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The Pro^{117} to glycine mutation of staphylococcal nuclease simplifies the unfolding–folding kinetics

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Kinetics of unfolding and refolding of a staphylococcal nuclease mutant, in which Pro^{117} is replaced by glycine, have been investigated by stopped-flow circular dichroism, and the results are compared with those for the wild-type protein. In contrast to the biphasic unfolding of the wild-type nuclease, the unfolding of the mutant is represented by a single-phase reaction, indicating that the biphasic unfolding for the wild-type protein is caused by *cis–trans* isomerization about the prolyl peptide bond in the native state. The proline mutation also simplifies the kinetic refolding. Importance of the results in elucidating the folding mechanism is discussed.

Staphylococcal nuclease; Folding mechanism; Proline isomerization; Stopped-flow technique; Circular dichroism; Site-directed mutagenesis

1. INTRODUCTION

Staphylococcal nuclease (SNase) has been studied for many years as a model for elucidating the mechanism of protein folding [1,2]. Our previous study was concerned with the kinetics of folding and unfolding of wild-type SNase measured by stopped-flow CD in the peptide region [3]. A remarkable feature of the kinetics found in that study was that there are two phases in unfolding for SNase. This forms a contrast with the fact that unfolding kinetics are represented as a single-phase reaction for most small globular proteins. The unfolding of SNase was induced by a concentration jump of urea and measured by the CD at 225 nm at 4.5°C. The biphasic kinetics were observed in the absence of pdTp, while the presence of pdTp was found to make the kinetics obey a single-phase reaction. This phenomenon has been interpreted by the presence of two distinct native species of SNase in the absence of pdTp.

Previous NMR studies by other researchers have indicated that there is a slow *cis–trans* interconversion about the Lys^{116}–Pro^{117} peptide bond in native SNase in the absence of pdTp, leading to the population of two native species, N_c (*cis*) and N_t (*trans*) [4,5]. Only N_c is populated in the SNase–Ca^{2+}–pdTp complex. Thus, the fast phase (15% in the amplitude) of the biphasic unfolding observed by CD has tentatively been assigned to unfolding of N_t that is the minor and less stable species, while the slow phase (85%) has been assigned to unfolding of N_c that is the major species. Nevertheless, a question may also be raised on the above explanation of the biphasic kinetics. The isomerization from N_c to N_t studied by NMR is known to be temperature-dependent with a ΔH of 45 to 54 kJ/mol [4,5]. The temperature dependence has suggested that only N_c is populated at 4.5°C where the biphasic unfolding was observed by CD.

The kinetic CD study has also shown that there are four phases in refolding of SNase in native conditions below 1 M urea [3]. If this behavior is also due to *cis–trans* isomerization of prolyl peptide bonds in the unfolded state as observed in many proteins [6], at least two prolyl residues are associated with the refolding kinetics; SNase contains 6 prolines.

In this study, the unfolding and refolding kinetics of a SNase mutant (P117G), in which Pro^{117} is replaced by glycine, has been investigated. We show that both the unfolding and refolding kinetics are simplified by the proline mutation. The relevance of the present results with the previous studies and importance of proline residues in elucidating the kinetic folding will be discussed.

Abbreviations: SNase, Staphylococcal nuclease; CD, circular dichroism; pdTp, thymidine 3',5'-diphosphate; NMR, nuclear magnetic resonance; N_c and N_t, two native species with the *cis* and *trans* forms, respectively, of the Lys^{116}–Pro^{117} peptide bond; P117G, the Pro^{117} to glycine mutant; IPTG, isopropyl-β-D-thiogalactopyranoside; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

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2. MATERIALS AND METHODS

The gene of SNase from the Foggi strain of *S. aureus* was obtained by *Sau*3AI digestion of a recombinant plasmid (pNU01) having the

SNase-encoding gene constructed in this laboratory (unpublished). An expression plasmid (pDRS113-SNase) carrying the coding sequence of SNase fused to the *E. coli* protease III signal sequence was constructed by insertion of the SNase gene fragment into the *BglII* cloning site of an expression-secretion vector (pDRS113) that contains the tandem lac-tac promoter followed by the ribosome-binding site and the signal sequence of protease III [7] (Kuwajima et al., in preparation); pDRS113 is a hybrid of pUC118 and a protease III expression plasmid (pDR42S) that was a gift of Dr S. Kanaya. The P117G mutant plasmid (pDRS113-SNase-P117G) was made from the single-stranded pDRS113-SNase and a mutagenic primer by the use of an oligonucleotide-directed in vitro mutagenesis system kit (Amersham, version 2). The mutation was confirmed by DNA sequencing of the mutant plasmid, and *E. coli* TG1 was transformed with pDRS113-SNase (or pDRS113-SNase-P117G) [8]. The protein was found to be exported into the periplasmic space, and the level of accumulation of exported SNase after 8-hour cultivation in LB broth with 1 mM IPTG was 10~20 mg per liter of culture. Periplasmic proteins including SNase (or its mutant) were extracted from the *E. coli* cells by the method of Sepersu et al. [9], and the purification method of the protein was essentially that of [3]. The amino acid sequences of the N-terminal 20 residues for purified SNase and P117G were determined by an Applied Biosystem 477A Protein Sequencer, and indicated correct processing of the protease III signal peptide in both purified proteins. Because the *Sau*3AI site used for excision of the SNase gene is located upstream compared to the N terminus of SNase A [10], the purified proteins contain an additional 6 residues in the N-terminal regions. These additional residues are disordered and have no significant effect on the stability and folding of the mature part of SNase.

The kinetics of unfolding and refolding of P117G were measured by CD spectroscopy, in the same manner as used previously for wild-type SNase, with a Jasco J-500A spectropolarimeter equipped with a laboratory-made stopped-flow apparatus (pH 7.0 and 4.5°C) [3]. The protein concentration was determined spectrophotometrically by using an extinction coefficient $E^{1\%}_{1cm} = 9.0$ considering the 6-residue extension at the N termini of the cloned proteins.

Other materials and methods were the same as reported previously [3].

3. RESULTS

The P117G mutant is fully unfolded above 4 M urea in the absence of pdTp and Ca^{2+} at pH 7.0 and 4.5°C (data not shown). The unfolding reaction of P117G was induced by a concentration jump of urea from 0 to 6 M, and the kinetics were measured by the ellipticity at 225 nm (θ_{225}) (Fig. 1a and b). In contrast to the biphasic unfolding of wild-type SNase, the unfolding of P117G is well represented by a single-phase reaction. The logarithm of $\theta(\infty)-\theta(t)$ linearly depends on time (Fig. 1b); $\theta(\infty)$ and $\theta(t)$ are the ellipticities at infinite time and time t, respectively. This is clear evidence that Pro^{117} is responsible for the biphasic unfolding previously observed for wild-type SNase.

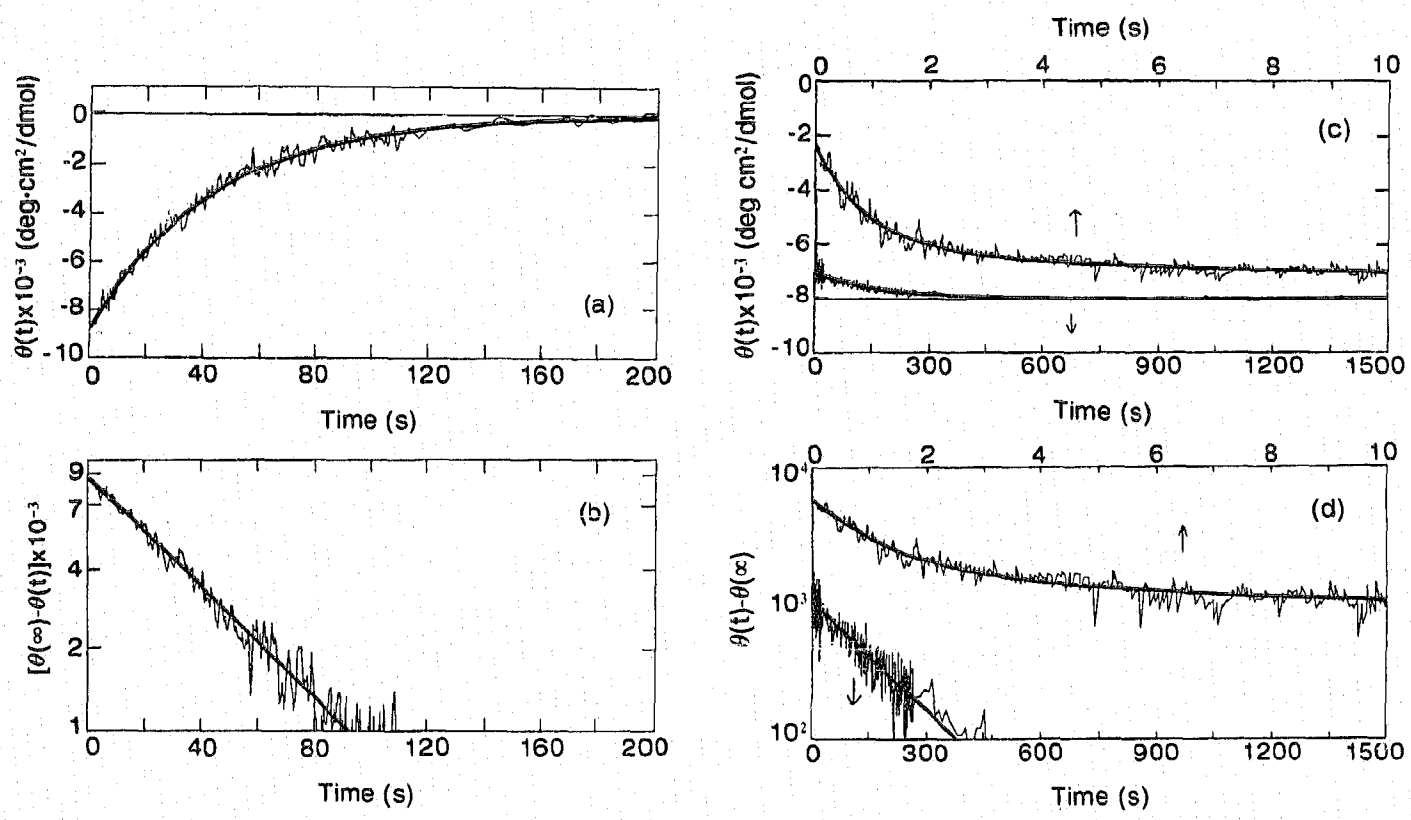

Fig. 1. Kinetic progress curves of unfolding ((a) and its logarithmic form in (b)) and refolding ((c) and its logarithmic form in (d)) of the P117G mutant measured by θ_{225} (pH 7.0 and 4.5°C). Final conditions: (a) and (b), 6 M urea and 1 mM EGTA; (c) and (d), 0.4 M urea and 1 mM EGTA. EGTA was added to the solutions to assure complete removal of protein-bound Ca^{2+}. Reaction curves in (c) and (d) are shown in two different time scales. The thick solid line in each figure represents the theoretical curve based on the parameter values of Table I.

The refolding reaction induced by a concentration jump of urea from 4 to 0.38 M was also measured by θ_{225} (Fig. 1c and d). Non-linear least-squares fitting of the observed curve to a multi-exponential function was done in the same manner as described in the previous paper [3]. The results have revealed that there are at least three phases in refolding of P117G. The θ (∞) obtained is identical with the ellipticity of native P117G, indicating the reversibility of unfolding.

The kinetic parameters for unfolding and refolding of P117G obtained in this study are summarized and compared with the corresponding parameters for wild-type SNase reported in the previous paper [3] (Table 1). For unfolding, the slow phase observed in wild-type SNase disappears in P117G, and the kinetics have a rate constant close to the value for the wild-type fast phase. As the Lys^{116}–Gly^{117} peptide bond of the mutant is expected to be in the *trans* only, the result is consistent with the proposal that the slow phase in the biphasic unfolding for wild-type SNase arises from unfolding of N_c. For refolding, the third or fourth phase of the four phases observed in wild-type SNase disappears in P117G, and the amplitude of the first phase is increased by the mutation. The presence of the three phases in refolding of P117G suggests that at least two prolyl residues other than Pro^{117} may be associated with the refolding kinetics.

4. DISCUSSION

The present results demonstrate that the biphasic unfolding previously observed for the wild-type protein has been caused by the presence of two native species (N_c and N_t) due to the *cis–trans* isomerization about the Pro^{117} peptide bond. Evans et al. [4] and Alexandrescu et al. [5] have studied the temperature dependence of the $N_c \rightleftharpoons N_t$ reaction of sNase by NMR spectroscopy, and the ΔH for the reaction has been estimated to be 45~54 kJ/mol at 40°C, which predicts that practically only N_c (>98%) might be populated at 4.5°C. The presence of the two forms, 85% for N_c and 15% for N_t in percent population, at 4.5°C shown by the previous and present studies demonstrates that the ΔH for $N_c \rightleftharpoons N_t$ obtained at a high temperature cannot be applied to the reaction at a low temperature, so that there must be a heat capacity change ΔC_p for the reaction. From van't Hoff plots for $N_c \rightleftharpoons N_t$ presented by Alexandrescu et al. [5] and the equilibrium constant $K = 0.18$ for $N_c \rightleftharpoons N_t$ at 4.5°C, we obtain a ΔC_p of 2.6 kJ/mol/K. As such a ΔC_p is caused most likely by exposure of buried hydrophobic groups [11], the N_c to N_t isomerization is accompanied with a partial unfolding of the molecule, i.e. exposure of hydrophobic side chains, presumably, in a region close to Pro^{117}. The ΔH for the *cis–trans* isomerization itself is known to be generally close to zero [6,12], so that the positive ΔH for $N_c \rightleftharpoons N_t$ may also arise from such a partial unfolding [11].

Studies of the kinetic intermediates and the transition state for folding are important for elucidating the mechanism of protein folding [13–15]. In such studies, however, it is also important that the kinetics observed are not rate-limited by proline isomerization. Ideally, it is best to study mutant proteins in which the kinetics of both folding and unfolding are free from proline isomerization and represented as single-phase reactions. Kinetic folding studies for SNase mutants in which more than 2 prolyl residues are replaced by others are thus being undertaken in this laboratory.

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Table I

Kinetic parameters of unfolding and refolding for wild-type SNase and the P117G mutant (pH 7.0, 4.5°C).

	Rate constant (s^{-1})				Ellipticity change (deg·cm²/dmol)				Final value (deg·cm²/dmol)
	k_1	k_2	k_3	k_4	$\Delta\theta_1$	$\Delta\theta_2$	$\Delta\theta_3$	$\Delta\theta_4$	$\theta(\infty)$
Unfolding[a]									
Wild-type	0.049	0.0073	–	–	−1230	−7480	–	–	−440
P117G mutant	0.024	–	–	–	−8690	–	–	–	−150
Refolding[b]									
Wild-type	2.7	0.45	0.022	0.0019	1020	4380	210	750	−9670
P117G mutant	1.2	0.29	0.0061	–	3500	1230	1020	–	−8040

The data for the wild-type protein were taken from [3]
[a]Urea concentration jump: 0→6 M.
[b]Urea concentration jump: 4→0.4 M.

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