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# Temperature-dependent Kinetics Study for Hydrogen Exchange of Type I Antifreeze Protein from Winter Flounder

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Antifreeze proteins (AFPs) are found in various organisms to survive at subzero temperatures by decreasing the freezing point of bodily fluids.[1-4] Type I AFPs have been found in Arctic and Antarctic fish such as winter flounder, yellowtail flounder, Alaskan plaice, shorthorn sculpin, and Arctic sculpin.[1,5] Winter flounder type I AFP is 37-residue isoform, which contains three 11-amino acid repeats of the sequence Thr-$X_2$-Asx-$X_7$, where X is generally alanine.[6] X-ray crystallographic studies revealed that the type I AFP is completely α-helical in conformation.[7] The hydroxyl and methyl groups of four threonine residues play key roles in the ice-binding properties of the type I AFP.[8] The mutants, A17L and A21L, where the substitution lies adjacent to the Thr-rich face, caused a significant decrement of the thermal hysteresis activity, whereas the mutants A19L and A20L exhibited wild-type activity.[9] Previously, we reported hydrogen exchange study on the winter flounder type I AFP and two mutants, A17L- and A20L-AFP performed at 15 °C.[10] This study suggested that the unusual conformational property of the type I AFP may be a key dynamic feature necessary to achieve wild-type thermal hysteresis activity.[10] However, this result cannot fully explain why these AFPs exhibit the distinct thermal hysteresis activities because the experimental temperature (15 °C) is not same with their biologically active temperature (subzero degree). Because aqueous solution could be frozen at subzero degree, it is required that the systematic NMR experiments should be carried out at various temperatures to understand physical properties of AFP at subzero degree. Here, to further understand the correlation between the dynamic properties and antifreeze activities of type I AFPs, we performed NMR hydrogen exchange experiments on the winter flounder type I AFP (Fig. 1, referred to as wt-AFP) at temperatures from 1-23 °C.

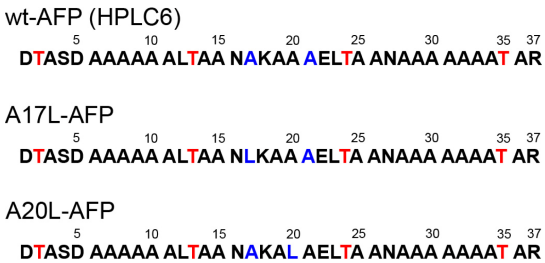


**Figure 1.** Amino acid sequence of winter flounder type I AFP (HPLC6 form) and its two mutants, A17L- and A20L-AFPs.

The winter flounder type I AFP and its mutants, A17L- and A20L-AFPs, were purchased from Cosmo Genetech Inc. (Seoul, Korea) and desalted using a Sephadex G-25 gel filtration column. Each protein sample (5 mg) was dissolved in 500 μL of 90% $H_2O$/10% $D_2O$ NMR buffer containing 10 mM sodium phosphate (pH 8.0) and 100 mM NaCl. Under basic condition, the amide protons of proteins can exchange easily with solvent water at low temperature.

All of the experiments were performed on an Agilent 700 MHz spectrometer (GNU, Jinju) equipped with a triple resonance probe. One dimensional NMR data were processed with FELIX2004 (FELIX NMR, San Diego) software. The hydrogen exchange rate constants ($k_{ex}$) of the type I AFP and its mutants at various temperatures were determined using a water magnetization transfer experiment as described previously.[10]

Supplementary data Figure S3 shows the temperature dependencies of the amide proton spectra for wt-, A17L-, and A20L-AFPs. The chemical shift is sensitive to the chemical environment of the observing nuclei and to temperature-associated structural or dynamic changes.[11] The $\Delta\delta/\Delta T$ values of the S4 amide protons in wt-AFP is −0.018 ± 0.001 ppm/°C. Similarly, the $\Delta\delta/\Delta T$ values of the S4 amide protons in A17L- and A20L-AFPs are −0.018 ± 0.001 and −0.017 ± 0.001 ppm/°C, respectively. These $\Delta\delta/\Delta T$ values are greater than the average $\Delta\delta/\Delta T$ value (−0.0275 ppm/°C) of hydrogen bonded amide protons based on statistical analysis of proteins and peptides,[12] indicating that the S4 amide protons of all three of the AFPs are not exchange-protected by hydrogen bonding interaction. However, the N16/N27 amide protons in wt-AFP have $\Delta\delta/\Delta T$ values of approximately −0.010 ppm/°C at ≤ 5 °C. Interestingly, when temperature is > 5 °C, the average $\Delta\delta/\Delta T$ value of the N16/N27 amide protons became −0.018 ± 0.001 ppm/°C. These results indicate that the N16 and N27 amide protons maintain hydrogen bonding interaction with the A20 and A31 carbonyl groups, respectively, at ≤ 5 °C but that these hydrogen bonding interactions are disrupted as temperature is increased to > 5 °C. The R37-Hη protons in all three of the AFPs exhibit hydrogen bonding interaction and have $\Delta\delta/\Delta T$ values of

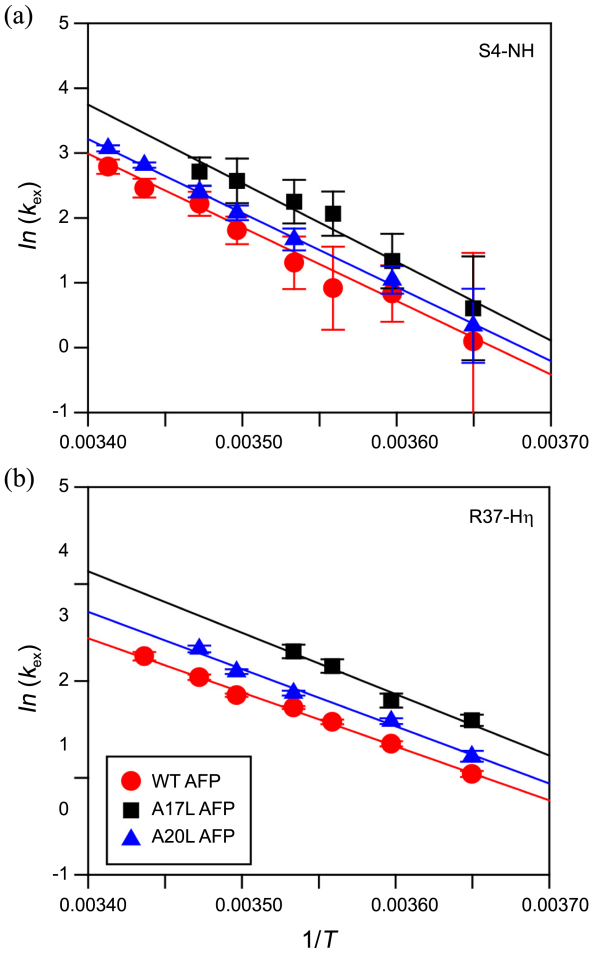


**Figure 2.** Logarithm scale of the $k_{ex}$ values for (A) the S4 amide proton and (B) R37-Hη side-chains of the wt- (red circle), A17L- (black square), and A20L-AFPs (blue triangle) versus the inverse of the temperature.

approximately −0.002 ppm/°C. Interestingly, in the wt- and A20L-AFPs, these R37-Hη resonances were observed as temperature was increased up to 20 °C and then disappeared at 25 °C (Supplementary data Fig. S3). However, in the A17L-AFP, these resonances disappeared at 15 °C, indicating that the R37-Hη proton in the A17L-AFP exchanged more rapidly compared to the other two AFPs.

Figure 2 shows the temperature-dependence of $k_{ex}$ values of the S4 amide protons and R37-Hη side-chain protons for wt-, A17L-, and A20L-AFPs. The linear correlation between ln($k_{ex}$) and 1/$T$ indicates the Arrhenius equation, and the slopes and $y$-intercepts of these lines yield the activation energies ($\Delta G_0^{\ddagger}$) and Arrhenius constants ($A$) of the hydrogen exchange process. The $\Delta G_0^{\ddagger}$ and $A$ values for hydrogen exchange of the S4 amide proton in wt-AFP are 22.6 kcal·mol$^{-1}$ and $1.1 \times 10^{18}$ s$^{-1}$, respectively (Table 1). In this plot, the S4 amide proton in the A20L-AFP exhibits very similar slope ($\Delta G_0^{\ddagger}$ = 22.7 kcal·mol$^{-1}$) and slightly larger $y$-intercept ($A$ = $1.8 \times 10^{18}$ s$^{-1}$) compared to wt-AFP (Fig. 2), indicating that hydrogen exchange of the S4 amide proton in type I AFP is minimally affected by the A-to-L substitution at position 20. Interestingly, the Arrhenius constant ($34.5 \times 10^{18}$ s$^{-1}$) of S4 amide proton exchange in A17L-AFP is 20-30-fold larger than those of wt- and A20L-AFPs (Table 1 and Fig. 2). These Arrhenius constant values indicate that the S4 amide protons in wt- and A20L-AFPs are well protected from collision with solvent water molecules compared to S4 amide protons of A17L-AFP. The R37-Hη proton in wt-AFP has similar $\Delta G_0^{\ddagger}$ (22.2 kcal·mol$^{-1}$) and $A$ values ($3.8 \times 10^{18}$ s$^{-1}$) for hydrogen exchange of the S4 amide proton (Table 1). In the A20L-AFP, the R37-Hη proton has a 20-fold greater $A$ value ($62.4 \times 10^{18}$ s$^{-1}$) than that of wt-AFP, whereas these protons have similar $\Delta G_0^{\ddagger}$ values (Table 1). Surprisingly, the R37-Hη proton in A17L-AFP exhibits a 1,000-fold greater Arrhenius constant ($2{,}680 \times 10^{18}$ s$^{-1}$) than that of wt-AFP, similarly to the S4 amide proton (Table 1). Taken together, we conclude that in contrast to the wt- and A20L-AFPs, both termini of A17L-AFP significantly expose to water molecules such that both the S4 amide proton (located near the N-terminal) and the R37-Hη proton (located at the C-terminal) are easily exchanged with solvent water.

**Table 1.** The activation energies ($\Delta G_0^{\ddagger}$) and Arrhenius constants ($A$) for hydrogen exchange of the indicated wt-, A17L-, and A20L-AFP protons. °C

| Protons | | wt | A17L | A20L |
|---|---|---|---|---|
| S4 amide | $\Delta G_0^{\ddagger}$ (kcal·mol$^{-1}$) | 22.6±1.1 | 24.1±2.3 | 22.7±0.5 |
| | $A$ ($\times 10^{18}$ s$^{-1}$) | 1.1±0.1 | 34.5±3.6 | 1.8±0.1 |
| R37 Hη | $\Delta G_0^{\ddagger}$ (kcal·mol$^{-1}$) | 22.2±0.6 | 25.2±3.3 | 23.5±1.4 |
| | $A$ ($\times 10^{18}$ s$^{-1}$) | 3.8±0.1 | 2,680±320 | 62.4±3.4 |

The S4 amide and R37-Hη protons have similar activation energies for hydrogen exchange in all three of the AFPs, but their Arrhenius constants are clearly different (Table 1). The larger Arrhenius constants of hydrogen exchange indicate that solvent water molecules can collide more easily with target protons to allow hydrogen exchange. The dimeric (or oligomeric) conformation of AFP, instead of its monomeric conformation, can explain the distinct hydrogen exchange rates caused by differences in Arrhenius constants, given that all three of the AFPs exhibited the same long stretch of α-helical conformation. In contrast to A17L-AFP, wt-AFP exhibits unique dimeric (or oligomeric) α-helical conformation; moreover the exchangeable protons of these protein forms, such as amide and R37-Hη protons, are well protected from collisions with water molecules. In addition, the linear correlation between ln($k_{ex}$) and 1/$T$ indicates that the same phenomena of solvent protection for these three AFPs occurred at temperatures ranging from 1-23 °C (Fig. 2). The unique conformations of the wt- and A20L-AFPs, where certain exchangeable protons have larger solvent protection effects (and smaller Arrhenius constants) than inactive A17L-AFP, may be key dynamic features necessary to achieve wild-type thermal hysteresis activity.

In summary, we determined hydrogen exchange rate constants of certain protons of wt-, A17L-, and A20L-AFPs as a function of temperature. This kinetics study confirmed the previous suggestion that wt- and A20L-AFPs exhibit unique

oligomeric α-helical conformation, in which several exchangeable protons are well protected from exchange with solvent water compared to inactive A17L-AFP.

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## References

1. Davies, P. L.; Hew, C. L. *FASEB J.* **1990**, *4*, 2460.
2. Breton, G.; Danyluk, J.; Ouellet, F.; Sarhan, F. *Biotechnol. Annu. Rev.* **2000**, *6*, 59.
3. Fletcher, G. L.; Hew, C. L.; Davies, P. L. *Annu. Rev. Physiol.* **2001**, *63*, 359.
4. Davies, P. L.; Baardsnes, J.; Kuiper, M. J.; Walker, V. K. *Philos. Trans. R. Soc. Lond. B* **2002**, *357*, 927.
5. Yeh, Y.; Feeney, R. E. *Chem. Rev.* **1996**, *96*, 601.
6. Harding, M. M.; Ward, L. G.; Haymet, A. D. *Eur. J. Biochem.* **1999**, *264*, 653.
7. Sicheri, F.; Yang, D. S. *Nature* **1995**, *375*, 427.
8. Zhang, W.; Laursen, R. A. *J. Biol. Chem.* **1998**, *273*, 34806.
9. Baardsnes, J.; Kondejewski, L. H.; Hodges, R. S.; Chao, H.; Kay, C.; Davies, P. L. *FEBS Lett.* **1999**, *463*, 87.
10. Kim, H.-E.; Lee, A.-R.; Lee, Y.-M.; Jeong, M.; Park, C.-J.; Lee, J.-H. *Bull. Korean Chem. Soc.* **2013**, *34*, 3137.
11. Ohnishi, M.; Urry, D. W. *Biochem. Biophys. Res. Commun.* **1969**, *36*, 194.
12. Andersen, N. H.; Tong, H. *J. Am. Chem. Soc.* **1997**, *119*, 8547.