Antifreeze Proteins of the Beetle *Dendroides canadensis* Enhance One Another's Activities†

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ABSTRACT: Larvae of the beetle *Dendroides canadensis* produce a family of 13 antifreeze proteins (DAFPs), four of which are in the hemolymph. Antifreeze proteins lower the noncolligative freezing point of water (in the presence of ice) below the melting point, producing a difference between the freezing and melting points termed thermal hysteresis. This activity (THA) is dependent upon DAFP specific activity, concentration, and the presence of enhancers. Enhancers may be low molecular mass enhancers, such as glycerol, or other proteins. The protein enhancers complex with the DAFPs, thereby blocking a larger surface area of the potential seed ice crystal and consequently lowering the freezing point. A yeast two-hybrid screen was performed using certain hemolymph DAFPs as "bait" in an effort to identify endogenous protein enhancers. Among the positive proteins identified as interacting with the bait DAFPs, and confirmed by co-immunoprecipitation, were other DAFPs. When pure DAFPs were added to one another, those identified by the yeast two-hybrid screen as interacting with one another exhibited a synergistic enhancement of thermal hysteresis activity. In contrast, those DAFPs which the screen indicated did not interact failed to enhance one anothers' activities. DAFPs-1 and -2 interact and enhance one another. Point mutations of one of the interacting DAFPs (DAFP-2) indicated that both of the two amino acid residues that differ between DAFPs-1 and -2 were required for interaction. Glycerol enhanced the THA of the DAFPs only when DAFPs known to interact were present in the test solution. Addition of glycerol to a test solution containing only one DAFP did not produce enhancement. Therefore, glycerol enhances activity by stimulating interactions between DAFPs.

Antifreeze proteins (AFPs)[1] are characterized by their ability to lower the nonequilibrium freezing point of water while not appreciably affecting the melting point, thereby producing a difference between the freezing and melting points which is termed thermal hysteresis (*1*). According to the generally accepted adsorption−inhibition mechanism of action (*2*, *3*), the AFPs depress the freezing point by adsorbing onto the surface of ice crystals at preferred growth sites (*4*, *5*) by means of hydrogen bonding (*6*, *7*), although hydrophobic and van der Waals interactions are probably involved in binding of some AFPs to ice (*3*, *8*−*10*). This adsorption forces ice crystal growth to occur between the AFPs in highly curved (high surface free energy) fronts rather than the preferred low curvature (low surface free energy) fronts. Consequently, the temperature must be lowered before ice growth can proceed.

Overwintering larvae of the beetle *Dendroides canadensis* produce antifreeze proteins, which are an important component of their freeze avoiding capabilities (*11*−*13*). A family of 13 AFPs are known from *D. canadensis* (*14*, *15*). These DAFPs (*Dendroides* AFPs) consist of varying numbers of 12- or 13-mer repeats with molecular masses of 7.3−16.2 kDa. Throughout the lengths of the DAFPs at least every sixth residue is a cysteine, which is disulfide bridged (*16*). Figure 1 shows the group I DAFPs. These are present in *D. canadensis* hemolymph. Groups II and III DAFPs are found in the gut fluid (*17*). There are both low (*18*) and high molecular mass enhancers (*19*) of DAFPs present in *Dendroides*, and these significantly increase thermal hysteresis activity. The mechanism of enhancement by high molecular weight enhancers probably results from the ability of the DAFPs and enhancers to bind to one another and of the DAFP−enhancer protein complex to block a larger surface area of the seed crystal than the DAFP alone, causing the hysteretic freezing point to be further depressed (*20*−*22*).

To initially identify potential protein enhancers of DAFPs, we used the yeast two-hybrid system. This provided evidence that certain of the group I DAFPs interact and enhance one another. This was then confirmed by determining activities of various DAFP combinations, with and without glycerol.

EXPERIMENTAL PROCEDURES

Construction of Bait Plasmids. The cDNAs encoding the mature proteins of *D. canadensis* DAFPs-1 and -4 were

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[1] Abbreviations: AFP, antifreeze protein; DAFP, *Dendroides canadensis* antifreeze protein; THA, thermal hysteresis activity; PCR, polymerase chain reaction; cDNA, complementary deoxyribonucleic acid; SDS, sodium dodecyl sulfate; RNA, ribonucleic acid; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; IPTG, isopropyl β-D-thiogalactoside; OD, optical density; Ade-, His-, Leu-, Trp-, requires adenine (Ade), histidine (His), leucine (Leu), or tryptophan (Trp) in the medium to grow and is autotrophic for at least one of these specific nutrients.

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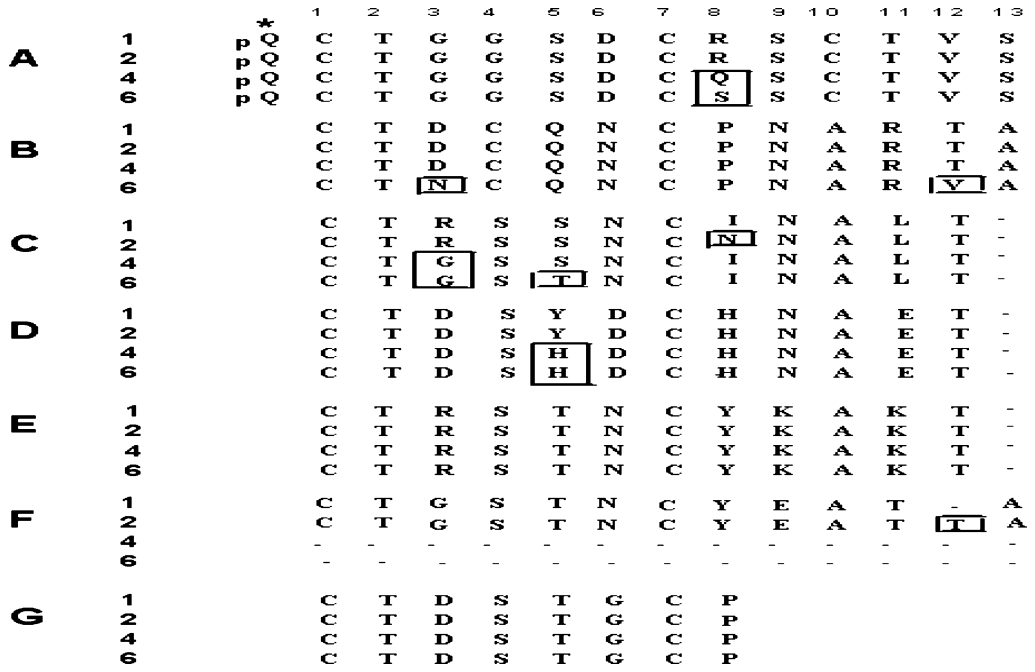

FIGURE 1: Comparison of amino acid sequences of *D. canadensis* hemolymph antifreeze proteins DAFPs-1, -2, -4, and -6. They consist of six or seven 12- and 13-mer repeat units (A−G). The sequences are highly conserved, and note that every sixth residue is a Cys. The N-termini of the DAFPs are blocked; pQ = pyroglutamate.

obtained by PCR from the pBluescript plasmid containing the appropriate sequence using the following primers, generated by PCR: 5′ primer, 5′-GCTGAATTCCAATGTACTG-GTGGTTCCGAT-3′, and 3′ primer, 5′-GCAGGATCCTG-GACATCCCGTGGAATCGGT-3′. Sequences were designed to include an *Eco*RI restriction site in the 5′ primer and a *Bam*HI restriction site in the 3′ primer. Amplified DNA was digested and cloned into pGBKT7, previously linearized with the same enzymes. The resulting plasmids, which contain the DAFP-1 or DAFP-4 coding sequences in-frame with the DNA binding domain of Gal4, were designated pGBKT7-DAFP-1 or pGBKT-7-DAFP-4, respectively. These constructs were used as baits for screening a *Dendroides* whole body cDNA library.

Western Blot Analysis. To verify that DAFP-1 and -4 expressed well in yeast strain AH109, the pGBKT7-DAFP-1 and pGBKT7-DAFP-4 were independently transformed into strain AH109, and the protein extracts were prepared from yeast by the urea/SDS method (Clontech yeast protocols handbook). The western blot was carried out with the antibody to DAFPs using purified DAFP-1 or DAFP-4 as positive controls and plain yeast strain AH109 protein extracts as a negative control.

Construction of the Prey cDNA Library. The *Dendroides* larvae were collected in late October. Total RNA was extracted from 3 g of *Dendroides* whole body, and part of it was used to extract mRNA and then synthesize the cDNA (Stratagene cDNA synthesis kit) with a designed *Eco*RI restriction site in the 5′ primer and *Xho*I restriction site in the 3′ primer. These cDNAs were then cloned into the pGADT7 and predigested with the same enzymes. The primary titer of the constructed cDNA library was 1.5×10^7 pfu/mL, and the titer of the amplified library was 2.4×10^{10} pfu/mL. The average size of inserts was 1.2 kb. The resulting plasmids, which contained the cDNAs of *Dendroides* whole body with the DNA activation domain of Gal4, were designated the pGADT7 library.

Yeast Two-Hybrid Screen. The yeast strain AH109 was simultaneously transformed using large-scale transformation with pGBKT7-DAFP-1 and the pGADT7 library using the lithium acetate method (Clontech BD Matchmaker two-hybrid system 3). The transformants were plated on synthetic medium lacking adenine, histidine, leucine, and tryptophan and with X-αGal. Colonies were picked at times from 4 to 8 days and replated on the second generation plates for 6−10 days with synthetic medium lacking histidine, leucine, and tryptophan and with X-αGal. The blue colonies that were well isolated, indicating segregation, were picked up and replated to synthetic medium lacking adenine, histidine, leucine, and tryptophan to verify that they maintain the correct phenotype. The yeast plasmids were purified using the lyticase method (Clontech yeast protocols handbook), transformed into *E. coli* using electroporation, and purified using Miniprep (Qiagen). The purified pGAD plasmids were retransformed into strain AH109 carrying pGBKT7-DAFP-1 to verify positive colonies. The positives were sequenced by automated DNA sequencing (ABI 3700).

In a similar fashion pGBKT7-DAFP-4 was used to screen the library for proteins interacting with DAFP-4.

In Vitro Transcription/Translation and Immunoprecipitation. The pGBKT7-DAFP-1, pGBKT7-DAFP-4, and pGAD plasmids from the yeast two-hybrid system were transcribed and translated using Promega's TNT T7 coupled reticulocyte lysate system in vitro to prepare ^{35}S-Met-labeled bait and prey proteins. The interactions between bait and prey proteins were confirmed and visualized by in vitro co-immunoprecipitation (Co-IP) using the BD Matchmaker Co-IP kit (Clontech) which is compatible with the BD Matchmaker two-hybrid system 3. The co-immunoprecipitates were separated by SDS−PAGE and analyzed by radioautography.

Construct of Mutated DAFP-2. DAFP-2 was mutated at the two positions that differ from DAFP-1. To construct DAFP-2 (N35I), the aspargine in the 35th position was mutated to isoleucine, and for DAFP-2 (−T75), the threonine

in the 75th position was removed. To construct DNA of DAFP-2 (N35I), DNA encoding the mature protein of DAFP-1 was amplified by PCR using the 5′ primer, 5′-ACGTCGGATCCACAATGTACTGGTGGTTCC-3′, and the 3′ primer, 5′-TGGACATCCCGTGGAATCGGTACAGGCTGTTGTAGCTTCGTA-3′. To construct DNA of DAFP-2 (−T75), DNA encoding mature protein of DAFP-2 was amplified by PCR using the 5′ primer, 5′-ACGTCGGATCCACAATGTACTGGTGGTTCC-3′, and the 3′ primer, 5′-TGGACATCCCGTGGAATCGGTACAGGCTGTAGCTTCGTA-3′.

Expression of Recombinant DAFPs. The DNAs encoding the mature DAFP-1, -2, -4, and -6, DAFP-2 (N35I), and DAFP-2 (−T75) were amplified by PCR using the 5′ primer, 5′-ACGTCGGATCCACAATGTACTGGTGGTTCC-3′, and the 3′ primer, 5′-CAGTGTGGTGGTGGTGGTGGTGTGGACATCCCGTGG-3′. Sequences were designed to include a *Bam*HI restriction site in the 5′ primer and an *Not*I restriction site in the 3′ primer. Amplified DNA was digested and cloned into pET-20b (Novagen), previously linearized with the same enzymes. The resulting plasmids were sequenced (ABI 3700). They were then transformed into *Escherichia coli* BL21(DE3) pLysS competent cells. The cells were grown in LB broth containing carbenicillin, ampicillin, and chloramphenicol. IPTG to a final concentration of 0.4 mM [for pet-20b (+)] or 1.0 mM [for pet-32b (+)] was added to the culture when the OD_{600} reached 0.6. Cells were grown at 37 °C for 3 h in the induction medium and collected by centrifugation at 6000 rpm for 20 min. The extracted crude protein was purified using the His-bind purification kit (Novagen). The purified protein was dialyzed, freeze-dried, and redissolved in sterilized distilled water.

Thermal Hysteresis Activity. The capillary technique was used to measure thermal hysteresis activity (*1*). The sample (∼4 μL) was placed in a sealed capillary tube, a small (∼0.25 mm) seed crystal was spray-frozen in the sample, and the capillary was placed into a refrigerated alcohol bath which was equipped with a viewing chamber through which the crystal could be observed with a microscope. The bath temperature was controlled to ±0.01 °C. The temperature was slowly raised (0.02 °C/5 min) until the crystal disappeared. This temperature was taken as the melting point of the sample. Another crystal was spray-frozen in the sample, the capillary again placed in the chamber, and the bath temperature lowered (0.1 °C/2.5 min) until the crystal grew. The temperature at which the seed crystal began to grow was taken as the hysteretic freezing point. Aqueous solutions, which do not contain thermal hysteresis antifreeze proteins, exhibit identical freezing and melting points. However, in the presence of these proteins the freezing point is often depressed several degrees below the melting point. This difference between the melting and freezing points is termed thermal hysteresis and is representive of the antifreeze protein activity of the sample. The THA of each sample was measured at least twice. Generally, the measurements were within 0.4 °C, or less, of one another. If the difference was greater, additional measurements were made.

RESULTS

To search for cDNA clones encoding proteins that interact with DAFP-1 using the yeast two-hybrid screen, the coding sequence for *D. canadensis* hemolymph DAFP-1 was fused with the Gal4 DNA binding domain, and a *D. canadensis* whole body library expressed from the pGADT7 vector was screened. Nineteen clones that displayed an *Ade+His+LacZ+* phenotype were isolated representing 15 different cDNAs as judged by cDNA sequencing, but only 5 turned out to be true positives. The interaction of DAFP-1 with these proteins was specific, as no interaction was observed between the identified proteins and the Gal4 DNA binding domain (BD) alone.

One positive yeast clone contained the complete cDNA of *D. canadensis* antifreeze protein DAFP-2. DAFP-2 belongs to the group I DAFPs present in hemolymph, all of which have sequences very similar to that of DAFP-1 (two amino acids different in the case of DAFP-2) (see Figure 1). Another positive yeast clone encoded the complete cDNA of *D. canadensis* antifreeze protein DAFP-4, another group I DAFP, which is also present in the hemolymph. DAFP-4 has one repeat less than DAFP-1, and three additional amino acids are different (see Figure 1). The interaction of DAFP-1 with both DAFP-2 and DAFP-4 was confirmed by in vitro transcription/translation and co-immunoprecipitations of bait protein (c-Myc-tagged DAFP-1) and prey proteins (HA-tagged DAFP-2 or DAFP-4). These co-immunoprecipitations were identified by HA-tagged (in pGADT7) polyclonal antibody and were separated by SDS−PAGE and analyzed by radioautography. Repeating the yeast two-hybrid procedure, DAFP-4 was also used as the bait protein. Two positives were identified, DAFP-1 (which is in accordance with the above result where DAFP-1 was used as bait) and DAFP-6, which is very similar to DAFP-4 (see Figure 1).

To determine if the interactions between these DAFPs, indicated by the yeast two-hybrid screens and co-immunoprecipitations, can enhance their thermal hysteresis activities, these DAFPs were expressed and purified, and the thermal hysteresis activity of individual DAFPs was tested at different physiological concentrations (*12*). The activities of combinations of DAFPs-1 and -2 were then compared with those of the individual DAFPs (Figure 2). If DAFP-1 and DAFP-2 cannot enhance one another, the activity of the combination should be between the activities of DAFP-1 and DAFP-2 alone, but we found that, up to at least 1.0 mg/mL, the activity of the combination was much higher than the activity of either DAFP-1 or DAFP-2 alone. The highest enhancement occurred at a total concentration of ∼0.5 mg/mL, where the THA of the combination is ∼4 °C, while the THA of DAFP-1 alone is 2.7 °C, and that of DAFP-2 is just 1.6 °C. The enhancement dropped gradually with increasing DAFP concentrations, and at 2 mg/mL there was no enhancement.

A known low molecular weight enhancer, glycerol (*13*, *18*), at a physiological winter hemolymph concentration of 0.5 M was added to the combination of DAFP-1 and -2 to determine if it can further enhance the activity of the combination (Figure 2). Glycerol was also added to a solution with DAFP-1 alone (Figure 3). The presence of glycerol did not enhance the activity of DAFP-1 alone (Figure 3), and glycerol likewise did not enhance DAFP-2 alone (data not shown). However, glycerol did enhance the activity of the combination of DAFP-1 and DAFP-2 (Figure 2). The enhancement increased dramatically up to a DAFP concentration of 1.0 mg/mL and then gradually dropped above 1.0 mg/mL.

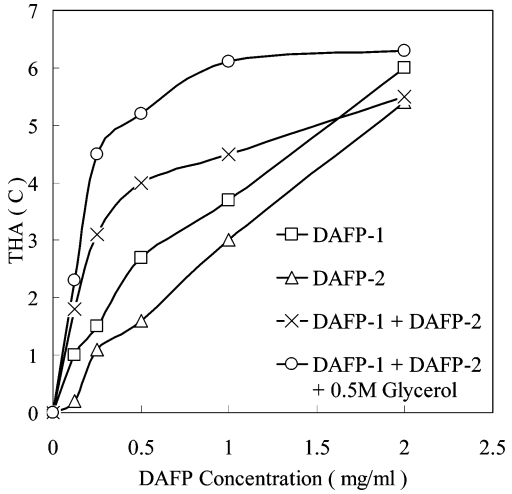

FIGURE 2: Effects of the combination of DAFP-1, DAFP-2, and glycerol on thermal hysteresis activity (THA). Equal concentrations of purified expressed DAFP-1 and DAFP-2 were mixed at indicated concentrations. The thermal hysteresis activity is compared with DAFP-1 or DAPF-2 alone. Glycerol was added at a final concentration of 0.5 M to the DAFP-1 and DAFP-2 combination solution. Note that the combination of DAFPs-1 and -2 resulted in increased THA at concentrations less than 2 mg/mL, and the addition of glycerol lead to additional enhancement. Key: □ = DAFP-1; △ = DAFP-2; × = DAFP-1 + DAFP-2; ○ = DAFP-1 + DAFP-2 + 0.5 M glycerol. Values are means of two or three measurements of the same sample. Standard deviation ranged from 0.00 to 0.21. Computer curve fitting (Excel) was used to connect points in the graph to guide the eye.

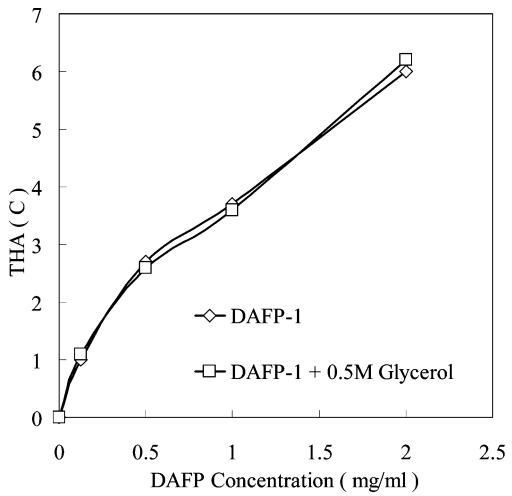

FIGURE 3: The addition of glycerol (0.5 M) to DAFP-1 did not result in enhancement of THA. Key: ○ = DAFP-1; □ = DAFP-1 + 0.5 M glycerol. Values are means of two or three measurements of the same sample. Standard deviation ranged from 0.00 to 0.14. Computer curve fitting (Excel) was used to connect points in the graph to guide the eye.

Using similar techniques, combinations of DAFP-1 and DAFP-4 were tested for enhancement of THA activity (Figure 4). Combinations of DAFP-1 and DAFP-4 showed no enhancement at DAFP concentrations of 0.25 mg/mL or less; however, there was considerable enhancement at 0.5 and 1.0 mg/mL. The enhancement disappeared at 2 mg/mL, and at 3 mg/mL the THA of DAFP-1 alone was greater than the combination of DAFPs-1 and -4. In fact, at 3 mg/mL the combination of DAFPs-1 and -4 produced THA halfway between those of DAFP-1 and DAFP-4 alone, as would be expected if the two failed to enhance one another. On the

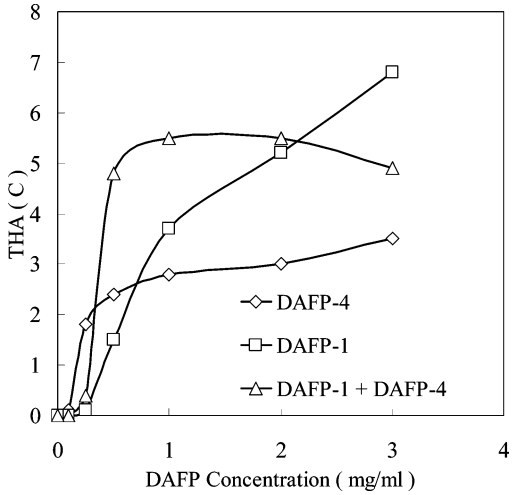

FIGURE 4: The combination of DAFP-1 and DAFP-4 had higher thermal hysteresis activity than DAFP-1 or DAFP-4 alone at concentrations below 2 mg/mL. Equal concentrations of purified expressed DAFP-1 and DAFP-4 were mixed at the indicated concentrations. Thermal hysteresis activity is compared with DAFP-1 or DAFP-4 alone. Key: □ = DAFP-1; ◇ = DAFP-4; △ = DAFP-1 + DAFP-4. Values are means of two or three measurements of the same sample. Standard deviation ranged from 0.00 to 0.21. Computer curve fitting (Excel) was used to connect points in the graph to guide the eye.

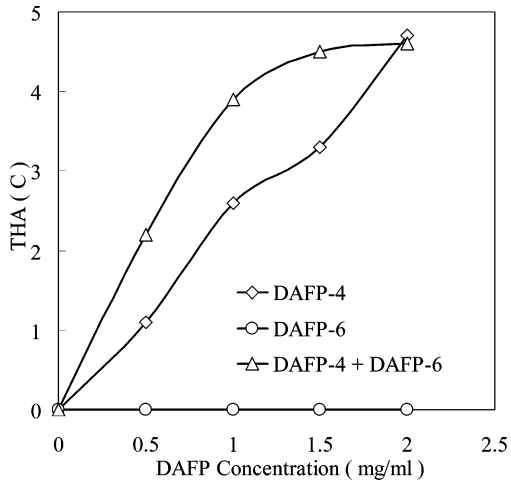

FIGURE 5: Addition of DAFP-6 to DAFP-4 resulted in enhancement of thermal hysteresis activity (THA) even though the expressed DAFP-6 lacked THA by itself. Key: ◇ = DAFP-4; ○ = DAFP-6; △ = DAFP-4 + DAFP-6. Equal concentrations of DAFP-4 and -6 were mixed at the indicated concentrations. Values are means of two or three measurements of the same sample. Standard deviation ranged from 0.00 to 0.21. Computer curve fitting (Excel) was used to connect points in the graph to guide the eye.

basis of the yeast two-hybrid results, using DAFP-1 as bait, DAFPs-1 and -6 do not interact, and when the THA activities of combinations of DAFPs-1 and -6 were tested, there was no enhancement (data not shown).

As expected, when DAFP-4 was used as the bait protein in the yeast two-hybrid screen, DAFP-1 (confirming the result when DAFP-1 was used as bait) and DAFP-6 were identified as positives. However, the screen did not identify DAFP-2, and when DAFP-2 and DAFP-4 were combined, there was no enhancement (data not shown). Figure 5 demonstrates that DAFP-6 enhances the THA of DAFP-4 at concentrations up to ~1.5 mg/mL. This is especially interesting as the expressed DAFP-6 lacked THA when tested

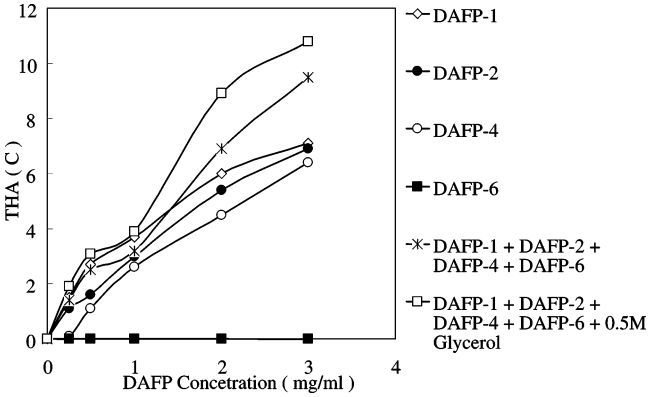

FIGURE 6: The combination of all group-1 DAFPs (1, 2, 4, and 6) resulted in significant enhancement of thermal hysteresis activity (THA) at total DAFP concentrations above 1 mg/mL. Glycerol at 0.5 M further enhanced THA. Equal concentrations of DAFPs-1, -2, -4, and -6 were mixed at the indicated concentrations. Key: ◇ = DAFP-1; ● = DAFP-2; ○ = DAFP-4; ■ = DAFP-6; × = DAFP-1 + DAFP-2 + DAFP-4 + DAFP-6; □ = DAFP-1 + DAFP-2 + DAFP-4 + DAFP-6 + 0.5 M glycerol. Values are means of two or three measurements of the same sample. Standard deviation ranged from 0.00 to 0.28. Computer curve fitting (Excel) was used to connect points in the graph to guide the eye.

alone even though (a) DAFP-6 was expressed and tested 10 times and (b) DAFPs-4 and -6 differ at only four positions (see Figure 1).

Figure 6 illustrates the activities of the group I DAFPs (1, 2, 4, and 6) individually, in combination, and in combination plus glycerol. (Note that the THA of individual DAFPs may be slightly different from those presented in earlier figures. This results from some variation in THA of different batches of expressed protein.) Between concentrations of 1.0−3.0 mg/mL the combination of all four group I DAFPs demonstrated high levels of enhancement. The combination of all the group I DAFPs produced total THAs over 9 °C at 3 mg/mL. Addition of glycerol to the combined DAFPs provided further enhancement, with THA of nearly 11 °C at 3 mg/mL. Note that the combination of all four DAFP's, both with and without glycerol, produced higher THAs than any of the DAFPs alone or in single combinations (i.e., DAFPs-1 and -2, DAFPs-1 and -4, or DAFP's -4 and -6). Recall that these other combinations at the higher concentrations produced THAs approximately identical to those of single DAFPs, or in the case of DAFP-1 and -4 the THA of the combination was actually less than DAFP-1 alone.

DAFP-1 and DAFP-2 are different at just two positions, the 8th position of repeat C (residue 35), which in DAFP-1 is an isoleucine but an asparagine in DAFP-2, and the 12th position in repeat F (residue 75), where DAFP-2 has an extra threonine (Figure 1). Asparagine 35 of DAFP-2 described above was mutated to isoleucine, named DAFP-2 (N35I), so this DAFP-2 differed from DAFP-1 only by the extra threonine at residue 75. In another mutagenesis, the extra threonine of DAFP-2 at position 75 was removed, named DAFP-2 (−T75), so this DAFP-2 differed from DAFP-1 only by the asparagine at position 35. To determine if there is still enhancement between DAFP-1 and each of the two mutated DAFP-2's, the mutated DAFP-2's were expressed, and each was combined (alone) with DAFP-1, thereby permitting testing of enhancement of DAFP-1 THA by the two mutated DAFP-2's that only differ from DAFP-1 at one

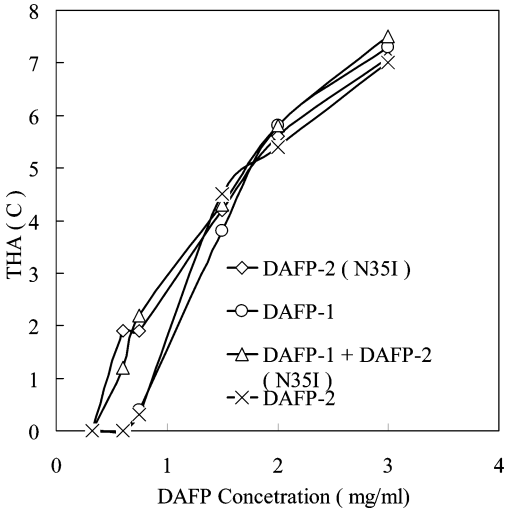

FIGURE 7: The combination of DAFP-1 and mutated DAFP-2 (N35I) showed no enhancement of thermal hysteresis activity (THA). Key: ◇ = DAFP-2 (N35I); ○ = DAFP-1; △ = DAFDP-1 + DAFP-2 (N35I); × = DAFP-2. Values are means of two or three measurements of the same sample. Standard deviation ranged from 0.00 to 0.14. Computer curve fitting (Excel) was used to connect points in the graph to guide the eye.

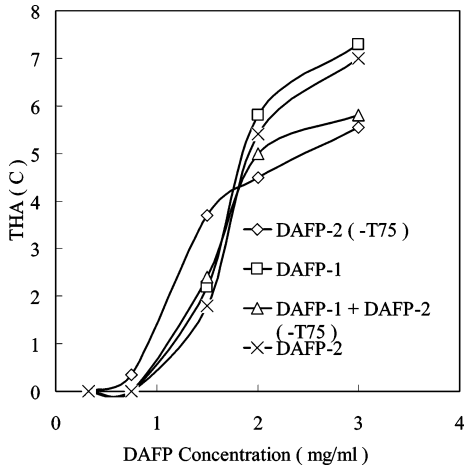

FIGURE 8: The combination of DAFP-1 and mutated DAFP-2 (−T75) showed no enhancement of thermal hysteresis activity (THA). Key: ◇ = DAFP-2 (−T75); □ = DAFP-1; △ = DAFP-1 + DAFP-2 (−T75); × = DAFP-2. Values are means of two or three measurements of the same sample. Standard deviation ranged from 0.00 to 0.21. Computer curve fitting (Excel) was used to connect points in the graph to guide the eye.

residue. Although the mutation did result in slight variations in THA of the mutated DAFP-2's when tested alone, more importantly neither was able to enhance DAFP-1 THA (Figures 7 and 8). Likewise, when the two mutated DAFP-2's were combined, the resulting THA was not greater than that of either of the mutated DAFPs alone, except at a concentration of 2 mg/mL (note that this latter point was checked three times) (Figure 9). Also, the simultaneous addition of both mutated DAFP-2's to DAFP-1 failed to enhance the THA of DAFP-1 (data not shown).

DISCUSSION

It has been shown that endogenous protein and low molecular mass enhancers (glycerol) are present and play important roles in increasing thermal hysteresis activity in the hemolymph of overwintering *D. canadensis* larvae (*17*).

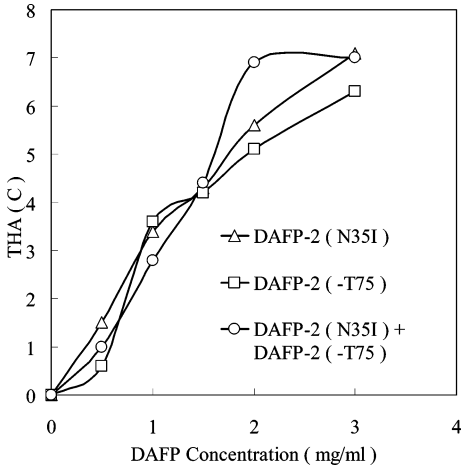

FIGURE 9: The combination of both mutated DAFP-2s lacked enhancement of thermal hysteresis activities compared to either DAFP-2 (N35I) or DAFP-2 (−T75) alone, except at 2 mg/mL where enhancement was detected. Key: △ = DAFP-2 (N35I); □ = DAFP-2 (−T75); ○ = DAFP-2 (N35I) + DAFP-2 (−T75). Values are means of two or three measurements of the same sample. Standard deviation ranged from 0.00 to 0.28. Computer curve fitting (Excel) was used to connect points in the graph to guide the eye.

Since protein enhancers of DAFPs, including IgGs specific for DAFPs, are known to bind to the DAFPs (*20*, *21*), the yeast two-hybrid system was used to identify endogenous *D. canadensis* proteins which interact with certain DAFPs and which therefore might be endogenous DAFP enhancers. Among the proteins identified by the yeast two-hybrid screen were other DAFPs. This binding between certain hemolymph DAFPs was confirmed by co-immunoprecipitation of bait proteins (c-Myc-tagged DAFPs) and prey proteins (HA-tagged DAFPs). Interestingly, neither DAFP-1 nor DAFP-4 when used as bait identified themselves, indicating that neither DAFP-1 nor DAFP-4 interact with themselves. It is also interesting that neither of the yeast two-hybrid screens using the group I DAFPs-1 and -4 identified any of the nine group II or III DAFPs, none of which are present in the hemolymph (*17*).

Using these techniques and employing DAFPs-1 and -4 as bait, it was shown that DAFP-1 can bind to DAFP-2 and DAFP-4, but not DAFP-6 or other DAFP-1's, while DAFP-4 can bind DAFP-1 and DAFP-6 but not DAFP-2 or other DAFP-4's. By expressing recombinant DAFP-1, DAFP-2, DAFP-4, and DAFP-6 (although we could not produce an active DAFP-6), these interactions were confirmed, as combinations of DAFPs that the yeast two-hybrid results indicated had protein−protein interactions always resulted in enhanced THA. In contrast, combinations of DAFPs that the yeast two-hybrid results indicated lacked protein−protein interactions always lacked enhancement of THA. A summary of these interactions and enhancements of THA is shown in Figure 10.

Certain of the THA values reported here, >9 °C, are the highest ever measured. These high levels of THA are especially noteworthy since the capillary technique (*1*) was used in these measurements, rather than the nanoliter osmometer. Although the two techniques use the same basic method, the capillary technique uses a much larger seed ice crystal (and possibly multiple crystals) than does the nanoliter osmometer. Since the level of THA measured with insect AFPs is inversely related to the size of the seed crystal

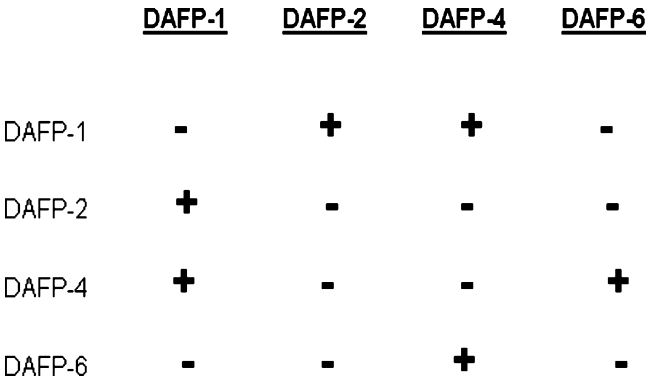

FIGURE 10: Summary of the interactions, or lack thereof, between the four group 1 DAFPs. Interactions include positive yeast two-hybrid screens using DAFPs-1 and -4 as bait, co-immunoprecipitation, and enhanced thermal hysteresis activity.

(*23*−*25*), the nanoliter osmometer reports larger THA than does the capillary technique. Also, since the sample volume was ∼5 μL in our measurements, the frozen fraction was small.

Our data showed that the combination of DAFP-1 and DAFP-2 can produce 1.5−2.5 °C higher thermal hysteresis activities than that of the single DAFPs (Figure 2), while the combination of DAFP-1 and DAFP-4 can produce 2.5−3.5 °C higher activity than either DAFP-1 or DAFP-4 alone (Figure 4). The higher enhancement resulting from these combinations of two DAFPs always occurred at lower concentrations, approximately 0.5−1.0 mg/mL. However, the combination of DAFPs-4 and -6 produced enhancement at all concentrations tested (Figure 5). (Recall that DAFP-6 alone lacks THA.) As might be expected, the greatest enhancement of THA occurred when all of the group I DAFPs were combined, resulting in THA of over 9 °C at 3 mg/mL and nearly 11 °C when 0.5 M glycerol was also added (Figure 6). This is the greatest THA yet reported for any antifreeze proteins. Interestingly, in contrast to the situations when just two DAFPs were combined (Figures 2, 4, and 5), when all four group I DAFPs were present, the enhancement occurred only at the higher concentrations (Figure 6). These results complicate attempts to explain the mechanism of the self-enhancement of the DAFPs.

According to the original adsorption−inhibition explanation of the mechanism of AFP activity (*2*), the AFPs adsorb onto the surface of ice, thereby forcing the crystal to grow between the adsorbed AFPs in high radius of curvature, high free energy fronts, rather than the preferred low radius of curvature fronts. Consequently, the temperature must be lowered below the equilibrium melting point, to the hysteretic freezing point, before the crystal can grow significantly. While this mechanism is still generally thought to be correct, certain complications are now recognized. For example, the presence of an ∼10 Å ice−water interface, where the properties of the water are in transition between those of water and ice (*26*), is now appreciated, indicating that the AFPs may interact both at the ice surface and the ice−water interface (*27*, *28*). Our previous ideas on the mechanism of enhancement of the DAFPs by other, non-AFP proteins were that the DAFP−enhancer protein complex blocks a larger surface area of the seed ice crystal than does the DAFPs alone and/or the larger complex is more difficult for the ice to overgrow. Consequently, the hysteretic freezing point is

further depressed (*20*−*22*). However, the mechanism by which different DAFPs enhance one another is more difficult to explain, except for situations involving DAFP-6 since this DAFP, like the non-AFP enhancer proteins, lacks THA. For one, the points raised in the preceding paragraph dealing with the observations of whether the DAFP combinations resulted in enhancement only at low or at high concentrations must be considered. Also, in the situation where two or more types of interacting DAFPs are present in solution, when some of these DAFPs combine to form a complex, the number of active antifreeze protein particles in solution actually decreases. It is not intuitively obvious that this would increase THA. This is presumably not the case when non-AFP proteins act as DAFP enhancers. Another complicating factor is that we do not know the number of DAFPs forming the complexes, nor do we know the shapes of the complexes. It is quite possible that the number of DAFPs comprising the complexes varies not only with the types of DAFPs present but also with DAFP concentration. This might help to explain results such as why enhancement of DAFPs-1 and -2 (Figure 2) or DAFPs-1 and -4 (Figure 4) only occurred at lower concentrations, yet when all four group I DAFPs were present, the enhancement only took place at higher concentrations. At this time we do not have a satisfactory explanation for the mechanism of self-enhancement of DAFPs.

The mutagenesis experiment showed that the two amino acid residues that are different between DAFP-1 and DAFP-2 are both important for the enhancement of THA. Comparing the protein sequences of DAFP-1 and DAFP-2 (Figure 1), there are only two amino acid differences: the 35th residue, which is Ile in DAFP-1 and Asn in DAFP-2, and after residue 74, where there is an extra Thr in DAFP-2. Mutated DAFP-2's were constructed such that each had only one amino acid difference from DAFP-1: N35I, and −T75. The THA activities of these two mutated DAFPs were similar to that of DAFP-2, which suggests that they still bind to ice with the mutated amino acid. However, we found that neither of the mutated DAFPs could enhance DAFP-1, which suggests that both sites are important for the interactions between DAFP-1 and DAFP-2.

Glycerol had previously been shown to enhance DAFP activity, both thermal hysteresis activity and the ability of DAFPs to inhibit ice nucleators (*17*, *18*). However, because DAFPs-1 and -2 and DAFPs-4 and -6 are so structurally similar, it has been impossible to separate DAFP-1 from DAFP-2 and DAFP-4 from DAFP-6 when purifying DAFPs from the hemolymph. Consequently, in earlier studies of glycerol enhancement combinations of DAFPs-1 and -2 or DAFPs-4 and -6 (*13*, *18*, *19*) were used. However, the current study used DAFPs from an *E. coli* expression system, thereby permitting the use of pure DAFP-1, etc. The inability of glycerol to enhance the activity of DAFP-1 (Figure 3) or DAFP-2 alone, while enhancing the activity of the combinations of DAFPs-1 and -2 (Figure 2) or all four DAFPs (Figure 6), provided insight into the previously unexplained mechanism by which glycerol enhances DAFP activity. Glycerol is known to promote protein−protein interactions, according to the preferential hydration model of Timasheff (*29*). Consequently, it is likely that high glycerol concentrations enhance the activity of DAFPs by stimulating increased DAFP interactions with one another. These DAFP complexes are then able to block a larger surface area of potential seed ice crystals, or ice nucleating surfaces, and consequently growth of ice is inhibited. In addition, the inability of glycerol to enhance DAFP-1 or -2 alone provides further evidence that DAFP-1 does not complex with itself, or if it does, glycerol cannot promote these interactions. Further evidence that DAFP-1 or DAFP-4 do not complex with themselves is that, as discussed earlier, when DAFP-1 or DAFP-4 were used as bait in the yeast two-hybrid screen, they did not identify themselves as positives.

This study demonstrated that protein−protein interactions between certain hemolymph DAFPs result in increased THA activity. Mutagenesis showed that the presence of an asparagine in DAFP-2 for an isoleucine in DAFP-1 and the presence of a second threonine in DAFP-2 at residue 75 are both required for the ability of DAFP-2 to enhance the activity of DAFP-1, or vice versa. In addition, it was demonstrated that glycerol enhances DAFP activity by promoting the interaction of the DAFPs. These interactions are crucial to the ability of the DAFPs to prevent freezing in overwintering *D. canadensis* larvae.

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