Unusually Slow Denaturation and Refolding Processes of Pyrrolidone Carboxyl Peptidase from a Hyperthermophile Are Highly Cooperative: Real-Time NMR Studies

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ABSTRACT: The refolding rate of heat-denatured cysteine-free pyrrolidone carboxyl peptidase (PCP-0SH) from *Pyrococcus furiosus* has been reported to be unusually slow under some conditions. To elucidate the structural basis of the unusually slow kinetics of the protein, the denaturation and refolding processes of the PCP-0SH were investigated using a real-time 2D $^{1}H-^{15}N$ HSQC and CD experiments. At 2 M urea denaturation of the PCP-0SH in the acidic region, all of the native peaks in the 2D HSQC spectrum completely disappeared. The conformation of the PCP-0SH just after removal of 6 M GuHCl could be observed as a stable intermediate (D_1 state) in 2D HSQC and CD experiments, which is similar to a molten globule structure. The D_1 state of the PCP-0SH, which is the initial state of refolding, corresponded to the state at 2 M urea and seemed to be the denatured state in equilibrium with the native state under the physiological conditions. The refolding of PCP-0SH from the D_1 state to the native state could be observed to be highly cooperative without any intermediates between them, even if the refolding rate was quite slow. In the higher concentration of denaturants, PCP-0SH showed HSQC and CD spectra characteristic of completely unfolded proteins called the D_2 state. The unusually slow refolding rate was discussed as originating in the conformations in the transition state and/or the retardation of reorganization in an ensemble of nonrandom denatured structures in the D_1 state.

Proteins obtained from hyperthermophiles, which can grow preferentially at extremely high temperatures near the boiling point of water, have been known to show remarkably high stability. To elucidate the mechanism of the extremely high stability of the proteins, the three-dimensional structures for many proteins have been determined, and the factors responsible for the high stability have been discussed (*1*, *2*). However, it is unknown whether a common mechanism responsible for the extra stability of hyperthermophile proteins exists. It is a challenging but difficult problem because protein conformations are only marginally maintained by many positive and negative factors for stabilization. The denaturation Gibbs energy (ΔG) of globular proteins is around 50 kJ/mol, corresponding to that of only a few hydrogen bonds (*3*). Hyperthermophile proteins seem to be also marginally stable under their physiological conditions near 100 °C (*4*).

The thermodynamic and kinetic characteristics of hyperthermophile proteins should be analyzed in detail to elucidate the stabilization mechanism. A considerable amount of data is now available on the unfolding kinetics and indicates the propensity of hyperthermophile proteins to denature with a highly slow rate constant limited by the high kinetic barrier (*5*−*14*). However, the thermodynamic characterization of hyperthermophile proteins has been hampered by the propensity of these proteins to undergo irreversible thermal denaturation except for a few successful reports (*15*−*19*). In the case of cysteine-free pyrrolidone carboxyl peptidase (PCP-0SH)[1] from a hyperthermophile, *Pyrococcus furiosus*, the thermal denaturation is reversible under some conditions (*19*, *20*), and the thermodynamic and kinetic properties are extensively analyzed (*19*). The refolding rate of heat denaturation for the PCP-0SH has been reported to be 7.17×10^{-6} s^{-1} (τ = 38.7 h) at 25 °C and 1.2×10^{-6} s^{-1} (τ = 231.5 h) at 18 °C, depending on the temperatures. This slow refolding rate compensates unusually slow denaturation rates, and it results in the appropriate equilibrium stability for the hyperthermophile protein at high temperatures. For understanding high stabilities of the protein, it is important to elucidate the mechanism of the slow rate on the structural basis of the PCP-0SH.

NMR spectroscopy is the best method to study the denaturation mechanism and partially denatured states in solution in a residue-specific manner (*21*). Protein folding,

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[1] Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; PCP-0SH, cysteine-free pyrrolidone carboxyl peptidase (Cys142/188Ser); HSQC, heteronuclear single-quantum coherence; GuHCl, guanidine hydrochloride.

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however, is generally very rapid and is difficult to be studied by measuring the real-time change in the NMR spectra. Therefore, the PCP-0SH with unusually slow relaxation kinetics of denaturation and refolding is a good sample to study protein folding by directly monitoring the individual amino acid residues using a real-time NMR method.

The pyrrolidone carboxyl peptidase (PCP) (E.C. 3.4.19.3) from a hyperthermophile, *P. furiosus*, catalyzes the cleavage of L-pyroglutamic acid from the N-terminus of polypeptide chains (*22*). The PCP is a homotetramer constituted of subunits with 208 residues (MW = 22800). The PCP contains two cysteines per subunit at positions 142 and 188. Because Cys188 partly forms an intersubunit disulfide bond, cysteine-free PCP has been used for refolding studies (*19*). The PCP-0SH (Cys142/188Ser) exists as a tetramer above pH 4.5, a dimer around pH 3.0, and a monomer below pH 2.7 and reversibly denatures in dimer or monomer forms (*20*). Therefore, the PCP-0SH in a monomer form provides an excellent system to study the kinetics and thermodynamics of the denaturation and refolding of a hyperthermophile protein.

In this paper, the denaturation and refolding processes of a PCP-0SH monomer were examined using real-time measurements of 2D ^{1}H−^{15}N HSQC and CD spectra in order to elucidate the structural basis of the unusually slow kinetics of the protein.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. Nonlabeled PCP-0SH was expressed in the *Escherichia coli* strain JM109/pPCP3022 cloned PCP-0SH gene and purified as described (*20*). Uniformly ^{15}N-labeled protein ([^{15}N]PCP-0SH) was made by incorporating ^{15}N-labeled $(NH_4)_2Cl_2$ as the sole nitrogen source in the M9 growth medium, using the same *E. coli* strain. Selectively [^{15}N]Leu-labeled protein ([^{15}N]-LeuPCP-0SH) was prepared by incorporating [^{15}N]Leu in a growth medium containing a mixture of the other unlabeled amino acids, using the same strain. Labeled proteins were also purified as described (*20*). The purified protein showed a single band on SDS−polyacrylamide gel electrophoresis. The protein concentration was determined using the absorption coefficient of 0.66 $(mg/mL)^{-1}$ with a cell of 1 cm light path length at 278 nm (*12*). Urea and guanidine hydrochloride (GuHCl) from Nacalai Tesque (Kyoto, Japan) were specially prepared reagent grade and were used without further purification. Other chemical reagents used were of analytical grade.

Sample Preparation for Refolding Experiments. Heat-denatured PCP-0SH hardly recovers at 4 °C and pH 2.4, although it completely does so at 32 °C (*19*). Therefore, samples for refolding experiments were prepared as follows: (1) PCP-0SH, [^{15}N]PCP-0SH, or [^{15}N]Leu PCP-0SH was incubated overnight in the presence of 6 M GuHCl in the 20 mM Gly buffer of pH 2.0 at 30 °C. (2) GuHCl was removed by gel filtration through a PD-10 column (Amersham Biosciences Corp.) in the 20 mM Gly buffer of pH 2.4 at 4 °C, where the denatured protein hardly recovers even after a 10 day incubation (*19*). For the experiment of the urea concentration dependence of refolding, the PCP-0SH after removal of 6 M GuHCl at 4 °C using a PD-10 was used as a denatured sample.

NMR Experiments. NMR spectra were recorded at 600.13 MHz on a Bruker Avance 600 DRX spectrometer equipped with a Bruker/SGI workstation. The two-dimensional ^{1}H−^{15}N HSQC (*23*) spectra were collected at various temperatures with 256 × 1024 points in t_1 and t_2 directions, using uniformly ^{15}N-labeled and selectively [^{15}N]Leu-labeled proteins. It took 39−160 min to record ^{1}H−^{15}N HSQC spectra. A volume of each cross-peak was calculated for peak intensity using Felix 98.0 software (Biosym/MSI).

Kinetic data were analyzed by the least-squares method to determine the rate constant using the equation

$$Y(t) = Y_o + \sum A_i e^{-k_i t} \quad (1)$$

where $Y(t)$ is the signal at any time (t), Y_o is the signal value when no further change is observed, k_i is the apparent rate constant, and A_i is the total amplitude of the ith kinetic phase. Nonlinear, least-squares calculation was carried out using Origin software (OriginLab).

CD Measurements. CD spectra were measured with a Jasco-J600 spectropolarimeter equipped with an NEC personal computer at 5 °C. Far- and near-UV CD spectra were scanned 16 times at a scan rate of 20 nm/min, using a time constant of 0.25 s. For the calculation of the mean residue ellipticity, the mean residue weight was assumed to be 109.3. The denaturation and refolding were also monitored by measuring the CD at 222 nm and at a given temperature after incubation for the desired time at different temperatures.

RESULTS

Uniformly ^{15}N-Labeled Protein. ^{1}H−^{15}N HSQC spectra were examined to monitor global structural changes during denaturation and refolding of the PCP-0SH from a hyperthermophile with unusually slow relaxation kinetics (*19*). Figure 1a shows the ^{1}H−^{15}N HSQC spectrum of [^{15}N]PCP-0SH in the native state at pH 2.4, in which observed cross-peaks are well dispersed along the ^{1}H resonance frequency except for the central part. This spectrum is characteristic of the protein closely folded. The resonance assignment of these peaks to specific residues has not yet been accomplished.

To follow the denaturation process of the PCP-0SH, the time course of ^{1}H−^{15}N HSQC spectra was recorded in the presence of 2 M urea solution at pH 2.4 and 30 °C. The spectra acquired at 3.0, 26.2, and 109.8 h after the initiation of denaturation are shown in panels b, c, and d of Figure 1, respectively. The spectrum at 3 h (Figure 1b) was quite similar to that of the native one (Figure 1a). The intensity of the well-dispersed cross-peaks substantially decreased after 26.2 h, and nativelike resonance peaks almost disappeared. Many sharp resonance peaks characteristic of denatured protein appeared at 109.8 h.

The denaturation process of the protein was followed in a residue-specific manner by monitoring the change in peak volumes of well-separated cross-peaks observed on ^{1}H−^{15}N HSQC spectra. Panels a and b of Figure 2 show the time-dependent changes in the peak volume for two representative cross-peaks boxed in Figure 1, panels b and d; one is the cross-peak originating from the residue in the native state and the other in the denatured state. Residue-specific relaxation times (τ) were determined by fitting the changes in volume as a function of time to a single-exponential decay

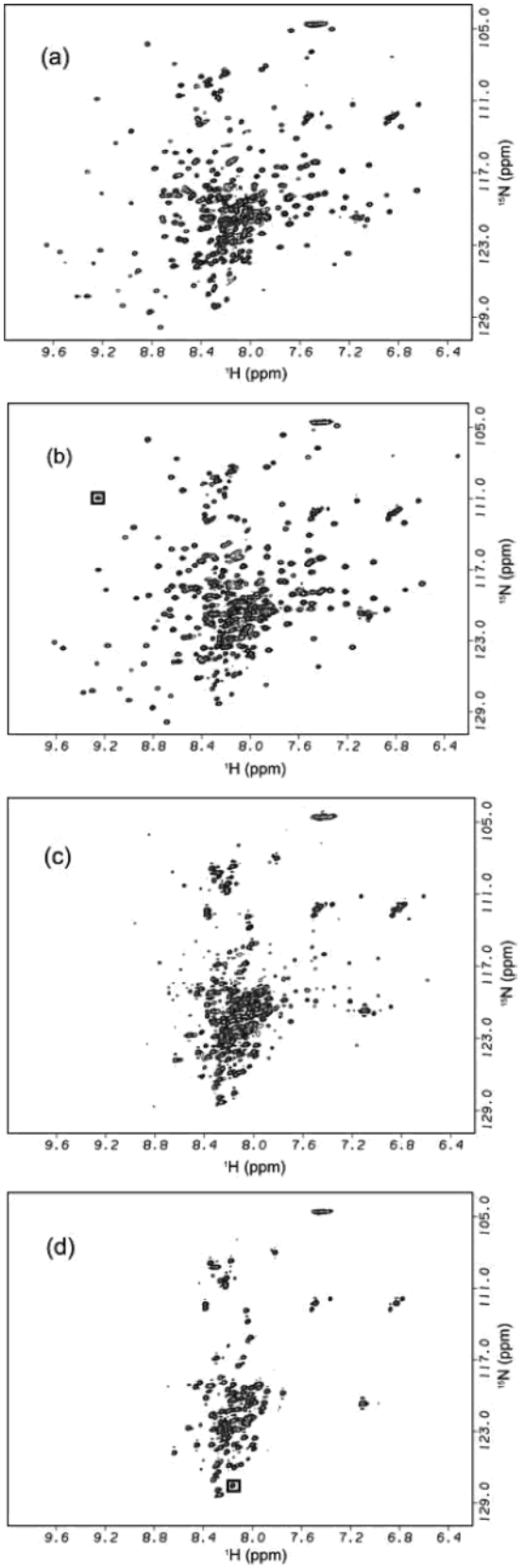

FIGURE 1: ^{1}H−^{15}N HSQC spectra of denaturation of the [^{15}N]PCP-0SH at pH 2.4 and 30 °C. (a) shows the spectrum in the native state. The time courses of the [^{15}N]PCP-0SH after incubation for 3.0, 26.2, and 109.8 h in the presence of 2 M urea are shown in (b), (c), and (d), respectively. These hours after the initiation of denaturation mean the half-point of NMR acquisition time. A cross-peak boxed in (b) or (d) was used as a representative one to follow time-dependent changes, as shown in (a) or (b) of Figure 2, respectively.

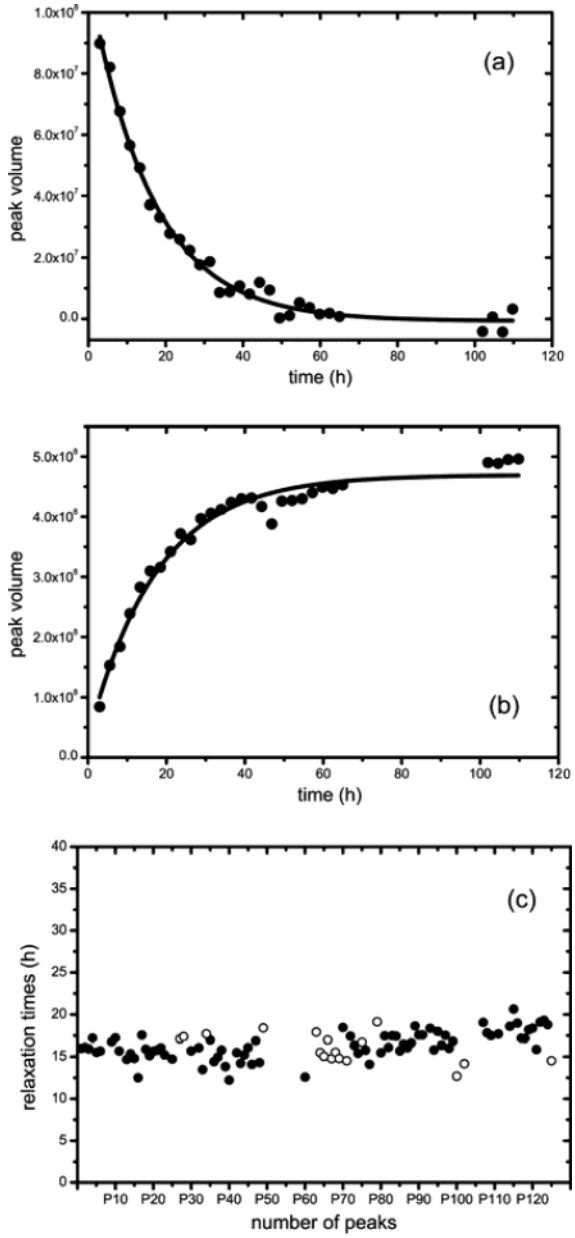

FIGURE 2: Time-dependent changes in the NMR data of individual residues of the PCP-0SH on 2 M urea denaturation at pH 2.4 and 30 °C. (a) A cross-peak boxed in Figure 1b was used as a representative residue from native state peaks. The relaxation time, τ, was 16 ± 1 h. (b) A cross-peak boxed in Figure 1d was used as a representative residue from denatured peaks. τ was 17 ± 1 h. (c) Relaxation times are of 95 representative residues; closed circles represent data from the native peak and open circles from the denatured peak. An average value of τ from native and denatured peaks was 16 ± 2 and 16 ± 2 h, respectively.

curve (eq 1). The average relaxation times of 78 well-separated cross-peaks in the native state and 17 peaks in the denatured state were 16 ± 2 and 16 ± 2 h, respectively (Figure 2c), indicating that all of the measured values are the same within experimental error. This means that the denaturation of protein under these conditions is highly cooperative.

After the [^{15}N]PCP-0SH was completely denatured in 6 M GuHCl at pH 2.0 and 30 °C, the denaturant was removed by gel filtration using a PD-10 equilibrated with 20 mM Gly buffer at pH 2.5 and 4 °C, where denatured PCP-0SH substantially does not refold (*19*). An HSQC spectrum just

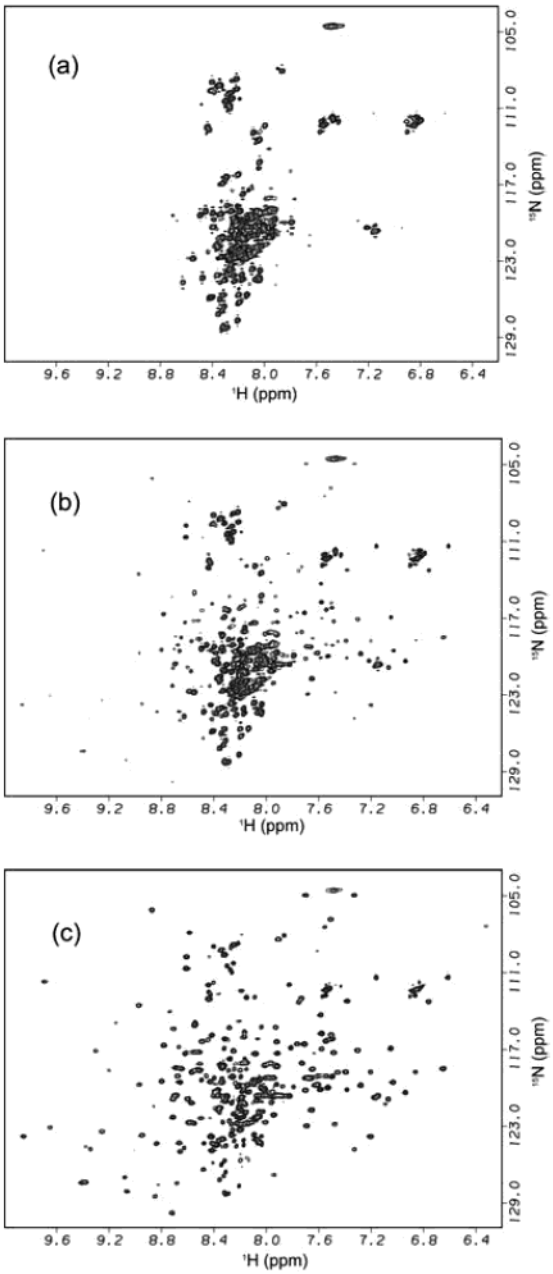

FIGURE 3: ^{1}H−^{15}N HSQC spectra recorded during refolding of the [^{15}N]PCP-0SH at pH 2.5 and 30 °C. (a), (b), and (c) represent the spectra at 1.1, 6.3, and 37.9 h, respectively, after the initiation of refolding. Refolding experiments were performed as described in Experimental Procedures.

after the initiation of refolding at 30 °C shows the degeneration of cross-peaks characteristic of native proteins (Figure 3a). It took 1.1 h until the midpoint of the NMR acquisition time after the initiation of refolding at 30 °C. It took 60 min to accomplish one NMR measurement. Cross-peaks in the native state began to appear in a spectrum measured at 6.3 h after the initiation of the refolding (Figure 3b). The spectrum of the folded protein was observed after 37.9 h as shown in Figure 3c. The refolding rate constants of individual residues were calculated from the time course of volumes of well-separated cross-peaks; the average relaxation times obtained from native peaks and denatured peaks were 7 ± 1 and 6.4 ± 0.3 h, respectively. These results suggest that the refolding process of the PCP-0SH is also highly cooperative between the native and denatured states as well as the denaturation process previously mentioned. The relaxation times of the PCP-0SH are comparable to those of the refolding from the thermally denatured state, which has been measured by calorimetric studies to be 10.6 h at pH 2.3 and 30 °C (*19*). This suggests that the initial structure of refolding in this study is similar to that in the heat-denatured state.

Selectively [^{15}N]Leu-Labeled Protein. Many resonance peaks were crowded into the central part of the ^{1}H−^{15}N HSQC spectrum of the uniformly ^{15}N-labeled PCP-0SH, as shown in Figure 1a. As a result, it was difficult to distinguish the native cross-peaks from cross-peaks originating from the denatured state or a transient intermediate state, if it does exist. To simplify the 2D spectra and to minimize the problems of signal overlap, the denaturation and refolding of the PCP-0SH were monitored by means of selective ^{15}N-labeling of Leu. The 16 Leu residues of PCP-0SH are located in distinctive regions with different types of secondary structures. As shown in Figure 4a, 15 resonance peaks in an HSQC spectrum of the [^{15}N]Leu-labeled PCP-0SH were well-separated, but two Leu residues overlapped each other as a single resonance peak. The relaxation rates of denaturation were examined using these probes. The values obtained were quite similar to those from uniformly ^{15}N-labeled PCP-0SH (not shown).

^{1}H−^{15}N HSQC spectra recorded during the refolding of the [^{15}N]Leu-labeled PCP-0SH are shown in Figure 4b−d. The native cross-peaks were not observed at 1.5 h just after the initiation of refolding, and only six cross-peaks were clearly observed (Figure 4b). Other resonance peaks are broadening, indicating slow conformational fluctuations in the structural ensemble. This is similar to NMR spectra observed for molten globule states (*24*). As shown in Figure 4c,d, native cross-peaks gradually appear depending on the time, but no resonance peak except for ones from the native and denatured states was observed, indicating that there is no intermediate state between them and that the native and denatured states are in slow exchange on the NMR time scale.

To compare the initial non-native state of the PCP-0SH in its refolding reaction with the completely denatured state in highly concentrated denaturants, the ^{1}H−^{15}N HSQC spectra of [^{15}N]Leu-labeled PCP-0SH were measured in various concentrations of urea (Figure 5). After a long incubation at 30 °C in 1.5 M urea, a few native resonance peaks remained with unchanged chemical shifts (Figure 5a). In 2.0 M urea, the native cross-peaks completely disappeared (Figure 5b), and the HSQC spectra were quite similar to those measured immediately after the initiation of refolding (Figure 4b). In 3.0 M urea, the spectrum was still similar to that in 2.0 M urea (Figure 5c), but in 7.1 M urea, 16 resonance peaks clearly isolated were sharp and intense (Figure 5d), indicating NMR signals characteristic of a completely unfolded protein. These results indicate that the initiation state of refolding for the PCP-0SH is quite similar to that in 2.0 M urea at pH 2.4, which is not in a fully unfolded state as in highly concentrated denaturants but in a non-native one as a molten globule.

CD Experiments. To examine the structural characteristics of the PCP-0SH in the initial state of refolding, CD spectra were measured in far- and near-UV regions. The near-UV spectrum immediately after the removal of the denaturant at 5 °C and pH 2.5 shows the loss of the tertiary structure

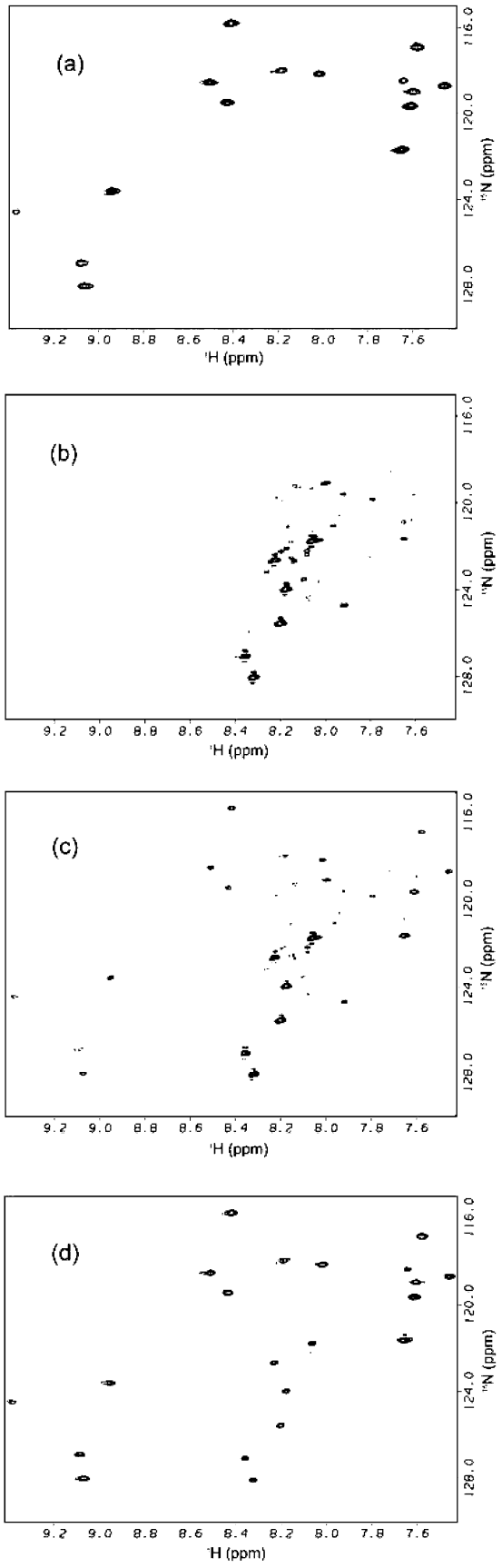

FIGURE 4: ^{1}H−^{15}N HSQC spectra recorded during refolding of [^{15}N]Leu-labeled PCP-0SH at pH 2.5 and 30 °C. (a) shows [^{15}N]-Leu-labeled PCP-0SH in the native state at pH 2.5; (b), (c), and (d) represent the spectra at 1.5, 4.9, and 59.6 h, respectively, after the initiation of refolding at 30 °C.

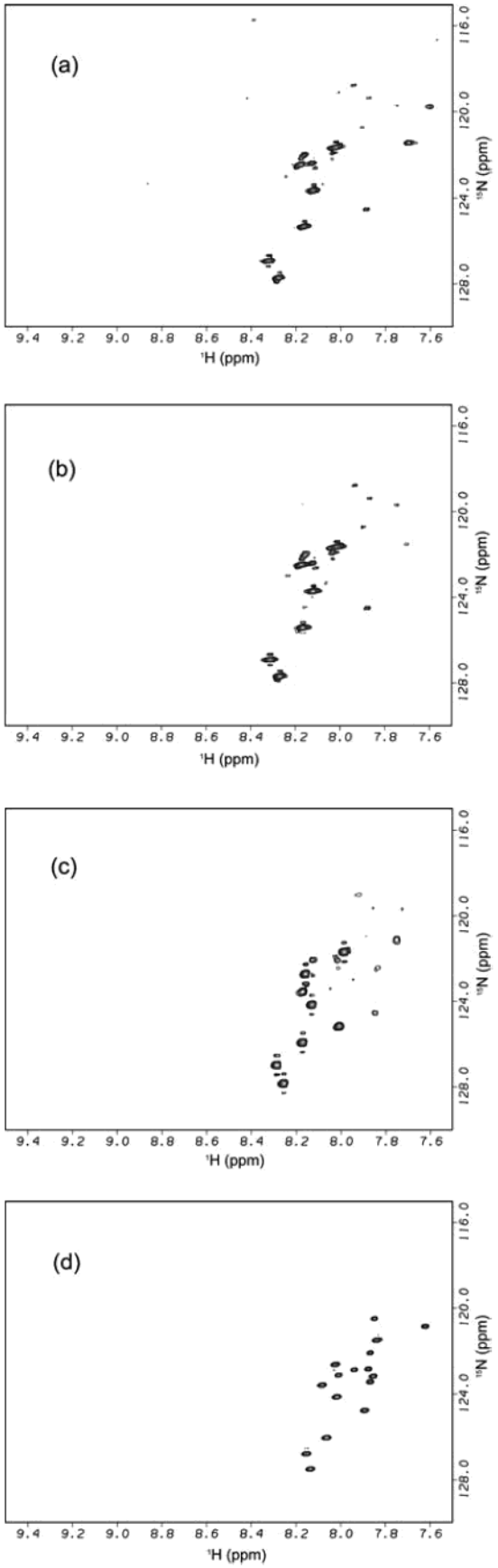

FIGURE 5: ^{1}H−^{15}N HSQC spectra of [^{15}N]Leu-labeled PCP-0SH in various concentrations of urea at pH 2.4 and 30 °C: (a) after 555 h in 1.5 M urea at pH 2.4, (b) after 424 h in 2.0 M urea at pH 2.4, (c) after 25.7 h in 3.0 M urea, and (d) after 20 h in 7.1 M urea.

judged from the CD spectrum originating from aromatic side chains (Figure 6b). This coincides with NMR data that show no resonance peak originating from the native conformation. However, judged from the spectrum in the far-UV region (Figure 6a), a fairly high content of secondary structures seems to be already restored in this state. These results indicate that the conformation of the PCP-0SH in the initial state of refolding is characteristic of a molten globule.

Urea-induced equilibrated denaturation and refolding of the PCP-0SH were monitored using the ellipticity of CD spectra at 222 nm. Refolding experiments were carried out after a 2 day incubation of the protein at 30 °C and a given concentration of urea (Figure 7) after removal of 6 M GuHCl with a PD-10 column. The urea-concentration-dependent curves of refolding seem to be biphasic. The transition of the first step is highly cooperative around 1.2 M urea, but the second step gradually changes above 2 M urea. The second phase should correspond to the transition from a molten globule-like state to the completely denatured state, because the ellipticity at 222 nm in 2 M urea is similar to that of the PCP-0SH in an initial state of refolding. Furthermore, the observation that the HSQC spectrum in equilibrium at 2 M urea contained no cross-peak in the native state strongly suggests that the equilibrium state at 2.0 M urea is not the mixture of the native and fully denatured proteins.

Assuming a three-state denaturation by urea, the Gibbs energy change (ΔG^{H_2O}) of denaturation for the PCP-0SH in water was evaluated according to the equation (*25*):

$$f_{app} = [\exp(-(\Delta G_{D1}{}^{H_2O} + m_1[C])/RT)\exp(-(\Delta G_{D2}{}^{H_2O} + m_2[C])/RT) + \alpha\exp(-(\Delta G_{D1}{}^{H_2O} + m_1[C])/RT)]/[1 + \exp(-(\Delta G_{D1}{}^{H_2O} + m_1[C])/RT)\exp(-(\Delta G_{D2}{}^{H_2O} + m_2[C])/RT) + \exp(-(\Delta G_{D1}{}^{H_2O} + m_1[C])/RT)] \quad (2)$$

where f_{app} represents the apparent fraction of denaturation and m the slope of the linear correlation between ΔG and urea concentrations [C]. Subscripts D1 and D2 mean changes between the native and intermediate states and between the intermediate and denatured states, respectively. α is the fraction of the total changes in $[\theta]_{222}$ that occurs in going from the native to intermediate states. The f_{app} in eq 2 is represented as a function of urea concentration [C]. Therefore, we used Origin software (OriginLab) to give a least-squares fit of the experimental data to eq 2 to obtain $\Delta G_{D1}{}^{H_2O}$ and $\Delta G_{D2}{}^{H_2O}$. The theoretical curve obtained fitted quite well into the experimental data as shown in Figure 7. The estimated parameters were the following: $\Delta G_{D1}{}^{H_2O}$ and $\Delta G_{D2}{}^{H_2O}$, 10 ± 1 and 11 ± 7 kJ mol^{-1}, respectively; m_1 = −11 ± 2 kJ mol^{-1} M^{-1} and m_2 = −5 ± 2 kJ mol^{-1} M^{-1}; α = 0.6 ± 0.2. The value of $\Delta G_{D1}{}^{H_2O}$ at 30 °C was comparable with a reported one estimated from the heat denaturation (*19*). The values of a parameter, *m*, suggest that the cooperativity of the second step is weaker than that of the first step.

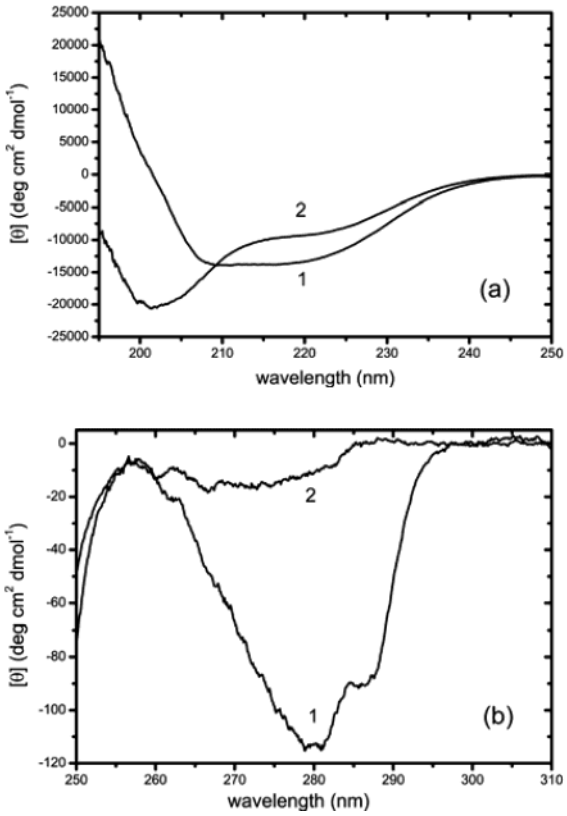

FIGURE 6: CD spectra of the PCP-0SH in the far- (a) and near- (b) UV region at 5 °C and pH 2.5. Curves 1 and 2 represent the spectra of the PCP-0SH in the native state and just after the initiation of refolding at pH 2.5, respectively.

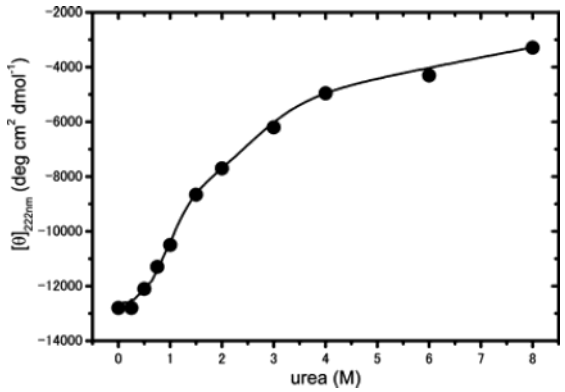

FIGURE 7: Refolding curve of the PCP-0SH as a function of urea concentration at pH 2.4 and 30 °C. Closed circles represent refolding data after 2 day incubation of the denatured sample in the presence of a given concentration of urea at 30 °C just after the removal of 6 M GuHCl. A solid curve represents a theoretical curve of urea denaturation for the PCP-0SH using eq 2. The obtained parameters were as follows: $\Delta G_1{}^{H_2O}$ and $\Delta G_2{}^{H_2O}$ were 9.6 and 10.5 kJ mol^{-1}, respectively; m_1 = −10.6 kJ mol^{-1} M^{-1} and m_2 = −4.6 kJ mol^{-1} M^{-1}; α = 0.57.

DISCUSSION

Real-Time NMR Studies. NMR experiments can provide detailed structural information at the resolution of each residue about protein denaturation and refolding. Since denaturation reactions sometimes take place over hours, denaturation kinetics has been monitored by real-time NMR techniques (*26*). On the other hand, it was difficult to study the refolding of denatured proteins by direct real-time NMR experiments, because the refolding rate is very fast in most cases. Some specific types of folding reactions are very slow due to the requirement of the prolyl cis−trans isomerization. Therefore, the real-time 2D NMR spectroscopy on refolding of proline mutant ribonuclease T1 (S54G/P55N) with only one cis Pro39 has been investigated (*27*) and explained that the entire region of the protein follows cooperatively the rate-limiting prolyl isomerization because all analyzed resonances

follow the same exponential kinetics within experimental error. Recently, Mizuguchi et al. (*28*) have reported that the folding of apoplastocyanin, initiated by the addition of salt and followed with the real-time 2D $^{1}H-^{15}N$ HSQC spectroscopy, is highly cooperative. The refolding of this protein from a denaturant-induced denatured state is known to be also slow due to the requirement for the isomerization of two proline residues from trans to cis at positions 16 and 35 (*29*).

The PCP-0SH has one cis proline at position 159 out of 16 proline residues. Although the effect of its cis proline on protein folding will be discussed later, the refolding rate of the PCP-0SH has been reported to be unusually slow depending on temperatures: the relaxation time of refolding is 38.7 h at 25 °C, but the refolding scarcely progresses at 4 °C (*19*). This large temperature dependence of the refolding rates is unlikely to suggest that the refolding rate is exclusively limited by the isomerization of the trans proline to the cis form.

This protein with unusually slow relaxation times was quite suitable to follow the refolding process using real-time 2D $^{1}H-^{15}N$ HSQC spectroscopy as shown in Figures 3 and 4. In present studies the refolding rate constants of individual residues could be calculated from the time course of volumes of well-separated cross-peaks. All of the measured relaxation times from native and denatured peaks were almost the same within experimental error, suggesting that the refolding process of the PCP-0SH is highly cooperative between the native state and denatured states. Although most cross-peaks of the $^{1}H-^{15}N$ HSQC spectrum of uniformly ^{15}N-labeled proteins were not well dispersed in a central part of the spectrum, almost all cross-peaks of selectively [^{15}N]Leu-labeled proteins were well isolated. This sample was quite suitable to determine whether there is any intermediate in a refolding process. All the events of the denaturation and refolding process of the PCP-0SH could be observed in real time in 2D $^{1}H-^{15}N$ HSQC spectroscopy.

An Initial State of Refolding of the PCP-0SH. The real-time 2D NMR experiments of the PCP-0SH clearly showed that the denaturation in 2 M urea was a two-state transition and there was no intermediate in the denaturation process. The denatured state of the PCP-0SH in 2 M urea was similar to the initial state of refolding just after the removal of 6 M GuHCl. The HSQC spectrum in 2 M urea (Figures 1d and 5b), which was quite different from that of a completely denatured state in 7.1 M urea (Figure 5d), was similar to that of the so-called molten globule state reported. As shown in Figure 7 the urea-induced denaturation curves showed two phases including an intermediate in an equilibrium state. The first transition step from the native to the stable intermediate state was highly cooperative near 1.2 M urea. The stable intermediate seems to correspond to the initial state of refolding just after the removal of the concentrated denaturant, because the NMR and CD spectra observed in the initial state of refolding were similar to those observed in an equilibrium state at 2 M urea. The denaturant-induced denaturation of the tryptophan synthase α-subunit has been reported to show a two-phase transition with a stable intermediate: the first phase is highly cooperative with substantial denaturation enthalpy, and the second phase comes from the transition from a molten globule to a highly disordered unfolded state with no denaturation enthalpy (*30*). The α-lactalbumin in the molten globule state also shows no heat transition, that is, no denaturation enthalpy (*31*). Therefore, the PCP-0SH in the presence of 2 M urea should correspond to the thermodynamically completely denatured state, because any nativelike cross-peak was not observed in the 2D HSQC spectrum measured in 2 M urea.

The compact intermediate state with a nativelike secondary structure but with the intramolecular mobility characteristic of proteins losing the tertiary structure has been originally termed a molten globule by Ogushi and Wada (*32*). Such intermediates in the refolding process often accumulate within the first few milliseconds after the removal or dilution of concentrated denaturants. The CD spectra in the far- and near-UV regions of the PCP-0SH just after removal of the denaturant also showed characteristics of a molten globule with a nativelike secondary structure but no tertiary structure (Figure 6). Therefore, we can summarize the denaturation and refolding processes of the PCP-0SH as follows:

$$N \leftrightarrow D_1 \leftrightarrow D_2$$

where N and D_2 represent the native state and completely unfolded state, respectively, of the PCP-0SH. D_1 represents the intermediate state found around 2 M urea, which is likely to correspond to the initial state of refolding just after the removal of the concentrated denaturant. The D_1 state may correspond to the denatured state in equilibrium with the native state under the physiological conditions because no cross-peak, except for those of N and D_1, was detected in both denaturation and refolding processes of the PCP-0SH as shown in Figures 1, 3, and 4.

Recently, increasing interest has focused on the properties of disordered structure or the denatured state, which is the starting point for the folding process (*33*). Generally, it is quite difficult to examine the denatured conformations present under conditions that favor the native state. However, the PCP-0SH with unusually slow refolding rates is a suitable protein for studying the denatured state under the native conditions, which may be the starting point of protein folding in a cell.

Origin of Unusually Slow Relaxation Kinetics of the PCP-0SH. It is important to examine the possible structural features responsible for the unusually slow relaxation kinetics of the unfolding and refolding of the PCP-0SH. Roder and Colon (*34*) have reported that either intrinsically slow conformational steps, such as cis/trans isomerization of peptide bonds, disulfide exchange, and the dissociation of non-native metal ligands, or non-native interactions between stable subdomains can give rise to large kinetic barriers.

In the PCP-0SH there is one cis proline residue at position 159 and no cysteine residue. The X-ray structures of three other kinds of pyrrolidone carboxyl peptidases (PCPs) from two hyperthermophiles, *Thermococcus litoralis* (*Tl*PCP) (*35*) and *Pyrococcus horikoshii* (*Pho*PCP) (*36*), and from a mesophile, *Bacillus amyloliquefaciens* (*Ba*PCP) (*37*), have been solved. The number of proline residues is 15 or 16 in four proteins, indicating that there is no difference in the stability due to proline content. On the other hand, Pro160 of *Tl*PCP and Pro156 of *Pho*PCP corresponding to cis Pro159 of the PCP-0SH are also cis configurations, but the cis Pro residue is not found in *Ba*PCP. When non-native prolyl isomers in the denatured state (in particular, trans isomers

of bonds that are cis in the native state) are present, folding is retarded from the millisecond to the minute time range (*38*). In the case of the PCP-0SH, however, it seems unlikely that only one cis proline residue is responsible for the unusually slow refolding rate such that the refolding hardly progresses at 4 °C (*19*).

When proteins in the denatured state were associated with each other, the refolding rate might be retarded. For the heat-denatured and refolded PCP-0SH, the sedimentation equilibrium analyses have been reported to give the apparent molecular weights of a monomer (*19*), indicating that the heat-denatured PCP-0SH does not aggregate and the protein exists in a monomer form at pH 2.3. Although the association of proteins at the initial state of refolding has not been examined, it cannot be the cause of the very slow relaxation time, because the refolding rates in the present experiments were quite similar to those reported in the refolding of the heat-denatured PCP-0SH (*19*).

In the interior of a PCP-0SH molecule, one polar amino acid, Glu192, has been found (*39*). The environment of Glu192 is completely surrounded by hydrophobic residues. This buried polar residue is conserved in hyperthermophiles, *Tl*PCP (Glu194) and *Pho*PCP (Glu190), in the hydrophobic surroundings, and its three-dimensional arrangement is also similar to that in the PCP-0SH. On the other hand, the corresponding residue of a mesophile, *Ba*PCP, is Ile189, also in the hydrophobic interior. This unfavorable residue, Glu192 of the PCP-0SH, might affect non-native collapsed structures (like a molten globule) in the D_1 state, in which the formation of the hydrophobic core is disturbed by the carboxyl ion of Glu192 when it is ionized. Although cis Pro159 is located far away from Glu192 in the native structure, both residues and/or other unfavorable residue(s), if it does exist, might be involved in non-native collapsed structures and stabilize them in the D_1 state, and they might result in the unusual retardation of the folding rate of PCP-0SH. Therefore, determining the structure in the D_1 state may be a key issue in understanding the mechanism of the unusually slow refolding rate of the PCP-0SH. It has been recently reported using NMR techniques that denatured proteins contain significant nonrandom structures under the denaturing conditions (*40*−*42*).

It has been reported that the folding rates for small two-state proteins are correlated with the contact order, meaning the average separation along the sequence in atomic contacts (*43*). This can explain that the barriers to folding contain significant contributions from entropic factors in the two-state folding of small proteins. On the other hand, in the case of the folding process that involves a rapid initial collapse (intermediates), the barriers to conformational reorganization within the intermediates limit the folding rate (*44*). The collapsed state, which is highly heterogeneous with many conformations, gives rise to complex rearrangement processes with high enthalpy barriers. Therefore, such proteins fold very slowly to the fully native state, relative to those whose compact collapsed states are less stable (*45*). The PCP-0SH in the D_1 state might correspond to the collapsed state with many conformations that have non-native character. In the new view of protein folding, which is referred to as energy landscapes (*46*, *47*), interconversion between conformations in the collapsed state is explained to be slower in the compact states than in more extended

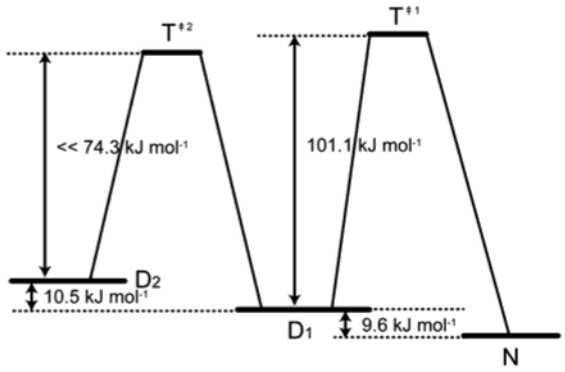

FIGURE 8: Energy coordinate diagrams between any states of the PCP-0SH in the denaturation and refolding process in the absence of denaturants (in water) at pH 2.4 and 30 °C. The values of equilibrium Gibbs energy (ΔG^{H_2O}) between native (N) and denatured (D_1) states and between denatured states D_1 and D_2 were obtained from the refolding curve in Figure 7. The values of activation Gibbs energy ($\Delta G^{\ddagger H_2O}$) were obtained from denaturation and refolding rates. A numeral, 74.3 kJ/mol, in a figure corresponds to the $\Delta G^{\ddagger H_2O}$ value when the relaxation time is assumed to be 1 s; the real value of $\Delta G^{\ddagger H_2O}$ should be smaller because the real value of the relaxation time is faster than 1 s due to the burst phase.

ones (*48*−*50*). In the refolding process of hen lysozyme, it has been known that the tendency to populate intermediates decreases as the temperature is raised (*51*). The fact that the folding rate of the PCP-0SH remarkably decreases as the temperature decreases (*19*) might correlate with the stabilization of non-native structures in the D_1 state of the PCP-0SH resulting in the delay of refolding. The interconversion of intermediates in simulation experiments of a 27-mer lattice model is also delayed by lowering the temperatures (*52*).

Energy Diagram of the Native, Transition, Intermediate (D_1), and Denatured (D_2) States. The value of the rate constants of denaturation and refolding can be converted to the activation Gibbs energy change ($\Delta G^{\ddagger}$) according to the transition state theory (*53*). The activation Gibbs energies in water ($\Delta G^{\ddagger H_2O}$) to the transition state from the native, intermediate, or denatured states can be calculated from the respective rate constant obtained in water using the equation:

$$\Delta G^{\ddagger H_2O} = RT \ln(k_B T/hk^{H_2O}) \qquad (3)$$

where k_B and h are the Boltzmann and Planck constants, respectively. The unfolding rate ($k_u^{H_2O}$) in water of the PCP-0SH could be estimated to be 1.24×10^{-6} s^{-1} from extrapolating to 0 M urea using unfolding rates in various urea concentrations at pH 2.4 and 30 °C. From this unfolding rate ($k_u^{H_2O}$) and the relaxation rate of the refolding experiment directly observed in water ($k_{obs}^{H_2O} = k_f^{H_2O} + k_u^{H_2O}$), the refolding rate in water ($k_f^{H_2O}$) was estimated to be 2.32×10^{-5} s^{-1}. Next, the activation Gibbs energy, $\Delta G^{T\ddagger 1-D1}$, from the D_1 to the transition states ($T^{\ddagger 1}$), and $\Delta G^{T\ddagger 1-N}$ from N to $T^{\ddagger 1}$ were calculated using the $k_f^{H_2O}$ and $k_u^{H_2O}$ to be 101.1 and 108.3 kJ/mol, respectively. The difference between two activation Gibbs energies, $\Delta G^{T\ddagger 1-N} - \Delta G^{T\ddagger 1-D1}$, is 7.2 kJ/mol and close to the change in Gibbs energy between D_1 and N states, $\Delta G_{D1}^{H_2O} = 9.6$ kJ/mol obtained from the equilibrium refolding experiments shown in Figure 7. This suggests that the analysis of equilibrium and kinetic data is entirely consistent with each other. The energy diagram between any two states is shown in Figure 8. Because the transition from D_2 to $T^{\ddagger 2}$ is a burst phase, the relaxation time

is likely to be much less than 1 s. Therefore, the activation energy from D_2 to $T^{\ddagger 2}$ should be $\ll$74.3 kJ/mol (the value $\Delta G^{\ddagger H_2O}$ of 74.3 kJ/mol corresponds to a relaxation time of 1 s) as shown in the diagram of Figure 8. Using this diagram we can propose the following mechanism for the PCP-0SH folding: The PCP-0SH in the D_1 state is an ensemble of nonrandom structures with high helical content, corresponding to the denatured state that is in equilibrium with the native state under the physiological conditions. Just after the removal of concentrated denaturants, proteins in the D_2 state rapidly move into the D_1 state, a burst phase upon the dilution of denaturants. The PCP-0SH in the D_1 state under physiological conditions (in water) needs a long time to reorganize its conformation and to reach the transition state, $T^{\ddagger 1}$, so that the refolding rate remarkably decreases. The transition state ($T^{\ddagger 1}$) of the PCP-0SH has been reported to be as compact as the native state (*19*). Therefore, it might be a reason for the unusually slow refolding that the formation of the transition state of the PCP-0SH takes place late in the folding process due to the specific conformation in the transition state ($T^{\ddagger 1}$) and/or the delay of reorganization among an ensemble of nonrandom structures in the denatured state (D_1).

CONCLUSIONS

Present real-time 2D NMR experiments of the PCP-0SH from a hyperthermophile show that the denaturation and the refolding of the protein are highly cooperative without any intermediates between the native and the denatured (D_1) states even if the relaxation rates are unusually slow. This means that the PCP-0SH in water (under physiological conditions) might be in equilibrium between the native and D_1 states, and the folding rate is remarkably slow because the barriers to structural reorganization within the D_1 state are quite high. Therefore, elucidating the nature of the denatured state in equilibrium with the native state under physiological conditions is a key issue in understanding the mechanism of the thermostabilization of this hyperthermophile protein.

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