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Identification of formation of initial native structure in onconase from an unfolded state

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Abstract

In the oxidative folding of onconase, the stabilization of intermediates early in the folding process gives rise to efficient formation of its biologically active form. To identify the residues responsible for initial formation of structured intermediates, the transition from an ensemble of unstructured three-disulfide species, 3S_U, to a single structured three-disulfide intermediate species, des-[30–75] or 3S_F, at pH 8.0, 25 °C was examined. This transition was first monitored by far-UV CD spectroscopy at pH 8.0, 25 °C, showing that it occurs with formation of secondary structure, presumably due to native interactions. The time-dependence of formation of native-like structure was then followed by NMR spectroscopy after arresting the transition at different times by lowering the pH to 3 and then acquiring ¹H, ¹⁵N – HSQC spectra at pH 3, 16 °C to identify amide hydrogens that become part of native-like structure. H/D exchange was utilized to reduce the intensity of resonances from backbone amide hydrogens not involved in structure, without allowing exchange of backbone amide hydrogens involved in initial structure. Six hydrogen-bonding residues, namely, Tyr38, Lys49, Ser82, Cys90, Glu91, and Ala94 were identified as involved in the earliest detectable native-like structure before complete formation of des-[30–75], and are further stabilized later in the formation of this intermediate through S-S/SH interchange. By observing the stabilization of the structures of these residues by their neighboring residues, the initial, native-like structural elements formed in this transition have been identified, providing details of the initial events in the oxidative folding of onconase.

Introduction

Previous investigations of the oxidative folding pathways of disulfide-bond-containing proteins have shown that the rate of recovery of the biologically active forms of these proteins from a reduced state containing no disulfide bonds is very slow compared to the recovery of these native structures when their disulfide bonds are intact (1, 2). Therefore, the oxidative folding process can be stopped at various times and analyzed to extract kinetic and thermodynamic information about intermediate species formed along the folding pathways. This study takes advantage of the ability to arrest disulfide-bond-dependent folding to determine the initial structure(s) formed during the recovery of native onconase (ONC), Figure 1. In previous studies (3–9), hydrogen/deuterium exchange, monitored by NMR or Mass spectroscopy, was used to analyze different stages of the folding process. In this study, NMR spectroscopy is used to determine the initial native-like structure(s) during disulfide-bond-dependent folding.

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Onconase is a 104-residue, four-disulfide homologue of bovine pancreatic ribonuclease A (RNase A). The following mechanism has been proposed for the oxidative folding of ONC (2):

(1)

where R is an ensemble of reduced onconase species, nS_U is an unstructured ensemble that contains n mostly non-native disulfide bonds, and $\text{des-}[x-y, \dots]$ is a structured species with native disulfide bonds but lacking the $[x-y, \dots]$ disulfide bond(s). The oxidative folding pathway, starting from R, begins with an oxidation to form the unstructured $1S_U$ ensemble. This $1S_U$ ensemble can be oxidized to produce the structured, two-disulfide species, $\text{des-}[19-68, 30-75]$, or the unstructured $2S_U$ ensemble in another pre-equilibrium. $\text{des-}[19-68, 30-75]$ can be oxidized to either $\text{des-}[19-68]$ or $\text{des-}[30-75]$, each of which can be oxidized to the native 104-residue form (N). $\text{des-}[19-68]$ can also be obtained by oxidation of the $2S_U$ ensemble (not shown). Of the pathways from the $1S_U$ ensemble, the oxidation to form $\text{des-}[19-68, 30-75]$ is more favored than the oxidation to the $2S_U$ ensemble. Because of the resulting small production of $2S_U$, no further oxidation to form the unstructured $3S_U$ ensemble from the $2S_U$ ensemble is observed in the oxidative folding pathway under a range of oxidation conditions (2). However, if folding were *initiated* from the unstructured $3S_U$ ensemble, four structured species, $3S_F$, namely, $\text{des-}[19-68]$, $\text{des-}[30-75]$, $\text{des-}[48-90]$, and $\text{des-}[87-104]$, could potentially be produced by S-S/SH interchange in the absence of an oxidizing or reducing agent. However, in the transition from $3S_U$ -to- $3S_F$ for ONC, only one structured species, $\text{des-}[30-75]$, is observed, presumably because it is the most thermodynamically favorable one of the four. This intermediate had been identified from previous work (10) and the formation of $\text{des-}[30-75]$ has been observed previously (11).

The goal of this investigation was to identify the region of onconase in which structure is formed, initially, in the passage from the $3S_U$ ensemble to a single $3S_F$ species, $\text{des-}[30-75]$. NMR spectroscopy was used to investigate the stepwise formation of native-like structure in the $3S_U$ -to- $3S_F$ transition in onconase, by starting with a $3S_U$ ensemble. A sample of $3S_U$ was prepared by reducing native ONC to obtain a maximum amount of only the $\text{des-}[30-75]$ species, purifying it, and reshuffling it in the presence of GdnHCl to a $3S_U$ ensemble by S-S/SH interchange. The progress of the $3S_U$ -to- $3S_F$ transition was followed by reducing the disulfide bonds of unstructured species but not the disulfide bonds of the structured $\text{des-}[30-75]$ product. The increase in the concentration of $\text{des-}[30-75]$ from the $3S_U$ ensemble is driven by formation of native-like structure, and is demonstrated here by the formation of native-like far-UV CD spectra that accompany the increase in the concentration of $\text{des-}[30-75]$, as well as by the appearance of resonances in ^{15}N , ^1H -HSQC spectra that correspond to those of native ONC as this transition proceeds. In addition, S-S-/SH reshuffling in this

transition was stopped at given times by lowering the pH to take advantage of the fact that the reactive species in S-S/SH reshuffling is the thiolate ion, whose concentration can be reduced at low pH by ~5 orders of magnitude by converting the thiolate ions to thiols. By stopping the folding progress before completion in this manner, the system can be analyzed by NMR spectroscopy (^1H , ^{15}N -HSQC). The goal of this investigation is to use NMR to determine the residues responsible for the initial formation of native-like structure in the 3S_F oxidative folding intermediate, des-[30–75], from an unfolded state (3S_U).

Materials and Methods

Materials

The plasmid for frog ONC was provided by R.J. Youle and used in the ^{15}N – enriched expression of ONC with the same protocol used previously (12). As in those methods, 97% enrichment was attained as determined by Mass Spectroscopy.

Stable isotopes (^{15}N ammonium sulfate, d_4 -acetic acid, and D_2O) were purchased from Cambridge Isotopes. All other reagents including Tris, Acetic Acid, and GdnHCl were purchased from Sigma-Aldrich and Fisher, and used without further purification.

Shigemi tubes were used for the NMR experiments, and Millipore concentrators, 3000 MWCO, were used to concentrate the samples for use in the NMR experiments.

Preparation of the 3S_U ensemble of ^{15}N – enriched ONC

A 3S_U ensemble was generated from des-[30–75], which was obtained by reductive unfolding of ^{15}N -enriched ONC (13) and purified by chromatography as shown below. The disulfide bonds of des-[30–75] were then reshuffled by S-S/SH interchange under denaturing conditions (7M GdnHCl, pH 8.3) to produce an ensemble of unfolded structures (3S_U) with mostly non-native disulfide bonds without any native conformations.

In the reductive-unfolding pathway (13), native onconase is reduced to the three-disulfide-bond-containing species, des-[30–75], which is further reduced to R. To maximize the amount of des-[30–75] by limited reduction, native ONC at 1 mg/mL was incubated in a pH 8.0, 0.1 M Tris buffer containing 10 mM DTT^{red}. After 2 hours, the reductive unfolding of ONC was stopped by adding glacial acetic acid. Des-[30–75] was purified by isolation from the reductive unfolding mixture by RP-HPLC using a water/acetonitrile buffer system with 0.09% TFA and a 25 cm $\times$ 4.6 mm SULPELCO Discovery® BIO Wide Pore C18, 5 μm particle size column with the eluting species monitored by absorbance at 210 nm. After the reductive unfolding mixture was loaded onto the column, a constant flow of 85% water/15% acetonitrile eluted the Tris and DTT^{red}. Then, a gradient of 30–45% acetonitrile over 60 minutes was sufficient to separate des-[30–75] from native ONC (10). After des-[30–75] was collected, it was frozen and then the TFA, water, and acetonitrile were removed by lyophilization. The lyophilized protein was reconstituted in a pH 3, 50 mM acetate buffer and stored at -70°C until further use.

To generate the 3S_U ensemble by S-S/SH reshuffling, the solution containing the des-[30–75] species at a protein concentration of 0.5 mg/mL was incubated in a pH 8.3, 0.1 M Tris buffer that contained 7 M GdnHCl that had been previously purged of dissolved oxygen by humidified argon. The S-S/SH reshuffling was allowed to proceed for two hours and stopped by addition of glacial acetic acid to lower the pH to 3. Buffer salts and GdnHCl were removed with a G25 size-exclusion column with pH 3, 50 mM acetic acid as the running buffer, and the eluting species were monitored at 280 nm. The desalted 3S_U ensemble was collected and frozen. Water and acetic acid were removed by lyophilization, and the 3S_U ensemble was reconstituted in 50 mM acetic acid at a concentration of 5 mg/

mL. A DTT^{red} reduction pulse was applied to an aliquot of the solution of the 3S_U ensemble to reduce any non-native disulfide bonds in the aliquot because there was no structure present to protect the disulfide bonds from reduction. A far-UV CD spectrum of the solution showed that no secondary structure was present (data not shown).

Monitoring the 3S_U to 3S_F transition of ONC by far-UV CD spectroscopy and RP-HPLC

The 3S_U-to-3S_F transition was then initiated by incubating the 3S_U ensemble under folding conditions that allow S-S/SH exchange to lead to the structured species, des-[30–75] preferentially. Far-UV CD spectroscopy was used initially to detect the formation of structure during the 3S_U-to-3S_F transition that could then be followed by NMR spectroscopy. To initiate the 3S_U-to-3S_F transition for the far-UV CD spectroscopic experiments, an aliquot of the 3S_U stock solution was diluted to a final protein concentration of 0.2 mg/mL in a 10 mM phosphate, pH 8.0 buffer (folding conditions) that was previously purged of dissolved oxygen using humidified argon. The mixture was transferred to a cuvette, which was then capped and sealed with parafilm, and the CD signal was monitored for folding at 205 nm for 30 minutes. A dead time of ~2 minutes from the preparation of the cuvette until the initial readings was incurred. An analysis by RP-HPLC of the final products showed the absence of oxidation during the transition; only S-S/SH interchange occurred in this 30-minute period.

As a control, the MRE values of unfolded and folded protein during the same time period were obtained. The unfolded signal was obtained by diluting the 3S_U stock into pH 3, 50 mM acetic acid to a final concentration of 0.35 mg/mL. Since there is no S-S/SH reshuffling at pH 3, no progress in the 3S_U to 3S_F transition can occur. For the folded signal, a sample of des-[30–75] was diluted into the same pH 3 buffer to a final concentration of 0.25 mg/mL. The pH of 3 was chosen to avoid S-S/SH reshuffling by thiolates, which are present at a very low concentration at this pH. At different times, the MRE signal at 205 nm of these two samples was averaged for 2 seconds and the average and standard deviation during this time was acquired.

In addition to monitoring the formation of secondary structure in this transition by CD, the distribution of 3S_U and 3S_F was also measured by RP-HPLC. To initiate the transition, an aliquot from the same 5 mg/mL stock solution of 3S_U was diluted to a final protein concentration of 0.2 mg/mL in a 100 mM Tris, pH 8.0 buffer that was previously purged of dissolved oxygen using humidified argon. At different times, an aliquot from this mixture was removed and subjected to 5 mM DTT^{red} for 2 minutes. Under these conditions, the disulfide bonds of unstructured species are reduced but the disulfide bonds of des-[30–75] are not reduced due to the protection of these bonds by the presence of structure in this species. The reduction was then quenched by addition of glacial acetic acid. Samples were immediately frozen at –70°C prior to analysis by RP-HPLC. The des-[30–75] was separated from other 3S_U species by RP-HPLC as described in the previous section. The results from the CD experiments and RP-HPLC analysis provided a basis for the time scale to use in the NMR experiments.

Acquisition of the NMR spectrum of native ¹⁵N –ONC (N-ONC) at 16 °C, pH 3

Varian 500 and 600 MHz NMR spectrometers were used to record ¹H,¹⁵N – HSQC spectra at 16 °C pH 3. Previous assignments of the ¹H and ¹⁵N resonances of N-ONC had been acquired at 37 °C, pH 3 (14). 3D ¹⁵N-NOESY-HSQC and 3D ¹⁵N-TOCSY-HSQC NMR spectra of a sample of N-ONC were taken at 37 °C, pH 3, prepared by using the procedure outlined in Figure 2A, to confirm previous assignments in ¹H,¹⁵N – HSQC spectra under these conditions.

In order to obtain these assignments at 16 °C, pH 3, where the ^1H , ^{15}N – HSQC spectra of acid-arrested 3S_U -to- 3S_F transition aliquots were acquired, the spectra of N-ONC were obtained in 3 – 4 degree decrements from 37 to 16 °C at pH 3 over a total time of 20 min for each spectrum. By following the continuous change in the positions of the peaks as the temperature was varied, 91 of the 97 previously assigned resonances (14) observed in the ^1H , ^{15}N – HSQC spectrum were assigned at 16 °C. These assignments were used to identify structure-stabilized resonances along the 3S_U -to- 3S_F pathway.

When acquiring these spectra at different temperatures, N-ONC was subjected to the same solvent conditions as used later for the aliquots of the solution undergoing the 3S_U -to- 3S_F transition, as outlined in Figure 2. A 100 μL aliquot of an 0.8 mM stock of N-ONC added to a 150 μL aliquot of a pH* 5.1, 50 mM phosphate buffer in D_2O , and hydrogen/deuterium exchange was allowed to proceed for 10 seconds at 25 °C at pH* 5.1 to decrease the intensity of cross peaks from unfolded regions yet maintain those from folded regions. After 10 seconds, the pH* of each of the exchanged solutions was lowered to 3 with d_4 -glacial acetic acid and immediately frozen until analysis by NMR.

Acquisition of NMR spectra during the 3S_U -to- 3S_F transition of ^{15}N – enriched ONC

The protocol to analyze the 3S_U -to- 3S_F transition of ^{15}N – enriched ONC at different times is shown in Figure 2B. The 3S_U -to- 3S_F transition was initiated by adding an aliquot of the 3S_U stock to an oxygen-purged solution of 0.1 M pH 8.0, Tris in H_2O to a final volume of 3.4 mL and a final protein concentration of 0.2 mg/mL. After the 3S_U -to- 3S_F transition was allowed to proceed for 180 seconds, half of the reaction mixture was removed, and the pH of this half was lowered to 3 by addition of 200 μL of glacial acetic acid and immediately put on ice. After 1200 seconds, to allow for the complete formation of 3S_F , 200 μL of glacial acetic acid was added to the other half of the reaction mixture and immediately put over ice. Using 3000 MWCO Millipore concentrators, the two aliquots were concentrated to a final volume of 100 μL at 4 °C. Each aliquot was subjected to hydrogen/deuterium exchange for 10 seconds at 25 °C as described in the previous section. The hydrogen/deuterium exchange time of 10 seconds is less than 1% of 1200 seconds, the time required to complete the 3S_U -to- 3S_F transition, and, therefore, it was assumed that there was no progress in the 3S_U -to- 3S_F transition during the hydrogen/deuterium exchange time. The protein concentration of each of the samples for the NMR experiments was 100 μM . The total time to obtain the spectrum of each aliquot of the 3S_U to 3S_F transition solution at 16 °C, pH 3 was 5.5 hours. For a control sample, in which no folding was allowed to proceed, 150 μL of the pH* 5.1, 50 mM phosphate buffer in D_2O was added directly to a 100 μL aliquot of the 5 mg/mL stock of the 3S_U ensemble, which was not exposed to pH 8.0, and H/D exchange was allowed to occur before analysis by NMR.

By lowering the pH to 3 of aliquots taken at 180 and 1200 seconds into the 3S_U -to- 3S_F transition, the progress of the transition was arrested. A control experiment, letting the aliquot taken after 180 seconds in the 3S_U -to- 3S_F transition remain under these low-pH arrested conditions at 16 °C for a longer time, showed that no progress in this folding transition was observed at this pH and temperature for 22 hours. During this time, no increase in the amount of 3S_F or air-oxidation was observed by examining the distribution of species by RP-HPLC.

Results

CD spectra of the 3S_U to 3S_F transition of ^{15}N – enriched ONC

Analysis of the 3S_U -to- 3S_F transition by RP-HPLC shows that des-[30–75], a native-like oxidative folding intermediate (2, 10, 11), is preferentially populated as the transition proceeds. In Figure 3A, unstructured species dominate at 360 seconds into the transition.

However, in Figures 3B and 3C, the structured intermediate, des-[30–75], dominates the distribution at 1040 and 1800 seconds. The identical ratio of des-[30–75] to unstructured species at 1040 and 1800 seconds suggests that the distribution of three-disulfide-bonded species has attained equilibrium. No des-[30–75] is observed after 1800 seconds when 3.5 M GdnHCl is present before initiation of the 3S_U-to-3S_F transition (Figure 3D). Analyzing this transition by far-UV CD spectra shows that the increase in the population of des-[30–75] correlates with an increase in secondary structure.

Figure 3E shows the time-dependence of the CD signal at 205 nm, indicating the formation of secondary structure. At this wavelength, a folded protein with secondary structure has a positive value but a protein with no secondary structure has a negative value (15). Two control spectra of folded and unfolded protein have MRE (Mean Residue Ellipticity) values of 1×10^3 and -9×10^3 , respectively. Therefore, any increase in the CD signal at this wavelength shows the formation of secondary structure. As can be seen in Figure 3E, this increase in the signal at 205 nm occurs over the same time scale in which the unstructured ensemble 3S_U is not allowed to undergo S-S/SH reshuffling at pH 3. Therefore, this increase is the result of accumulation of secondary structure. The formation of secondary structure is slow enough to obtain structural information about the distribution of disulfide-bonded species at discrete times, which correspond to different stages in this folding transition. The initial formation of native-like structure in des-[30–75] can be observed 180 seconds into the transition while details of the further stabilization of des-[30–75] can be studied at longer times into the transition. The discrepancy between the initial signal of the kinetic trace (filled black circles) and the fully unfolded signal (unfilled circles) is the result of a dead time of ~2 min to mix the 3S_U stock solution in the appropriate buffer and prepare the anaerobic cuvette prior to data acquisition. This dead time is not an issue when preparing samples for NMR analysis because all of the preparation is performed at pH 3, a condition under which there is no folding progress. The decrease in pH is essentially instantaneous on the folding timescale (less than 1 second) so that the time during the transition to which the NMR spectra correspond is known accurately. According to the CD spectrum in Figure 3E, the MRE signal remains relatively constant after 1040 seconds until 1800 seconds. This indicates that an equilibrium distribution is being attained between structured and unstructured species formed during this transition, which is consistent with the previous analysis by RP-HPLC. Consequently, the kinetic trace does not fully reach the purely folded signal (filled triangles) because of the negative contribution of the unfolded signal at this time. Therefore, analysis of the progress at 1200 seconds into this transition could identify the residues responsible for a stable distribution of three-disulfide-bonded species dominated by des-[30–75]. ¹H, ¹⁵N-HSQC spectra were taken at 180 and 1200 seconds to identify the residues involved in these two stages along the transition.

Analysis by NMR spectroscopy of the 3S_U to 3S_F transition of ¹⁵N – enriched ONC

The ¹H, ¹⁵N-HSQC spectrum of the 3S_U ensemble (Figure 4A) illustrates the characteristics of an unstructured protein with little chemical shift dispersion. The corresponding spectrum of native ONC (Figure 4B) illustrates the characteristic chemical shift dispersion of a structured protein. Both samples were subjected to a pulse of D₂O to decrease the contribution of the unstructured residues, although residual peaks from unstructured residues can be observed in Figure 4A due to back exchange of protons with remaining H₂O. The ¹H, ¹⁵N-HSQC spectra of native ONC and of the unstructured ensemble of 3S_U were used to identify amide hydrogens that are stabilized 180, and 1200 seconds into the 3S_U-to-3S_F transition, since there is a mixture of structured and unstructured species at these two times.

Panels A and B of Figure 5 show ¹H, ¹⁵N-HSQC spectra of samples for which the 3S_U-to-3S_F transition was arrested at 180 and 1200 seconds, respectively. Fig. 5A shows six

(circled) resonances arising from structured regions, i.e., they overlap with resonances observed in native ONC (Fig. 4B). These six resonances appear outside the region of the spectrum of Fig. 4A that is associated with unfolded species. Additional resonances in Fig. 5B, including those circled in Fig. 5A, are observed corresponding to the spectrum of the native protein shown in Fig. 4B. The regions containing these resonances are circled in Fig. 5B.

Fig. 6 shows four regions of the superposition of ^1H , ^{15}N -HSQC spectra of Figs. 4B (green, native), 5A (red, acquired after 180 seconds), and 5B (purple, acquired after 1200 seconds). Panels A, B, C and D are different regions in the ^1H , ^{15}N -HSQC spectra. In all of the panels of Figure 6, the purple (1200 s) and red (180 s) resonances are superposed on the green resonances of the spectrum of the native protein, which indicates that the corresponding residues are involved in native-like structure. In the regions shown in Figure 6, no resonances from the spectra of the unfolded protein (Fig. 4A) are superimposed onto resonances from the native spectrum, or on the 180 second, and 1200 second spectra, indicating that these resonances correspond to amide protons of a folded protein.

Figure 7 shows the intensities of the peaks in the ^1H , ^{15}N -HSQC spectra of Figs. 5A and 5B corresponding to the residues listed in Tables 1 and 2, respectively. The relative intensities of the peaks at 180 and 1200 seconds suggest that some regions of ONC are stabilized at 180 seconds while other regions show native structure only at 1200 seconds. Six peaks (Table 1) exhibit chemical shifts corresponding to the native structure at 180 seconds, whereas 37 peaks (Table 2) exhibit chemical shifts consistent with native structure at 1200 seconds. One possibility is that, rather than representing an intermediate structure, the six peaks observed at 180 seconds arise from des-[30–75] and are merely the most intense in the spectrum. However, this is probably not the case because these six peaks, observed at 1200 seconds, are not the most intense in the spectrum, and have intensities similar to the 31 remaining peaks. This suggests that an intermediate is populated at 180 seconds but a significant amount of des-[30–75] is observed at 1200 seconds, and that the intermediate structure is populated in regions that are stabilized more efficiently.

Since the observed amide protons belonging to stabilized residues have the same chemical shifts as those found in the spectrum of the native protein, and thus the same chemical environment, the interactions displayed in the crystal structure that show contacts with these amide protons can be identified as those that are involved in the initial formation of native-like structure during this transition. The NMR-derived structure (14) agrees with the crystal structure within a 0.74 Å RMSD. These contacts would include hydrogen-bonding partners to the amide hydrogens as well as other atoms from side chains of neighboring residues. Tables 1 and 2 list the residues that contain amide protons that are involved in native-like structure after 180 and 1200 seconds, respectively. The hydrogen-bonding partner for each amide proton, namely, the carbonyl oxygen of a neighboring residue, as well as the secondary-structural element that contains this hydrogen bond are also listed. Figure 8 shows the location of these amide protons 180 and 1200 seconds into the 3S_{U} -to- 3S_{F} transition, as well as the four disulfide bonds in ONC.

Structure stabilized at 180 seconds

Three of the residues listed in Table 1, stabilized at 180 seconds into the 3S_{U} -to- 3S_{F} transition, are in the vicinity of the C-terminus of the protein and are involved in a loop consisting of residues 90–94, namely Cys90, Glu91, and Ala94 (Fig. 8A, 8B). These residues form complementary hydrogen bonds leading to anti-parallel β strands. Glu91 and Ala94 are involved in two hydrogen bonds with each other, where the amide proton of Glu91 is hydrogen bonded to the carbonyl oxygen of Ala94 and the amide proton of Ala94 is hydrogen bonded to the carbonyl oxygen of Glu91 (Figure 9A). These two residues close

the four-residue loop (Glu91, Asn92, Gln93, Ala94) in this β strand. While the amide proton of Cys90 is not involved in the hydrogen bonding of this strand, interactions with the side chain of the neighboring residue, Thr89, presumably help stabilize the hydrogen bond between the amide proton of Cys90 and the carbonyl oxygen of Lys55 (Figure 9B). Also, since this loop is in close proximity to the 48–90 disulfide bond, this structural element could very likely protect the 48–90 disulfide bond from S-S/SH interchange.

Of all the loop motifs in this protein, this four-residue loop motif is the shortest. Therefore, formation of these hydrogen bonds involves the smallest entropic penalty in loop closure. Thus, it is especially favorable for these hydrogen bonds to form early in the folding process to initiate formation of this loop. This motif brings Cys87 and Cys104 into proximity to form a disulfide bond (Fig. 8 E, F), which is formed in the first structured intermediate in the oxidative folding pathway, des-[19–68, 30–75].

Hydrophobic interactions are presumably responsible for stabilizing native-like structure surrounding the hydrogen bond between the amide hydrogen of Lys49 and the carbonyl oxygen of Ala46. The hydrophobic methylenes of the side chain of Lys45 contact the two ring $-\text{CH}_2$'s of Pro95 and the $-\text{CH}_3$ group of Ala94 (Figure 9C) and help shield the amide proton of Lys49 from H/D exchange. Also, this contact is in the vicinity of the 48–90 disulfide bond and would presumably help stabilize it from S-S/SH interchange. The stabilization of the 48–90 disulfide bond early in the folding pathway is consistent with the fact that the 48–90 disulfide bond is also formed in the first structured species, des-[19–68, 30–75] in the oxidative folding pathway.

While Tyr38 and Ser82 are separated by many residues in the amino acid sequence, native-like structure in the vicinity of these amide hydrogens are formed by hydrophobic contacts with the side chain of Arg15 after 180 seconds (Figure 9D). Tyr38 and Ser82 are involved in hydrogen bonds with Tyr64 and Leu65, respectively, in the crystal structure. These two hydrogen bonds are not in the vicinity of disulfide bonds, in contrast to the other four residues listed in the left-hand column of Table 1. Therefore, they must be stabilized by interactions with the neighboring side chains of Arg15, Tyr38, Tyr65, and Lys81. The guanidino group of Arg15 contacts the amide hydrogen of Ser82. This contact would presumably be stabilized by additional contacts of the *hydrophobic methylenes* of the side chain of Arg15 observed in the crystal structure with the aromatic rings in the side chains of Tyr38 and Tyr65. In addition, the hydrophobic methylenes in the side chain of Lys81 also contact the aliphatic carbons of the side chain of Arg15.

Structure stabilized after 1200 seconds

Those interactions formed after 180 seconds provide a scaffold for additional native structure that is stabilized after 1200 seconds. The amide protons observed after 1200 seconds are listed in Table 2. Further structure includes a loop containing Asn92 and Gln93, which enables additional hydrogens bonds to form in the anti-parallel β -Sheet consisting of residues 87–100 and, further, stabilizes the 87–104 disulfide bond (Fig. 8 E,F). Another structural element that is stabilized after 1200 seconds is the loop containing residues 49–55. The carbonyl oxygens of Ile51 and Ile52 of this loop form hydrogen bonds with the amide hydrogen atoms of the loop containing Gln93 and Asn92, respectively (Fig. 8 C, D), with mutual cooperating stabilization of these two loops and the disulfide bond between Cys48 and Cys90. After 1200 seconds, the interactions in the vicinity of the side chain of Arg15 initialize the formation of the three-stranded anti-parallel β -Sheet in the core structure of the protein consisting of residues 34–37, 64–69 and 78–83.

The stabilization of the secondary-structural elements listed in Table 2 shows how the des-[30–75] species is selectively populated along the 3S_U -to- 3S_F transition. According to Table

2, there are no resonances in the vicinity of the disulfide bond, 30–75, shown in yellow in Figures 8E and 8F, which indicates that this disulfide bond is not formed in 1200 seconds. The observation that no residues in the vicinity of Cys30 and Cys75 have stabilized protons is consistent with the RP-HPLC analysis of this transition that no disulfide is formed between residues Cys30 and Cys75. In other words, thermodynamically favorable interactions involving the side chains of non-cysteine residues are presumably responsible for the stabilization of des-[30–75] rather than any from the addition of a disulfide bond.

Discussion

The formation of the oxidative folding intermediate, des-[30–75] of ONC, through S-S/SH reshuffling at pH 8.0 without oxidation to the four-disulfide form, from an ensemble of unstructured three-disulfide species that contain native and non-native disulfide bonds was examined. The native-disulfide-bond connectivity of des-[30–75] results from thermodynamically favorable interactions. Since the increase in the concentration of des-[30–75] by S-S/SH reshuffling could be arrested at different times by lowering the pH to 3, the stabilized portions of the protein during the 3S_U-to-3S_F transition were identified (Figure 8). ¹H, ¹⁵N-HSQC spectra, collected at different times, identified amide protons of stabilized residues with chemical shifts found in the spectrum of native ONC, which had been assigned previously (14). From these interactions, folding events in the initial formation of native-like structure have been identified.

Comparison with previous research on RNase A and ONC

Previous NMR studies of three-disulfide-bond-containing oxidative folding intermediates of RNase A, des-[65–72] and des-[40–95] (12, 16), are consistent with the NMR experiments performed on des-[30–75] of ONC in this study, in the sense that these intermediates of RNase A have native-like structure except in the vicinity of the 65–72 and 40–95 disulfide bond, respectively. Residues in the vicinity of these missing disulfide bonds in RNase A have substantial differences in chemical shifts from those in the wild-type protein, and experience an increased rate of H/D exchange (12, 16). Similarly, with des-[30–75] of ONC, a substantial number of resonances in the ¹H, ¹⁵N - HSQC spectra of des-[30–75] correspond to resonances in the spectrum of native ONC, and no resonances are observed that correspond to residues in the vicinity of the 30–75 disulfide bond.

In an earlier study of the disulfide-bond-intact folding pathway of guanidine-denatured ONC, quench flow methods coupled with H/D exchange and 2D-NMR spectroscopy identified 31 amide hydrogens of stabilized residues from the protein backbone as the earliest detectable structure in ONC after 250 ms and folding was complete in ~5 seconds (17). Because folding was so rapid in this study (17), it was not possible to obtain much detail about early folding events. By contrast, in our oxidative-folding study, by allowing S-S/SH interchange to occur along the 3S_U-to-3S_F transition, the formation of native structure occurs on a much longer time-scale, 1200 seconds. This made it possible to detect the first 6 amide hydrogens to be involved in native-like structure after 180 seconds. Almost all of the amide hydrogens of residues stabilized after 1200 seconds are the same as those first detected in the previous work (17). Therefore, the present experiments have enabled us to study much earlier events in the folding of onconase. The formation of secondary structure monitored by H/D exchange and Mass Spectroscopy on a longer timescale during disulfide-dependent folding has also been observed in an all- α -helical protein (18). In that study, intermediates with different numbers of disulfide bonds were isolated from the oxidative folding of LR³IGF-I. As the number of disulfide bonds increased in each intermediate, less H/D exchange occurred, which is indicative of increasing secondary structure. Therefore, slow folding behavior is not restricted to ONC or other proteins that have β -Sheets and the interactions discussed below can be applicable to folding events in other proteins.

Comparison between the Structure Stabilized after 180 seconds and Chain Folding Initiation (CFI) Site in RNase A

The observation of early folding events in ONC after 180 seconds has enabled us to compare the mechanism of formation of chain-folding initiation (CFI) sites in ONC to those of proposed structurally homologous CFI sites in RNase A. Of special interest is the strand-loop-strand motif forming an anti-parallel β -sheet consisting of residues 87–96 (CVTCENQAPV). This sheet in the C-terminal region of ONC is in a structurally homologous position to the C-terminal anti-parallel β -sheet consisting of residues 106–118 (IIVACEGNPYVPV) in RNase A, which was proposed to be the CFI site, “F” (19, 20). Hydrophobic interactions among non-polar residues, Ile106, Ile107, Val108, Val116, Val118, of RNase A bring the strands together to form a loop. ONC does not contain a similar number of nonpolar residues in this motif, and must use other interactions, such as the hydrogen bonds involving the 91–94 loop, to stabilize it. Another difference between these motifs is that there are six loop residues (EGNPYV) within the CFI site in RNase A while there are four loop residues in ONC (ENQA). We found that the 91–94 loop region in ONC is stabilized by hydrogen bonds between residues Glu91 and Ala94 in the C-terminal anti-parallel β -sheet of ONC after 180 seconds, and that this sheet is stabilized further after 1200 seconds. This suggests that, in this sheet in ONC, the loop region is stabilized first, before inter-strand hydrogen bonds are formed, which is a different pathway than in RNase A to stabilize this anti-parallel β -sheet, i.e., hydrophobic interactions that initiate the folding of these β -strands in RNase A are further away from the loop region in this anti-parallel β -sheet than in ONC.

Hydrophobic interactions play a role in stabilizing a region in ONC structurally homologous to the CFI site, “E” of RNase A. This site in RNase A is comprised of residues 71–90, and 91–111, which form a β -Sheet in the core of the protein. Strands 3 and 4 in ONC form a similar β -Sheet. The interactions between the aliphatic carbons in the side chain of Arg15 and the aromatic rings in the side chains of Tyr38 and Tyr64, as well as the interactions between the guanidino group in the side chain of Arg15 and the aliphatic carbons in the side chain of Lys81 in ONC (Figure 9) bring strands 3 and 4 together in 180 seconds, with further stabilization provided after 1200 seconds. In addition, Loop 4, which contains Tyr38, is also initially stabilized by this interaction. Similar hydrophobic packing that spans these strands is not found in the structurally homologous region in RNase A. Therefore, this mechanism to form this β -Sheet in ONC is different from that in RNase A.

Hydrophobic interactions also bring together two regions in ONC that are structurally homologous to the CFI sites “D” and “F” in RNase A. The two ring $-\text{CH}_2$'s of Pro95 and the $-\text{CH}_3$ group of Ala94 of Strand 6 interact with the hydrophobic methylenes in the side chain of Lys45 of Helix 3 to bring these two structural elements together. Helix 3 in ONC is part of the structurally homologous region in the CFI site “D” in RNase A, which is comprised of residues 53–79 in RNase A, and Strand 6 in ONC is part of the structurally homologous CFI site “F” in RNase A, which is comprised of residues 106–118. Ala94 is part of a loop region (Glu91-Asn92-Gln93-Ala91) that is stabilized after 180 seconds into the transition. Bringing two regions of ONC that adopt native-like structure into close proximity early in the folding process helps ONC adopt native structure efficiently.

Conclusions

^1H , ^{15}N – HSQC spectra acquired at different stages of the 3S_{U} -to- 3S_{F} transition identified backbone amide protons that are involved in the initial formation of native-like structure. By identifying the backbone amide protons of stabilized residues at different stages in the folding process that are involved in forming the initial native-like structure, initial folding events from an unfolded state have been identified.

Favorable hydrogen-bond interactions between two residues (Glu91 and Ala94) in a short loop, and favorable interactions with non-polar groups on side chains, e.g., Arg15 with Tyr38, Ser82 and Lys81, as well as Lys45 with Pro95 and in long loops, stabilize six amide hydrogens (Table 1, Figure 9). These long loops could be formed because they contain favorable interactions that offset the larger entropic penalty to close them, compared to the smaller entropic penalty involved in closing a shorter loop. By observing the formation of these loops very early in the protein folding pathway, these favorable interactions illustrate how transient structure can be present in partially unfolded proteins or how secondary structure, especially β -sheets, starts to form. In addition, by identifying these redox-independent interactions that are formed in the folding pathway, insight is gained into the events that are important in the successful recognition by quality control mechanisms in cells. If these interactions cannot form at a given oxidation potential in a cell, which is carefully regulated, the protein may misfold and be shuttled to a degradation pathway (21).

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Abbreviations

ONC	onconase
nS_U	an ensemble of unstructured n-disulfide-bonded species that contain a distribution of native and non-native disulfide bonds
3S_F	a structured three-disulfide-bonded intermediate species all of whose disulfide bonds are native
des-[x-y]	a species that contains native disulfide bonds but lacks the (x-y) disulfide bond
HDX	hydrogen/deuterium exchange
RNase A	bovine pancreatic ribonuclease A
¹H, ¹⁵N – HSQC	heteronuclear single-quantum coherence between ¹⁵ N and ¹ H atoms
RP-HPLC	reversed phase HPLC
MRE	mean residue ellipticity
pH*	uncorrected pH reading of a D ₂ O buffer
GdnHCl	Guanidinium Hydrochloride
Tris	Tris[hydroxymethyl]aminomethane hydrochloride
TFA	trifluoroacetic acid
DTT^{red}	reduced dithiothreitol

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Figure 1.
X-ray structure of ONC (PDB code: 1ONC). (A) and (B) are two views of ONC that show numbered helices (red), strands (yellow), and loops (green).

Figure 2.

(A) Procedure to obtain ^{15}N -HSQC NMR spectrum of native ONC at 37 $^\circ\text{C}$. (B) Procedure to follow 3S_U -to- 3S_F transition by NMR at 16 $^\circ\text{C}$ for 180 seconds and 1200 seconds, respectively. H/D exchange reduced the intensity of peaks arising from unfolded resonances without affecting those from structured residues.

Figure 3.

(A–C) RP-HPLC analysis of the distribution of structured and unstructured species after a reduction pulse to reduce the disulfide bonds of unstructured species yet keeping disulfide bonds of structured species intact after 360 (A), 1040 (B) and 1800 (C) seconds, is shown on the upper panels. As the amount of secondary structure increases, indicated by CD, the concentration of des-[30–75] also increases, indicated by the RP-HPLC diagrams, as a result of increasing native interactions. The identical distribution of species in (B) and (C) indicates that a stable distribution is formed after 1040 seconds into this transition. The initial addition of GdnHCl before starting the 3S_U-to-3S_F transition prevents native interactions from forming and, consequently, des-[30–75] does not appear (D). (E) Kinetic

trace of the $3S_U$ -to- $3S_F$ transition of ONC, monitored by CD spectroscopy at 205 nm at pH 8.0, 25 °C. The increase in the MRE signal (filled black circles) indicates an increase in secondary structure. A CD spectrum of an unfolded protein has a negative signal at 205 nm. The CD signal from an unfolded protein (unfilled circles) comes from the unstructured $3S_U$ ensemble at pH 3, a condition that does not allow S-S/SH reshuffling, and consequently, no progress in the $3S_U$ to $3S_F$ transition. However, the signal from proteins with secondary structure would be positive. The CD signal from a folded protein (filled triangles) comes from des-[30–75] at pH 3. The error bars from the folded and unfolded signals come from the standard deviation from averaging the signal for 2 seconds. A stable positive signal is observed 1040 seconds into this transition. 1H , ^{15}N -HSQC spectra were acquired after 180 seconds and 1200 seconds, respectively, into the $3S_U$ -to- $3S_F$ transition to identify the initial and later native-like structure formed, respectively.

Figure 4.

Panel A is an ^1H , ^{15}N -HSQC spectrum of the 3S_U ensemble prepared as described in Figure 2B without allowing the 3S_U -to- 3S_F transition to proceed. The close localization of the peaks is indicative of an unfolded protein. Panel B is an ^1H , ^{15}N -HSQC spectrum of native ONC prepared as described in Figure 2A. The native structure gives rise to diverse chemical environments for the amide hydrogens as indicated by the distribution of the peaks in this spectrum. The circled resonances are observed in Fig. 5A, which identifies the initial native-like structure observed in this transition.

Figure 5.

Panel A is an ^1H , ^{15}N -HSQC spectrum of the folding transition after 180 seconds into the 3S_{U} -to- 3S_{F} transition. More resonances are observed outside the unfolded region observed in Panel A in Figure 4. The circled resonances are not observed in Panel A of Fig. 4. These resonances are also circled in Fig. 4B. Panel B is a ^1H , ^{15}N -HSQC spectrum taken after 1200 seconds into the 3S_{U} -to- 3S_{F} transition. The circled regions in this panel indicate resonances that are found in Figure 4B. The presence of these peaks indicates the formation of native-like structure during this transition.

Figure 6.

Panels A, B, C and D are superposed ^1H , ^{15}N -HSQC spectra from different regions of Figures 4A, 4B, 5A and 5B to identify resonances that indicate the initial formation of structure. The green resonances are from Panel B of Figure 4, which corresponds to the native structure. The purple resonances are from Panel B of Figure 5, which corresponds to spectra acquired after 1200 seconds. The red resonances are from Panel A of Figure 5, which correspond to spectra acquired after 180 seconds. No resonances from the unfolded spectrum of Panel A of Figure 4 are superposed on these resonances indicating that these resonances correspond to amide hydrogens of structured species.

Figure 7.

Plot of the intensities of resonances detected in the ^1H , ^{15}N -HSQC spectra of Figures 5A and 5B that indicate an intermediate with chemical shifts similar to those in the native-like structure at the corresponding residue numbers, which are listed in Tables 1 and 2.

Resonances detected after 180 seconds into the 3S_{U} -to- 3S_{F} transition are shown as unfilled circles and those detected after 1200 seconds as filled circles. The noise level for each of the spectra is 4.5×10^3 . The elements of secondary structure, of which each residue is a part, are listed below and are numbered according to Figure 1. Helices are abbreviated as "H" and are represented as red cylinders. Strands are abbreviated as "S" and are represented as yellow arrows.

Figure 8.

X-ray structure of ONC (PDB code: 1ONC). (A) and (B) are two views of ONC that show, in red, the residues that contain stabilized amide protons after 180 seconds in the $3S_U$ -to- $3S_F$ transition. (C) and (D) are two views in the same position as (A) and (B), respectively, which show stabilized amide protons not only after 180 seconds but also after 1200 seconds in purple. (E) and (F) show the disulfide bonds in ONC in space-filled spheres in the positions identical to (C) and (D), respectively.

Figure 9.

Contacts surrounding the hydrogen bonds that contain the amide protons of residues detected after 180 seconds into the transition and involved in native-like structure (PDB code: 1ONC). Showing the van der Waals radii of the atoms would bring out the packing of these contacts more clearly, but, at the same time, would complicate the view of the hydrogen bonds. To show the orientation of the residues surrounding the hydrogen bonds, the structures are represented as stick models. Carbon atoms are in green, nitrogen atoms are in blue, hydrogen atoms are white, oxygen atoms are in red and sulfur atoms are in yellow. Hydrogen bonds are represented as dotted orange lines. The residues that flank the donors and acceptors of the hydrogen bonds in the amino acid sequence are also shown. (A) The

complimentary hydrogen bonds between Glu91 and Ala94 close the shortest loop region (Glu91-Asn92-Gln93-Ala94) in ONC. This structural element presumably helps shield the 48–90 disulfide bond, indicated by an arrow, from S-S/SH reshuffling. (B) The side chain –CH₃ of Thr89, which contacts the amide proton of Cys90, presumably stabilizes the hydrogen bond between the amide proton of Cys90 and the carbonyl oxygen of Lys55. (C) The contacts between two ring –CH₂'s of Pro95, the –CH₃ group of Ala94 and the hydrophobic methylenes in the side chain of Lys45 presumably stabilize the hydrogen bond between Lys49 and Ala46. Also, these contacts are in the vicinity of the 48–90 disulfide bond, indicated by an arrow, and, as a result, would stabilize this disulfide bond by preventing S-S/SH reshuffling. (D) The guanidino group of Arg15, indicated by an arrow, is in the vicinity of the amide proton of Ser82. The guanidino group is presumably oriented by hydrophobic contacts between the aromatic rings of Tyr38 and Tyr64, and the hydrophobic methylenes in the side chain of Lys81 with the methylenes of the side-chain of Arg15, all indicated by arrows. The hydrophobic contacts involving the side chains of Arg15, Lys81, Tyr38 and Tyr64 would stabilize the hydrogen bonds between the amide proton of Tyr38 and the carbonyl oxygen of Tyr64 as well as between the amide proton of Ser82 and the carbonyl oxygen of Leu65.

Table 1

Residues^a that contain amide protons identified from resonances observed in a ¹H,¹⁵N-HSQC spectrum taken 180 seconds into the 3S_U-to-3S_F transition, and are indicative of native-like structure. The numbered loops and strands refer to Figure 1

Amide Proton ^a	Carbonyl Oxygen ^b	Structural Element ^c
38 Tyr	64 Tyr	Loop 4
49 Lys	46 Ala	Loop 5
82 Ser	65 Leu	Strand 4
90 Cys	55 Lys	Strand 5
91 Glu	94 Ala	Loop 9
94 Ala	91 Glu	Loop 9

^a) Hydrogen bond donors

^b) Hydrogen-bond acceptors chosen by assuming native structure (PDB Code:1ONC)

^c) Secondary-structural element that contains the hydrogen bond to residue in the left column, also by assuming native structure.

Table 2

Detectable amide protons involved in native-like structure after 1200 seconds, as seen by a $^1\text{H}, ^{15}\text{N}$ -HSQC spectrum.

Amide Protons ^a	Carbonyl Oxygens ^b	Structural Element ^c
3 Trp	...	Loop 1
12 Thr	37 Ile	Loop 2
17 Val	15 Arg	Loop 2
20 Asp	...	Helix 2
22 Ile	18 Arg	Helix 2
37 Ile	10 His	Sheet 1
38 Tyr	64 Tyr	Loop 4
39 Ser	12 Thr	Loop 4
40 Arg	...	Loop 4
44 Val	40 Arg	Helix 3
46 Ala	42 Glu	Helix 3
47 Ile	44 Val	Helix 3
48 Cys	45 Lys	Helix 3
49 Lys	46 Ala	Loop 5
51 Ile	48 Cys	Loop 5
52 Ile	50 Gly	Loop 5
53 Ala	...	Loop 5
57 Val	88 Val	Strand 2
58 Leu	...	Loop 6
59 Thr	86 Phe	Loop 6
64 Tyr	38 Tyr	Strand 3
65 Leu	82 Ser	Strand 3
66 Ser	36 Phe	Strand 3
67 Asp	80 Lys	Strand 3
80 Lys	67 Asp	Strand 4
82 Ser	65 Leu	Strand 4
85 Lys	...	Loop 8
87 Cys	100 Gly	Strand 5
88 Val	57 Val	Strand 5
90 Cys	55 Lys	Strand 5
91 Glu	94 Ala	Loop 9
92 Asn	52 Ile	Loop 9
93 Gln	51 Ile	Loop 9
94 Ala	91 Glu	Loop 9
96 Val	89 Thr	Strand 6
100 Gly	87 Cys	Strand 6
101 Val	...	Loop 10

^{a)} Amide protons that adopt native-like structure after 180 seconds are also observed after 1200 seconds

b) Hydrogen-bond acceptors chosen by assuming native structure (PDB Code:1ONC)

c) Secondary-structural element, labeled in Figure 1, that contains this hydrogen bond to residues in left column, also by assuming native structure