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Thermal stabilization of ribonuclease T_1 by carboxymethylation at Glu-58 as revealed by ^1H nuclear magnetic resonance spectroscopy

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Abstract Ribonuclease T_1 (RNase T_1) carboxymethylated at the γ-carboxyl group of Glu-58 with iodoacetic acid is known to be completely inactive while it retains an almost full substrate-binding ability. In order to further clarify the effects of the carboxymethylation, the thermal stabilities of intact and Glu-58-carboxymethylated (CM-) RNase T_1 were compared by measuring ^1H NMR spectra at various temperatures. The transition curves of unfolding were obtained by plotting, as a function of temperature, the peak areas for the α and δ protons of Asn-81 and Ile-90, respectively, which are well apart from each other in the three-dimensional structure of the enzyme. For each of intact and CM-RNase T_1, the transition curve of the Asn-81 α proton was identical with that of the Ile-90 δ methyl protons, suggesting that the thermal unfolding occurred simultaneously in every part of the molecule of CM-RNase T_1 as well as of intact RNase T_1. The midpoint of unfolding was 52°C for intact RNase T_1, and was increased by 9°C upon carboxymethylation at Glu-58. This marked stabilization by carboxymethylation is thought to be due to formation of a salt bridge between the introduced carboxymethyl group and the neighboring guanidium group of Arg-77.

Key words: RNase T_1; Carboxymethylated RNase T_1; NMR; Thermal stability; Unfolding; Transition enthalpy

1. Introduction

RNase T_1 (EC 3.1.27.3) is a guanyloribonuclease secreted by *Aspergillus oryzae* [1–3]. It is a single-chain globular protein of 104 amino acid residues and is unfolded reversibly by heating or addition of denaturants. Therefore, a number of studies on the stability and unfolding/refolding have been made by using differential scanning calorimetry and spectroscopic methods including circular dichroism and fluorescence measurements [4–11]. The thermodynamic analysis indicated that the unfolding equilibrium of RNase T_1 is described by a two-state model [4,5,8]. The parameters obtained by the above methods, however, represent only the averaged characters of the whole molecule. On the other hand, NMR enables us to investigate precisely the local conformational changes and microenvironments of proteins at the atomic level [12]. The NMR spectra of RNase T_1 have been analyzed in detail and most of the ^1H NMR signals have been assigned [13].

When RNase T_1 is treated with iodoacetic acid under mild conditions, Glu-58 is specifically carboxymethylated, optimally at pH 5.5 [14]. Upon carboxymethylation the enzyme loses the catalytic activity thoroughly although it still retains almost completely the nucleotide-binding ability [15]. The carboxymethylation of Glu-58 only extends the length of the side chain about 4 Å ($-COO^- \rightarrow -COOCH_2COO^-$) and no other changes occur in the primary structure including the charge distribution. In order to clarify further the effects of the carboxymethylation, we investigated, in the present study, the thermal unfolding of intact and CM-RNase T_1 by 600 MHz ^1H NMR using the signals of the Asn-81 α proton and the Ile-90 δ methyl protons, which are isolated well from the other signals in the NMR spectra and are located in a significant distance apart from each other in the molecule of RNase T_1 (Fig. 1A). The results confirmed the two-state unfolding model, and showed that the unfolding temperature of RNase T_1 was increased markedly (by 9°C) upon carboxymethylation at Glu-58. This suggested that the negatively charged carboxymethyl group introduced forms a strong ionic pair with a neighboring positively charged group, Arg-77, to stabilize CM-RNase T_1. To our knowledge, this is the first case that such a marked stabilization of an enzyme molecule was found to occur by introduction of a single salt bridge.

2. Materials and methods

RNase T_1 was kindly supplied by Dr. H. Tamaoki (Sankyo Co.). CM-RNase T_1 was prepared as described [14]. NMR samples were prepared by dissolving lyophilized intact RNase T_1 or CM-RNase T_1 at 1 to 2 mM in 99.95% D_2O in the absence of salt. The pH values of all sample solutions were adjusted to 4.4 with a small amount of DCl. The pH values (direct pH meter readings) were determined in a Radiometer PHM84 pH meter equipped with a 180 mm Ingold combination electrode.

600 MHz ^1H NMR spectra were recorded on a Bruker AMX600 spectrometer at various temperatures. 32K data points were acquired with a spectral width of 10 kHz. 64 transients were accumulated for each measurement with a relaxation delay of 1.0 s. Line broadening of 3 Hz was applied to free induction decays prior to Fourier transformation in the absolute intensity mode. The proton chemical shifts were described downfield from the external sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Data processing and representation were also performed by using the software Felix (Biosym Technologies) on IRIS Indigo Elan and Indigo2 workstations (Silicon Graphics).

The transition temperature was determined by plotting the peak areas of the signals of the Asn-81 α proton and the Ile-90 δ methyl protons as a function of temperature. These signals were isolated from the envelopes of signals and therefore permitted correct determination of their peak areas. The assignments of these signals were confirmed by the analysis of two-dimensional NMR spectra (H. Miyano et al.,

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Abbreviations: CM-Glu, γ-carboxymethylated Glu; CM-RNase T_1, ribonuclease T_1 carboxymethylated at Glu-58.

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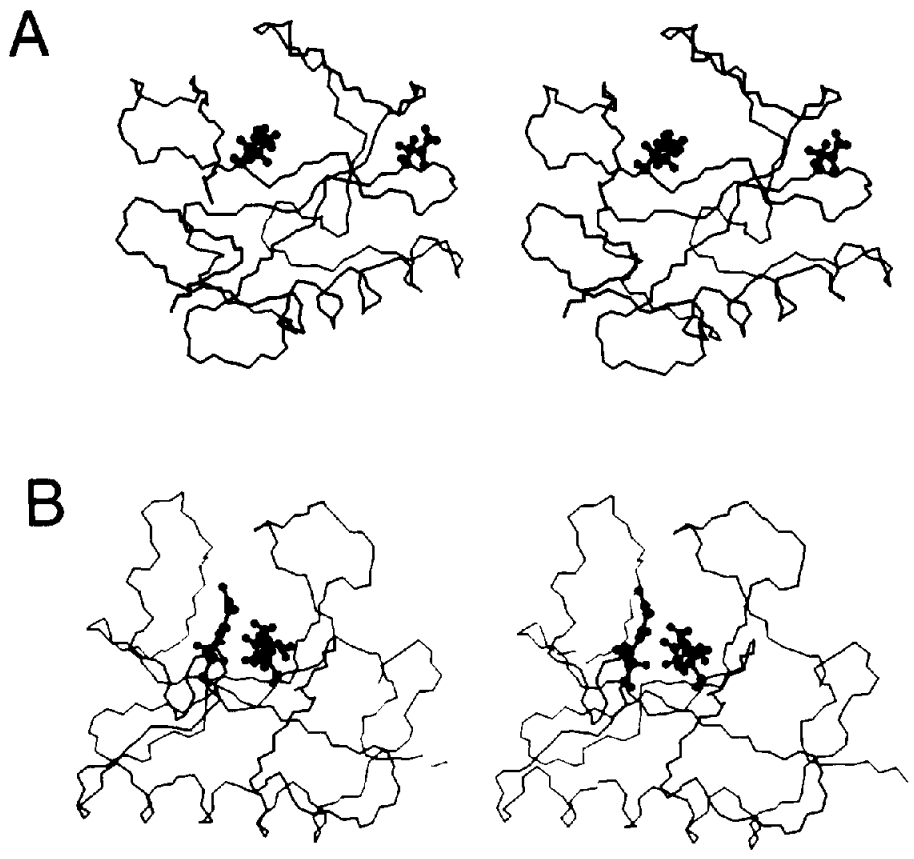

Fig. 1. (A) Stereo view of RNase T_1 illustrated in a wire model. The side chain atoms of Asn-81 and Ile-90 are expressed in a ball-and-stick model. Asn-81 is located on the right and Ile-90 on the left. (B) Stereo view of CM-RNase T_1. The side chains of CM-Glu-58 and Arg-77 are drawn in a ball-and-stick model, and the main chain of the molecule in a wire model. CM-Glu-58 is located on the left and Arg-77 on the right.

unpublished). The transition enthalpy of unfolding (ΔH) was estimated by plotting the logarithm of the equilibrium constant (K) of unfolding at the absolute temperature T against $1/T$, on the basis of the van 't Hoff relationship

$$\ln K = -\Delta H/RT + \Delta S/R$$

where R is the gas constant and ΔS is the transition entropy. K values were estimated from Fig. 4. The standard deviations in midpoint temperature and transition enthalpy were estimated directly from the above equation using the linear least-squares method.

To illustrate the three-dimensional structures of intact and CM-RNase T_1, the coordinates of the complex of Lys-25 RNase T_1 and 2'-GMP [16] were adopted from the data stored in the 1RNT file of the Protein Data Bank (Brookhaven National Laboratory), in which the coordinates of Glu-102 were given as those of Ala. Lys-25 and Ala-102 were replaced with Gln and Glu, respectively, and besides, Glu-58 was replaced with CM-Glu for CM-RNase T_1. Using these structures as the initial ones, the energy-minimized structures of intact and CM-RNase T_1 were calculated by molecular dynamics and energy minimization calculations in the same manner as in a previous paper [17]. All the calculations were completely independent of experimental data. These treatments and the visualization of the structure were performed by using the program BIOGRAF (Molecular Simulations). The computers used for calculation and graphics were Indigo Elan and Indigo^2 workstations (Silicon Graphics).

3. Results

Fig. 2 shows the ^{1}H NMR spectra in the native and the unfolded states of intact RNase T_1 and in the native state of CM-RNase T_1. The spectrum in the unfolded state of CM-RNase T_1 was very similar to that of intact RNase T_1 (data not shown). Among the peaks in the native states, the signals of the Ile-90 δ methyl protons (indicated by an arrow at −0.8 ppm) and the Asn-81 α proton (indicated by an arrow at 6.1 ppm) were isolated from others, and were shifted extremely from the chemical shift values of the random-coil state (0.89 ppm for the Ile δ methyl protons and 4.75 ppm for the Asn α proton) [12]. Therefore, these peaks were characteristic of the native state. Fig. 3 shows parts of the spectra around these signals for intact and CM-RNase T_1 at various temperatures. For intact RNase T_1, the peak intensity of the Asn-81 α proton was decreased in a similar way to that of the Ile-90 δ methyl protons as the temperature was increased. Other signals also showed similar behaviors to these peaks in Fig. 3A, which indicates that the thermal unfolding of intact RNase T_1 is highly cooperative. It is also the case for CM-RNase T_1.

In order to analyze the unfolding profile more quantitatively, the peak areas of these two signals were plotted against the observed temperatures (Fig. 4). The peak areas are normalized by the average value of those at lower temperatures for each resonance signal to express per cent proportion of the native state. As shown in this figure, the temperature-dependent transition curve of the Ile-90 δ methyl protons was indistinguishable from that of the Asn-81 α proton for both intact and CM-RNase T_1. The midpoint of the transition curve was 52.3 ± 0.3°C for intact RNase T_1. This value almost agreed

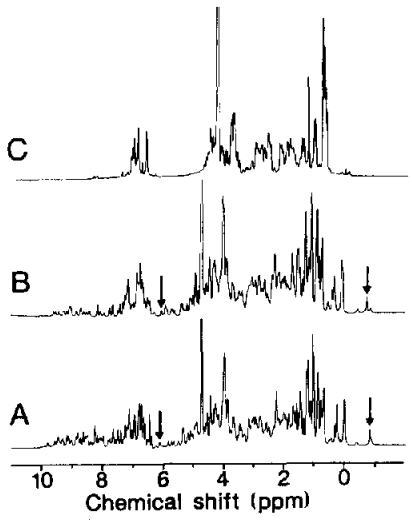

Fig. 2. ^{1}H NMR spectra of (A) intact RNase T_1 in the native state at 30°C, (B) CM-RNase T_1 in the native state at 30°C, and (C) intact RNase T_1 in the unfolded state at 60°C. All the spectra were measured in D_2O in the absence of salt at pH 4.4. Arrows at −0.8 ppm and at 6.1 ppm in the spectra A and B indicate the signals of the Ile-90 δ methyl protons and the Asn-81 α proton, respectively.

with that obtained in the previous studies (about 55°C) [4,10,11]. On the other hand, the midpoint of the transition curve was 60.8 ± 0.3°C for CM-RNase T_1, 9°C higher than that for intact RNase T_1. The enthalpy changes obtained by plotting as shown in the inset of Fig. 4 were about 530 ± 40 kJ/mol for intact RNase T_1 and about 780 ± 50 kJ/mol for CM-RNase T_1. The value estimated for intact RNase T_1 was consistent with that (490–520 kJ/mol at pH 5) obtained by other methods [4,8,10,11]. Thus, the carboxymethylation also makes the thermal unfolding more cooperative.

4. Discussion

In the present study, the changes in the signals of the Asn-81 α proton and the Ile-90 δ methyl protons were followed to study the temperature-dependent transition of CM-RNase T_1 as well as RNase T_1 in detail. The Asn-81 α proton is a main chain proton, whereas the Ile-90 δ methyl protons are located at the end of a side chain. In addition, Asn-81 and Ile-90 belong to different β-strands in the three-dimensional structure [16], and the Asn-81 α proton is located distant from the Ile-90 δ methyl protons in the whole molecule (Fig. 1A). These two signals are thus good probes to examine the behaviors of distinct portions of the molecule. The thermal transition curves of these signals, which showed the same behavior (Fig. 4), suggest that practically all parts of the main and side chains were unfolded simultaneously for both intact and CM-RNase T_1. The midpoint temperature of intact RNase T_1 was a little lower than those obtained by calorimetry, fluorescence and CD measurements [4,8,10,11]. NMR spectroscopy detects local conformational changes at the atomic level, while other methods provide global information of the molecule. NMR may, therefore, detect the conformational changes at lower temperature more sensitively than the other methods.

The marked elevation of the transition temperature indicates that carboxymethylation at Glu-58 stabilizes the RNase T_1 molecule. A similar increase in transition temperature upon carboxymethylation had been observed previously as examined by changes in absorbance at 278 nm of the enzyme solution [1]. It has been suggested that a salt bridge was formed between the side chains of CM-Glu-58 and Arg-77 based on the pH titration curve of ^{13}C chemical shift of the carboxymethyl group in CM-RNase T_1 [18]. The modeling of the molecular structure of CM-RNase T_1 supports this supposition (Fig. 1B). In CM-RNase T_1, the carboxylate anion of CM-Glu-58 is located at such a position that a salt bridge can be formed with the Arg-77 guanidinium cation which is about 2 Å apart from the carboxylate anion. As shown in Fig. 1B, the RNase T_1 molecule appears to be separated roughly into two domains. Glu-58 is located in one domain and Arg-77 in the other, and the polypeptide chain intersects four times between the two domains. In CM-RNase T_1, the formation of an additional salt bridge between CM-Glu-58 and Arg-77, hence between the two domains, is thought to stabilize the whole molecule. This explanation is in line with the fact that the conformation of RNase T_1 is extremely stabilized by salts [4–6,8,10].

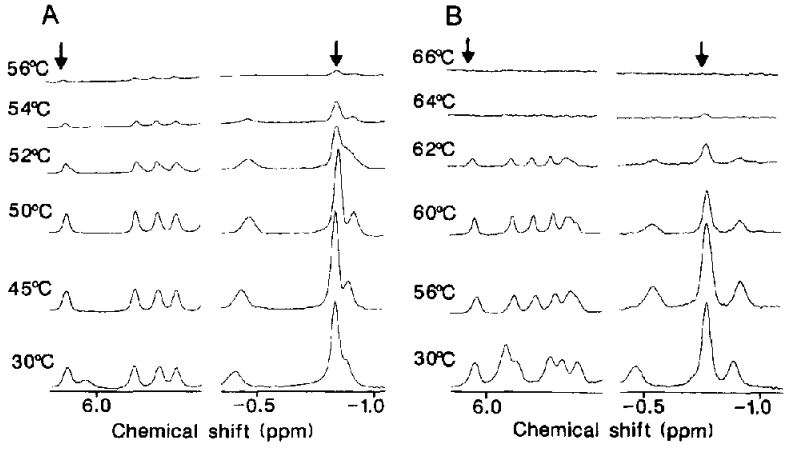

Fig. 3. Parts of the NMR spectra around the signals of the Asn-81 α proton and the Ile-90 δ methyl protons in (A) intact RNase T_1 and (B) CM-RNase T_1 at the designated temperatures. The positions of the signals of the Asn-81 α proton (6.1 ppm) and the Ile-90 δ methyl protons (−0.8 ppm) are indicated by arrows.

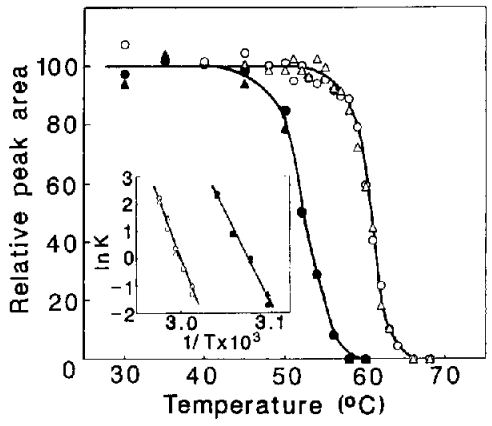

Fig. 4. Temperature-dependent transition curves of intact and CM-RNase T_1. ●, Ile-90 δ methyl protons in intact RNase T_1; ○, Ile-90 δ methyl protons in CM-RNase T_1; ▲, Asn-81 α proton in intact RNase T_1; and △, Asn-81 α proton in CM-RNase T_1. For each proton signal, the peak areas are normalized by the average value of those at lower temperatures. Inset: Van 't Hoff plots of unfolding curves of intact RNase T_1 (●, ▲) and CM-RNase T_1 (○, △), as observed with the Ile-90 δ methyl protons (●, ○) and the Asn-81 α proton (▲, △).

In addition, a local conformational change induced by the carboxymethylation may also contribute somewhat to the stabilization. A geometrical change in the β-sheet structure induced by the salt bridge formation appears to bring the two β-strands closer to each other. NOE and deuterium exchange experiments revealed that the center portion of the β-sheet of CM-RNase T_1 is a little stiffer than that of RNase T_1 (H. Miyano et al., unpublished). Further, the hydrophobic core formation may take place around the methylene group of the carboxymethyl group of CM-Glu-58. Indeed, ^{13}C-edited NOESY spectrum of [2-^{13}C]CM-Glu-58-RNase T_1 shows that His-40, Tyr-42, and Phe-100 are close to the methylene group of the carboxymethyl group of CM-Glu-58 (H. Miyano et al., unpublished). These results appear to be consistent with a previous observation for the Glu-58 → Ala mutant that the mutant protein is 3.4 kJ/mol less stable than wild-type RNase T_1 [6]. This destabilization may also be due in part to the loss of hydrophobic interaction as well as electrostatic interaction.

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