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Identification of the ice-binding face of antifreeze protein from *Tenebrio molitor*

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Abstract The beetle *Tenebrio molitor* produces several isoforms of a highly disulfide-bonded β-helical antifreeze protein with one surface comprised of an array of Thr residues that putatively interacts with ice. In order to use mutagenesis to identify the ice-binding face, we have selected an isoform that folds well and is tolerant of amino acid substitution, and have developed a heating test to monitor refolding. Three different types of steric mutations made to the putative ice-binding face reduced thermal hysteresis activity substantially while a steric mutation on an orthogonal surface had little effect. NMR spectra indicated that all mutations affected protein folding to a similar degree and demonstrated that most of the protein folded well. The large reductions in activity associated with steric mutations in the Thr array strongly suggest that this face of the protein is responsible for ice binding.

Key words: Antifreeze protein; Folding; Insect; Mutagenesis; NMR; Thermal hysteresis

1. Introduction

The beetle *Tenebrio molitor* (Tm) produces several isoforms of a potent antifreeze protein (AFP) [1,2]. AFPs bind to the surface of ice crystals to inhibit their growth, thereby lowering the freezing temperature of the surrounding solution below the melting temperature [3,4]. The difference (°C) between this depressed freezing temperature (non-equilibrium freezing point) and the unaltered colligative melting temperature in a solution containing ice is referred to as thermal hysteresis (TH). For this reason, insect AFPs are often referred to as thermal hysteresis proteins (THPs) [5].

T. molitor THP isoforms are comprised of seven or eight tandem repeats of a 12-a.a. sequence (TCTxSxxCxxAx), although a 10-repeat sequence has also been seen at the cDNA level [1,2]. The structure of an isoform with seven repeats, 'Tm 2-14', has been solved by both X-ray crystallography (PDB 1EZG) and nuclear magnetic resonance (PDB 1L1I) and is an extremely regular right-handed β-helix [6,7]. Each 12-a.a. repeat forms a loop (or coil) of the helix that is internally disulfide-bonded, although the first two repeats at the N-terminal end deviate somewhat from the consensus sequence and contain an additional disulfide bridge. The repeats are stacked side by side such that the protein presents a regular, two-dimensional array of Thr residues on one side of the molecule (Fig. 1A).

The spacing of the hydroxyl groups of the Thr residues on this face of the protein closely resembles that found on the prism plane of ice ({10-10}), suggesting that this surface of the protein is complementary to ice and may be responsible for ice binding. The average distance between TCT motifs in adjacent loops is 4.64 Å, while the average distance between Thr residues within each TCT motif is 7.44 Å [6]. This spacing makes a very good match to the distances on the prism plane of ice, where the O-atom spacing found along the *a*-axis is 4.52 Å and the spacing in the direction perpendicular to the *a*-axis is 7.35 Å [6]. The spacing of the Thr array also approximates the spacing of O-atoms on the basal plane of ice, which is 4.52 Å by 7.83 Å.

Although Thr arrays have been implicated in the binding to ice of type I AFP from flounder [8–11] and spruce budworm AFP [12], regularly spaced Thr residues are not a requirement in all AFPs. Shorthorn sculpin (shs) produces Ala-rich α-helical type I AFPs that lack repeating Thr residues. These helices are amphipathic with a lysine-rich face that has been modeled as the ice-binding face [13]. However, it has recently been demonstrated that, like flounder AFP [14], shsAFP binds to ice through its Ala-rich hydrophobic surface [15]. These examples illustrate that it cannot be assumed that an ice-binding face requires Thr residues or that regularly spaced hydroxyl groups necessarily constitute the ice-binding surface of an AFP.

To determine if the Thr array of TmAFP constitutes the ice-binding face of this protein, a number of Thr residues were mutated to bulkier amino acids (Leu, Lys and Tyr) that would be expected to sterically interfere with ice binding and diminish TH activity. It has been hypothesized that AFPs dock directly to the surface of the ice lattice (e.g. [3,8,10]) using the analogy of a receptor (AFP):ligand (ice) interaction [16]. Because docking occurs between the relatively 'flat' ice-binding face of the AFP and a complementary surface of ice, the

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Abbreviations: AFP, antifreeze protein; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; Osm, osmols; PDB, protein database; shs, shorthorn sculpin; TH, thermal hysteresis; Tm, *Tenebrio molitor*; TOCSY, total correlation spectroscopy

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introduction of a bulky residue protruding from the ice-binding face of the AFP should prevent the simultaneous engagement to ice of all ice-binding residues. Steric mutations have been employed to delineate the ice-binding faces of several AFPs [12,14,15]. Due to the possibility that a mutation might reduce TH activity by disrupting the tertiary structure of the protein, nuclear magnetic resonance (NMR) spectroscopy was used to confirm that mutant TmAFPs were properly folded.

2. Materials and methods

2.1. Thermal hysteresis activity assay

TH activity was assayed using a nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA) [17]. This standard procedure was slightly modified as described by Liou et al. [2] to accommodate the high activity of insect AFPs. All assays were done in 100 mM NH_4HCO_3 and protein concentrations were determined by amino acid analysis (Advanced Protein Technology Centre, Hospital for Sick Children, Toronto, ON). TH is defined as the difference between the melting point and the non-equilibrium freezing point in a solution containing ice. This freezing point is determined by slowly cooling a drop of AFP solution that contains a small seed ice crystal until the ice grows. In order to improve the consistency of the assay, all seed crystals were manipulated by another cycle of freezing and melting to attain a very small crystal with a smooth and uniform surface before measuring the freezing temperature.

2.2. Recombinant expression and mutagenesis of TmAFP

As described previously [18], the Tm 2-14 gene was cloned into the expression vector pET 20b(+) and transfected into *E. coli* BL21 (DE3). Mutations were produced using the method of Kunkel [19] and all mutations were confirmed by DNA sequencing. Cultures containing wild-type or mutant genes were grown at 37°C in 4 l LB broth with 100 μg/ml ampicillin to an OD_{600nm} of ~1.0 and protein expression was induced with 100 μg/ml isopropyl-β-D-thiogalactopyranoside (IPTG). After incubation for 3 h at 37°C, the culture was left at room temperature overnight, and then cells were harvested by centrifugation, resuspended in 50 ml 100 mM Tris–HCl (pH 9.0), 0.1 mM phenylmethyl-sulfonyl fluoride (PMSF) and lysed by sonication. Cellular debris was removed by centrifugation. The Tm 4-9 gene was cloned into pET 24a(+) and expressed similarly, but in 100 μg/ml kanamycin.

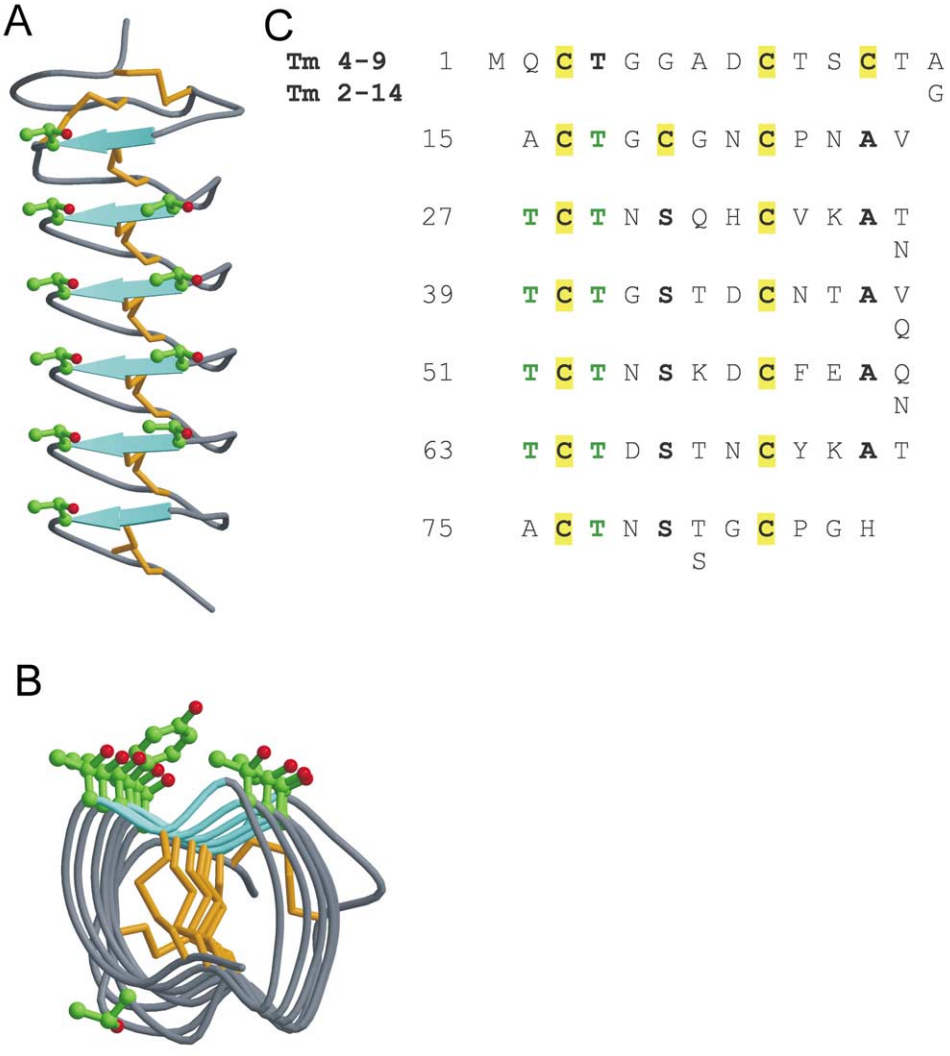

Fig. 1. Models and sequence alignments of TmAFP. (A) An energy minimized model of Tm 4-9 based on the structure of Tm 2-14 [6] (SYBYL, Tripos, St. Louis, MO, USA). (B) A model of the Tm 4-9 steric mutant T41Y, illustrating how the introduction of the Tyr residue disrupts the Thr array. The residue T44, which was replaced with Tyr as a 'control' mutation, is also shown. Green: Thr and Tyr side-chains with O-atoms in red; orange: disulfide bonds; cyan: β-strands. (C) Sequence alignment of the TmAFP isoforms 4-9 and 2-14. The sequence of Tm 4-9 is shown in the top line. At the five positions that vary between these two isoforms, the residue found in 2-14 is shown below. Residues that are highly repetitive between loops are bold, with Cys residues highlighted in yellow and the Thr residues of the Thr array in green.

2.3. Refolding and purification

Immediately after sonication, the cell lysate typically shows little or no TH activity. To refold the protein, the cell lysate was allowed to oxidize for several weeks at 4°C in 50 ml tissue-covered tubes and the TH activity was monitored periodically [18]. To improve the yield of well-folded TmAFP, some modifications were made to this previously described procedure. To maximize disulfide bonding, 25 mM oxidized glutathione (GSSG) was added to the cell lysates of the expressions of wild-type Tm 4-9 and mutants once substantial TH activity was measured (~1000 mOsm for wild-type protein) or when TH activity appeared to reach a plateau (for mutants with low activity). Following the addition of glutathione, TH measurements were continued periodically until the TH activity stopped increasing. At this point, we tested the stability of the protein by heating the sample to 50°C for 15 min or leaving it overnight at 25°C. We have found that completely disulfide-bonded protein is very stable and its TH activity is not affected by heating. Partially folded protein can have considerable activity but is less resistant to heating. When the TH activity of the lysate was no longer significantly reduced by heating, the protein was purified. The supernatant was loaded on a Sephadex G-75 size exclusion chromatography column (5.0×100 cm, Pharmacia) and eluted with 10 mM Tris–HCl (pH 9.0) at a flow rate of 1.2 ml/min [18]. Active fractions were pooled, lyophilized, resuspended in 0.05% TFA, and separated by reversed-phase high performance liquid chromatography (HPLC) on a preparative C-18 column with a linear gradient (10–40%) of buffer B (80% acetonitrile, 0.05% TFA) over 30 min at a flow rate of 8 ml/min.

2.4. NMR spectroscopy

In order to determine the degree of folding of the wild-type TmAFP protein and the selected mutants, NMR spectra were obtained. Samples were prepared by dissolving approximately 2 mg of each protein in 500 μl of 90% H_2O/10% D_2O (by volume). The pH was adjusted to 5.3 with μl aliquots of 100 mM NaOD or DCl as required, and 0.1 mM 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) was added as an NMR chemical shift reference. All spectra were acquired at 30°C on a Varian Unity 600 MHz spectrometer equipped with a 5-mm triple resonance probe and *z*-axis pulsed field gradients. The experiments included two-dimensional ^{1}H homonuclear total correlation spectroscopy (TOCSY) spectra [20,21] with a mixing time of 54 ms. The acquired data consisted of 1024 complex data points in the acquisition domain and 256 complex data points in the indirectly detected domain. The spectral width for both dimensions was 7000.4 Hz. The NMR spectra were processed using VNMR 6.1B software on a Sun workstation.

3. Results and discussion

3.1. Isoform Tm 4-9 folds better than 2-14

The folding of recombinant TmAFP isoform 2-14 takes several weeks and has an appreciable failure rate, with approximately one in four preparations yielding low TH activity. Nevertheless, steric mutations were initially made to the Tm 2-14 isoform because the crystal structure of this protein had been solved [6]. When various bulky residues were substituted for Thr residues in two different positions in the putative ice-binding face, the TH activity of the mutants was low, as expected. During purification, the elution peaks from HPLC were typically less sharp than those of the wild-type, and NMR spectra indicated that these mutations produced misfolded proteins (not shown).

Because Tm 2-14 was difficult to refold, we compared the expression of another TmAFP isoform, Tm 4-9. Tm 4-9 protein is much more abundant than 2-14 in the hemolymph of *T. molitor* [2] and, although it only differs from 2-14 at five residues (Fig. 1C), recombinant Tm 4-9 was found to fold more rapidly and reliably than 2-14. Fig. 2 shows the development over one month of TH activity in the cell lysates from 4 l cultures of Tm 2-14 and 4-9 grown on the same day. The 4-9 lysate exhibits higher TH activity shortly after cell lysis (2 h), subsequently gains activity more rapidly, and reaches a higher TH activity than 2-14 after an extended period of folding. Aliquots were withdrawn and left at room temperature overnight at various times during the refolding process. As TmAFP refolding approaches completion, its activity becomes more resistant to incubation at room temperature (Fig. 2). This led to the development of a 50°C heating test to assess folding. Based on a limited number of expressions, it appears that Tm 4-9 preparations have a lower failure rate than 2-14.

The two-dimensional ^{1}H TOCSY spectra of both the Tm 2-14 and Tm 4-9 isoforms displayed in Fig. 3 are of excellent quality and are typical of high resolution spectra of small, stable and well-folded proteins. In particular, the wide chemical shift dispersion for the proton resonances in the individual amino acid spin systems indicates the folded nature of the protein. The observed resonance linewidths are typical for a protein of 8.5 kDa, suggesting there is no aggregation of the protein in solution. We have previously used two-dimensional ^{1}H TOCSY NMR spectra to identify improperly folded protein (see [18] and Section 3.2). In these spectra there is little indication of resonances that would be caused by incorrectly folded protein or by the protein existing in multiple conformations. Finally, the spectrum of the Tm 4-9 isoform is almost identical in chemical shift dispersion and resonance linewidth to that of Tm 2-14, which suggests that although the folding pathways may differ in rate and efficiency, the well-folded form of each isoform is ultimately very similar.

3.2. Tm 4-9 mutations

The improved folding observed for Tm 4-9 suggested that this isoform might be more tolerant of mutation than Tm 2-14. Six steric mutations (T41Y, T41L, T41K, T63Y, T27Y, T39Y) were made to the putative ice-binding face to sample four different positions and three types of replacements at one position. The NMR spectra show that the wild-type protein (Fig. 4A), as well as the samples of two representative ice-binding face mutants (T27Y and T39Y, Fig. 4B and C), consist primarily of properly folded protein with a small fraction of unfolded protein present. This is

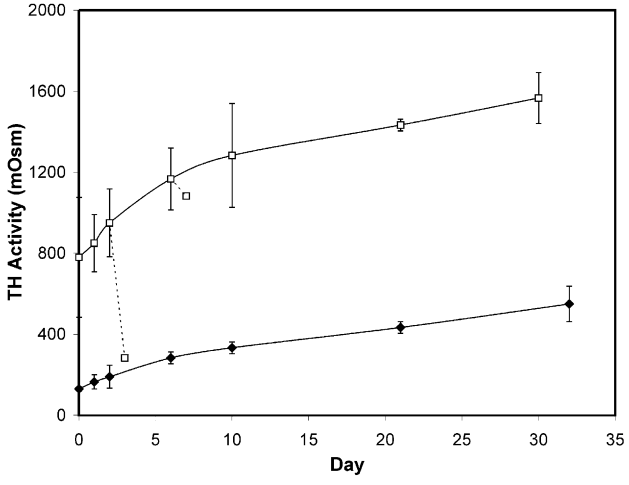

Fig. 2. Development of TH activity during refolding of Tm 2-14 (♦) and 4-9 (□). Both isoforms were expressed concurrently in *E. coli* and the TH activity of the cell lysate, which was allowed to oxidize at 4°C, was monitored periodically over the course of several weeks. On days 2 and 6, aliquots were heated to 25°C overnight, and re-assayed the next day (dotted lines).

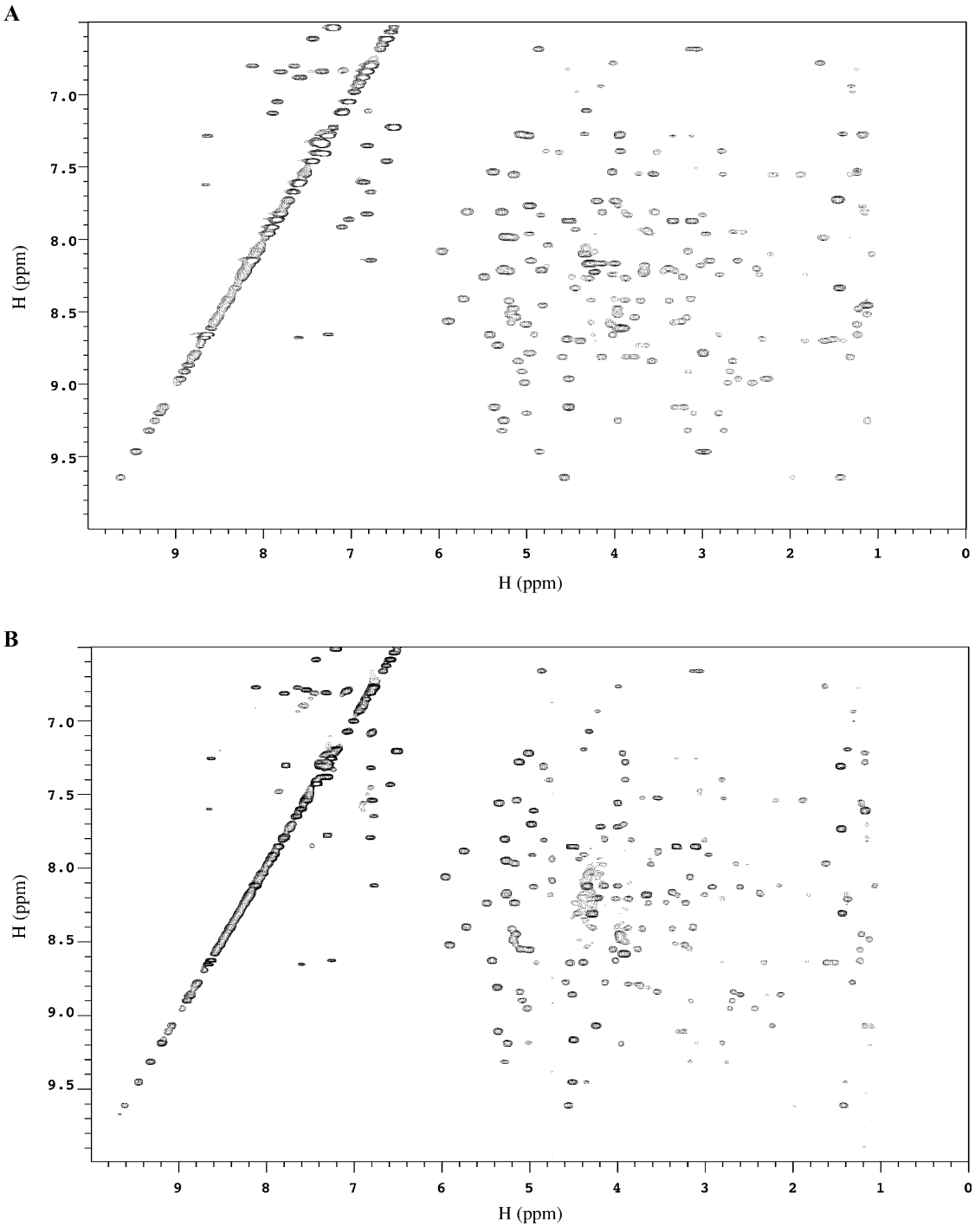

Fig. 3. Regions of the two-dimensional TOCSY ^{1}H NMR spectra showing correlations of NH and aromatic protons to protons in the rest of the spectrum for (A) TmAFP isoform 2-14 and (B) TmAFP isoform 4-9.

evidenced by the existence of a poorly defined broad underlying component in the 'fingerprint' region of the two-dimensional ^{1}H TOCSY spectrum, which contains some of the amide NH-αCH-βCH connections via the amino acid spin systems. The broad, undefined region from 8.0 to 8.5 and 4.0 to 4.5 ppm is indicative of a small amount of unfolded, random coil protein [18]. While reversed-phase HPLC is somewhat effective at separating well-folded TmAFP from improperly folded forms, it is difficult to remove all traces of unfolded protein.

Fig. 1B is a model of a steric mutant (T41Y), illustrating how the presence of a Tyr residue would disrupt the flatness of the ice-binding face. We selected Tyr as a steric substitute for Thr in a number of positions because Tyr and Thr have similar Kyte–Doolittle hydrophobicity values [22]. Tyr also has a high propensity for formation of β-sheet [23,24] and the pairing of a Tyr residue with a Thr residue across adjacent β-strands has been demonstrated to form a particularly stable interaction [25].

All six mutant proteins were purified and their TH activity

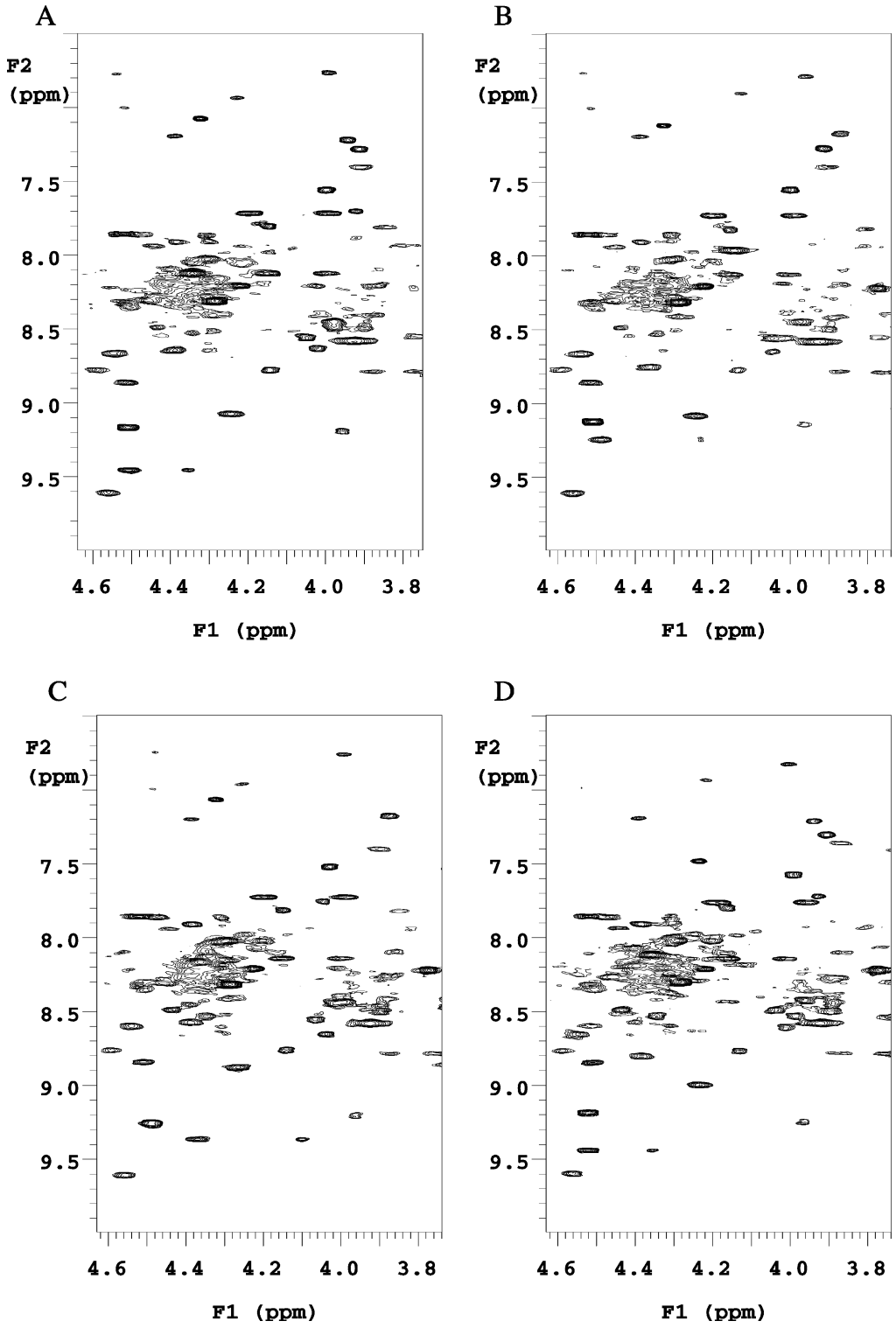

Fig. 4. Comparative two-dimensional TOCSY NMR spectra focusing on the fingerprint region of the protein spectrum, which shows correlations of NH protons to αCH (and some βCH) protons. This region is diagnostic of the misfolded TmAFP isoforms. (A) Wild-type TmAFP 4-9, (B) T27Y mutant, (C) T39Y mutant and (D) T44Y mutant.

was assayed over a range of concentrations (typically 0.1 to 1.5 mg/ml). A mutation near the center of the long rank of six Thr residues, T41Y, exhibited about 10% of the TH activity of wild-type Tm 4-9. To investigate whether particular positions in the Thr array are more important than others for TH activity, three more Thr residues within the array (27, 39 and 63) were each changed to Tyr. T39 is in the same loop as T41 but lies in the rank that contains four Thr and two Ala residues rather than six Thr (Fig. 1C). As seen in Fig. 5A, the T41Y and T39Y mutations both decreased activity by ~90%, indicating that although the 'long' rank contains two more Thr residues, it is no more important for TH activity than

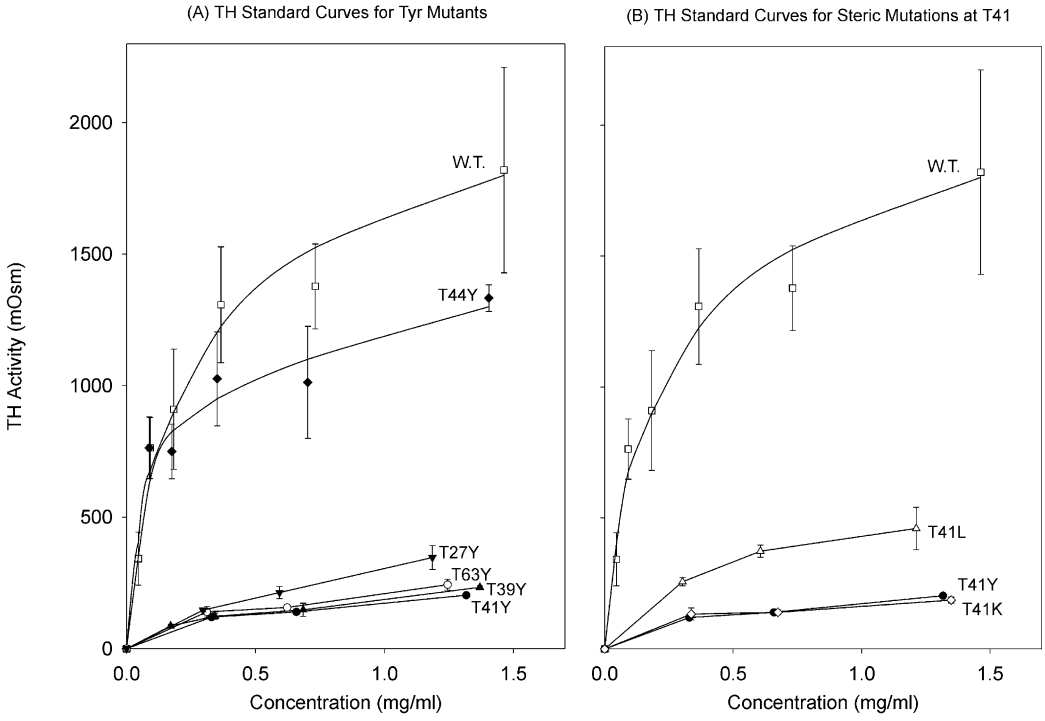

Fig. 5. Standard activity curves for wild-type Tm 4-9 and steric mutants. (A) TH activity (mOsm, where 1 Osm equals 1.86°C) versus concentration (mg/ml) for wild-type Tm 4-9 (□) and mutants with Thr to Tyr substitutions at five different sites (T41Y ●, T39Y ▲, T63Y ○, T27Y ▼ and T44Y ♦). T41, T39, T63 and T27 are part of the putative ice-binding Thr array while T44Y is on another face of the protein. (B) Standard activity curves for wild-type Tm 4-9 and three mutants with amino acid substitutions at position 41 (T41Y ●, T41L △ and T41K ◇).

the 'short' rank. The mutation to Tyr of residues T63 and T27, the terminal Thr residues at either end of the shorter rank, decreased activity by ~90% and ~80%, respectively, suggesting that the ends of the Thr array are critical for TH activity.

To check that the loss of activity seen for Thr to Tyr mutants is not peculiar to the amino acid Tyr, two other residues were substituted for Thr at position 41. The bulky residues Leu and Lys were chosen because they have been employed previously to map the ice-binding faces of other AFPs [12,14,15]. The introduction of a Lys residue caused a reduction in activity similar to Tyr (~90%), while mutation to Leu reduced activity by ~75% (Fig. 5B). It appears that activity loss may be related to the size of the amino acid introduced to the ice-binding face.

To determine whether the loss of activity associated with steric mutations of Thr residues is specific to the putative ice-binding face, a Thr residue on a face of the protein that is orthogonal to the ice-binding face (Fig. 1B) was also mutated to Tyr. The position T44 was chosen because this residue is part of the same loop as T41 and T39, which were also mutated to Tyr, and because this residue is surrounded by fewer bulky groups and is more exposed than the other Thr residues found outside the Thr array. The 'fingerprint' region of the two-dimensional ^{1}H TOCSY NMR spectrum (Fig. 4D) of this mutant is similar to the others shown, indicating that the sample also consists primarily of well-folded protein with a small percentage of unfolded protein. The mutation T44Y resulted in only ~20% loss of TH activity, suggesting that this Thr residue, and by inference the surrounding region of the β-helix, are not crucial for ice binding.

The major challenge to the production of recombinant TmAFP lies in folding this protein. The slow pace of this process may be attributable to TmAFP's unusual structural features. It has no hydrophobic core, but is held together by an extraordinarily dense arrangement of disulfide bonds and an extensive network of intra- and inter-loop hydrogen bonds. The 12-a.a. repeats of which TmAFP is comprised represent the smallest 'loop' found to date in a β-helix [6], most of which are formed from loops of at least 15 amino acids. Creating a complete loop from 12 amino acids requires the formation of a γ- or β-turn at each of the four corners of the rectangular cross section and an internal disulfide bond.

Refolding wild-type Tm 2-14 approaches the limit of what can be accomplished, and mutations in this protein made proper refolding beyond reach. Fortunately, isoform 4-9 folded more rapidly and also proved more tolerant of mutation. To minimize the amount of incompletely folded protein co-purifying with the well-folded AFP, the extent of folding was assessed prior to purification by measuring the TH activity remaining after a period of heat treatment. This is important because the final purification step (reversed-phase HPLC) does not completely separate partially folded from fully folded protein. NMR analysis confirmed that the incompletely folded form was a minor component of the purified AFP preparations and that the fraction of protein that was not well folded was fairly constant from one mutant to another. Therefore, it is reasonable to conclude that the ~90% loss of activity seen for the steric mutants T41Y and T39Y, versus the ~20% loss seen for the control mutation T44Y, reflects steric interference with the binding of the Thr array to ice.

These mutations are analogous to steric mutations used to map the ice-binding faces of other AFPs like the spruce budworm AFP [12], and type I AFPs from winter flounder and

shorthorn sculpin [14,15]. In type I AFPs, where the introduction of a Leu or Lys residue onto the Ala-rich ice-binding surface drastically reduced TH activity, the same mutation on the opposite side of the helix had little effect [14,15].

The redefinition of the ice-binding site in the type I AFPs raises an interesting parallel to this study. In essence, the ice-binding site of these AFPs proved to be the perfectly conserved alanine-containing surface of the helix rather than the hydrophilic, less well-conserved face containing some regularly spaced Thr and Asx. In retrospect, sequence conservation should have been the first clue to identifying the key structural element of this protein. The same argument can be made here. In the sequence of the 12-a.a. repeats, TCTxSxxCxxAx, four of the consensus residues have side-chains directed into the helix core. Only the two threonines have solvent-accessible side-chains. Thus, once again, the most highly conserved residues on the surface of the AFP are implicated in ice binding. Identifying the ice-binding face of TmAFP is a prerequisite to modeling its interaction with ice.

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