Trifluoroethanol Increases the Stability of Δ⁵-3-Ketosteroid Isomerase

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Twenty-five years ago it was observed that the enzyme stability increases by 2.5 kcal/mol in the presence of 5% trifluoroethanol (TFE). To elucidate the increased enzyme stability by TFE, the backbone dynamics of Δ⁵-3-ketosteroid isomerase were studied in the presence and absence of 5% TFE by 15N NMR relaxation measurements, and the motional parameters (S², τe, and Rₑₑ) were extracted from the relaxation data using the model-free formalism. The presence of 5% TFE causes little change or a slight increase in the order parameters (S²) for a number of residues, which are located mainly in the dimer interface region. However, the majority of the residues exhibit reduced order parameters in the presence of 5% TFE, indicating that high frequency (pico- to nanosecond) motions are generally enhanced by TFE. The results suggest that the entropy can be an important factor for the enzyme stability, and the increase in entropy by TFE is partially responsible for the increased stability of Δ⁵-3-ketosteroid isomerase.

2,2,2-Trifluoroethanol (TFE) has been widely used for many decades as a co-solvent to stabilize structures in peptides and to denature proteins. Small amounts of TFE (5–10%) are known to destabilize native proteins by disrupting the hydrophobic interactions, thereby acting as a denaturant of tertiary and quaternary structures (1–4). Such destabilization of native proteins by TFE has been observed in cytochrome c, hen egg white lysozyme, ribonuclease A, and carbonic anhydrase (5–6). On the other hand, TFE induces many partly folded states at high concentration (5–10%) by stabilizing various secondary structures in peptides and quaternary structures (1–4). Such destabilization of native proteins by TFE has been observed in cyano-myo-globin, hen egg white lysozyme, and carbonic anhydrase (5–6). On the other hand, TFE induces many partly folded states at high concentration (5–10%) by stabilizing various secondary structures in peptides and quaternary structures (1–4). Such destabilization of native proteins by TFE has been observed in cyano-myo-globin, hen egg white lysozyme, ribonuclease A, and carbonic anhydrase (5–6). On the other hand, TFE induces many partly folded states at high concentration (5–10%) by stabilizing various secondary structures in peptides and quaternary structures (1–4). Such destabilization of native proteins by TFE has been observed in cyano-myo-globin, hen egg white lysozyme, ribonuclease A, and carbonic anhydrase (5–6). On the other hand, TFE induces many partly folded states at high concentration (5–10%) by stabilizing various secondary structures in peptides and quaternary structures (1–4). Such destabilization of native proteins by TFE has been observed in cyano-myo-globin, hen egg white lysozyme, ribonuclease A, and carbonic anhydrase (5–6). On the other hand, TFE induces many partly folded states at high concentration (5–10%)...
is the free energy change in the absence of urea, and \( m \) represents a measure of the \( \Delta G_f \) dependence on urea concentration. The data from a urea denaturation curve were fitted to Equation 4 by nonlinear least-squares analysis (28) using a graphics program, Kaleidagraph version 2.6 (Abelbeck Software).

\[
E = E_0(1 - E_0/\exp(m[\text{urea}]) - \Delta G_f^{15N}/RT) \times (1 + 8P_e/\exp(m[\text{urea}]) - \Delta G_f^{15N}/RT)^{1/2} - 1/4P_e \quad \text{(Eq. 4)}
\]

The difference in the free-energy change for the unfolding, \( \Delta G_{f,UE} \), between the presence and absence of 5% TFE was obtained with the following equation.

\[
\Delta G_{f,UE} = \Delta G_{f} - \Delta G_{f}^{15N} \quad \text{(Eq. 5)}
\]

where \( \Delta G_{f}^{15N} \) and \( \Delta G_{f} \) are the free energy changes for unfolding of the KSI in the presence and absence of 5% TFE, respectively.

**Preparation of NMR Sample—** NMR samples used in this study were prepared in 2 M urea solution. The NMR sample without 5% TFE was prepared to contain 1 mM uniformly 15N-labeled protein in 2 M urea with 90% \( \text{H}_2\text{O} \) and 10% \( \text{D}_2\text{O} \). The pH of the sample was adjusted to 7.0. The NMR sample with 5% TFE was obtained by an addition of 20 \( \mu \)l of TFE to 380 \( \mu \)l of 2 M urea sample.

**NMR Measurements and Processing—** All NMR data were collected at 27°C on a Bruker DRX500 spectrometer (500.13 MHz for \( ^1\text{H} \) and 50.7 MHz for \( ^15\text{N} \)) equipped with a triple resonance, pulse field gradient probe with actively shielded 2 axis gradients and a gradient amplifier unit. The pulse sequences used to record \( ^1\text{H} \) and \( ^15\text{N} \) NOE spectra were those described by Barbato et al. (29) with a slight modification to include water flip back (30) and WATERGATE (water suppression by gradient-tailored excitation; Ref. 31) techniques for eliminating the water resonance. Decoupling of \( ^1\text{H} \) spins during acquisition was performed using WALTZ-16 composite pulse sequence with a field strength of 2 kHz. The observed \( ^1\text{H} \) chemical shifts were determined relatively to internal reference, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), which was reported to be insensitive to changes in temperature. Indirect referencing was used for \( ^15\text{N} \) chemical shifts using the frequency ratio of \( m_{\text{DSS}}/m_{\text{KSI}} = 0.101329118 \) for DSS in water (32).

The \( T_1 \) and \( T_2 \) measurements were performed using total 48 transients per \( T_1 \) experiment. 128 \( \times \) 2048 complex points were acquired in the \( t_1 \times t_2 \) dimensions. A total of 9 data sets were collected to measure \( T_2 \) with delay values of 8, 150, 300, 500, 700, 900, 1100, 1400, and 1700 ms. A total of 12 data sets were collected to measure \( T_1 \) with delay values of 0.60, 27.4, 89.5, 298.8, 900, 1700, 3000, 5000, and 8000 ms. The NOE measurements were performed using total 96 transients per \( T_1 \) experiment. The \( t_1 \) dimension was zero-filled to 256 real data points, and 90° phase-shifted sine bell window function was applied before Fourier transformation and base-line correction in both dimensions.

**Analysis of the Relaxation Parameters—** \( ^15\text{N} \) relaxation parameters were analyzed with the model-free method (23, 24) by using the program Modelfree version 4.0 (36, 37). The cross-peak intensities were measured as peak volumes to increase sensitivity (38) and the relaxation parameters of too much overlapped peaks were not measured. The \( T_1 \) and \( T_2 \) were obtained by non-linear fitting of single exponential decays to the experimental data. The error levels in \( T_1 \) and \( T_2 \) were estimated by a 500 Monte Carlo simulation (36). The errors in the peak intensities for the simulation were determined from two independently measured data sets. NOE values were determined as the ratio of the steady-state intensities measured in the presence and absence of saturation of the proton magnetization, respectively. The molecular rotational diffusion tensor (\( D \)) was estimated from the measured \( T_1/T_2 \) values and the solution structure of free wild type KSI (17) as input structures. The calculations were performed by using the program R2R1_difffusion (36, 37) for the cases of isotaxic and axially symmetric.
Effects of TFE on the Stability of KSI

Effects of 5% TFE on 1H and 15N Chemical Shifts of KSI—To understand the effects of TFE on the enzyme stability, 1H-detected 15N NMR were carried out for the backbone amides of KSI in the presence and absence of 5% TFE. All the NMR experiments were performed in 2 M urea solution, since the resolution was much better in this condition than in the native samples, probably due to enhanced flexibility of KSI in 2 M urea. The assignments of 1H and 15N chemical shifts were initially carried out by comparing cross-peaks from heteronuclear single-quantum coherence spectra with the assignments of previous paper for the native KSI (46). The assignments were confirmed for most of the resolved peaks by the analysis of a three-dimensional 15N-edited nuclear Overhauser exchange spectroscopy and total correlation spectroscopy. Ambiguous peaks and too many overlapped peaks were eliminated during the assignment procedure.

Fig. 3 shows the backbone 1H and 15N chemical shift differences between the KSI samples in the presence and absence of 5% TFE. The residues having larger than 0.10 ppm chemical shift differences (weighted sum of 1H and 15N) are identified as 22, 57, 58, 63, 78, 85, 86, 99, 100, 103, 108, 109, 115, and 116. Among them, the residues 57, 58, 85, 86, 99, 100, and 103 are located in the central part of the secondary structure elements. However, there is no simple correlation between the enzyme activity and the chemical shift changes of the catalytic residues. Thus, no change in the enzyme activity occurred in the presence of 5% TFE, whereas one of the key catalytic residues, Asp-99, shows a relatively large chemical shift change of 0.10 ppm, and the other two catalytic residues, Tyr-14 and Asp-38, show marginal changes of 0.05 and 0.02 ppm, respectively.

Molecular Rotational Diffusion Analysis of KSI—To obtain the model-free parameters, S2, τr, and Rext, it is necessary to estimate an exact value for the overall rotational correlation time, τrot, from the 15N T1/2 ratio because small amounts of rotational anisotropy may contribute to Rext (40). Ignoring rotational anisotropy can also lead to a distortion of the internal correlation time when the extended model-free formalism is required for an adequate fit (47, 54). Therefore, an anisotropic model for rotational diffusion is essential in the case of highly asymmetric or multidomain proteins (54–56). KSI is highly asymmetric, having a relative ratio of 1.00:0.95:0.55 for the principal components of the inertia tensor based on the solution structure (17).

RESULTS

Equilibrium Unfolding of KSI by Urea in the Presence of 5% TFE—The folding of KSI is almost completely reversible and can be described as a two-state mechanism of folded dimer and unfolded monomer (49). Fig. 2 shows the urea-induced equilibrium unfolding curves of KSI in the presence and absence of 5% TFE measured by far UV-CD (A) and fluorescence spectroscopy (B). The unfolding curves both in the presence and absence of 5% TFE represent a typical two-state transition between native dimer and unfolded monomer, which is evidenced by the superposition of the unfolding curves of far-UV CD and fluorescence measurements. However, in the presence of 5% TFE, the denaturant molarity at the midpoint of the unfolding transition increases from 3.80 to 5.57 M, indicating that 5% TFE increases the stability of KSI. The free energy changes for KSI unfolding can be estimated from Equations 1–5 and are summarized in Table I. The difference in free energy change, ΔG\text{f}^\text{f} for KSI unfolding between the enzymes in the presence and absence of 5% TFE is -2.5 kcal/mol. This increase in the enzyme stability is somewhat unexpected, since the dimer interface region of KSI is mainly stabilized by the hydrophobic interactions (17, 18). The results also contradict the previous observations that a small amount of TFE disrupts the hydrophobic interactions, thereby acting as a denaturant of tertiary and quaternary structures (1–4).

On the other hand, the enzyme did not show any gross changes in the CD spectrum up to the TFE concentration of 15% (data not shown) nor did the enzyme dissociate into monomers, indicating that conformational changes of the enzyme are negligible at low TFE concentration. The CD spectrum begins to change from 15% TFE and shows significant α-helix content at 40% TFE (data not shown), presumably due to non-native partially folded states at high TFE concentration (50–51). In addition, the activity of KSI was measured in the presence and absence of 5% TFE, since it has been speculated that there is a trade-off between the stability and activity (52) of enzyme. However, TFE does not cause any changes in the activity of KSI, unlike other proteins such as another dimeric enzyme, aminoacylase, which loses 60% of its activity at 5% TFE (53). This result suggests that the increased stability of KSI by 5% TFE may not be related to specific interactions in the active site of the enzyme.

![Fig. 2. Urea-induced equilibrium unfolding curves of KSI in the presence and absence of 5% TFE measured by (A) CD and (B) fluorescence spectroscopy. Data were measured at pH 7 (10 mM potassium phosphate buffer) and 25 °C. The circles and squares represent the data points in the presence and absence of 5% TFE, respectively.](image-url)

### Table I

| Concentration of TFE | ΔGf°f kcal/mol | m | [urea]50% |
|----------------------|----------------|---|-----------|
| 0                    | 22.0           | 4.00 | 3.80      |
| 5                    | 24.5           | 3.17 | 5.57      |

Concentration of TFE and the 15N chemical shift differences detected by 15N NMR resonance assignments were correlated with the backbone amides of the NMR spectra. The assignments of 1H and 15N chemical shifts were initially carried out by a combination of cross-peak analysis from heteronuclear single-quantum coherence spectra with the assignments of previous paper for the native KSI (46). The assignments were confirmed for most of the resolved peaks by the analysis of a three-dimensional 15N-edited nuclear Overhauser exchange spectroscopy and total correlation spectroscopy. Ambiguous peaks and too many overlapped peaks were eliminated during the assignment procedure.

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Effects of TFE on the Stability of KSI

For an initial estimate of the rotational correlation time ($\tau_m$) and diffusion tensor ($D$), residues were selected on the basis of criteria outlined by Tjandra (40). From a total of 91 and 97 residues whose relaxation parameters were measured in the presence and absence of 5% TFE, respectively, 57 and 70 residues were used to calculate $\tau_m$ and the diffusion parameters. The statistical F-test indicates that the relaxation data are best described by an axially symmetric rotational diffusion tensor in both cases. Hence, the $\tau_m$ and $D/D_{1}$ values from the axially symmetric model were used to analyze the internal motions of KSI. After selection of the appropriate internal dynamics models and final model free calculations, the optimized effective $\tau_m$ was 18.26 ± 0.09 ns with a diffusion anisotropy $D/D_{1} = 1.15 \pm 0.03$ for KSI in the presence of 5% TFE and 17.17 ± 0.05 ns with $D/D_{1} = 1.24 \pm 0.02$ for KSI in the absence of 5% TFE. These values of $\tau_m$ and $D/D_{1}$ are in good agreement with the values of 19.23 ± 0.08 ns and 1.26 ± 0.03 for the native KSI (46), indicating that the enzyme does not dissociate into monomeric forms or change its overall shape by the presence of 5% TFE or 2 M urea. Sedimentation analysis also shows that the enzyme exists in dimeric forms in 2 M urea solution (49).

Backbone Dynamics of KSI in the Absence of 5% TFE — $^{15}$N relaxation parameters were measured for 97 of 120 backbone amides (excluding the N-terminal methionine and 4 prolines). The residual 23 peaks were excluded from the analysis due to resonance overlap. The relaxation parameters for KSI in the absence of 5% TFE are shown in Fig. 4, A–C. For most residues, the $T_1$, $T_2$, and heteronuclear NOE lie in the ranges of 0.8 – 1.1 s, 30 – 50 ms, and 0.6 – 0.8, respectively, except for several residues that are located between the secondary structure elements or at the C terminus.

The model-free analysis was performed with an axially symmetric rotational diffusion tensor. The model-free parameters $S^2$, $\tau_e$, and $R_{ex}$ for KSI in the absence of 5% TFE are shown in Fig. 5, A–C. The spectral density models used to fit the relaxation data are summarized in Table II. The well defined secondary structure elements, $\alpha$-helices and $\beta$-strands, have average values for the order parameters ($S^2$) of 0.95 and 0.93, respectively, which are relatively larger than those found in other proteins, indicating that the internal motions of the backbone residues are highly restrained on the picosecond to nanosecond time scales.

Effective correlation times ($\tau_e$) for internal motions were detected for 16 residues having the values in the range of 100 – 1200 ps. Most of these residues are located between the secondary structure elements or at the C terminus. Slower motions of $\tau_e$ on the time scale greater than 500 ps were found for

![Figure 3](image1.png)

**Fig. 3.** Backbone chemical shift differences between the KSI samples in the presence and absence of 5% TFE as a function of residue number. A, amide $^1$H chemical shift differences. B, amide $^{15}$N chemical shift differences. C, sum of the absolute magnitude of $^1$H and $^{15}$N chemical shift differences that were weighted according to the backbone amide chemical shift dispersion in the $^1$H and $^{15}$N dimensions (3.11 and 27.3 ppm). The values for KSI in the absence of 5% TFE are subtracted from those for KSI in the presence of 5% TFE.

![Figure 4](image2.png)

**Fig. 4.** Plots of the measured $^{15}$N relaxation parameters and their uncertainties as a function of residue number for the KSI in the absence (A–C) and presence (D–E) of 5% TFE. A and D, longitudinal relaxation time, $T_1$. B and E, transverse relaxation time, $T_2$. C and F, Heteronuclear NOE.

![Figure 5](image3.png)

**Fig. 5.** Plots of the model-free parameters and their uncertainties as a function of residue number for the KSI in the absence (A–C) and presence (D–F) of 5% TFE. A and D, the generalized order parameter, $S^2$. B and E, the effective correlation time, $\tau_e$. C and F, the chemical exchange contribution, $R_{ex}$. 
residues 88, 89, 90, 91, and the two C-terminal residues (124 and 125). Among them, the residues in the turn (89 and 90) at the end of the strand B4 needed comparable magnitude of \( \tau_e \) values around 800 ps. The conformational exchange terms (\( R_{ex} \)), which reflect the existence of an exchange process on the micro- to millisecond time scale, were detected for 10 residues having the values in the range of 2 – 16 Hz.

**Backbone Dynamics of KSI in the Presence of 5% TFE**—Relaxation parameters were measured for 91 of 120 backbone amides (excluding the N-terminal methionine and 4 prolines). The residual 29 peaks were excluded from the analysis due to resonance overlap or lack of signal intensity. The relaxation parameters for KSI in the presence of 5% TFE are shown in Fig. 4, \( D - F \). The \( T_1 \), \( T_2 \), and heteronuclear NOE for most residues are in the ranges of 0.8 – 1.2 s, 30 – 40 ms, and 0.6 – 0.8, respectively.

The model-free parameters for KSI in the presence of 5% TFE are shown in Fig. 5, \( D - F \). Table III lists the average order parameters (\( S^2 \)) for each of the secondary structure elements of KSI both in the absence and presence of 5% TFE. The average \( S^2 \) for most secondary elements of KSI are significantly smaller in the presence of 5% TFE than in the absence of 5% TFE, indicating that KSI has a high degree of internal motions on the pico- to nanosecond time scales in the presence of TFE.

Effective correlation times (\( \tau_e \)) for internal motions were found for 17 residues in the range of 40 – 1200 ps. Slower motions of \( \tau_e \) on the time scale greater than 500 ps were detected for the residues at positions of 22, 61, 90, 91, 92, 124, and 125. Most residues that require the \( \tau_e \) values for adequate fitting were found at the similar locations to the case of KSI without TFE. The exchange terms (\( R_{ex} \)) were detected for 9 residues, which is also similar to the case of KSI without TFE, indicating that the motions on the micro- to millisecond time scales are relatively limited for KSI both in the absence and presence of 5% TFE.

**DISCUSSION**

Table II summarizes the spectral density models used to fit the relaxation data of KSI in the presence and absence of 5% TFE. Relaxation data for most residues in both cases are fit well using a simple model (\( S^2 \) only). Fig. 6 shows the differences between the order parameters for KSI in the presence and absence of 5% TFE. The differences in the order parameters between the two cases are larger than 0.08 in most residues, which are significant considering the average uncertainty of 0.036 in \( S^2 \). As can be seen in Fig. 6, the majority of the residues have reduced order parameters in the presence of 5% TFE, indicating the increased high frequency (pico- to nanosecond) motions of KSI by TFE. However, a number of residues exhibit increased order parameters in the presence of 5% TFE, which are mainly located in the secondary structure elements A3, B2, B3, B5, and B6. Table III lists the average order parameters (\( S^2 \)) for each of the secondary structure elements of KSI both in the absence and presence of 5% TFE. As might be expected from Fig. 6, the average order parameters of A1, A2, B1, and B4 decrease significantly in the presence of 5% TFE, whereas those of A3, B2, B3, B5, and B6 remain the same or decrease slightly. Such distinctive changes in the average order parameters for the secondary structure elements may provide a clue for better understanding the relationship between the internal dynamics and the stability of KSI.

Two most important parts for the function of KSI are the dimer interface region and the hydrophobic cavity containing the active site. The active site of KSI comprises a conical cleft of 14 – 16 Å deep to be effective for substrate binding, which is hydrophobic in nature. As can be seen in Fig. 1, one side of this hydrophobic cavity is lined with residues from the front faces of the strands B3, B4, B5, and B6, whereas the other side of the cavity is composed of the residues that reside on A1, A2, and B1. On the other hand, the strands B3, B5, and B6 play an essential role in forming the dimer interface of KSI by having most residues in them participate in the dimer interactions. Thus, the changes in the average order parameters for the secondary structure elements may reflect the effects of TFE on KSI, where a small amount of TFE enhances the flexibility of the hydrophobic cavity while not affecting the dimer interface region. This result may be compared with those of recent folding study (49) that the dimerization plays an essential role in maintaining the conformational stability of KSI and contributes to the recovery of full enzyme activity.

TFE is known to stabilize the secondary structures by strengthening the intramolecular hydrogen bonds and to destabilize the proteins by disrupting the hydrophobic interactions in the tertiary or quaternary structures (1–6). Our results indicate that 5% TFE does not affect the backbone dynamics in the dimer interface region of KSI, where the interactions are

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**Table II**

Summary of spectral density function models used to fit \( T_1 \), \( T_2 \) and NOE data for KSI in the presence and absence of 5% TFE.

| Model parameters | 0% TFE | 5% TFE |
|------------------|--------|--------|
| \( S^2 \)        | 70     | 69     |
| \( S^2 \) and \( \tau_e \) | 9      | 7      |
| \( S^2 \) and \( R_{ex} \) | 1      | 4      |
| \( S^2 \), \( \tau_e \), and \( R_{ex} \) | 6      | 6      |
| Not fit          | 2      | 0      |
| Total            | 97     | 91     |

**Table III**

Average order parameters for secondary structure elements of KSI in the presence and absence of 5% TFE.

| Secondary structure | Residue | \( S^2 \) (0% TFE) | \( S^2 \) (5% TFE) |
|---------------------|---------|--------------------|--------------------|
| Helix A1            | 3–20    | 0.980 (0.026)      | 0.926 (0.052)      |
| Helix A2            | 22–30   | 0.955 (0.040)      | 0.844 (0.105)      |
| Strand B1           | 34–38   | 0.946 (0.041)      | 0.853 (0.047)      |
| Strand B2           | 43–47   | 0.894 (0.020)      | 0.884 (0.089)      |
| Helix A3            | 48–61   | 0.916 (0.064)      | 0.914 (0.071)      |
| Strand B3           | 63–74   | 0.922 (0.077)      | 0.910 (0.060)      |
| Strand B4           | 77–88   | 0.944 (0.080)      | 0.885 (0.059)      |
| Strand B5           | 91–104  | 0.916 (0.079)      | 0.899 (0.095)      |
| Strand B6           | 108–123 | 0.950 (0.048)      | 0.922 (0.053)      |
mostly between side chains and hydrophobic in nature. Then it can be
assumed that the strengthened hydrogen bonds by TFE in the
secondary structure elements are primarily responsible for the increased stability of KSI. However, since the free
energy is a sum of the enthalpic and entropic terms, it is
necessary to consider the entropic contribution to the enzyme
stability. The entropic contribution to free energy can be easily
estimated from the order parameters and quantitatively de-
scribed by Equation 6 as proposed by Akke et al. (25).

\[
\Delta G = \left(G_{\text{TFE}} - G_{\text{5\%TFE}}\right) = -RT \sum_{i=1}^{N} \ln[(1 - S_{i}^{2,\text{TFE}})/(1 - S_{i}^{2,\text{5\%TFE}})] \tag{Eq. 6}
\]

Here, \(\Delta G\) is the difference in free energy between the KSI
samples in the presence and absence of 5% TFE, which results
only from the changes in high frequency motions of the back-
bone amides. The number of residues whose order parameters
are determined both in the presence and absence of 5% TFE is
81 of the total 125. Using Equation 6 and the order parameters
for 81 residues, the \(\Delta G\) for KSI is calculated to be \(-34.79\)
kal/mol, which is much larger than the measured value of
4.16 kcal/mol in 2 M urea condition (equivalent to \(-2.5\) kcal/
 mol in the native state). This result suggests that the enthalpic
contribution to \(\Delta G\) for KSI could be a large positive number
(\(+30.63\) kcal/mol). Thus, the negative enthalpic effect of TFE
on the enzyme stability (destruction of the hydrophobic inter-
actions, especially in the tertiary structures) could be dominant
over the positive effect (strengthening of the hydrogen bonds in
the secondary structures), and the enzyme as a whole would
not be stabilized enthalpically in the presence of 5% TFE. Of
course, the entropic contribution to \(\Delta G\) by the remaining 44
residues, whose order parameters could not be measured, can
be a large positive number. Furthermore, the dynamics of the side chains are not considered here, which can also contribute
to \(\Delta G\) as a positive number. Nevertheless, the results show that the entropic contribution to free energy alone can be a main
source for the increased stability of KSI by 5% TFE.

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