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SHORT COMMUNICATIONS

Observation of a Pressure-Induced Unfolding Intermediate of Thermolysin by Using Pressure-Jump Method

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Thermolysin (EC 3.4.24.27: TLN) is a 34.6-kDa thermophilic zinc-containing neutral protease, secreted by *Bacillus thermoproteolyticus*.[1] This enzyme is one of the most studied members of the M4 protease family. Its three-dimensional structure, as well as its stability against many kinds of physical and chemical perturbation have been amply characterized.[2–6] It is constituted of two structural domains of equal size (residues 1–157 and 158–316) with a functional zinc ion, four calcium ions, and it does not contain thiol or disulfide groups.[3] These domains seem to have differential stability against physical and chemical perturbations and, in addition, certain partial sequences of these domains are known to fold autonomously under moderate conditions.[7–10]

We have reported the effects of high pressure on TLN activity and spectroscopic properties over a range of temperature and pressure.[4–6] The pressure-induced spectroscopic changes of TLN were explained by a simple two-state transition model, accompanied with a large and negative reaction volume change, ΔV. The strongly diminished activity and the altered spectroscopic profile after decompression indicated that the pressure induced changes were partially irreversible. Pressure-induced denaturation of TLN is likely to occur *via* multi-state mechanism, since the domains have differential tolerance against pressure. Relaxation methods using physical or chemical perturbations are useful technique to investigate the mechanism of sequential reactions of protein folding/unfolding and their transition states. Comparing with temperature or chemical perturbations, pressure is very useful, since it is easy to modulate the degree of denaturation without change of chemical constituents and undesirable reactions. Recently, pressure jump method has been used to characterize a transition state of some enzymes of small size, and it provided useful information on the transition states.[11, 12] In this study, we analyzed the kinetics of unfolding after pressure jump at various temperatures and pressures, and characterized a possible transition state of TLN in order to reveal a pressure-induced intermediate of TLN unfolding.

EXPERIMENTAL

TLN was obtained from Daiwa Kasei (Osaka, Japan; lot number T4DB191). Other chemicals were commercially available regent grade. The enzyme was stocked as a concentrated aqueous solution at 4°C (in *ca.* 0.1 mM in a 50 mM Hepes/NaOH buffer: pH 6.5, 10 mM $CaCl_2$). The protein concentrations were determined from the absorbance at 280 nm.[4] Using A (1%, 1 cm) = 13.6 and M_r = 34500 after centrifugation at 10000 g for 1 min at 4°C. Deionized and distilled water was used throughout the experiments.

The denaturation process of TLN was detected by following the intrinsic fluorescence from Trp residues. TLN has several fluorophores; 3 Trp and 28 Tyr. When excited at 295 nm, Trp is mainly excited and the conformational changes around the Trp residues can be monitored.

Pressure jump kinetics was carried out by using a Vacomax high pressure cell with a high-pressure generation system, fundamentally similar to that described by the literature.[13] UV multifiber bundle (Oriel Corp., Statford, CT, USA) with focusing optics was used to bring the exciting light (295 nm) from the monochromater to the pressure cell. Trp emission was monitored at an angle of 90° through a Corion 360 nm high pass filter. Pressure jumps of 50–100 MPa were made by closing the value to the sample compartment, pumping to the desired new pressure into the reservoir, and

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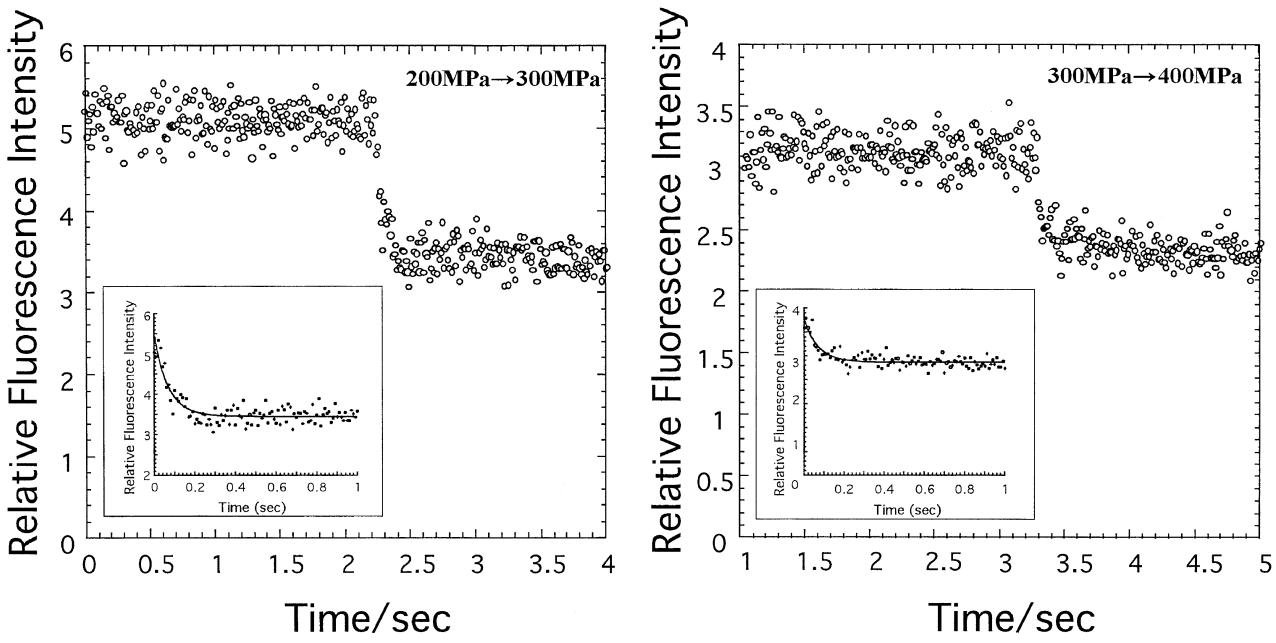

Figure 1. Typical time course profiles of the fluorescence intensity change of TLN by pressure-jump denaturation at 25°C. Top, 200 MPa→300MPa. Bottom, 300 MPa →400 MPa. Excitation and emission wavelengths were 295 nm and 340 nm, respectively. [TLN] = 10 μM in 50 mM Hepes/NaOH (pH 6.5) and 10 mM $CaCl_2$. Inset shows the results of the single exponential non-liner curve fitting.

then rapidly reopening the electro-magnetically driven valve to the sample compartment. The circulating thermostated water very quickly removed the possible increase in temperature of the sample due to the pseudo-adiabatic compression and the effects were practically negligible in the experiments. Fluorescence intensity was recorded with 10 ms integration times.

The fluorescence decay profiles were fitted to the following single exponential equation.

$$I(t) = I_0 \exp(-t/\tau) + C \quad (1)$$

$$1/\tau = k_{+1} + k_{-1} \quad (2)$$

where $I(t)$ is the fluorescence intensity at time t, I_0, the intensity at time zero, τ, the relaxation time, C, the asymptotic constant value, k_{+1}, the apparent unfolding rate constant, and k_{-1}, the apparent refolding rate constant. Relaxation times were measured over a range of pressure (100 to 400 MPa) and temperature (5 to 25°C). The volume of activation ($\Delta V^{\ddagger}$) for the denaturation process was obtained by the following equation.

$$\ln(k_p) = \ln(k_0) - P\Delta V^{\ddagger}/RT \quad (3)$$

where k_p and k_0 indicate the rate constants at experimental pressure (P) and at atmospheric pressure, respectively. The kinetic data were analyzed by fitting by using DELTA GRAPH® PRO in order to calculate both k and $\Delta V^{\ddagger}$.

RESULTS AND DISCUSSION

Figure 1 shows typical fluorescence change kinetics for the denaturation caused by pressure-jump[15] (300 MPa → 400 MPa). Upon a sudden increase of pressure, a decrease in fluorescence emission intensity was observed. This decrease was due to an exposition of Trp residues to water. The kinetics could be well fitted to a single exponential decay (eq 1). There was no indications for an additional initial burst phase or for a second slow phase.

When the pressure jump was negative (from high to low pressure) no significant fluorescence change was observed, except for a very fast one originated from a small temperature change due to adiabatic decompression (data not shown). Thus, only the pressure-induced unfolding processes (positive p-jumps) were analyzed. Hence, the contribution from the reverse reaction to the observed relaxation time of denaturation was negligible (*i.e.*, $\tau^{-1} = k_{+1}$). The time-scale of the relaxation kinetics after the p-jump was in 100 ms, which was rather fast, when compared to the reported results of some other proteins.[12, 13]

The logarithmic value of the rate constant, ln (k_{+1}), was plotted against pressure for selected temperatures (Figure 2). As pressure increased, the unfolding rate decreased, while the temperature dependence was somehow complex. The results were analyzed by using eq 3 and the activation volumes ($\Delta V^{\ddagger}$) were estimated as 12, 5, and 3 mL mol^{-1} at 5°C, 15°C, and 25°C, respectively. The $\Delta V^{\ddagger}$values were positive and became smaller with increasing temperature. The activation expansibility, $\Delta\alpha^{\ddagger}$, was evaluated to be about −0.45 mL mol^{-1} MPa^{-1}.

In the present results, the relaxation kinetics after

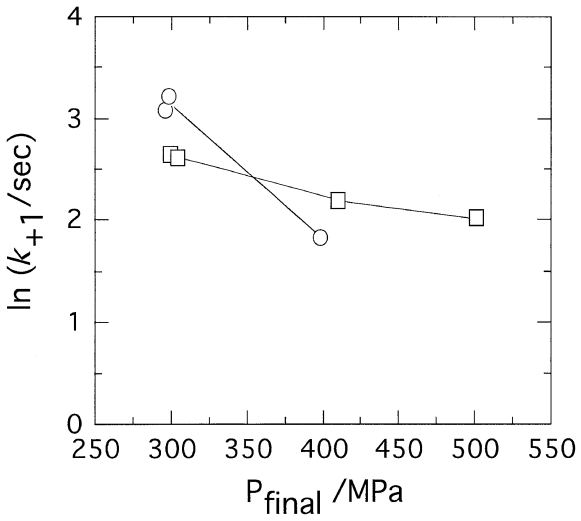

Figure 2. Pressure dependence of the apparent denaturation rate constant (k_{+1}) of TLN at 5°C (○), 15°C (△), and 25°C (□). [TLN] = 10 μM in 50 mM Hepes/NaOH (pH 6.5) and 10 mM $CaCl_2$.

p-jumps were well fitted to single exponential decays (eq 1), which is in accord to our previous pseudo-equilibrium study, where a single two-state change was observed.[5] However, at higher temperature, *e.g.*, 40°C,[6] we had found a multi-step (at least two steps) fluorescence change. Due to limitations in the time-scale, the attempts for kinetic observation at higher temperatures were not possible in the present study.

The apparent relaxation time of the pressure-induced denaturation of TLN is 10–100 times faster than that of, *e.g.*, *Staphylococcal* Nuclease (Snase), a well-documented monomeric globular protein.[11] In our previous study,[5] CD measurements revealed that pressure denatured TLN at 400 MPa was not completely denatured, but retained some secondary structure. This indicates that the perturbation by high pressure leads to a partial unfolding, *i.e.*, to a collapse of only one sub-domain; a partial sequence of the domain can form a native-like conformation at moderate condition. This incompleteness of denaturation is one of the main differences between TLN and other small globular proteins, such as Snase or chymotrypsin inhibitor 2.[11,14] Because of their structural simplicity, the unfolding of these small globular proteins occurs within a two-state cooperative unfolding process and do not form an unfolding intermediate, which is categorized in the "global collapse" unfolding model.[14] On the other hand, the unfolding process of multi-domain proteins depends on sub-domain tolerances against perturbation. They are classified into "framework" unfolding model, which is initiated by the unfolding of one local sub-domain and forms unfolding intermediate in some cases. TLN is likely to be grouped into the latter class. The present results are apparently attributed to the simple two-sate transition model and are consistent with our previous steady–state kinetics studies. However, absolute values of ΔV were significantly small, comparing with that of our previous steady–state kinetic data. It will be suggested that pressure-jump technique tracked an early step of denaturation process of TLN and that our previous steady–state kinetic observation contained several steps including this early step. The present results support an existence of pressure-induced intermediate and will be an additional proof of the framework model of TLN. It is known that certain C-terminal fragment of TLN are able to refold automatically under moderate conditions.[9,10] Moreover, it was reported that the early stage of unfolding process of two domains of proteases in the TLN super-family started in parallel at elevated temperatures, and the multiple temperature-susceptible sites in the C-terminal domain were predominantly digested by autolysis.[15] It is also reasonable to interpret the present results as that the sub-domain sensitivities of the two domains of TLN towards are also different, and their unfolding is much independent.[16] The deformation of the less stable domain was observed by the present pressure-jump technique. To elucidate the stability of each domain and the relationship with above-mentioned thermal stability, selective Trp substitution in each domain of TLN (one in N-terminal domain and two in C-terminal domain) by genetic manipulation is underway in our laboratories.

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