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Mutational effects on cold denaturation and hydration of a protein, *Streptomyces* subtilisin inhibitor[1]

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Abstract

Effects of mutations of a protein, *Streptomyces* subtilisin inhibitor, on cold denaturation have been examined by means of differential scanning calorimetry. Cold denaturation was observed in a mutation-independent manner, while heat denaturation was clearly dependent on the mutations, suggesting that hydration of polar peptide bonds are responsible for the transition of a protein structure at low temperature. The hydration effect was also examined by extrapolating thermodynamic data previously obtained for amino acid replacements at Met73, which is completely exposed to solvent. Enthalpic changes induced by the mutations were shown to be nearly compensated by the entropic changes at 25°C, whereas the deviation became larger at 100°C, suggesting that the hydration is a primary cause for the compensation. © 1998 Elsevier Science B.V.

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1. Introduction

It has been known that the native structure of a protein can be denatured not only by increasing the temperature but also by decreasing it [1–3]. The latter phenomenon, called cold denaturation, is due to the increased heat capacity change upon denaturation of a native protein structure. Recently, detailed thermodynamic studies on cold denaturation of some proteins such as myoglobin [4], staphylococcal nuclease [5], and β-lactoglobulin [6] have been undertaken. Cold denaturation of a dimeric protein *Streptomyces* subtilisin inhibitor (SSI) [7] was found to be observed at relatively high temperature, i.e., at around 10°C, [8,9], making this protein an ideal choice for investigating the effects of mutations on cold denaturation.

Hydrophobic effect, especially in view of hydration, has been a focus of understanding the protein stability [10,11]. The increase in heat capacity upon denaturation appears to be caused by the increased water–protein interaction or hydration of polar as well as non-polar residues [12]. Therefore, cold denaturation can be regarded as a representation of hydration effect.

Reverse hydrophobic effect, or higher hydrophobicity of a replacing amino acid side-chain showing a correlation with lower stability, was first reported on λ cro [13] and detailed calorimetric study was undertaken on a series of mutants at a position Met73 of SSI [14]. This position is unique in that the solvent accessibility is unusually high, i.e., 106% compared to that of a tripeptide Ala-Met-Ala [15]. This hyper-exposure

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of the side-chain of Met73 is maintained by the rest of the native structure without interacting with other portion of the protein and thus only water-side-chain interactions can exist. Therefore, the mutations at Met73 is an excellent selection in studying the water–protein interactions.

In the present paper, effects of mutations on the hydration were investigated by observing the cold denaturation and analyzing the thermodynamic data of Met73 mutants. In addition, thermodynamic data for other mutants at position Val13 located at the hydrophobic interface of dimer formation and Met103 in the hydrophobic core were also analyzed.

2. Experimental

2.1. Protein samples

The wild-type SSI was obtained by cultivating *Streptomyces albogriseolus* S-3253 as originally proposed [16]. Mutant proteins were harvested from *Streptomyces lividans* 66 transformed by plasmids mutated by site-directed mutagenesis [17]. The secreted proteins were precipitated with ammonium sulfate and then purified by passing through an anion-exchange (DE52, Whatman) and a gel filtration (Sephacryl S-200, Pharmacia) column. Protein concentrations were determined spectrophotometrically at pH 7.0 and 280 nm with an absorbance of 0.796 for a 1.00 mg ml^{-1} solution [7] as an average value for three measurements. No light-scattering was observed that reduced the accuracy in concentration measurements. All the sample solutions were centrifuged for 10 min at 15,000 rpm and precipitants, if any, were removed before the calorimetric measurements.

2.2. Differential scanning calorimetry (DSC)

Calorimetric measurements were made with a Nano-DSC (CSC, Utah, USA). A scan rate of 1°C/min was used in either the upscan (0 to 80°C) or the downscan (45 to 0°C) mode. The protein samples were dialyzed overnight against two changes of a 25 mM glycine buffer adjusted with HCl to the desired pH. Instrumental baselines were determined prior to scanning each sample with both cells filled with dialysate. The final dialysate was used as indication of pH as well as the reference solution in the calorimeter. The denaturations were found to be fully reversible provided that the first heating or cooling was not carried beyond 95% completion of the denaturation.

3. Results

3.1. Cold denaturation of mutants

DSC scans for mutants Val13Leu (V13L), Met73Asp (M73D), and Met103Leu (M103L) from 3 to 79°C at pH 2.51 are shown in Fig. 1(a). For mutants

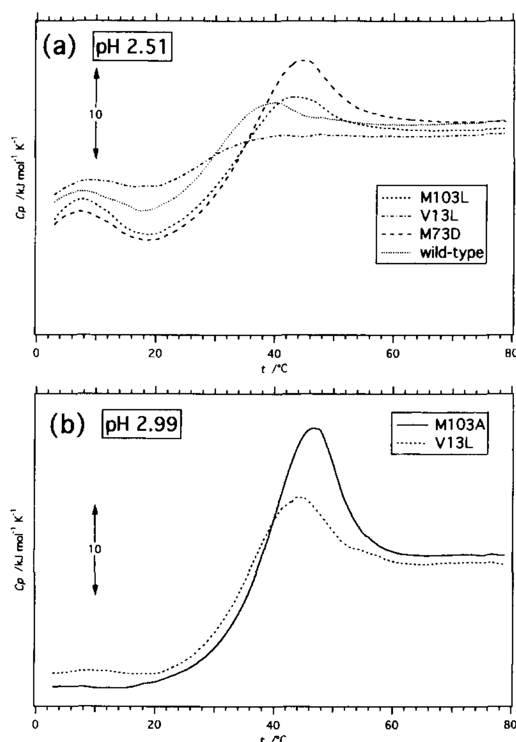

Fig. 1. (a) DSC scans from 3 to 79°C for the solutions with the wild-type SSI (3.15 mg ml^{-1}), and mutant forms Val13Leu (V13L, 3.69 mg ml^{-1}), Met73Asp (M73D, 4.12 mg ml^{-1}) and Met103Leu (M103L, 3.31 mg ml^{-1}) at pH 2.51. Peak temperatures graphically evaluated for heat denaturation are 39.9°C (wild-type), too unstable to get a peak temperature (V13L), 44.8°C (M73D), and 43.5°C (M103L). Peak temperatures for cold denaturation are 8.0°C (wild-type), 9.2°C (V13L), 7.5°C (M73D), and 7.6°C (M103L). (b) DSC scans for the solutions with Met103Ala (M103A, 4.50 mg ml^{-1}) and Val13Leu (V13L, 4.09 mg ml^{-1}) at pH 2.99. Peak temperatures for heat denaturation are 46.7°C (M103A) and 44.1°C (V13L).

Table 1
Differences in thermodynamic parameters between the wild-type SSI and mutant forms calculated at 25°C. (units in kJ mol^{-1} for $\Delta\Delta H$ and $\Delta\Delta G^0$, and J mol^{-1} K^{-1} for $\Delta\Delta S^0$)

	V13A	V13G	V13F	V13I	V13L	V13M		
$\Delta\Delta H$	37.6	−98.6	−73.6	−14.2	−25.1	−20.1		
$\Delta\Delta S^0$	191.4	−151.3	−135.0	−24.2	−35.1	19.2		
$\Delta\Delta G^0$	−19.2	−53.5	−33.4	−6.7	−14.6	−25.5		
	M73K	M73D	M73E	M73G	M73A	M73V	M73L	M73I
$\Delta\Delta H$	−4.2	−27.6	−0.4	1.7	−16.3	11.3	3.8	18.0
$\Delta\Delta S^0$	−18.8	−93.2	−7.9	5.9	−48.9	40.1	12.1	63.1
$\Delta\Delta G^0$	1.3	0.4	1.7	0.0	−1.7	−0.4	0.3	−0.8
	M103G	M103A	M103V	M103I	M103L			
$\Delta\Delta H$	22.6	30.1	14.2	−13.0	5.4			
$\Delta\Delta S^0$	148.4	112.0	68.6	−6.3	23.8			
$\Delta\Delta G^0$	−21.7	−3.3	−6.3	−11.3	−1.3			

Val13Leu (V13L) and Met103Ala (M103A), DSC scans for solutions at pH 2.99 are shown in Fig. 1(b).

3.2. Extrapolation of thermodynamic parameters to 25 and 100°C

Thermodynamic data obtained previously in the range 43 to 83°C for the mutants [14,18,19] were extrapolated to lower (25°C) or higher (100°C) temperature according to the following relations:

$$\Delta H(T) = \Delta H(T_{1/2}) + \Delta C_p(T - T_{1/2}) \quad (1)$$

$$\Delta S^0(T) = \Delta S^0(T_{1/2}) + \Delta C_p \ln\left(\frac{T}{T_{1/2}}\right) \quad (2)$$

$$\Delta G^0(T) = \Delta H(T_{1/2}) - \frac{T}{T_{1/2}}(\Delta H(T_{1/2}) - \Delta G^0(T_{1/2})) + \Delta C_p\left(T - T_{1/2} - T\ln\left(\frac{T}{T_{1/2}}\right)\right) \quad (3)$$

where ΔC_p is temperature independent and adopted values previously reported [14,18,19]. The thermodynamic parameters, ΔH, ΔS^0, and ΔG^0 are summarized as differences from the wild-type in Table 1 (25°C) and Table 2 (100°C) with

$$\Delta\Delta Y = \Delta Y(\text{mutant}) - \Delta Y(\text{wild-type}) \quad (4)$$

Table 2
Differences in thermodynamic parameters between the wild type SSI and mutant forms calculated at 100°C. (units in kJ mol^{-1} for $\Delta\Delta H$ and $\Delta\Delta G^0$, and J mol^{-1} K^{-1} for $\Delta\Delta S^0$)

	V13A	V13G	V13F	V13I	V13L	V13M		
$\Delta\Delta H$	10.5	−125.8	−101.2	−41.4	−74.0	−47.2		
$\Delta\Delta S^0$	109.5	−233.2	−216.9	−106.2	−181.8	−62.7		
$\Delta\Delta G^0$	−30.4	−39.1	−20.1	−1.8	−6.3	−23.8		
	M73K	M73D	M73E	M73G	M73A	M73V	M73L	M73I
$\Delta\Delta H$	12.1	−41.4	−30.9	−1.3	−35.1	10.9	13.0	9.6
$\Delta\Delta S^0$	28.8	−135.4	−99.9	−3.3	−104.9	38.5	38.5	37.7
$\Delta\Delta G^0$	1.0	9.0	6.0	−0.2	4.1	−3.4	−1.7	−4.6
	M103G	M103A	M103V	M103I	M103L			
$\Delta\Delta H$	−4.9	31.8	−20.1	−54.8	−43.5			
$\Delta\Delta S^0$	66.5	116.2	−35.1	−131.7	−122.9			
$\Delta\Delta G^0$	−29.8	−11.8	−7.2	−5.8	2.5			

where Y is H, S^0, or G^0. The thermodynamic parameters for the wild-type SSI were calculated based on the data previously obtained [20] as: At 25°C; $\Delta H = 98.2$ kJ mol^{-1}, $\Delta S^0 = 28.0$ J mol^{-1} K^{-1}, $\Delta G^0 = 89.9$ kJ mol^{-1}. At 100°C; $\Delta H = 1012.4$ kJ mol^{-1}, $\Delta S^0 = 2763.4$ J mol^{-1} K^{-1}, $\Delta G^0 = -18.7$ kJ mol^{-1}.

4. Discussion

DSC scans for the solutions with the wild-type SSI and mutants V13L, M73D, and M103L at pH 2.51 (Fig. 1(a)) show that the peak temperatures for cold denaturation are relatively insensitive to the mutations whereas those for heat denaturation vary significantly depending on the mutations. At pH 2.99, at which no peak corresponding to cold denaturation appeared in the case of the wild-type [8], no peaks for cold denaturation were observed in the cases of M103A and V13L (Fig. 1(b)) even though these proteins were sufficiently destabilized by the mutations so that cold denaturation is expected to appear. In fact, the peak temperature for the heat denaturation of V13L is almost the same as that of M73D in Fig. 1(a), where cold denaturation is clearly observed. This phenomenon was also confirmed by downscanning from 45 to 0°C (data not shown), except for shifts of transitions toward lower temperature due presumably to slow folding–unfolding processes. These observations lead to a conclusion that the cold denaturation is pH sensitive and mutation insensitive, whereas the heat denaturation is dependent on both the pH and mutations.

Although, it was earlier assumed [4,11] that cold denaturation was caused by hydration of non-polar groups, it now appears that the role of polar groups is more significant than that of non-polar groups because hydration of polar groups has a Gibbs energy of negative sign while the Gibbs energy of hydration of non-polar aliphatic groups is positive, and the increase in the hydration of these groups with decreasing temperature leads only to a decrease of its value. The above conclusion can be explained if the cold denaturation is induced mainly by hydration of polar groups or main chain peptide bonds as recently proposed [12], since the mutations intrinsically keep the main chain intact.

Based on model compounds and detailed analyses of thermodynamic data of proteins, it is concluded that the hydration is effective at low temperature according to the following reasoning [11]. In a simple scheme, native protein $\rightleftharpoons$ denatured protein, Gibbs free energy can be written down when the logarithm term is expanded with an assumption of constant heat capacity change as:

$$\Delta G^0(T) \cong \Delta H(T_X) - T\Delta S(T_X) - \Delta C_p \frac{1}{2}\left(\frac{T_X - T}{T}\right)^2 \qquad (5)$$

where T_X (110°C or higher) is the temperature at which the enthalpy and entropy of hydration becomes zero, although it is now concluded that T_X is not the same among non-polar, polar, and aromatic residues [12]. The last term, which is the only one term including the heat capacity change, includes all possible contribution but the hydration effect might be the major factor. This is always negative and becomes more negative as the temperature decreases. The first term, which is positive and constant at any temperature, represents the total enthalpy of hydrogen bonds and van der Waals interactions in protein. The second term, which is the action of dissipative forces, is negative and increased in absolute value as the temperature increases. Therefore, cold denaturation is caused only by the third term, or the hydration effect, which becomes significant in a low temperature region. On the other hand, the hydration effect becomes negligible at high temperature approaching T_X.

In order to see how the hydration affects the stability of the protein, thermodynamic parameters were extrapolated to 25 and 100°C for reevaluation of the data (Tables 1 and 2, respectively). Further extrapolation can induce significant errors due to the intrinsic uncertainty of the heat capacity changes and was thus avoided.

Special attention should be paid to M73X mutant forms, where X is a replacing amino acid residue in one-letter symbol, since the side-chain at the position 73 is perfectly exposed to solvent and no side-chain interactions exist in the native structure [21] and no such interactions can be expected for the denatured state, either. Therefore, the majority of the changes in thermodynamic parameters is due to the changes in the

water-side-chain (at the position 73) interactions. Reverse hydrophobic effect can be read from Table 2 at 100°C qualitatively. Quantitative evaluation, for example by setting $\Delta\Delta G^0$ of $-3\ \mathrm{kJ\,mol}^{-1}$ for a methylene group, $+2\ \mathrm{kJ\,mol}^{-1}$ for a sulfate atom (in methionine), $+5\ \mathrm{kJ\,mol}^{-1}$ for COO^- and $+6\ \mathrm{kJ\,mol}^{-1}$ for NH_{3+}, results in a partial agreement (in this case, deviation is significant for M73G and M73L whereas good agreements were attained for the rest of the mutants). No other hydrophobic scale showed perfect correlation and this situation also remained for $\Delta\Delta H$ for $T\Delta\Delta S^0$.

Correlation between the changes in enthalpy and entropy was explored by plotting $\Delta\Delta H$ against $T\Delta\Delta S^0$ for M73X in Fig. 2(a). At 25°C, where the hydration term is effective. The plot leads to the following least-squares fitted line

$$T\Delta\Delta S^0 = 0.00(\pm 0.40) + 1.00(\pm 0.03)\Delta\Delta H \tag{6}$$

indicating that the changes in enthalpy and entropy are nearly perfectly compensated. On the other hand, at 100°C where the hydration effect is small, the plot (filled symbols in Fig. 2(a)) shows deviation from the line draws on the basis of Eq. (6).

For other mutants, similar plots were shown in Fig. 2(b) together with the line of Eq. (6). These plots do not give a clear indication as to whether the data at 25°C are better correlated or not, showing another example that we are far from a firm understanding of the primarily non-covalent forces that hold proteins in their native structure and thus fully satisfactory quantitative explanations of the experimental results obtained with mutant proteins can very seldom be given [22]. Still, I believe that the present thermodynamic results obtained by investigating the mutational effects on the cold denaturation and on the changes in the thermodynamic parameters at a unique position of Met73 can be a step forward to understanding of the protein thermodynamics.

Fig. 2. Plots of $T\Delta\Delta S^0$ (◇,◆) and $\Delta\Delta G^0$ (○,●) against $\Delta\Delta H$ for the thermal denaturation of M73X mutant forms at 25°C (open symbols) and at 100°C (filled symbols). The lines are drawn as results of the linear-least squares fitting: the continuous line, $T\Delta\Delta S^0 = 0.00 + 1.00\Delta\Delta H$; and the broken line, $\Delta\Delta G^0 = -0.16 - 0.02\Delta\Delta H$. (b) Plots of $T\Delta\Delta S^0$ against $\Delta\Delta H$ for the thermal denaturation of V13X (○,●) and M103X (◇,◆) mutant forms at 25°C (open symbols) and at 100°C (filled symbols). The line is drawn based on Eq. (6) in the text, which is the same as in (a) but with an extended range to accommodate all the points.

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