified to represent that of INP. An energy minimization of 3000 steps was performed on the 144-residue structure to correct again for improper bond geometries and steric clashes.

After the formation of this β-helical model, a solvated MD simulation was performed using Sybyl to examine the stability of the proposed structure. Solvent and a periodic boundary condition box were introduced as implemented in the program. The latter was present to prevent waters from escaping the box. Waters located inside the protein model (as defined by the surface of the protein) were removed because their presence would cause the subsequent MD simulation to fail. No restraints were added to the MD simulation. To obtain a reasonable sampling of conformational space, the simulation was run for a duration of 300 ps with a step size of 1 fs, and snapshots were taken every 1500 fs for analysis. The pressure was held constant and the temperature was set to 300 K.

RESULTS AND DISCUSSION

We set out to determine a possible structure of bacterial INP. The hypothesis was developed by analyzing the INP sequence, which consists of ~60 16-residue repeats that are similar in length to the left-handed, 15-residue loops of spruce budworm AFP (sbwAFP) (Tyshenko et al., 1997). In addition, these two proteins and the right-handed, 12-residue loops of *Tenebrio molitor* AFP (TmAFP) contain a motif of TXT (where X is any amino acid) in every repeat (Wolber and Warren, 1989; Tyshenko et al., 1997; Graham et al., 1997). Mutation of the threonine residues in the TXT motif of sbwAFP to leucine caused a >70% loss in antifreeze activity, whereas mutations of threonines away from this site caused only a <30% loss, suggesting that the TXT motif constitutes the ice-binding site of the protein (Graether et al., 2000). Because both of the insect AFPs and INPs contain the TXT repeat, and the proteins contain a sequence repeat of similar length, we were interested in knowing whether bacterial INP could also have a β-helical structure.

On the basis of this initial proposal, we synthesized a 48-residue peptide of the sequence outlined in Material and Methods to determine whether it could fold into a β-helix. We performed circular dichroism (CD) and NMR experiments with the peptide and compared the results with the data from earlier CD (Ala et al., 1993) and NMR (Tsuda et al., 1997) experiments. In agreement with the previous CD analysis, we did not detect any secondary structure at room temperature or 4°C (data not shown). In contrast to the previous NMR experiments, where the authors suggested that there is a β-hairpin structure, we did not observe any NOE contacts. The difference between the two NMR studies may be that our analysis could be performed only at room temperature or higher due to equipment constraints, whereas that of the previous one was done at 4°C. However, our CD analysis of the peptide at both temperatures suggests that the observed β-hairpin loops may be only a transitory structure and not representative of the larger number of 16-residue repeats formed in whole INP. Therefore, it is very likely that this 48-residue peptide might not be long enough to form a stable tertiary structure on its own.

Given that analysis of a large membrane-associated protein by x-ray crystallography is difficult, and that analysis of the 48-residue peptide failed to find a stable structure, we decided to model the INP structure and use solvated MD to test the stability of the resultant structure. Performing homology modeling would have been a more systematic approach, but a search of the PDB database did not find any β-helical proteins with loops that were 16 residues long. In addition, a search for the sequence AGYGSTXTAXXX-SXLX (where X is any amino acid) in the PDB database failed to find any matching experimental structures. These approaches were therefore unsuccessful in finding a structure that could be used directly as a starting template.

It was decided to use an 18-residue loop of the UDP-acetylglucosamine acyltransferase, a left-handed β-helical protein, and delete two residues from the loop. The rationale for using an 18-residue loop, rather than, for example, the smaller 15-residue sbwAFP, was that the 18-residue repeat and 16-residue repeat are both even numbered and the structure of the loops is more regular compared with that of several other β-helical proteins. Therefore, the 48-residue repeat of INP would simply consist of three turns of the 16-residue repeat.

Naturally we cannot, without experimental data, predict whether a β-helical INP should consist of left- or right-handed loops. Because spruce budworm AFP is a left-handed β-helix, and *Tenebrio molitor* AFP is a right-handed β-helix, we can conclude that handedness is not important in the interaction between protein and ice. Because the starting template is left-handed, the INP model is left with the same handedness.

Residues 36–53 of the acyltransferase were chosen because it contained a GQG at a corner of the loop, which would serve as a starting site for the GYG repeat of INP. To apply the INP sequence to the acyltransferase, three rules were employed. First, as mentioned, given the propensity of Gly to be found in β-helical turns, the GYG repeat would be placed at the turn of the loop corresponding to the GQG sequence of the template. Second, the Thr residues of the TXT motif were placed so that they would be facing the solvent and the X residues (usually Gln or Gly) would be facing inward and that this motif would not be located near a turn in the loop. This would give these residues a similarly stacked arrangement as in sbwAFP and TmAFP (Graether et al., 2000; Liou et al., 2000). Third, the Leu residue, the only hydrophobic amino acid that is consistently present in the 16-residue repeats, would be located in the core of the protein, analogous to the leucine stacks found in the hydrophobic core of this acyltransferase and other β-helical proteins (Heffron et al., 1998).

As expected, the resulting 16-residue loop (Fig. 2 *A*) resembles the loops of the 18-residue starting template in several ways (Fig. 2 *B*). The conserved Gly residues of GYG and the often-present TAG repeat are at the turns in the loop, and the Leu residues are inside the protein. The third turn has a Ser residue, which is also found in one turn of the acyltransferase (Raetz and Roderick, 1995). Each side of the starting, 18-residue template has six residues. For the 16-residue loop, the TXT side contains six residues, whereas the two flanking faces have five residues each. Although other arrangements are possible, this one does not violate the rules outlined above and reasonably resembles the arrangement of the source β-helical protein.

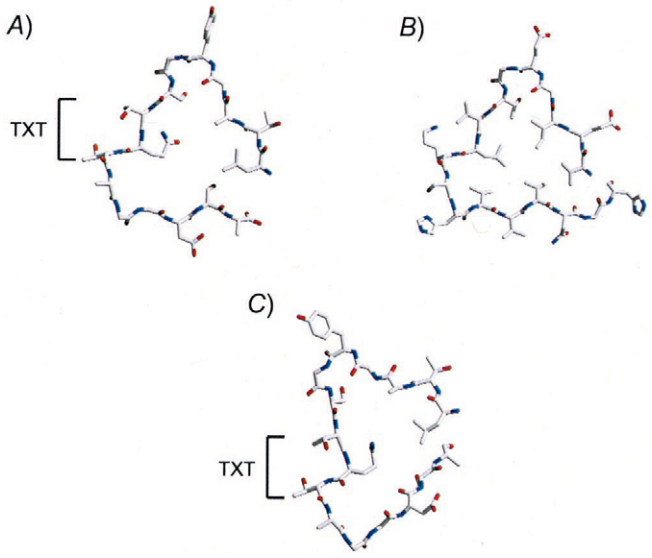

FIGURE 2 Cross section of modeled INP and a β-helical protein. All figures show a wire frame representation of one loop. (*A*) Cross section of the modeled INP after 100 steps of energy minimization. (*B*) Cross section of the modeled INP after 3000 steps of energy minimization. (*C*) Cross section of UDP-acetylglucosamine acyltransferase, residues 36–53.

Subsequently, the 16-residue template loop was repeated three times and the separate loops were linked together by the formation of a peptide bond. The loops were aligned such that successive ones were placed 4.5 Å apart, a spacing similar to that of the insect AFPs (Graether et al., 2000; Liou et al., 2000). An energy minimization of 100 steps was performed on the 48-residue structure to allow peptide bonds of acceptable geometry to form between adjacent loops. Finally, the 48-residue structure was repeated twice to form a 144-residue β-helix structure, and a longer energy minimization of 3000 steps was performed.

Interestingly, the cross section of the loop of the β-helical fold after the 3000-step energy minimization shows that the approximately triangular shape had collapsed to a more oval one (compare Fig. 2, *A* versus *C*). The cross section now looked less like the triangle found in the 15-residue loop sbwAFP (Graether et al., 2000) or the 18-residue loop UDP-acetylglucosamine acyltransferase (Fig. 2 *B*), but somewhat akin to the 12-residue loop of TmAFP (Liou et al., 2000). The TmAFP lacks a hydrophobic core and is mainly held together by interloop disulfide bonds. INPs may have compensated for the lack of a hydrophobic core and disulfide bonds by the presence of Gln in approximately two of every three TXT motifs. This Gln repeat is similar to the Asn ladder seen in the core of other β-helical proteins (Yoder et al., 1993), which may stabilize the protein by forming hydrogen bonds to backbone nitrogen and oxygen atoms on the other side of the loop. A Gly (and occasionally a Ser) residue is present in every third repeat. The presence of a small amino acid may help keep the β-helical axis straight and therefore the ice-template flat. Such an explanation agrees with what has been observed for the fish type III AFP, where flatness is said to be important for effective ice binding (Jia et al., 1996; Yang et al., 1998).

A 300-ps solvated MD simulation was performed to test the stability of the energy-minimized model. Overall, the protein essentially retained its β-helical fold despite the lack of any constraints in the simulation (Fig. 3). The figure shows that the C-terminal region has unraveled somewhat. This is not surprising, because the whole INP would consist of approximately five more of the 144-residue repeats followed by a C-terminal domain. The C-terminus may act as a capping structure to prevent the repeats from unfolding. The total energy of the system at the end of the simulation versus the average value decreased during the simulation by

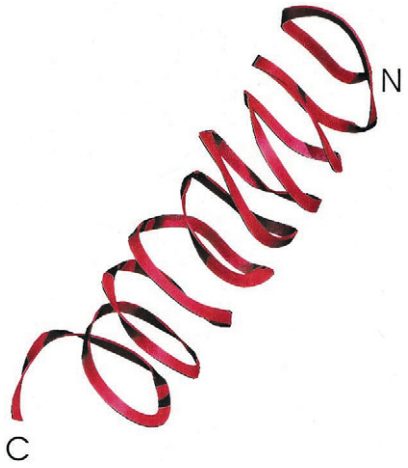

FIGURE 3 Ribbon representation of a β-helical INP, depicting the structure of the 144-residue INP model after 300 ps of solvated MD.

~130 kcal/mol, and Ramachandran analysis using PROCHECK (Laskowski et al., 1993) shows that 94% of the residues are in allowed ϕ, ψ regions. In addition, a 3D/1D profile score was calculated to ensure that the model is structurally reasonable (Luthy et al., 1992). The calculation shows that the average profile score is 0.35, with only three residues having a score below 0.2 (residue 121, 0.18; residue 127, 0.19; and residue 129, 0.17). Therefore, according to this method, the INP model is completely plausible.

In the absence of experimental data, there is naturally no reliable way to assess the validity of our β-helical INP model. Previously, CD experiments were carried out using whole INP, but the presence of solubilizing detergent led to a somewhat noisy spectrum (Schmid et al., 1997). Although these data cannot prove that the INP is β-helical, it does confirm that there is a considerable amount of β-sheet in the structure. In another CD experiment, the β-helical pectate lyases were examined to see whether they had a unique spectral profile that made a distinction between the β-helical proteins and proteins with anti-parallel β-sheets (Sieber et al., 1995). Unfortunately, to distinguish the two types of structures unequivocally, the authors calculated a derived spectrum from the pectate lyases, which required taking the percentage of the various secondary structures as defined by x-ray crystallographic structures into account. A very recent study has also excluded the use of Fourier-transformed infrared spectroscopy as a method to distinguish β-helical proteins from other folds containing β-sheets (Khurana and Fink, 2000).

The presence of TXT motifs in both insect AFPs and bacterial INPs suggests that the surfaces consist of a repetitive arrangement of threonyl oxygens that mimic ice. However, studies of the fish type I AFP show that hydrogen bonds are insufficient in explaining the ice-binding interaction, and the actual binding mechanism may be considerably more complex (Chao et al., 1997; Haymet et al., 1998; Zhang and Laursen, 1998). Similar observations were made for type III AFP (Chen and Jia, 1999; Graether et al., 1999). More studies will be required before we can understand the interaction between ice and AFPs or INPs. Simply from geometric considerations, however, an ice-binding model was developed for the TXT-containing TmAFP, where TXT can essentially match the ice lattice and participate in the hydrogen-bonding network by replacing the corresponding oxygen atoms of the ice section (Liou et al., 2000). If this model is correct, it would not be surprising that INP could also have a similar interaction with ice.

If some AFPs and INPs have a similar structure and ice-binding or ice-forming TXT motifs, what property would then distinguish the two activities? The difference may lie in the disparity in size between the two classes of proteins. The sbwAFP and TmAFP have ~6 TXT motifs, whereas a single INP has 61 TXT motifs, or over 10 times the number compared with the AFPs. For an ice embryo to continue growing at −12°C (the approximate ice-nucleating temperature of one INP), it would have to be a sphere with a surface area of 20,100 Å^2 (Hobbs, 1974). The TXT face of the modeled INP has a surface area of ~4200 Å^2. To elevate the ice-nucleation temperature to −2°C, approximately 125 copies of INP are required (Govindarajan and Lindow, 1988). With this arrangement, the effective ice-forming surface of INP would be 525,000 Å^2, and the minimal ice-embryo spherical surface area would be 1.02×10^6 Å^2. Although the mathematical agreement between the surface area of INP and the required ice-embryo surface area is not perfect (nearly a fivefold and twofold difference, respectively), the values are certainly much closer in agreement than that between one AFP and an ice embryo. For the β-helical AFPs from spruce budworm and *Tenebrio molitor*, the TXT motif surface area is ~250 Å^2, 1/80 the size required to nucleate ice at −12°C and 1/4000 the size required to nucleate ice at −2°C. If the water molecules bound to an AFP surface were to form an ice-like arrangement, this embryo would be far too small to be stable and would melt rather than grow. AFPs may therefore bind to ice by mimicking an ice surface, but unlike INPs, they are not able to promote its formation.

The two insect AFPs and the bacterial INPs may not be the only ice-interacting proteins with a β-helical fold. Recently, a carrot AFP has been cloned by two groups (Meyer et al., 1999; Smallwood et al., 1999). Both noted that the 23-residue repeat sequence is rich in Leu residues. Interestingly, Leu-rich repeats have been found in other β-helical proteins (Heffron et al., 1998). Based on this information, preliminary modeling suggests that carrot AFPs could also form a β-helix with 23-residue loops (Z. Jia and V. Petrounevitch, unpublished). Furthermore, an AFP isolated from ryegrass also has an intriguing seven-residue repeat rich in a number of residues, including Ser and Thr (Sidebottom et al., 2000). Despite the diversity of fish AFP folds, these protein sequences suggest that the β-helical fold may

be found in several insect and plant AFPs and bacterial INPs.

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