Effect of Prolyl Isomerase on the Folding Reactions of Staphylococcal Nuclease†

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ABSTRACT: The low-temperature fluorescence-detected refolding of staphylococcal nuclease (SNase) can be described by three slow kinetic phases. The slowest phase is absent in the P117G mutant of SNase. Peptidyl prolyl *cis*−*trans* isomerase (cyclophilin), which has been shown to catalyze the slow folding reactions of some proteins, was employed to determine which of the refolding reactions of SNase and P117G SNase involve proline isomerization. We report here that all three folding phases of the wild type and the slower phase of P117G SNase are catalyzed by prolyl isomerase, indicating that proline isomerization is involved in these fluorescence-detected phases in the refolding of SNase. Since the rates of these phases are denaturant-dependent, we conclude that the slow folding steps involve isomerization of non-native *cis* proline peptide bonds and are tightly coupled to denaturant-sensitive structural changes.

Some proteins that contain prolyl residues can exist in alternative folded states due to *cis*−*trans* isomerization of X−Pro peptide bonds (Brandts et al., 1975; Kim & Baldwin, 1982; Langsetmo et al., 1989; Creighton, 1984; Kiefhaber et al., 1992). Evidence supporting this has been obtained using NMR techniques (Fox et al., 1986; Chazin et al., 1989; Alexandrescu et al., 1989). Non-native proline conformations in the unfolded protein have been found to generate slow refolding phases in several proteins (Brandts et al., 1975; Schmid & Baldwin, 1978; Kelley & Richards, 1987; Wood et al., 1988; Kiefhaber et al., 1990).

Staphylococcal nuclease (SNase)[1] exemplifies a well-characterized model system for studies of protein folding. It is a Ca^{2+}-dependent nuclease of 149 amino acids lacking disulfide bonds and free cysteinyl residues (Tucker et al., 1978). Isomerization about the Lys116−Pro117 bond of SNase has been shown to produce multiple conformations in the folded and unfolded states (Evans et al., 1987, 1989; Alexandrescu et al., 1989; Raleigh et al., 1992; Kautz & Fox, 1993; Hodel et al., 1993). The *cis*−*trans* isomerization about the Lys116−Pro117 peptide bond is responsible for the slowest refolding phase (Nakano & Fink, 1990; Kuwajima et al., 1991; Nakano et al., 1993) as this phase is eliminated in P117G SNase.

Refolding phases that involve proline *cis*−*trans* isomerization are characterized by rate processes that have high activation enthalpies (∼20 kcal/mol). Generally, such reactions are also independent of denaturant concentration and are catalyzed by strong acids (Garel et al., 1976; Schmid & Baldwin, 1978). The elimination of a slow folding phase following the site-specific replacement of a prolyl residue with a non-prolyl residue establishes the involvement of the prolyl residue in the refolding phase. Peptidyl prolyl *cis*−*trans* isomerase (PPI), which was first discovered in 1984 (Fischer et al., 1984), catalyzes prolyl isomerization in peptide substrates and some *in vitro* protein folding reactions (Fischer & Bang, 1985; Lang et al., 1987; Lin et al., 1988; Davis et al., 1989; Harrison & Stein, 1990; Jackson & Fersht, 1991; Veeraraghavan & Nall, 1994). Thus, catalysis by PPI may be used as a test for folding reactions arising from proline isomerization, although this does not allow assignment of a kinetic phase to a particular proline. We describe the use of PPI in characterizing the folding reactions of staphylococcal nuclease and two of its site-specific mutants. The folding process of the P117G SNase lacks the slowest of the three fluorescence-detected slow phases of the wild type SNase. The NCA S28G SNase contains a six-amino acid type I β-turn substitute (in place of a five-amino acid segment of wild type SNase) from concanavalin A, in which the second serine of the inserted segment has been mutated to glycine (Hynes et al., 1989). The resulting protein is rather unstable and exhibits cold denaturation at neutral pH (Antonio et al., 1991; Eftink et al., 1991).

MATERIALS AND METHODS

Materials

Staphylococcal Nuclease A. Wild type ($M_r \approx 16\,800$) and mutant forms of SNase were produced via recombinant expression in *Escherichia coli* and purified according to Shortle (1986). The P117G and NCA S28G SNase proteins

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[1] Abbreviations: PPI, peptidyl prolyl *cis*−*trans* isomerase; SNase, staphyloccal nuclease A; P117G SNase, SNase in which proline 117 is replaced by glycine; NCA S28G, SNase−concanavalin A hybrid mutant containing a six-amino acid type I β-turn sequence from conA with the second serine in the sequence mutated to a glycine (Hynes et al., 1989); succinyl-AAPF-*p*-nitroanilide, tetrapeptide substrate used in the determination of the specific activity of PPI (succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide); Gdn.HCl, guanidine hydrochloride; CD, circular dichroism; k_{cat}/K_m, catalytic specificity of PPI; $(k_{cat}/K_m)_{pep}$, catalytic efficiency of PPI toward the peptide substrate; $(k_{cat}/K_m)_{prot}$, catalytic efficiency of PPI toward protein substrate; $(k_{cat}/K_m)_{rel}$, catalytic efficiency of PPI toward the protein substrate relative to peptide substrate.

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were generously provided by R. O. Fox. The protein concentration was determined using the molar extinction coefficient E_{280} of 15 624 M^{-1} cm^{-1} (Fuchs et al., 1967).

Prolyl Isomerase. PPI was purified from pHNJ/XA90 *E. coli* cells in the presence of reducing agent according to Liu et al. (1990), chromatographed by binding to CM-Sephadex cation exchange resin, and eluted with a 0 to 0.25 M sodium chloride gradient. Purified PPI was stored at −70 °C and used within 6 months. The enzyme concentration was determined by Bradford assay (Bradford, 1976) and/or by using the calculated molar extinction coefficient (Gill & Von Hippel, 1989) E_{280} of 8730 M^{-1} cm^{-1}. The specific activity of PPI, $(k_{cat}/K_m)_{pep}$, was measured at 20 °C as described previously (Veeraraghavan & Nall 1990). $(k_{cat}/K_m)_{pep}$ was measured at 2.5 °C to measure $(k_{cat}/K_m)_{rel}$. Cyclosporin A, used to inhibit PPI-catalyzed protein folding, was a gift from the Sandoz Research Institute (East Hanover, NJ).

Preparation of the Guanidinium Hydrochloride Stock. A 10 mM solution of sodium cacodylate buffer (Sigma) at pH 7.0 was used to prepare a 3 M guanidinium hydrochloride (Gdn.HCl) stock solution. The molar concentration of Gdn.HCl solutions was determined from the change in the refractive index relative to the buffer (ΔN) using the formula

$$[\text{Gdn.HCl}] = 57.147(\Delta N) + 38.68(\Delta N)^2 - 91.6(\Delta N)^3$$

(Pace, 1986). Buffer and Gdn.HCl solutions were filtered using 0.45 μm Millipore filters (type HA).

Methods

SNase Refolding Reactions. Stock solutions of SNase at about 100 or 300 μM were prepared by dissolving lyophilized protein in 3 M Gdn.HCl and 10 mM sodium cacodylate buffer at pH 7.0. Samples were vortexed gently to aid dissolution and clarified by centrifugation at 14000*g* for 10 min. All refolding reactions were initiated by manual mixing and carried out at 2.5 ± 0.1 °C (temperature of the cuvette containing 2.5 mL of the sample). The sample chamber was purged with dry air or nitrogen to prevent condensation. Refolding was initiated by diluting a stock solution of SNase into pre-equilibrated buffer containing varying concentrations of PPI so that the final concentration of SNase was 6 μM. The buffer also contained sufficient Gdn.HCl to obtain the desired final denaturant concentration. Fluorescence emission changes at 330 nm were measured using an SPF 500 C spectrofluorometer (SLM, Inc.) by excitation of the samples at 295 nm. Excitation and emission slits were 5 and 7.5 nm, respectively. The dead time for manual mixing was approximately 5 s.

A stock solution of cyclosporin A was prepared at 3 mM in 95% ethanol and stored at 4 °C. Fluorescence-detected refolding of SNase was conducted in the presence of CsA as follows. Buffer containing PPI and CsA was equilibrated at 2.5 °C for 10 min in a quartz cuvette. An appropriate amount of 3 M Gdn.HCl was added to this solution. Refolding was initiated by the dilution of an unfolded SNase stock (94 μM) into buffer containing CsA. The final solution contained 5 mM CsA, 5 mM PPI, 6 μM SNase, 0.4 M Gdn.HCl, and 10 mM sodium cacodylate at pH 7.0 and 2.5 °C.

Data Analysis. Kinetic data, from fluorescence-detected refolding experiments, were analyzed using both BioKine (Molecular Kinetics) and Origin (Microcal) software. Following baseline subtraction, the apparent first-order rate constants (k_i^{app}) for the *i*th phase and corresponding amplitudes (ΔF) were obtained by multiexponential nonlinear least-squares fits of the data using Pade-Laplace or Simplex routines according to the equation

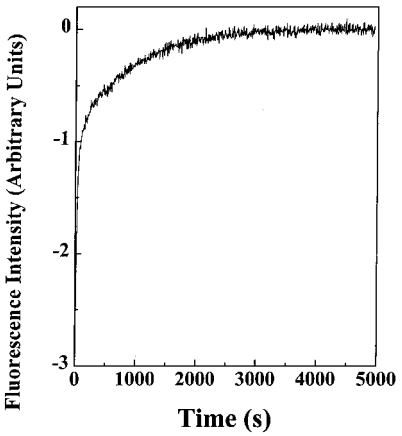

FIGURE 1: The fluorescence-detected slow folding reactions of wild type staphylococcal nuclease with 0.4 M Gdn.HCl at 2.5 °C and pH 7.0. A trace of the baseline-subtracted data is shown. The data were best fit by a three-exponential first-order rate equation with the following rate constants (k) and relative amplitudes (a): k_1^{app} = 0.001 s^{-1}, k_2^{app} = 0.006 s^{-1}, k_3^{app} = 0.06 s^{-1}, a_1 = 0.3, a_2 = 0.26, and a_3 = 0.44.

$$F(t) = F_\infty + \sum_i \Delta F_i \exp(-k_i^{app} t) \quad (1)$$

where k_i^{app} is the apparent rate constant for a given phase *i*, F_∞ the final fluorescence, $F(t)$ the measurement at time *t*, and ΔF_i the amplitude associated with the *i*th phase. Rate constants for the folding reactions in the absence of added prolyl isomerase are denoted by $k_{i,0}^{app}$.

The specific activity, $(k_{cat}/K_m)_{pep}$, was determined using the peptide substrate, succinyl-AAPF-*p*-nitroanilide, according to the method of Harrison and Stein (1990), and is given by

$$(k_{cat}/K_m)_{pep} = (k_{obs} - k_u)/[\text{PPI}]_o \quad (2)$$

where k_{obs} is the observed first-order rate constant (at 390 nm), k_u the uncatalyzed rate, and $[\text{PPI}]_o$ the total concentration of PPI used in the assay. Thus, the specific activity is determined from the initial linear portion of a plot of k_{obs} versus $[\text{PPI}]_o$. The catalytic efficiency of PPI toward the protein substrate was determined similarly from the observed refolding rates at various PPI concentrations. Under the experimental conditions employed to investigate the effects of PPI on SNase refolding reactions, PPI maintained its specific activity. Hence, the uncorrected catalytic efficiency of PPI toward the protein substrate is presented.

RESULTS

Fluorescence-Detected Refolding of Wild Type SNase. The equilibrium and kinetic data for the refolding of wild type SNase, monitored by Trp fluorescence, have been published (Nakano et al., 1993). In agreement with these data, we observed three distinct kinetic phases in refolding SNase at a final Gdn.HCl concentration of 0.4 M, with 10 mM sodium cacodylate at pH 7.0 and 2.5 °C (Figure 1). The rate constants of these reactions, in the absence of PPI,

Table 1: Rate Constants and Amplitudes for PPI-Catalyzed Refolding of Staphylococcal Nuclease[a]

	SNase							P117G SNase[b]			
	k_i^{app} (s^{-1}) (rate constant)			a_i^{app} (% relative amplitude)				k_i^{app} (s^{-1}) (rate constant)		a_i^{app} (% relative amplitude)	
PPI (μM)	k_1^{app}	k_2^{app}	k_3^{app}	a_1^{app}	a_2^{app}	a_3^{app}	PPI (μM)	k_2^{app}	k_3^{app}	a_2^{app}	a_3^{app}
0	0.001	0.006	0.06	30.0	26.0	44.0	0	0.001	0.07	36.3	63.7
0.05	0.002	0.009	0.068	28.5	33.3	38.1	0.05	0.003	0.064	38.9	61.1
0.1	0.004	0.016	0.069	30.1	29.6	40.3	0.1	0.004	0.076	31.2	68.8
0.2	0.004	0.011	0.073	28.2	30.2	41.6	0.2	0.006	0.07	25.6	74.4
0.4	0.006	0.012	0.072	29.1	34.5	36.4	0.3	0.008	0.079	24.9	75.1
0.6	0.008	0.020	0.076	31.3	29.2	39.5	0.4	0.009	0.072	24.4	75.6
1.2	0.008	0.028	0.114	36.3	25.9	37.8	0.8	0.018	0.079	31.4	68.6
3	0.009	0.033	0.104	28.7	29.5	41.8	1.5	0.026	0.098	21.6	78.4
							2	0.026	0.096	25.9	74.1
3 (+5 mM CsA)	0.001	0.007	0.047	28.7	28.4	42.9					

[a] Rate constants and amplitudes were obtained by curve fitting the refolding data as described under Data Reduction. Final conditions were as follows: 0.4 M Gdn.HCl, 6 μM SNase, 10 mM sodium cacodylate, pH 7.0, and 2.5 °C. [b] P117G SNase refolding proceeds via two phases. The slowest refolding phase ($k_1^{app} = 1/\tau_1$), which is present in SNase, is eliminated as a result of the proline mutation.

are $k_{1,0}^{app} = 0.001\ s^{-1}$, $k_{2,0}^{app} = 0.006\ s^{-1}$, and $k_{3,0}^{app} = 0.060\ s^{-1}$. The relative amplitudes of these phases are 0.3 (a_1), 0.26 (a_2), and 0.44 (a_3), respectively (Table 1). Errors in the rate constants are in the range of 1−2% of the standard deviation, while errors in the amplitudes are up to 30%. A detailed kinetic analysis was carried out for all kinetics experiments, including comparing curve fits to one, two, or three exponentials, to confirm that there were three phases and that the best values for the observed rate constants were obtained. Excellent agreement was obtained using two different software analysis programs. In all cases, the best fit was obtained with three exponentials; this was ascertained by the goodness of fit, minimum χ^2 values, and minimum residuals. In addition, fixing two of the three rate constants to those in the absence of PPI and curve fitting the data as a function of PPI concentration gave very poor fits. At least one faster phase is detected using stopped-flow fluorescence under these experimental conditions. The rates of the two faster phases are denaturant concentration-dependent, whereas the slowest phase is not. As the PPI concentration is increased (0 to 3 μM), each of the refolding rate constants increases linearly and then plateaus at what appears to be a maximum value (Figures 2 and 3). The apparent rate constants ($k_i^{app} = 1/\tau_i$) were found to plateau at PPI concentrations of 1−2 μM. The maximum observed rate constants ($k_{i,max}^{app}$), in the presence of high concentrations of PPI, for the three phases were 0.009, 0.033, and 0.10 s^{-1}, respectively. These are ~9-, 5-, and 2-fold faster than the rates measured in the absence of PPI, respectively (Figure 3, Table 1). The amplitudes remain unchanged for PPI-catalyzed refolding. In the presence of the inhibitor CsA (5 μM), PPI does not catalyze the refolding reactions, and the k_i^{app} and amplitudes of refolding are nearly the same as $k_{i,0}^{app}$ (Table 1).

Fluorescence-Detected Refolding of NCA S28G SNase. This mutant displays biphasic refolding kinetics (Table 2). At a final Gdn.HCl concentration of 0.08 M, with 10 mM sodium cacodylate at pH 7.0 and 2.5 °C, the rate constants of these reactions[2] are 0.001 and 0.013 s^{-1} with an a_1 of ≅0.85 for the slowest phase, in agreement with Antonio (1991). The addition of up to 3 μM PPI does not catalyze either of these two slow folding reactions (Table 2).

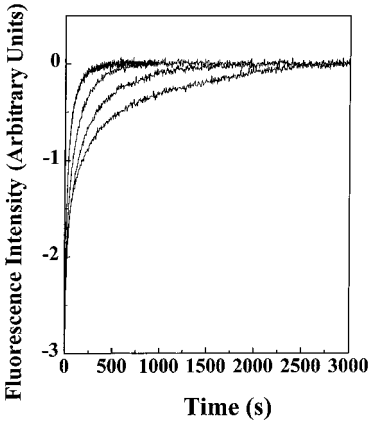

FIGURE 2: Catalysis of fluorescence-detected slow folding reactions of wild type staphylococcal nuclease. Kinetic traces (bottom to top) of the slow folding reactions of SNase are shown at PPI concentrations of 0, 0.05, 0.6, and 1.2 μM, respectively. Final conditions of the experiment were as follows: 6 μM SNase, 0.4 M guanidinium hydrochloride, 10 mM sodium cacodylate buffer, pH 7.0, and 2.5 °C. Refolding in the presence of PPI inhibited with cyclosporin A resulted in a trace with rates and amplitudes very similar to that with no added enzyme (Table 1).

Fluorescence-Detected Refolding of P117G SNase. The effects of the P117G mutation on the equilibrium and kinetics of SNase folding have been described previously (Kuwajima et al., 1991; Nakano et al., 1993). Here, we report that, when SNase is refolded in the absence of PPI at a final Gdn.HCl concentration of 0.4 M, biphasic kinetics are observed for the P117G SNase mutant (Table 1, Figure 4). These fluorescence-detected phases have rate constants of 0.001 s^{-1} (k_2^{app}) and 0.07 s^{-1} (k_3^{app}). In the presence of PPI, the intermediate refolding phase (τ_2) is catalyzed ~25-fold relative to its normal folding rate in the absence of PPI, $k_{2,0}^{app}$ (Figure 4, Table 2). The faster phase (τ_3) was minimally affected by the addition of PPI (about 50% increase in rate).

DISCUSSION

Catalysis of SNase Refolding by Prolyl Isomerase. The folding kinetics of SNase, P117G SNase, and NCA S28G

[2] The rate of the slow phase measured for the P117G mutant is similar to that of the slowest phase of the wild type in the experiments reported here because of differences in the stabilities of the proteins and consequent changes in the rates as a function the denaturant concentration. The slowest observed refolding phase for P117G corresponds to τ_2 of the wild type (Antonino, 1991; Nakano et al., 1993).

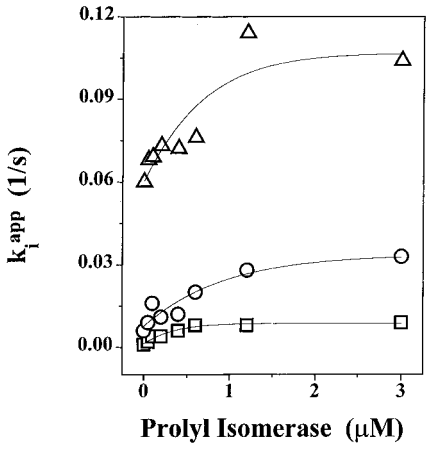

FIGURE 3: Effect of PPI on the refolding of SNase. First-order rate constants, k_i^{app}, for the fluorescence-detected slow refolding phases of SNase are plotted as a function of final prolyl isomerase concentration. The effect of PPI on the slowest (k_1^{app}, □), intermediate (k_2^{app}, ○), and fast (k_3^{app}, △) refolding phases is shown. The curves indicate the general trend in the data and have no theoretical significance. The catalytic efficiency of prolyl isomerase, $(k_{cat}/K_m)_i$, determined from a least-squares linear fit of the data between 0 and 0.8 μM PPI is presented in Table 2. Final conditions of the experiment were as follows: 6 μM SNase, 0.4 M Gdn.HCl, 10 mM sodium cacodylate buffer, pH 7.0, and 2.5 °C.

SNase have been determined previously in guanidinium thiocyanate, urea, or guanidinium hydrochloride denaturant solvent systems (Sugawara et al., 1991; Kuwajima et al., 1991; Nakano et al., 1993). The equilibrium unfolding and refolding experiments (Antonio et al., 1991; Eftink et al., 1991; Nakano et al., 1993) indicate that the NCA S28G mutant is much less stable than the wild type: $C_m \sim 0.14$ M Gdn.HCl and $\Delta G = 1.27 \pm 0.3$ kcal/mol for the mutant, compared to $C_m = 0.8$ M Gdn.HCl and $\Delta G = 5.5 \pm 0.3$ kcal/mol for the wild type (Antonio et al., 1991; Eftink et al., 1991). Furthermore, this mutant exhibits cold denaturation at neutral pH in the absence of denaturant (Antonino et al., 1991). Under the refolding conditions used here (0.08 M Gdn.HCl at 2.5 °C), at least 75% of the NCA S28G SNase is estimated to refold to the native state.

PPI (human cyclophilin) catalyzes all the observed refolding phases of wild type SNase and P117G SNase at 2.5 °C, and catalysis is most efficient for the slowest phases (see Table 1). The maximum rate enhancement of refolding, in the presence of 2 or 3 μM PPI, varies between about 25-fold for the slowest phase of the P117G and 2-fold for the fastest phase of the wild type SNase, relative to the corresponding uncatalyzed rates. The catalytic efficiency of PPI, $(k_{cat}/K_m)_i$, is on the order of 10^4 M^{-1} s^{-1}; this value is very similar to that observed in 0.3 M Gdn.HCl for yeast isocytochrome *c*. In the presence of cyclosporin A, catalysis of the refolding phases of wild type SNase by PPI is eliminated. This suggests catalysis of these folding reactions is by proline *cis*−*trans* isomerization activity of PPI and not by a chaperonin-like activity (Freskgård et al., 1992). Further, recent investigations by Kern et al. (1994) have shown that the only PPI-catalyzed folding reaction that was thought to be due to a chaperonin-like activity of PPI is in fact a result of prolyl isomerase activity of the enzyme. One faster folding phase, with a time constant of around 250 ms, is observed by stopped flow for wild type SNase under the experimental conditions used here, in agreement with previous reports (Epstein et al., 1971; Sugawara et al., 1991).

In refolding of the wild type SNase, the PPI catalysis of the denaturant-independent τ_1 phase is not surprising since this reaction has many hallmarks of a folding reaction involving proline isomerization. The τ_1 phase is attributed to the isomerization of the K116−P117 bond (Kuwajima et al., 1991; Nakano et al., 1993). Generally, rate constants of the slow folding phases involving proline isomerization do not show strong denaturant dependence since prolyl isomerization is believed to occur more readily in the unfolded or partly folded states of a protein. The denaturant-dependent intermediate phase (τ_2) of the wild type is also believed to involve proline isomerization, from the *cis* isomer in the unfolded state to the *trans* isomer in the native state (Nakano et al., 1993). This is confirmed by catalysis of the rate of this reaction by PPI. Further, the denaturant dependence of τ_2 implies that proline isomerization is not directly rate-limiting, but is coupled to denaturant-sensitive structural changes in a partially folded intermediate.

The observation that the fastest fluorescence-detected phase (τ_3) is also catalyzed by PPI was unexpected, due to the strong denaturant dependence of the rate constant. Other folding reactions with similar rate constants [for example, the fluorescence-detected slow folding phase of iso-2-cytochrome *c*; see Veeraraghavan and Nall (1994)], albeit at 20 °C, are catalyzed by PPI. However, the fast folding reaction of SNase ($k_{3,0}^{app} = 0.06$ s^{-1}) was followed at 2.5 °C; the same reaction would proceed much faster at ambient temperature ($k_{3,0}^{app} = 1.9$ s^{-1} by stopped flow at 20 °C) and not be presumed to involve prolyl isomerization *a priori*. The guanidine dependence of τ_3 is readily rationalized by the same arguments as those for τ_2, namely that the reaction involves structure-forming reactions coupled to proline isomerization. This is supported by the results of hydrogen exchange studies which demonstrate the presence of structured intermediates early in the folding reaction (Jacobs & Fox, 1994).

The small variation in k_{cat}/K_m of PPI for phases $\tau_3-\tau_1$ suggests differences in the accessibility of PPI to prolines within partially folded intermediates. The differences in accessibility may be static, or they may reflect differences in the stability of kinetically trapped intermediates (Veeraraghavan & Nall, 1994; Veeraraghavan et al., 1995). The coupling between proline isomerization and denaturant-sensitive structure in the observed faster phases (τ_2 and τ_3) probably reflects situations where the non-native proline isomer hinders the correct interaction between two regions of the molecule and thus slows conversion to the next intermediate on the folding pathway (Nakano et al., 1993). It is unclear if the three phases observed in fluorescence-detected refolding experiments correspond to three parallel pathways, emanating from three different conformations of the unfolded state (Nakano et al., 1993), arising from different degrees of proline isomerization or to successive intermediates along a sequential folding pathway (Chen et al., 1992a). However, the effects of PPI are most consistent with a mechanism involving parallel pathways.

The refolding kinetics for NCA S28G SNase mutant are biphasic (Antonio, 1991), and the slower phase (τ_1) is insensitive to Gdn.HCl. It also has a high activation energy (22 kcal/mol) consistent with a reaction involving proline *cis*−*trans* isomerization. Surprisingly, however, neither this phase (τ_1) nor the faster phase (τ_2) of this mutant is catalyzed by PPI. This indicates that, if the phases are linked to proline

Table 2: Catalytic Efficiencies of PPI toward the Fluorescence-Detected Slow Refolding Phases of Staphylococcal Nuclease[a]

	$k_{i,0}^{app}$ (s^{-1})			$k_{i,max}^{app}$ [b] (s^{-1})			$k_{i,0}^{app}/k_{i,max}^{app}$			$(k_{cat}/K_m)_i$ x 10^{-4} (M^{-1} s^{-1})		
protein	$i = 1$[c]	$i = 2$	$i = 3$	$i = 1$	$i = 2$	$i = 3$	$i = 1$	$i = 2$	$i = 3$	$i = 1$	$i = 2$	$i = 3$
SNase (wild type)	0.001	0.006	0.060	0.009	0.034	0.100	9	5	2	1.0	1.6	2.0
P117G SNase	−	0.001	0.070		0.025	0.100	−	25	1.5	−	2.0	1.8
NCA S28G SNase	0.001	0.013	−	0.001	0.013	−	1	1	−	−	−	−

[a] The rate of slow folding in the absence of PPI is equal to $k_{i,0}^{app}$. Values of k_{cat}/K_m are described by the slopes between 0 and 0.8 μM PPI and reflect the catalytic efficiency of PPI toward a particular protein substrate. The final concentration of SNase and its mutants was 6 μM. Refolding was initiated by the dilution of a concentrated protein stock solution (~100 or 300 μM) in 3 M Gdn.HCl into 10 mM sodium cacodylate buffer containing sufficient Gdn.HCl at pH 7.0 and 2.5 °C. The final Gdn.HCl concentrations were 0.4 M for wild type and P117G SNase and 0.08 M for NCA S28G SNase. [b] $k_{i,max}^{app}$ refers to the maximum rate of refolding in the presence of a large amount of PPI. [c] *i* refers to the identity of the slow folding phase for which the parameters are described.

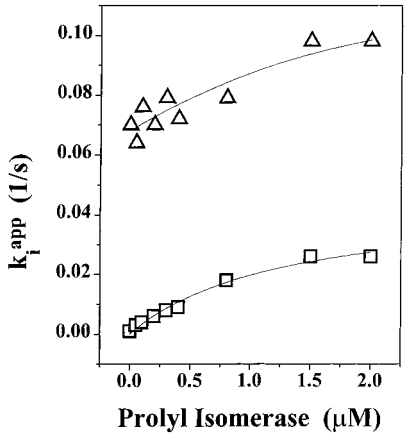

FIGURE 4: Effect of PPI on the slow folding reactions of P117G SNase observed by fluorescence. The curves indicate the general trend in the slow (□) and fast (△) refolding phase data and have no theoretical significance. The catalytic efficiency of PPI was determined from the slope of the plot between 0 and 0.6 μM PPI. Final concentrations of P117G SNase and Gdn.HCl were 6 μM and 0.4 M, respectively, and the PPI concentration was varied. Other conditions were as follows: 10 mM sodium cacodylate buffer, pH 7.0, and 2.5 °C.

cis−*trans* isomerization, either the prolines are inaccessible with respect to prolyl isomerase (PPI) or the reaction is complicated by the mixing in of faster folding phases, the rates of which may be limited by structure formation. The X-ray crystallographic structure (Hynes et al., 1989) shows that the final folded conformation of NCA S28G is similar to that of the wild type protein. Thus, the inability of PPI to catalyze the folding reactions of NCA S28G SNase may mean that the structure of the folding intermediates for this mutant differs from those of wild type SNase. An alternative possibility is that processes other than proline isomerization are responsible for the slow folding reactions of this mutant. Since there are no cysteine residues, the most likely alternative is that the mutation strongly destabilizes a key early intermediate in the folding process, and effectively shifts the transition state for folding to a stage much earlier than that for the wild type; i.e. the folding itself is rate-limiting.

The catalytic efficiency of PPI toward the different slow folding phases can be described by the corresponding $(k_{cat}/K_m)_i$ values. The $(k_{cat}/K_m)_i$ values for each phase, determined from the linear (initial) portion of the k_{obs} versus PPI concentration curves (Figures 3 and 4), are listed in Table 1. Differences in the catalytic efficiencies may arise from differences in the amino acid residue preceding the critical proline(s) or from the extent of (non-native or native-like) secondary and tertiary structure present near the critical proline. Investigations with unstructured peptides have demonstrated that the specific activity of PPI remains the same regardless of the type of amino acid preceding a critical proline (Harrison & Stein, 1990). The catalytic efficiency of PPI toward a number of protein substrates is on the order of 10^4 M^{-1} s^{-1} (Table 3) and suggests that PPI may also lack sequence specificity for protein substrates. Therefore, the roughly 100−1000-fold difference between k_{cat}/K_m for peptide substrates (~10^7 M^{-1} s^{-1}) and protein substrate (~10^4 M^{-1} s^{-1}) is probably due to differences in accessibility of PPI to the critical prolines. In other words, structural differences (varying degrees of compactness) near the critical prolines in the folding intermediates determine the catalytic efficiency of PPI toward a particular folding reaction. We suggest that differences in the $(k_{cat}/K_m)_i$, for the different folding phases of wild type and P117G SNase, reflect differences in the extent of structure near the critical prolines within the slow folding intermediates. Further, the larger the k_{cat}/K_m value, the less stable the structure near the critical proline residue(s) in the folding intermediate. Support for this hypothesis is provided by measurements of the denaturant dependence of k_{cat}/K_m for PPI-catalyzed folding of yeast iso-2-cytochrome *c* (Veeraraghavan & Nall, 1994; Veeraraghavan et al., 1995).

Table 3: k_{cat}/K_m Values for Slow Folding Reactions of Various Proteins[a]

substrate	k_{cat}/K_m (M^{-1} s^{-1})	reaction conditions
succinyl-AAPF-*p*NA (peptide)	1×10^7	0 M Gdn.HCl, pH 8, and 10 °C
immunoglobulin light chain[b]	4.5×10^4	0.25 M urea, pH 7.8, and 10 °C
chymotrypsin inhibitor 2[c]		0 M Gdn.HCl, pH 8, and 25 °C
slow phase	2.8×10^5	
fast phase	9.7×10^5	
RNase A, S-protein fragment[d]	2.9×10^2	0.25 M urea, pH 8, and 10 °C
porcine pancreatic RNase[d]	9.4×10^2	0.25 M urea, pH 8, and 10 °C
RNase T1[e]		0.05 M urea, pH 5.9, and 11 °C
τ_2 phase	1.2×10^4	
τ_1 phase	5×10^2	
type III collagen[f]	5.4×10^2	0.2 M NaCl, pH 7.5, and 25 °C
yeast iso-2-cytochrome *c*[g]		0.3 M Gdn.HCl, pH 6.0, and 20 °C
fluorescence-detected phase	1.1×10^4	
absorbance-detected phase	4.9×10^3	

[a] Values were calculated from data available in the literature. [b] Lang and Schmid (1988). [c] Jackson and Fersht (1991). [d] Lang et al. (1987). [e] Lin et al. (1988). [f] Bächinger (1987). [g] Veeraraghavan and Nall (1994).

CONCLUSIONS

Prolyl isomerase catalyzes the three fluorescence-detected slow folding phases of wild type SNase with catalytic efficiencies $[(k_{cat}/K_m)_i]$ of up to 2×10^4 M^{-1} s^{-1}. This is similar to the catalytic efficiency of PPI toward other protein substrates. The slow refolding phase of the P117G SNase mutant is also catalyzed with similar catalytic efficiency. We conclude that each of these phases arises from proline *cis*−*trans* isomerization in the unfolded state. Under the conditions of the experiments, the slow folding reaction NCA S28G mutant of SNase is not catalyzed by PPI, and hence cannot be ascribed to proline isomerization.

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