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Parallel folding pathway of proline-free staphylococcal nuclease studied by the stopped-flow double-jump method

Kiyoto Kamagata and Kunihiro Kuwajima *

Department of Physics, School of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033 Japan

Abstract. The folding mechanism of proline-free staphylococcal nuclease (SNase (pro$^-$)) (P11A, P31A, P42A, P47T, P56A, P117G) was investigated using the double-jump stopped-flow method (interrupted refolding). This method has enabled us to specifically monitor the amount of the native molecules during the refolding. The results indicate that the middle and slow phases observed in the refolding kinetics represent the formation of the native state ($I_M \rightarrow N$, $I_S \rightarrow N$) and that the folding mechanism of SNase (pro$^-$) is not represented by a single sequential pathway, but at least two parallel pathways are required for interpreting the results.

1. Introduction

For small globular proteins, the biologically active native state is formed on a millisecond to second timescale, starting from a highly disordered unfolded state. Most proteins have been found to accumulate transient partially-folded intermediates during the refolding from the unfolded state to the native state. The existence of the partially-folded intermediates indicates that there are local minima, other than the unique global minimum, in the conformational free-energy landscape. How does a protein reach the unique global free-energy minimum (the native state) going through local free-energy minima (partially-folded intermediates) in the conformational free-energy landscape? This is an unresolved question regarding protein folding.

Staphylococcal nuclease (SNase) is a favorable system to investigate the shape of the conformational free-energy landscape of protein folding. The X-ray crystallographic structure of this small $\alpha+\beta$ protein has been reported [1], its high expression in *Escherichia coli* (*E. coli*) has been established [2,3], and this protein has been well characterized as a typical model globular protein for protein folding studies for some time [2–7]. The proline-free mutant of SNase, in which all proline residues were replaced with other residues, has been constructed for the purpose of investigating the folding reaction of the protein without the intervention of slow *cis*/*trans* isomerizations of peptidyl proline bonds. Even the folding of this proline-free mutant has been found to be still a complex process, in which at least three phases of refolding (fast, middle, and slow phases) are observed [3,8]. This requires at least two transient intermediates which accumulate during the folding. Studies by the combination of pulsed hydrogen exchange and NMR have shown that the early transient intermediate contains a β-hairpin formed by strands 2 and

*Corresponding author. Tel.: +81 3 5841 4128; Fax: +81 3 5841 4512; E-mail: kuwajima@phys.s.u-tokyo.ac.jp.

3 and a structure near the C-terminus [9]. This result indicates that the early transient intermediate may have native-like partially folded structure.

The question is regarding the folding pathway of SNase. Walkenhorst et al. (1997) have suggested a parallel folding pathway (fast, middle, and slow phases) by investigating the proline-free SNase folding [8]. They have built a model from the GdnHCl-dependence of the apparent rate constant of the three phases. On the other hand, Nishimura et al. (2000) have suggested a sequential-pathway model (middle, slow, and very slow phases) by investigating wild-type SNase folding [10]. They have proposed that these equilibrium intermediates, which are stabilized by suitable anions in the acidic condition (pH 2), are similar to the transient intermediates. There is, however, no sufficient direct evidence in favor of either model, corroborated by experimental observations. Therefore, it is still an open question whether the folding pathway of SNase is parallel or sequential.

The purpose of the present study is to determine the folding mechanism of SNase (pro$^-$) (P11A, P31A, P42A, P47T, P56A, P117G) in which all proline residues are replaced with the other residues (Ala, Gly, Thr). First, the acid-induced unfolding transition of SNase (pro$^-$) was studied by fluorescence spectroscopy. Second, the direct refolding and unfolding kinetics were also performed by stopped-flow fluorescence so as to investigate the kinetic mechanism. Finally, we performed the double-jump experiment (interrupted refolding) and determined whether the SNase (pro$^-$) folds through a sequential or parallel pathway. As a result, we show that the folding mechanism of SNase (pro$^-$) is represented by either of two parallel-pathway models.

2. Materials and methods

2.1. Expression and purification of SNase (pro$^-$)

The expression and purification of SNase (pro$^-$) were carried out as described previously [2], except that a Sephacryl S-100 column equilibrated with 300 mM ammonium hydrogencarbonate (pH 8.0) was used instead of 100 mM ammonium acetate (pH 8.0) in gel filtrations.

2.2. Sample preparation

The sample preparation for the equilibrium measurements were carried out as described previously [3] with small modification. The lyophilized SNase (pro$^-$) was dissolved into a 7 M urea solution (50 mM sodium acetate plus 50 mM NaCl, or 50 mM sodium cacodylate (above pH 5.5) plus 50 mM NaCl and 1.0 mM [ethylenebis](oxyethlenenitrilo)tetraacetic acid (EGTA)) and the solution was filtrated through a Millipore membrane filter (Millex-HV) with a pore size of 0.45 μm. It was passed through a NAP^{TM} 10 column which had been equilibrated with 50 mM sodium acetate or 50 mM sodium cacodylate, both of which contained 50 mM NaCl, and the latter contained additionally 1.0 mM EGTA. The protein concentration of SNase (pro$^-$) was determined from the UV absorbance with an extinction coefficient $E^{1\%}_{1\ \mathrm{cm}}$ of 9.3 at 280 nm. Then the stock of SNase (pro$^-$) was diluted into various pH solutions (50 mM sodium acetate plus 50 mM NaCl (below pH 5.5), or 50 mM sodium cacodylate plus 50 mM NaCl and 1.0 mM EGTA (above pH 5.5)), and the final protein concentration was adjusted to 0.01 mg/ml.

For direct unfolding experiments, the preparation method was essentially the same as that for the equilibrium preparation. The final protein concentration was adjusted to 0.02 mg/ml, and the buffer (pH 6.0, 50 mM sodium cacodylate, 50 mM NaCl) was used.

For refolding and double-jump experiments, the lyophilized SNase (pro$^-$) was dissolved into pH 2.0 solution (100 mM HCl/NaCl) and the solution was filtrated through a Millipore membrane filter (Millex-HV) with a pore size of 0.45 μm. The protein concentration of SNase (pro$^-$) was determined as described above and was adjusted to 0.02 mg/ml (direct refolding measurements) or 0.04 mg/ml (double-jump measurements).

2.3. Equilibrium measurements

The pH-induced unfolding transition was studied by measuring the intrinsic tryptophan fluorescence of 21 equilibrium samples (pH 1.8–6.8). The equilibrium transition curve was measured in a stopped-flow fluorescence apparatus 'SX.18MV' (20 $\pm$ 0.1°C). The excitation wavelength was 295 nm. The emission of the fluorescence was detected using a 320-nm cut off filter to detect only the fluorescence of tryptophan. The optical path length was 0.2 cm.

2.4. Kinetic measurements

Direct refolding and unfolding kinetics were monitored by the change in intrinsic tryptophan fluorescence with excitation at 295 nm. The kinetics were measured in the stopped-flow fluorescence apparatus 'SX.18MV' by monitoring the change in fluorescence above 320 nm (20$\pm$0.1°C). The optical path length was 0.2 cm. Typically, 4–6 traces were averaged. The dead time of the apparatus was approximately 4 ms either using the pseudo first-order reaction that 5,5′-dithiobis(2-nitrobenzonic acid) changed to 2-nitro-5-mercaptobenzoic acid in the presence of a large amount of thioglycerol [11] or using the kinetic reaction for formation of Mg^{2+}-8-hydroxyquinoline chelate [12].

2.5. Double-jump experiments (interrupted refolding)

Interrupted refolding experiments were performed to monitor the time course of formation of fully native molecules using the stopped-flow fluorescence apparatus 'SX.18MV' (20 $\pm$ 0.1°C). Acid-induced denatured SNase (pro$^-$) (pH 2.0, 100 mM HCl/NaCl, 0.04 mg/ml) was mixed with the refolding buffer (pH 6.2, 100 mM sodium cacodylate, 10 mM EGTA) at the ratio of 1 : 1, which initiated the refolding reaction (pH 6.0). After a delay time t_d, the second mixing with the unfolding solution (pH 1.1, HCl/NaCl) at the ratio of 1 : 1 prevented a protein from further folding (final pH 1.6, 0.01 mg/ml) and unfolded the already folded protein at various delay-times. A series of the interrupted-refolding reactions with different delay-times thus measured the unfolding from the folded state. All unfolding reactions were fitted to a single exponential (the apparent rate constant 31 $\pm$ 1 s^{-1}). This indicated that only the unfolding from the fully native state was observed, and the unfolding from transient intermediates was not observed. It is likely that the unfolding from the transient intermediates is too fast to detect and occurs within the dead time of the apparatus (4 ms). The relative difference between $F(0)$ and $F(\infty)$ is thus a measure of the accumulation of the native state, where $F(0)$ is the fluorescence value of the fitting curve extrapolated to the unfolding-reaction time 0 and $F(\infty)$ is the fluorescence value at unfolding-reaction time ∞.

2.6. Data fitting

Kinetic data were fitted by the non-linear least-squares method with an equation.

$$F(t) = F(\infty) + \sum_{i=1}^{n} \Delta F_i \cdot \exp(-k_i t), \tag{1}$$

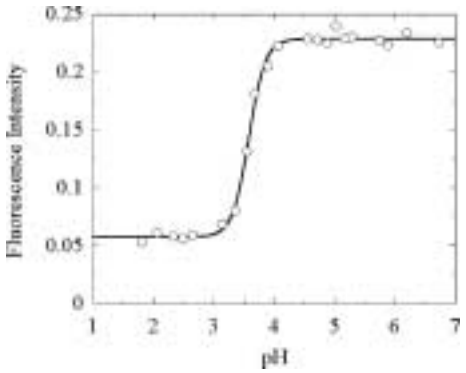


Fig. 1. The acid-induced unfolding transition curve of SNase (pro$^-$) at 20°C. The transition curve was monitored by the change in intrinsic tryptophan fluorescence. The protein concentration was 0.01 mg/ml. The solid line represents the best-fit curve obtained by Eq. (3).

where $F(t)$ and $F(\infty)$ are the observed fluorescence at time t and infinite time, respectively. ΔF_i and k_i are the fluorescence amplitude and the rate constant, respectively, of the ith phase. These analyses were performed using KaleidaGraph (Synergy Software) and SigmaPlot (SPSS Science).

3. Results

3.1. Equilibrium unfolding transition

Figure 1 shows the pH-induced unfolding transition of SNase (pro$^-$) (0.01 mg/ml, 20°C). The equilibrium unfolding curve was analyzed by a two-state approximation in which only the native state (N) and the acid-induced denatured state (A) were assumed to be populated in the transition zone, as

$$\mathrm{N} + n\mathrm{H}^+ \rightleftharpoons \mathrm{A}, \tag{2}$$

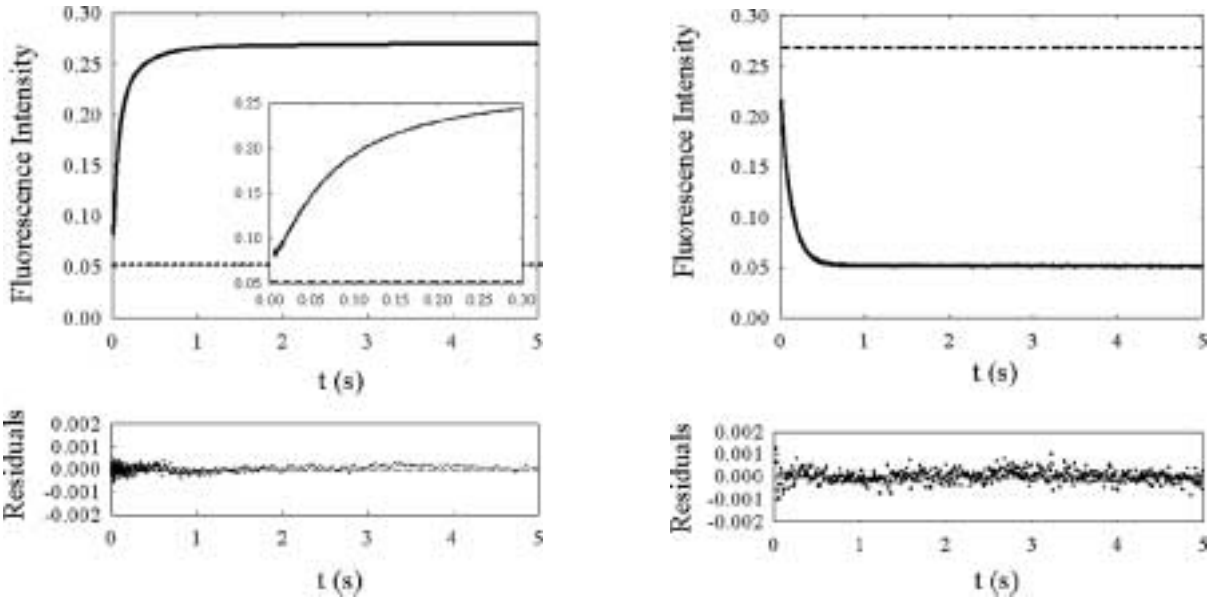
where n and H^+ represent the number of a bound proton and a free proton, respectively. The observed value of fluorescence of SNase (pro$^-$) at various values of pH, F, are thus given by

$$F = F_\mathrm{A} \cdot \frac{K_\mathrm{n} \cdot 10^{-n\cdot\mathrm{pH}}}{1 + K_\mathrm{n} \cdot 10^{-n\cdot\mathrm{pH}}} + F_\mathrm{N} \cdot \frac{1}{1 + K_\mathrm{n} \cdot 10^{-n\cdot\mathrm{pH}}}, \tag{3}$$

where K_n, F_N and F_A represent the apparent equilibrium constant and the fluorescence values in the native and the denatured states, respectively. F_N and F_A were assumed to be constant and to be independent of pH. The pH-induced unfolding transition curve was analyzed using Eq. (3) by non-linear least-squares method (KareidaGraph). F was the observable value, and K_n, F_N, n and F_A were the valuable parameters. As a result, the minimum number, n, of bound protons which induced the unfolding transition of SNase (pro$^-$) was 3.3 ± 0.3.

3.2. Direct refolding and unfolding kinetics

Figures 2(a) and (b) show the direct kinetic refolding and unfolding reaction curves of SNase (pro$^-$), respectively. Refolding kinetics (from pH 2.0 to pH 6.0) is described by the sum of 4 exponentials



(a) (b)

Fig. 2. Direct refolding and unfolding kinetics of SNase (pro−). Both reactions were monitored by the change in intrinsic tryptophan fluorescence. The final protein concentration was 0.01 mg/ml. (a) Refolding was initiated by pH-jump from pH 2.0 to pH 6.0 and was monitored at 20°C. The refolding reaction is best described by the sum of four exponentials with apparent rate constants of 150 s^{-1} (−13%), 16 s^{-1} (66%), 3.6 s^{-1} (29%), and 0.3 s^{-1} (2.6%). The solid line represents a quaternary exponential fit of the data. The dashed line represents the fluorescence intensity of the initial condition (pH 2.0). The inset shows the early time region for the direct refolding reaction. (b) Unfolding was initiated by pH-jump from pH 6.0 to pH 2.0 and was monitored at 20°C. The unfolding reaction is best described by a single exponential with the apparent rate constant of 7.9 s^{-1}. The solid line represents a single exponential fit of the data. The dashed line represents the fluorescence intensity of the initial condition (pH 6.0).

(Eq. (1)). There are four phases: fast (rate constant of 150 s^{-1}, relative fluorescence amplitude of −13%), middle (16 s^{-1}, 66%), slow (3.6 s^{-1}, 29%), and very slow phases (0.3 s^{-1}, 2.6%). The middle and slow phases were major phases, and the sum of the relative fluorescence amplitude was about 95%. The relative fluorescence amplitude of the fast phase was about −13%, and was opposite in sign to that of the middle and slow phases. This indicates that this fast phase does not represent the folding directly into the native state. The sum of the amplitudes of the four phases represent most of the fluorescence change (∼85%) expected from the equilibrium unfolding curve. This refolding kinetic result also shows that there are at least two transient intermediates during the refolding process if the very slow phase is not taken into account because of the minor relative amplitude.

Unfolding kinetics (from pH 6.0 to pH 2.0) is described by a single exponential with the apparent rate constant of 7.9 s^{-1} (Eq. (1)). The relative amplitude of this phase is 80%, indicating that there may be transient unfolding intermediates during the unfolding process or the fluorescence intensity of the native state may change after the rapid mixing from the native condition to the unfolding condition.

3.3. Time course of accumulation of the native state measured by the double-jump method

To investigate the formation of the N state (the native state) from the denatured A state (the acid-induced denatured state), we performed the stopped-flow double-jump experiments (interrupted refolding). The denatured SNase (pro^-) (pH 2.0) was mixed with the refolding buffer (pH 6.2), which made

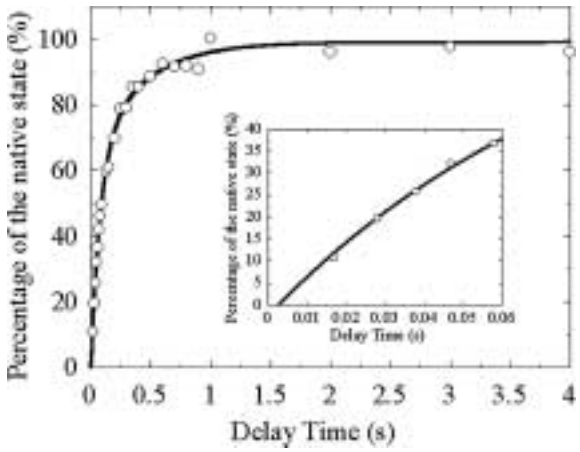


Fig. 3. The time course for the accumulation of the native state measured by the interrupted refolding experiments at 20°C. Open circles (○) represent the accumulation of the native state (pH 6.0). The formation of the native state is better described by the sum of two exponentials with the rate constants (the amplitudes) of $12.9 \pm 2.4\ s^{-1}$ ($65 \pm 9\%$) and of $2.5 \pm 0.6\ s^{-1}$ ($35 \pm 10\%$). The solid line represents a double-exponential fit of the data. The inset shows the early time region. The percentage of the native state cannot be extrapolated to zero at delay time 0, indicating the presence of a lag phase.

the denatured protein fold into the N state. After a delay time t_d, the second mixing with the unfolding solution (pH 1.1) prevented the protein from further folding and unfolded the already folded protein (final pH 1.6).

All unfolding reactions after the second mixing in various delay times can be analyzed by a single exponential (rate constant $31 \pm 1\ s^{-1}$). Therefore, only the unfolding from the N state was observed, whereas the unfolding from transient intermediates was not observed. It is likely that the unfolding from the transient intermediates occurs within the dead time (4 ms) of the stopped-flow apparatus.

Figure 3 thus shows the percentage of the N state accumulated at the delay time during refolding caused by the first mixing. The formation curve for the N state is described by the sum of the two exponentials with rate constants of $12.9 \pm 2.4\ s^{-1}$ ($65 \pm 9\%$) and of $2.5 \pm 0.6\ s^{-1}$ ($35 \pm 10\%$). The two phases coincide with the middle and slow phases observed in the direct refolding reaction. This indicates that the fluorescence changes of both the middle and slow phases represent the accumulation of the N state ($I_M \rightarrow N$, $I_S \rightarrow N$). A sequential pathway model shown below thus can not explain the observed accumulations of the N state in both phases.

Sequential folding pathway scheme

$$A \xrightarrow{\text{fast}} I_1 \xrightarrow{\text{middle}} I_2 \xrightarrow{\text{slow}} N$$

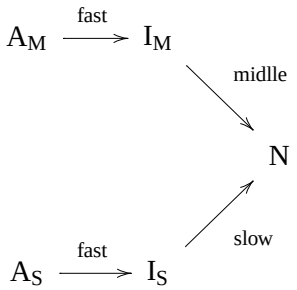
In this scheme, the denatured A state folds into the N state through two transient intermediates (I_1 and I_2) sequentially.

The percentage of the N state is not extrapolated to zero at delay time 0, indicating the presence of a lag phase. The presence of the lag phase indicates that the denatured A state does not fold directly into the N state. The lag phase seems to be equivalent to the fast phase observed in the direct refolding reaction.

Two simple folding schemes are possible as a model to explain both results of the direct refolding and the interrupted refolding experiments of SNase (pro^-); these schemes are simplified by the approxima-

tion that the microscopic rate constants are equal to the apparent rate constant observed in the kinetic measurements. The first is parallel folding pathway Scheme (1). There are at least two heterogeneous populations in the acid-denatured A state, A_M and A_S, which fold through the middle and slow phases, respectively.

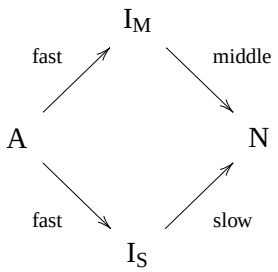
Parallel folding pathway Scheme (1)



Here, I_M and I_S represent the intermediates which are on-pathway intermediates formed in the fast phase and lead the N state through the middle or slow pathway, respectively. In this scheme, it is assumed that the interconversion between two unfolded states (A_M and A_S) is slower than the refolding reaction, indicating a significant free-energy barrier between A_M and A_S.

The second scheme is parallel folding pathway Scheme (2). In this scheme, there is kinetic partitioning between the middle and slow pathways during the fast phase. There are no energetically separated heterogeneous populations in the denatured ensemble, and the free-energy barrier between the two populations (I_M and I_S) is produced only during the fast phase.

Parallel folding pathway Scheme (2)



It is, however, difficult to distinguish between the two parallel-pathway schemes on the basis of the direct refolding kinetics and the interrupted refolding experiments.

4. Discussion

The acid-induced equilibrium unfolding transition of SNase (pro^-) was studied by fluorescence spectroscopy. The direct refolding and unfolding experiments were performed by stopped-flow fluorescence so as to investigate the kinetic mechanism of folding. We also performed the double-jump experiments

(interrupted refolding) to determine the mechanism of folding. The results have shown that the refolding of SNase is represented by either of the two parallel-folding pathway schemes. Here, we discuss the folding mechanisms of wild-type SNase and SNase (pro$^-$), and possible reasons for the parallel folding pathways.

4.1. Folding mechanisms of wild-type SNase and SNase (pro$^-$)

The folding of wild-type SNase has been studied by various probes including fluorescence, circular dichroism (CD), and H/D-exchange pulse-labeling with NMR for more than the past 30 years [4–7]. Its folding is complicated owing to the *cis*/*trans* isomerizations of the four proline residues present in SNase [2,3]. The *cis*/*trans* proline isomerization causes heterogeneity in the unfolded state and give rise to additional slow folding reactions [13]. SNase (pro$^-$) was thus constructed to simplify the folding kinetics, and the refolding of SNase (pro$^-$) has been studied by fluorescence and CD spectroscopy [3, 8]. However, even SNase (pro$^-$) still shows a complex kinetics with at least three phases of refolding (fast, middle, and slow phases), which have been ascribed to the presence of at least two parallel folding pathways in the present study.

4.2. Possible reasons for the parallel folding pathway of SNase (pro$^-$)

There are three possible reasons for the parallel folding-pathway as follows.

(1) Nonprolyl *cis* peptide bonds in the unfolded state lead to heterogeneity of the unfolded molecules and may give rise to the fast and slow folding pathways [14]. This reason is supported by the experimental data that 5% of the unfolded state in proline-free tendamistat folds slowly due to nonprolyl *cis* peptide bonds [14]. Scherer et al. (1998) indicates that there is 0.1–0.2% *cis* isomer per peptide bond using several model peptides such as AAYA and AAYAA [15]. In SNase (pro$^-$), the population of the *cis* isomer per peptide bond may be multiplied by the number of peptide bonds, that is, $0.15\% \times 148 = 22\%$. The population of SNase (pro$^-$) that has one *cis* isomer may thus be approximately 22%. This seems to be a rough estimate to explain the population of the slow-folding species in the denatured A state. The rate constants of nonprolyl *cis* $\rightarrow$ *trans* isomerization are 0.3–2.4 s^{-1} (25°C) for several peptides, 0.9–1.4 s^{-1} (25°C) for RNase T_1 (P39A) mutant, 0.7 s^{-1} (15°C) for RNase A variant, and 2.5 s^{-1} for proline-free tendamistat (25°C) [14–16]. The rate constant of the slow phase of SNase (pro$^-$) (3.6 s^{-1} (20°C)) is thus close to those previously observed for the nonprolyl *cis* $\rightarrow$ *trans* isomerizations. This seems to indicate that the slow phase may be caused by nonprolyl *cis* isomers of SNase (pro$^-$). If this is true, the folding mechanism of SNase (pro$^-$) could be represented by parallel-pathway Scheme (1).

(2) Our purified recombinant SNase (pro$^-$) might not be completely homogeneous, which gives rise to apparently parallel folding pathways. For example, the N-terminal sequence of a recombinant protein expressed in *E. coli* is widely recognized to start with formyl-methionine [17], which is in most cases subsequently processed by deformylase enzyme [18,19] and removed by methionine aminopeptidase to finally produce the N-terminal methionine-free recombinant protein. Removal of the N-terminal extra methionine, however, does not always occur. This fact is supported by the result that recombinant interleukin-2 and ricin A produced from *E. coli* contained 70% and 40% of molecules with N-terminal extra methionine [20]. Therefore, it might be possible that the extra methionine of our purified recombinant SNase (pro$^-$) has not been removed completely and that our purified recombinant SNase (pro$^-$) consists of two forms with and without the N-terminal extra methionine. In bovine α-lactalbumin, the N-terminal methionyl residue destabilizes the thermal stability [21]. Furthermore, in goat α-lactalbumin,

the N-terminal methionyl residue destabilizes the GdnHCl-dependent stability and increases the unfolding rate [22]. These results indicate that the N-terminal methionyl residue can change the energy landscape of protein folding and that one of the two pathways may be caused by molecules with the N-terminal extra methionyl residue.

(3) There may be real different folding pathways for SNase (pro$^-$), due to the presence of at least two accessible routes on the free energy landscape of SNase (pro$^-$) folding. If this is true, this is one of rare cases for protein folding. Most small globular proteins fold through a single pathway, and it is not necessary to consider any additional folding pathway other than caused by proline residues. There is, however, an exception that hen egg lysozyme folds through two competing pathways under a particular condition (pH 5.2) [23–25]. In the lysozyme folding, the fast pathway leads directly from the unfolded state to the near-native protein, whereas folding on the slow pathway proceeds through a partially folded intermediate. If there are at least two accessible routes on the free energy landscape of SNase (pro$^-$) folding, the free energy landscape of SNase (pro$^-$) folding seems to be similar to that of hen egg lysozyme folding. If this is true, the folding mechanism of SNase (pro$^-$) can be represented by parallel-pathway Scheme (2).

5. Conclusions

To investigate the mechanism of SNase folding, we performed the double-jump experiments (interrupted refolding) of SNase (pro$^-$) as well as the direct folding and unfolding experiments by stopped-flow fluorescence spectroscopy. The results have shown that the middle and slow phases observed during the direct refolding kinetics represent the formation of the fully native molecule ($I_M \rightarrow N$ and $I_S \rightarrow N$), so that the folding mechanism of SNase (pro$^-$) is represented by either of the two parallel-pathway models. This is one of rare cases for folding of small globular proteins. Most small globular proteins are known to fold through a single pathway if the additional pathways caused by prolyl isomerizations are not taken into account. SNase (pro$^-$), however, folds through at least two parallel pathways although it has no proline residues. Possible reasons that lead to the parallel-pathway folding have been discussed.

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