Stabilization by Fusion to the C-terminus of Hyperthermophile Sulfolobus tokodaii RNase HI: A Possibility of Protein Stabilization Tag

Kazufumi Takano1,2*, Tomohiro Okamoto1, Jun Okada1, Shun-ichi Tanaka1, Clement Angkawidjaja1, Yuichi Koga1, Shigenori Kanaya1
1Department of Material and Life Science, Osaka University, Osaka, Japan, 2Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Osaka, Japan

Abstract

RNase HI from the hyperthermophile Sulfolobus tokodaii (Sto-RNase HI) is stabilized by its C-terminal residues. In this work, the stabilization effect of the Sto-RNase HI C-terminal residues was investigated in detail by thermodynamic measurements of the stability of variants lacking the disulfide bond (C58/145A), or the six C-terminal residues (ΔC6). The results showed that the C-terminal does not affect overall structure and stabilization is caused by local interactions of the C-terminal, suggesting that the C-terminal residues could be used as a "stabilization tag." The Sto-RNase HI C-terminal residues (IGCIILT) were introduced as a tag on three proteins. Each chimeric protein was more stable than its wild-type protein. These results suggested the possibility of a simple stabilization technique using a stabilization tag such as Sto-RNase HI C-terminal residues.

Introduction

An important goal of protein engineering is designing variants that enhance the conformational stability of proteins. Structure-based design [1,2], sequence alignment approaches [3,4], and random mutagenesis [5,6] are all used to stabilize proteins by mutagenesis, but problems still exist, especially in finding simple and general techniques.

Protein tags, which are peptide sequences genetically grafted onto the N- or C-terminus of recombinant proteins, are widely used experimentally because they are easy to manipulate. Tags are attached to proteins for various purposes, such as purification [7,8], solubilization [9,10], and fluorescent imaging [11,12]. Development of a "stabilization tag" will allow researchers to attach to proteins for various purposes, such as purification [7,8], solubilization [9,10], and fluorescent imaging [11,12]. Development of a "stabilization tag" will allow researchers to develop a universal stabilization tag or optimal stabilization tags for individual proteins.

Methods

Protein purification

Wild-type, C58/145A and ΔC6 Sto-RNase HI were overproduced and purified as previously described [13,14]. RNase HI from the psychrotrophic bacterium Shewanella oneidensis MR-1 (So-RNase HI) [16], or from Escherichia coli (Ec-RNase HI) [17], and esterase from Sulfolobus tokodaii (Sto-esterase) [Angkawidjaja et al., in preparation] were overproduced and purified as described. Plasmids for the overexpression of variants of So-RNase HI, Ec-RNase HI, and Sto-esterase were constructed from the wild-type genes using standard recombinant DNA techniques. Overproduction and purification of the chimeric proteins was as for the wild-type proteins. Protein purity was confirmed using SDS-PAGE.

Biophysical analyses

Circular dichroism (CD) spectra measurements, equilibrium and kinetic guanidine hydrochloride (GdnHCl)-induced, and heat-
induced unfolding were as previously described [15]. Buffers were 20 mM glycine-HCl pH 3.0 for Sto-RNase HI, 10 mM acetate pH 5.5 with 1 M GdnHCl for So-RNase HI, 10 mM glycine-HCl pH 3.0 for Ec-RNase HI, and 25 mM CHES-NaOH pH 9.0 with 1 M GdnHCl for Sto-esterase.

Equilibrium experiments on GdnHCl-induced unfolding were examined by monitoring the CD at 220 nm. Protein solutions were incubated in GdnHCl at different concentrations and at different temperatures for unfolding. The GdnHCl-induced unfolding curves were determined, and a nonlinear least-squares analysis was used to fit the data to

\[
y = \frac{\{(b_0^n + a_n[D]) + (b_0^u + a_u[D])\exp(\Delta G(H_2O) - m[D])/RT\}/\{1 + \exp(\Delta G(H_2O) - m[D])/RT\}}.
\]

(1)

\[C_m = \frac{\Delta G(H_2O)}{m}\]  

(2)

where \(y\) is the observed CD signal at a given concentration of GdnHCl, \([D]\) is the concentration of GdnHCl, \(b_0^n\) is the CD signal for the native state, \(b_0^u\) is the CD signal for the unfolded states, \(a_n\) is the slope of the pre-transition of the baseline, and \(a_u\) is the slope of the posttransition of the baseline. \(\Delta G(H_2O)\) is the Gibbs energy change (\(\Delta G\)) of the unfolding in the absence of GdnHCl, \(m\) is the slope of the linear correlation between \(\Delta G\) and the GdnHCl concentration \([D]\), and \(C_m\) is the GdnHCl concentration at the midpoint of the curve. Two or three replicates were measured for each condition. The raw experiment data were directly fitted to Eq. (1) using SigmaPlot (Jandel Scientific). Stability profiles (temperature dependence of \(\Delta G(H_2O)\)) were fitted to the Gibbs-Helmholtz equation, Eq. (3).

\[
\Delta G(H_2O) = \Delta H(T_0) - T\Delta S(T_0) + \Delta C_p[T - T_0 - T\ln(T/T_0)]
\]

(3)

Figure 1. Crystal structure of wild-type Sto-RNase HI. C-terminal seven residues (cyan); hydrophobic side-chains (blue); hydrogen bonds (thin red lines); and disulfide bond (thick red line).

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| Table 1. Statistics on data processing and structure determination of \(\Delta C_6\) Sto-RNase HI. |
|---------------------------------|----------------|
| Wavelength (Å)                 | 1.0            |
| Temperature (K)                | 100            |
| Space group                    | P1             |
| Unit cell edges (Å)            | 40.98, 41.02, 43.53 |
| Unit cell angles (Å)           | 89.72, 89.71, 86.76 |
| Resolution (Å)                 | 50.0–1.66 (1.72–1.66) |
| No. measured reflections       | 244,376        |
| No. unique reflections         | 32,375         |
| \(R_{merge}\) (%)             | 3.4 (21.8)     |
| Completeness (%)               | 96.2 (94.3)    |
| \(<I>/<\sigma(I)>\)           | 37.2 (6.1)     |
| \(R_{work}/R_{free}\) (%)     | 19.7/25.0      |
| Total atoms (protein/solvent)  | 2244/256       |
| Root-mean-square deviation     |                |
| Bond length (Å)                | 0.011          |
| Bond angles (deg.)             | 1.391          |
| Ramachandran plot              |                |
| Most favored (%)               | 94.6           |
| Additionally allowed (%)       | 5.4            |
| Disallowed (%)                 | 0              |

Values in parentheses are the highest-resolution bin of respective data.

\(R_{merge} = \sum_{hkl} |I_{hkl} - <I_{hkl}>|/\sum_{hkl} <I_{hkl}>, \) where \(<I_{hkl}>\) is the intensity measurement for reflection with indices \(hkl\) and \(<I_{hkl}>\) is the mean intensity for multiply recorded reflections.

\(R_{work}, free = \sum_{\{I_{work} - |F_{calc}|\}^2/\sum_{\{I_{work}\}^2}, \) where the \(R\)-factors are calculated using the working and free reflection sets, respectively. The free reflections comprise a random 10% of the data held aside for unbiased cross-validation throughout refinement.

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where \( \Delta H(T_o) \) and \( \Delta S(T_o) \) are the enthalpy and entropy of unfolding at the reference temperature \( T_o \), and \( \Delta C_p \) is the difference in heat capacity between the native and unfolded states.

Kinetic experiments on GdnHCl-induced unfolding were followed by CD spectra measurement at 220 nm. The unfolding reactions of proteins were induced by a concentration jump in GdnHCl, with various differing concentrations. The kinetic data were analyzed using Eq. (4).

\[
A(t) - A(\infty) = Ae^{kt}
\]  

(4)

Here, \( A(t) \) is the value of the CD signal at a given time \( t \), \( A(\infty) \) is the value when no further change is observed, \( k \) is the apparent rate constant, and \( A \) is the amplitude. Two or three replicates were measured for each condition. The GdnHCl concentration dependence of the logarithms of the apparent rate constant (\( k_{app} \)) for unfolding was also examined. The rate constants for unfolding in the absence of GdnHCl (\( k_u(H_2O) \)) were calculated by fitting to Eq. (5):

\[
\ln k_{app} = \ln k_u(H_2O) + m_u[D]
\]  

(5)

where \([D]\) is the concentration of GdnHCl and \( m_u \) represents the slopes of the linear correlations of \( \ln k_u \) with the GdnHCl concentration.

Heat-induced unfolding was examined by monitoring the CD at 220 nm. All experiments were carried out at a scan rate of 1 °C min \(^{-1}\). A nonlinear least-squares analysis was used to fit the data to

\[
y = \{b_n + a_n[T] + (b_u + a_u[T])\exp(\Delta H_m/RT)((T - T_m)/T_m)\} / \{1 + \exp(\Delta H_m/RT) - ((T - T_m)/T_m)\}
\]  

(6)

where \( y \) is the observed CD signal at a given temperature \([T]\), \( b_n \) is the CD signal for the native state, \( b_u \) is the CD signal for the unfolded states, \( a_n \) is the slope of the pretransition of the baseline, \( a_u \) is the slope of the postransition of the baseline, \( \Delta H_m \) is the enthalpy of unfolding at the transition midpoint temperature \( T_m \), \( T \) is the temperature, and \( R \) is the gas constant. Curve fitting was performed using SigmaPlot. Two or three replicates were measured for each condition.

**Structural analysis**

Crystals of \( \Delta C6 \) Sto-RNase HI were grown in 20% PEG 3000, 0.1 M citrate pH 5.5, including 6–7 mg mL \(^{-1}\) protein at 4 °C. All full diffraction sets were collected at 100 K without cryoprotectants on a SPring-8 BL38B1. Diffraction data were indexed, integrated, and scaled using the HKL2000 program suite [18]. The crystal structure was solved by the molecular replacement method using MOLREP [19] in the CCP4 program suite [20], with the wild-type structure (2EHG) as a starting model. Structure refinement was with the programs Coot and REFMAC in the CCP4 program suite [21,22]. Progress in structure refinement was evaluated at each stage by the free R-factor and by inspecting stereochemical parameters calculated by the program PROCHECK [23]. Collected and refined data are in Table 1. Figures were prepared using PyMol (http://www.pymol.org).

Figure 2. CD spectra and crystal structure of Sto-RNase HI variants. (A) CD spectra of wild-type (solid line), CS8/145A (dashed line), and \( \Delta C6 \) (dot-dashed line) Sto-RNase HI. (B) Crystal structure of \( \Delta C6 \) Sto-RNase HI. (C) Crystal structure of wild-type Sto-RNase HI. The C-terminal seven residues are in cyan.

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The coordinates and structure factors of DC6 Sto-RNase HI have been deposited in the RCSB Protein Data Bank under ID code 3ALY.

Results and Discussion

CD spectra and crystal structure of Sto-RNase HI variants

CD spectra of the wild-type, C58/145A, and DC6 Sto-RNase HI were measured in the far-UV region to examine the effect of the mutations on the overall secondary structure. As shown in Figure 2A, the shape of the spectra was almost the same for the wild-type and variant proteins. The crystal structure of DC6 Sto-RNase HI was solved at a resolution of 1.66 Å, as shown in Figure 2B. Two molecules are contained per asymmetric unit. The root-mean-square deviations of the Ca atoms for the A and B chains of the DC6 variant against the wild-type protein were 0.446 and 0.436 Å. These results showed that the overall structures of both C58/145A and DC6 Sto-RNase HI resembled the wild-type protein. C-terminal residues 142 and 143 of DC6 Sto-RNase HI were not observed because of disorder, indicating that deletion of the previous C-terminal residues made the new C-terminus flexible.

Stability of Sto-RNase HI variants

Heat-induced unfolding of the wild-type, C58/145A, and DC6 Sto-RNase HI variants was previously analyzed by differential scanning calorimetry (DSC) at pH 3.0 [14]. The denaturation temperature is 102°C for the wild-type, 93°C for C58/145A and 78°C for DC6 Sto-RNase HI. These results indicated that Sto-

| Table 2. Thermodynamic parameters for denaturation of wild-type, C58/145A and DC6 Sto-RNase HI. |
|------|---------|---------|---------|
|       | $T_m$ (°C) | $\Delta H(T_m)$ (kJ mol$^{-1}$) | $\Delta C_p$ (kJ mol$^{-1}$ K$^{-1}$) |
| Wild-type | 102$^a$  | 890±21$^b$  | 12.8±0.6$^b$  |
| C58/145A | 87.3$^a$ | 827±24$^b$  | 11.0±0.7$^b$  |
| DC6    | 79.1$^a$ | 508±21$^b$  | 7.9±0.7$^b$   |

$^a$Data from [14].
$^b$Errors are standard error values from the data fitting using Eq. (3).
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Protein data bank accession number

The coordinates and structure factors of DC6 Sto-RNase HI have been deposited in the RCSB Protein Data Bank under ID code 3ALY.
RNase HI is a hyperthermostable protein with a denaturation temperature beyond the boiling temperature, and is destabilized by 9°C by elimination of the disulfide bond and by 24°C by C-terminal truncation. In this work, we confirmed stability changes in the variant proteins using GdnHCl-induced equilibrium unfolding experiments at various temperatures (Figure 3A). GdnHCl denaturation was reversible under all conditions examined. The ΔG(H2O) value at each temperature was calculated, and the resultant values are plotted as a function of temperature for a stability profile in Figure 3B. When fitting these values to Eq. (3), the Tm value, which is the thermal denaturation temperature obtained from the heat-induced unfolding experiment [14], was used (ΔG(H2O) = 0 at Tm). The thermodynamic parameters are in Table 2. For C58/145A Sto-RNase HI, the curve shifts towards a lower temperature, indicating that destabilization is caused by entropic penalty [24]. This suggests that elimination of the disulfide bond mainly affected the conformation of C58/145A Sto-RNase HI in the denatured state. In contrast, the ΔC6 variant shifted the curve downward and flattened it. This is the result of decreases in ΔH and ΔCp. Because the C-terminal truncation eliminates hydrogen bonds and hydrophobic interactions, this result suggested that elimination of these forces at the C-terminal region was mainly responsible for the decreases in ΔH and ΔCp, resulting in the destabilization of ΔC6 Sto-RNase HI. We concluded that the C-terminal residues of Sto-RNase HI contributed to stability through local interactions.

Unfolding kinetics of Sto-RNase HI variants

Sto-RNase HI is highly stable, as indicated by its remarkably slow unfolding [14,15]. To understand the stabilization mechanism of the C-terminus of Sto-RNase HI, we performed GdnHCl-induced kinetic unfolding of the variant proteins at 25°C (Figures 4A and 4B). The reaction was initiated by jumps to various GdnHCl concentrations followed by CD measurements. All kinetic traces were described by a single exponential. We calculated k(H2O), which is the rate constant for unfolding in the absence of GdnHCl, from the GdnHCl concentration dependence of the logarithms of the apparent unfolding rate constant (kapp), which is the linear correlation of ln kapp with GdnHCl concentration. The k(H2O) was 5.7 × 10^-11 s^-1 for the wild-type, 1.0 × 10^-10 s^-1 for C58/145A, and 1.7 × 10^-5 s^-1 for ΔC6 Sto-RNase HI. Both variant proteins unfolded much faster than the wild-type protein. These results suggested that the C-terminal residues of Sto-RNase HI also contribute to the slow unfolding of this protein through hydrophobic interactions, because hydrophobic effects are one reason for the slow unfolding of ribonuclease HIII from hyperthermophilic archaeon Thermococcus kodakaraensis [25,26].

Attachment of the C-terminal residues of Sto-RNase HI to other proteins

As described above, the C-terminal residues of Sto-RNase HI contribute to stability through local hydrogen bonds, hydrophobic interactions, and a disulfide bond. This suggests the possibility of their use as a stabilization tag, because they structurally affect only their local region, but thermodynamically affect overall stability. We tested the effect on stability of attaching the C-terminal residues of Sto-RNase HI to So-RNase HI, Ec-RNase HI, and Sto-esterase. So-RNase HI and Ec-RNase HI are homologous to Sto-RNase HI (amino acid sequence identity of 19 and 18% to Sto-RNase HI) but lacking a C-terminal anchoring (Figures 5A and 5B), so a positive effect on stabilization was expected. Since So-RNase HI is from a psychrotrophic bacterium, it may be particularly easy to stabilize. In contrast, Sto-esterase (Figure 5C) is a hyperthermophilic protein and non-homologous with Sto-RNase HI, and might be difficult to stabilize.

Figure 5. Crystal structure of So-RNase HI, Ec-RNase HI, and Sto-esterase. (A) So-RNase HI, (B) Ec-RNase HI and (C) Sto-esterase. doi:10.1371/journal.pone.0016226.g005
We designed chimeric proteins with the C-terminal seven residues [IGCIILT] of Sto-RNase HI fused to the original C-terminal of So-RNase HI, Ec-RNase HI, or Sto-esterase. Overproduction and purification of the chimeric proteins was carried out as for the wild-type proteins, as shown in Figure 6A. Although the attached residues were somewhat hydrophobic, the proteins did not aggregate from the decrease in solubility. The heat-induced unfolding curves of the chimeric proteins are depicted in Figure 6B. The apparent fraction of unfolded protein is shown as a function of temperature. Curves 1, 2 and 3 represent the unfolding traces of chimeric So-RNase HI (closed circles), Ec-RNase HI (open circles) and Sto-esterase (cross). Lines are best fits to a two-state equation.

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In conclusion, we showed the validity of a stabilization tag using the C-terminal residues of Sto-RNase HI. A stabilization tag could be easy to use because genes can be modified without structural information about the proteins they encode. We do not suggest that the C-terminal residues used here are the best stabilization tag. A universal stabilization tag may exist that stabilizes all proteins, or an optimal stabilization tag could be designed for individual proteins.

Author Contributions
Conceived and designed the experiments: KT. Performed the experiments: TO. Analyzed the data: JO ST CA. Contributed reagents/materials/analysis tools: YK SK. Wrote the paper: KT. Helped in interpretation of data and discussion of results: YK SK.

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