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Article

Increased thermodynamic stability by hydrophilic amino acid substitutions on the surface of *Streptomyces* subtilisin inhibitor

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***Streptomyces* subtilisin inhibitor (SSI) is a homodimer composed of 113 amino acid residues in a subunit and contains three Met residues at the positions of 70, 73, and 103. Met73 is the P1 site residue on the surface of SSI and is crucial for inhibitory activity. We have studied the relationship between the thermal stability and hydrophobicity of the P1 site residue. Mutant proteins were prepared by the substitution of Met73 with Gly, Ala, Val, Leu, Ile, Lys, Asp, or Glu. The melting temperatures of the mutants were determined by monitoring the temperature dependence of the ^{1}H NMR signal intensities of Met70 and Met103. The results showed that mutants whose P1 residue is hydrophilic are more stable than the wild-type SSI. A good correlation was obtained between the increase of the thermal stability and the decrease of the hydrophobicity of the side-chain of the P1 site, which would be a "reverse hydrophobic effect", as first reported by Pakula and Sauer (Nature 344, 363-364, 1990).**

Keywords: NMR, protein engineering, *Streptomyces* subtilisin inhibitor, thermal stability

Introduction

Streptomyces subtilisin inhibitor (SSI) was first isolated from *Streptomyces albogriseolus* S-3253, and strongly inhibits alkaline serine proteases such as subtilisin BPN' [1]. SSI has a unique homodimeric structure with 11.5 kDa subunits and has been the subject of thorough studies, especially as the target of protein engineering in the 1980s and 1990s [2,3]. The three-dimensional structures of both free SSI and its complex with subtilisin BPN' show that the two subunits face each other through predominantly hydrophobic residues on four-stranded β-sheets, which are tightly bound and make almost a single large hydrophobic core (Fig. 1) [4,5]. This arrangement of the two subunits makes the protein quite stable, causing the thermal transition of the wild-type to be over 80°C at neutral pH [6,7]. SSI contains three Met residues, which are all located at crucial sites for its structure and function (Fig. 1). Met73 constitutes the P1 site in the reactive-site of the inhibitor, and Met70 constitutes the P4 site of the enzyme-binding segment, in which, in both cases, the side-chains are well exposed to the solvent. The effects of the Met70 and Met73 mutations on the inhibitory activity and structure of SSI have been studied in some detail [8-13]. In contrast to the surface residues of Met70 and Met73, Met103 is deeply buried in the hydrophobic core of each subunit and is located at a crucial position to define the stability of the core. We previously reported the effects of Met103 replacement with other amino acids on the

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The abbreviations used are: SSI, *Streptomyces* subtilisin inhibitor; DSC, differential scanning calorimetry; δ, chemical shift (in ppm) down field from the internal reference, sodium 3-trimethylsilylpropionate-2,2,3,3-d_4; T_m, midpoint transition temperature for denaturation: ΔG, Gibbs free energy change; ΔH_{vH}, van't Hoff enthalpy change for denaturation; OMH, optimal matching hydrophobicity

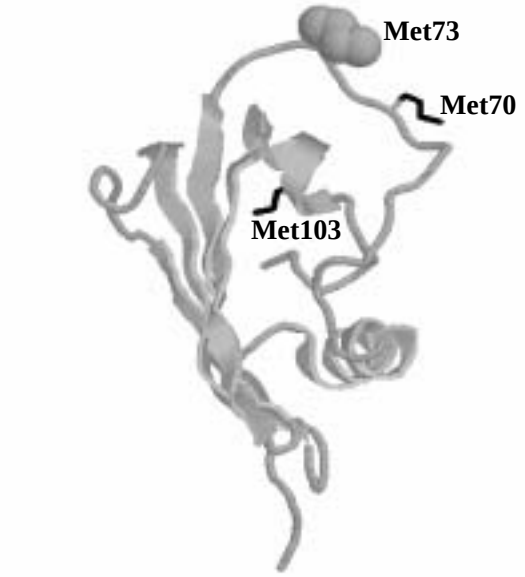

Figure 1. Backbone structure of an SSI subunit with the three Met residues, based on the crystallographic coordinates (PDB code, 3SSI). The side-chain of Met73 is represented as a spacefill model, and those of Met70 and Met103 are indicated as stick models.

inhibitory activity, structure, and stability, analyzed by fluorescence, ^{1}H NMR, and differential scanning calorimetry (DSC) [7,14]. In this study, we analyzed the effects of amino acid residues at the P1 site, the hyper-exposed surface of SSI, on the thermal stability by ^{1}H NMR, and re-evaluated the hydrophobic effects on the protein surface with regard to stability using a well-analyzed protein, SSI.

Materials and Methods

Protein preparation

The SSI proteins were expressed and purified as described previously [7]. The purified proteins were lyophilized and redissolved in 40 mM sodium deuterated phosphate buffer (pH 7.0) containing 100 mM NaCl. Here the name of each mutant protein is indicated as, for example, M73G for the mutation that replaces Met73 with Gly.

NMR measurements

^{1}H NMR spectra were measured on a JEOL GX-400 spectrometer with standard 5 mm probes. The lyophilized proteins were redissolved to a final protein concentration of 5 mg ml^{-1}. Chemical shifts (δ) were measured relative to an internal reference, sodium 3-trimethylsilylpropionate-2,2,3,3-d$_4$. The midpoint transition temperature for denaturation (T_m) is defined as the temperature at which the peaks of the native and denatured proteins have equal intensities. The van't Hoff enthalpy change for denaturation (ΔH_{vH}) was calculated from the conventional linear van't Hoff plot, using a two state model for the scheme $N_2 \leftrightarrow 2D$ [7].

Results

The ^{1}H NMR spectra of the mutant proteins at 30°C were similar to that of wild-type SSI, indicating that the three-dimensional structures of mutants, other than at the mutation site itself, are the same as that of the wild-type, which is also supported by X-ray crystallography analysis [11,12]. The thermal stabilities were analyzed based on the methyl proton signals of Met residues obtained as a function of the temperature at pH 7.0. The three methyl proton signals of Met70, Met73, and Met103 of the native wild-type SSI are separately observed and were previously assigned as follows: Met103 in the native state at δ = 1.78, Met70 in the native state at δ = 2.08, Met73 in the native state at δ = 2.13, Met73 and Met103 in the denatured state at δ = 2.04, and Met70 in the denatured state at δ = 2.06 [15]. In the denatured state, the signals of Met73 and Met103 overlap, but the signal of Met70 is separately observed and can be used to monitor the denatured fraction [7,15]. The chemical shifts of Met70 and Met103 of the mutants, M73V, M73I, M73K, M73E, and M73D, were similar to those of the wild-type in both the native and denatured states (Fig. 2). The transition could be analyzed based on either the Met70 or Met103 signal, and the T_m and ΔH_{vH} values were determined from the van't Hoff analysis based on the respective signals. For M73G, M73A, and M73L mutants, because the signals of Met70 and Met103 in the denatured state overlap, the T_m and ΔH_{vH} values were determined on the basis of the sum of these signals (Fig. 3). Table 1 summarizes the thermodynamic parameters of the wild-type and eight mutant proteins studied at pH 7.0. The T_m values of M73G, M73V, M73L, and M73I are lower than that of the wild-type, while those of M73A, M73K, M73D, and M73E are higher than that of the wild-type. The mutant with higher T_m tends to have higher ΔH_{vH}.

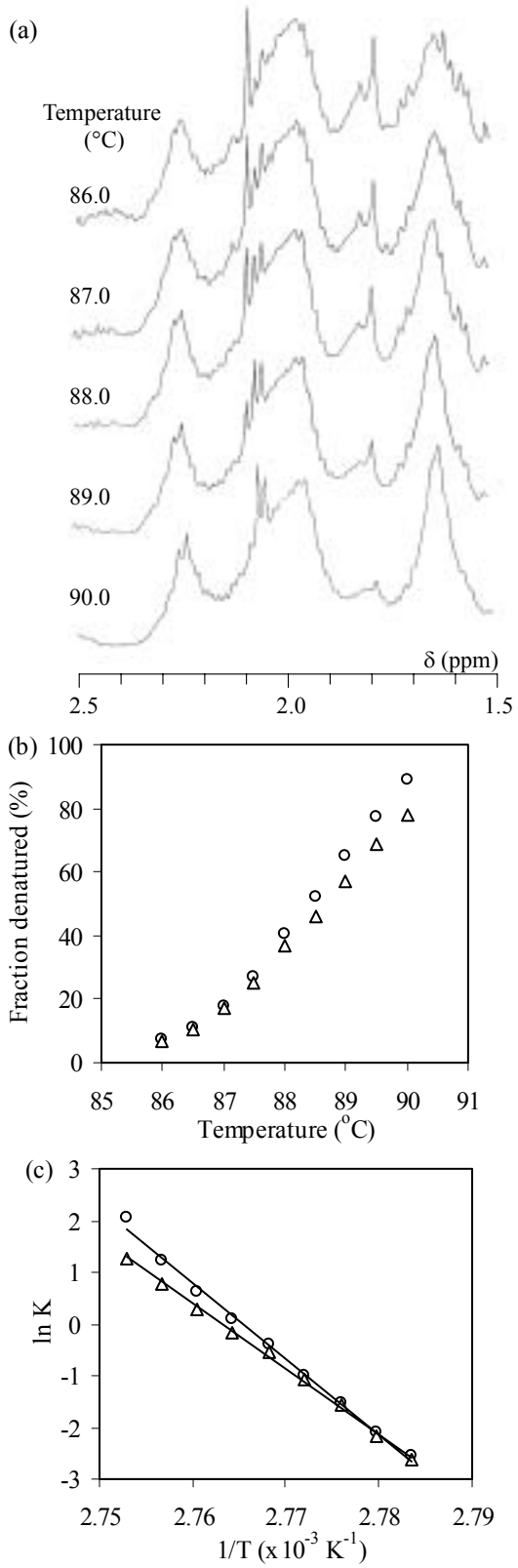

Figure 2. Thermodynamics of M73E mutant for denaturation at pH 7.0. (a) Temperature dependence of the ^{1}H NMR spectra in the methyl proton region. (b) Denatured fraction analyzed based on Met70 (○) and Met103 (Δ) signals. (c) van't Hoff plots on the basis of the data in (b).

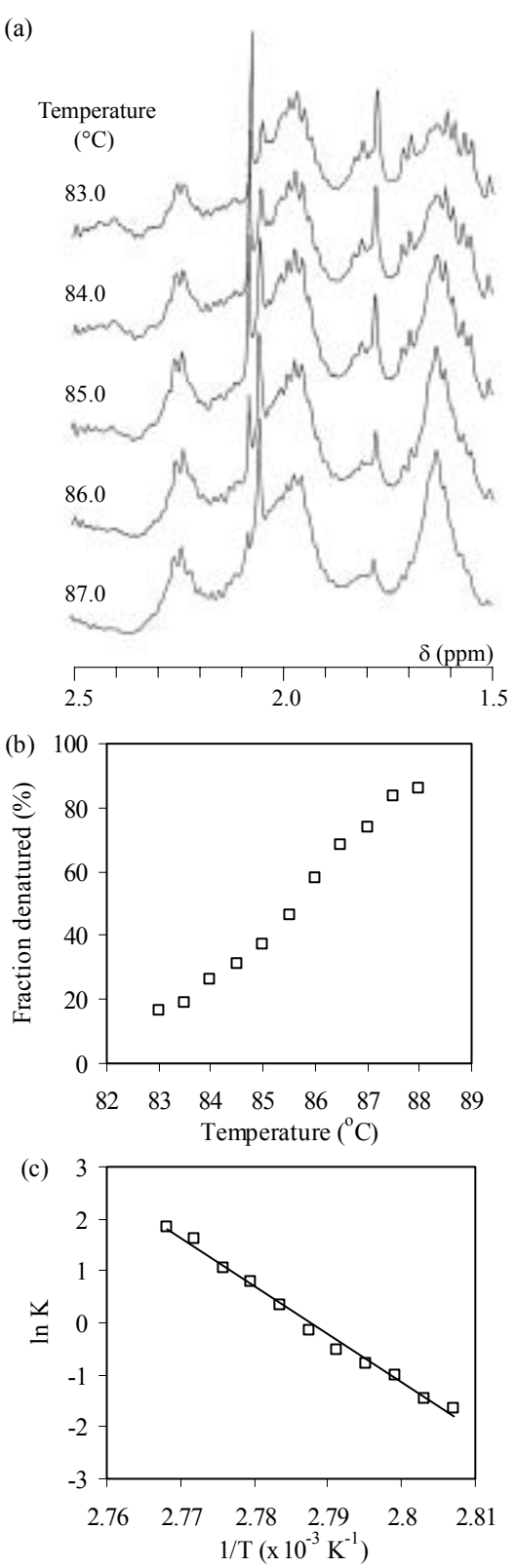

Figure 3. Thermodynamics of M73G mutant for denaturation at pH 7.0. (a) Temperature dependence of the ^{1}H NMR spectra in the methyl proton region. (b) Denatured fraction analyzed based on the sum of Met70 and Met103 signals. (c) van't Hoff plots on the basis of the data in (b).

Table 1. Thermodynamic parameters of wild-type SSI and mutant proteins

Protein	T_m (°C)	ΔT_m [b] (°C)	ΔH_{vH} (kcal mol^{-1})	$T_{1/2}$ [c] (°C)
wild-type [a]	86.2 ± 0.2	0	177.1 ± 5.5	82.2
M73G	85.6	-0.6	185.3	82.0
M73A	87.2	1.0	203.6	83.4
M73V [a]	85.1 ± 0.1	-1.1	231.1 ± 19.5	81.0
M73L	85.6	-0.6	164.4	81.8
M73I [a]	84.8 ± 0.1	-1.4	206.2 ± 12.8	80.4
M73K [a]	87.1 ± 0.1	0.9	221.7 ± 21.6	82.9
M73D [a]	89.0 ± 0.2	2.8	283.5 ± 47.0	85.3
M73E [a]	88.6 ± 0.1	2.4	271.5 ± 28.5	84.2

[a] T_m and ΔH_{vH} are the average values for the methyl proton signals of Met residues, as mentioned in the Results.
[b] ΔT_m is the change in T_m relative to that of the wild-type.
[c] Calorimetric data in ^1H_2O taken from Tamura and Sturtevant [16].

Discussion

The thermal stability of each mutant protein could be correlated with the hydrophobicity of the substituted amino acid at the position of 73 (Fig. 4). This would be a "reverse hydrophobic effect" observed in the mutational study in λ Cro [17]. The "reverse hydrophobic effect" means that the protein stability can be increased by the amino acid substitution with decreasing side-chain hydrophobicity at a protein surface. The hydrophobic group of the hyper-exposed residue can alter the equilibrium ratio of native to denatured protein. The replacement of Met73 by Asp, which has the lowest optimal matching hydrophobicity (OMH) [18], generates the mutant, M73D, with the highest thermal stability among the mutants analyzed in this study. The stability of M73G seems to be lower than that predicted from the Gly hydrophobicity. The coefficient of determination for the linear fitting to the data in Fig. 4 actually changes from 0.70 for all data to 0.90 for the data except that for M73G. The lower stability of M73G should be due to the increased entropy in the denatured state. Gly lacks β-carbon and has more backbone conformational flexibility. In order to increase protein stability in general, it will be effective to replace Gly with other amino acids [19]. Furthermore, this can also be applied to the enhanced binding affinity for protein interaction [20].

The thermal stabilities of Met73 mutants were also analyzed by means of DSC [16]. Although the $T_{1/2}$ values determined by DSC are lower than the T_m values determined by NMR, the relative thermal stability of each mutant is comparable for both measurements (Table 1). Because the solvents used for DSC and NMR were ^1H_2O and ^2H_2O, respectively, the higher T_m values determined by NMR would be due to the solvent isotope effects between ^1H_2O and ^2H_2O. Several reports have shown the relatively higher stability in ^2H_2O and discussed the solvent isotope effects [21,22]. In the present case of Met73 mutants, the T_m values in ^2H_2O at pH 7.0 were 4.0 ± 0.4°C higher than those in ^1H_2O; these results are similar to those of Met103 mutants, reported previously [7]. While calorimetric analyses can provide precise thermodynamic parameters, NMR analyses can monitor the transitions of the respective residues.

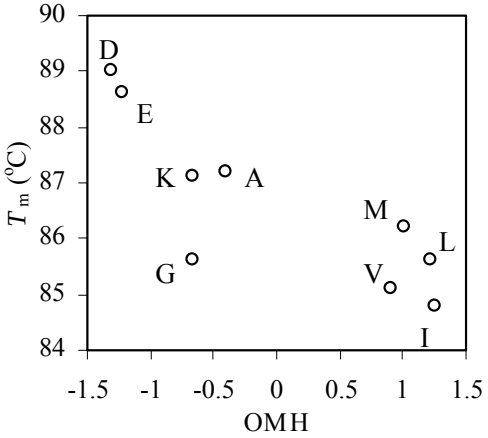

Figure 4. The correlation of thermodynamic stability, T_m, with hydrophobicity of substituted amino acid at 73. The values at x-axis are the optimal matching hydrophobicity (OMH), as reported by Sweet and Eisenberg [18]. The name of introduced amino acid into position 73 is shown in the figure as one-letter symbol.

The respective NMR signals, Met70 and Met103, located at the surface and in the hydrophobic core, respectively, transit from the native to the denatured states concomitantly (Fig. 2 and Table 1), indicating that the unfolding transition of SSI is cooperative not only within the core part consisting of the five-stranded β-sheet and two short helices but also within the reactive segment carrying the P1 and P4 sties.

Protein stability is affected not only by the protein structure itself but also by hydration. While the residue at 103 of SSI contributes to the native structure [7], the residue at 73 can determine the stability without changing the native structure. Although the residue 73 is critical for the inhibitory function, the M73K mutant, for example, showed almost the same inhibitory activity as the wild-type [10,13]. The design strategy to replace surface residues with more hydrophilic ones will be effective to increase the protein stability with causing very little perturbation to the protein structure and function.

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