

Oxidative Folding and N-Terminal Cyclization of Onconase[†]

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Received December 4, 2006; Revised Manuscript Received March 13, 2007

ABSTRACT: Cyclization of the N-terminal glutamine residue to pyroglutamic acid in onconase, an anti-cancer chemotherapeutic agent, increases the activity and stability of the protein. Here, we examine the correlated effects of the folding/unfolding process and the formation of this N-terminal pyroglutamic acid. The results in this study indicate that cyclization of the N-terminal glutamine has no significant effect on the rate of either reductive unfolding or oxidative folding of the protein. Both the cyclized and uncyclized proteins seem to follow the same oxidative folding pathways; however, cyclization altered the relative flux of the protein in these two pathways by increasing the rate of formation of a kinetically trapped intermediate. Glutaminyl cyclase (QC) catalyzed the cyclization of the unfolded, reduced protein but had no effect on the disulfide-intact, uncyclized, folded protein. The structured intermediates of uncyclized onconase were also resistant to QC catalysis, consistent with their having a native-like fold. These observations suggest that, *in vivo*, cyclization takes place during the initial stages of oxidative folding, specifically, before the formation of structured intermediates. The competition between oxidative folding and QC-mediated cyclization suggests that QC-catalyzed cyclization of the N-terminal glutamine in onconase occurs in the endoplasmic reticulum, probably co-translationally.

The N-terminal glutamine residue of peptides and proteins undergoes *nonenzymatic* spontaneous cyclization, resulting in the formation of pyroglutamic acid (1). This is a slow process requiring days for completion, depending on the conditions (1). Examples of peptides and proteins with an N-terminal pyroglutamic acid include gonadotropin-releasing hormone, thyrotropin-releasing hormone, neurotensin, and so forth, for which biological activity depends on the presence of pyroglutamic acid at their N-termini (2, 3). Loss or modification of the N-terminal pyroglutamic acid residue leads to a decrease in biological activity (4, 5). This implies the existence of an enzyme that accelerates this cyclization *in vivo* (6–8). Indeed, enzymes with glutaminyl cyclase (QC¹) activity have been isolated from many sources (9–13), and the cDNA of QC was identified in many organisms (14–16).

Prior to QC catalysis, prohormone convertase must unmask the N-terminal glutamine during the secretion of several

peptide hormones by removing the precursor peptide that precedes the glutamine residue on the prohormone (17). This requires that QC acts in the regulated secretory pathways, subsequently to prohormone convertase. Subcellular distribution studies have confirmed the presence of QC in pituitary secretory granules (18). In the case of proteins, however, in which there is no need to unmask the N-terminal glutamine, very little is known about where and when QC-mediated catalysis takes place in relation to other post-translational events such as folding and disulfide-bond formation.

Onconase (ONC; registered trademark of Alfacell Corp., Bloomfield, NJ) has an N-terminal glutamine. It is a very stable 104-residue frog ribonuclease containing four disulfide bonds. Its amino acid sequence (19) and three-dimensional (3D) structure (20) are homologous to those of bovine pancreatic ribonuclease A (RNase A). The cyclized pyroglutamic acid-containing form has significantly higher ribonuclease activity (21) and is slightly more stable than its uncyclized form (unONC (22)). ONC is toxic to tumor cells

[†] This research was supported by NIH Grant GM-24893, HSRF Grant NF61431, Marie Curie IRG-04515 and EIF-011636, and by the National Office for Research and Technology Hungary RET-08/2004. E.W. is an HHMI international scholar.

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¹ Abbreviations: ONC, frog (*Rana pipiens*) onconase; N-ONC, native onconase; R-ONC, fully-reduced onconase; unONC, uncyclized onconase; I₁, I₂, and I₃, intermediates with one, two, and three native disulfides, respectively, formed during the oxidative folding of ONC; RNase A, bovine pancreatic ribonuclease A; des [30–75], an ONC intermediate having all native disulfide bonds but lacking the (30–75) disulfide bond; QC, glutaminyl cyclase; ER, endoplasmic reticulum; WT, wild-type; AEMTS, 2-aminoethylmethylthiosulfonate; DTT^{red} and DTT^{ox}, reduced and oxidized dithiothreitol, respectively; GdnHCl, guanidine hydrochloride; EDTA, ethylenediamine-tetra-acetic acid; Tris-HCl, tris(hydroxymethyl) aminomethane hydrochloride; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Fmoc, 9-fluorenylmethyloxycarbonyl; MAP, methionine amino peptidase; PBS, phosphate buffered saline.

and is in Phase III clinical trials for the treatment of malignant mesothelioma, an asbestos-induced lung cancer (23–25). The anti-cancer activity of ONC (due to the absence of a specific intracellular inhibitor such as one that inhibits the activity of RNase A) is related to its ribonuclease activity (19, 20, 26–29).

In this study, we examine the influence of the cyclization of N-terminal glutamine on the reductive unfolding and oxidative folding profiles (rates and pathways) in onconase. We also investigate the effect of formation of the native structure (which is coupled to disulfide-bond formation) on the QC-catalyzed cyclization of the protein.

Experimental reductive unfolding (30–39) and oxidative folding studies (40–53) of proteins, *in vitro* and *in vivo*, have contributed to a better understanding of the relationship between protein structure and folding/unfolding characteristics. The reductive unfolding and oxidative folding of ONC have been examined in detail (54–56). The reductive unfolding of ONC is a two-stage process that is initiated with the rapid reduction of the first relatively exposed disulfide bond, resulting in the quantitative conversion of the native protein to des [30–75], an intermediate containing three native disulfide bonds (54, 56). However, the identity of the disulfide bond to be reduced in the next stage is yet to be determined.

The oxidative folding of ONC was compared to that of RNase A under several identical conditions (55). Three peaks (I_1 , I_2 , and I_3) corresponding to structured intermediates of ONC were detected by HPLC (55). I_1 has two disulfide bonds, whereas I_2 and I_3 each have three (Narayan et al., unpublished work). This is in contrast to RNase A, which has no structured two-disulfide-bond-containing intermediate (35, 41, 42). The overall oxidative folding rates of ONC and RNase A do not differ significantly at 15 and 25 °C at pH 8 and 25 mM DTT^{ox} (55). However, the oxidative folding rate of ONC is 100-fold faster at 37 °C, where the disulfide-secure intermediates of RNase A begin to melt (41). The higher rate has been attributed to the higher stability of the disulfide-secure (57) intermediate(s) of ONC (55).

EXPERIMENTAL PROCEDURES

Materials. WT-ONC cDNA in a pET11 expression vector (21) was amplified by PCR and cloned to a pET 22b(+) vector in-frame with the pelB signal sequence without the starting methionine residue. WT-ONC was expressed in BL21(DE3) cells and purified as described earlier (26), except that the protein was refolded in the presence of 0.5 M arginine to prevent precipitation. The folded protein (ONC or unONC) was purified on a strong cation-exchange column after concentration, dialyzed against 20 mM acetic acid solution at 4 °C, and then lyophilized as described previously (54). This preparation of unONC was found to contain 10% of the cyclized species. The nonenzymatic conversion of the N-terminal glutamine to pyroglutamic acid to produce 100% cyclized protein was carried out by treating the uncyclized folded form with a 200 mM, pH 7 phosphate buffer at either room temperature or 37 °C for more than 2 days.

A model peptide corresponding to the six N-terminal residues of unONC, H-Gln-Asp-Trp-Leu-Thr-Phe-NH₂, was synthesized using standard Fmoc chemistry and purified by reversed-phase HPLC.

AEMTS (>99% pure) was purchased from Anatrace and used without further purification. DTT^{ox} and DTT^{red} were obtained from Sigma. DTT^{red} was used without further purification, whereas DTT^{ox} was purified to remove traces of contaminating DTT^{red} by reversed-phase HPLC (40). All other chemicals were of the highest grade commercially available.

N-terminal sequencing and mass spectrometry analyses were carried out by the Cornell Biotechnology Resource Center.

Preparation of Reduced ONC/unONC. Lyophilized onconase (with a cyclized or uncyclized N-terminal Gln) was dissolved in a buffer (pH 8, 25 mM Tris-HCl, 1 mM EDTA) containing 6 M GdnHCl and 100 mM DTT^{red}, and incubated at room temperature for 2 h. The reducing agent and salts were removed from the reduced protein by desalting the sample using a G25 size-exclusion column (with 50 mM acetic acid as the running buffer) followed by lyophilization. The lyophilized, reduced onconase (R-ONC and R-unONC) was stored at –20 °C until further use.

Reductive Unfolding of ONC/unONC. Lyophilized native onconase (with a cyclized or uncyclized N-terminal Gln) was dissolved in an acetic acid buffer (50 mM) at room temperature to obtain a stock solution (5 mg/mL), and aliquots (0.2 mL each) of this solution were kept frozen at –20 °C until used. Reduction experiments were initiated by introducing one such aliquot of ONC or unONC into a solution (pH 8, 100 mM Tris-HCl and 1 mM EDTA at 15 °C) containing DTT^{red} (final concentration 10 mM) such that the final protein concentration was 0.5 mg/mL.

Aliquots of the reaction mixture were withdrawn periodically, and any free thiols were blocked immediately with excess AEMTS (final concentration 50 mM). After 5 min, the pH of the mixture was reduced to 3 by addition of 20 μ L of glacial acetic acid. The AEMTS-blocked, acid-quenched samples were then desalted on a Hi-Trap G25 gel filtration desalting column and analyzed by HPLC. HPLC runs were carried out on a Rainin-Hydropore strong cation-exchange column using a salt gradient (50–150 mM NaCl over 150 min).

Reduction of ONC/unONC was also carried out at a higher concentration of the reducing agent (100 mM DTT^{red}).

Oxidative Folding of ONC/unONC. Lyophilized R-ONC/R-unONC was dissolved into a 50 mM acetic acid solution to obtain a stock solution of protein (10 mg/mL). Oxidative folding of ONC/unONC was initiated by introducing an aliquot of R-ONC/R-unONC into a solution containing 25 mM DTT^{ox} (final protein concentration was 22 μ M, pH 8, 100 mM Tris-HCl, and 1 mM EDTA at 25 °C).

Aliquots of the reaction mixture were withdrawn periodically, and any free thiols were blocked immediately with excess AEMTS (final concentration 40–50 mM). The AEMTS-blocked samples were desalted, and analyzed by cation-exchange HPLC as described above. In some experiments, the blocked, desalted aliquots were divided into two halves and incubated with and without QC, respectively, in phosphate buffered saline (PBS) at pH 7.2 at room temperature and analyzed by cation-exchange chromatography.

Some aliquots were subjected to a reduction pulse (42) by the addition of 5 mM DTT^{red} for a period of 2 min at 25 °C before being blocked with AEMTS.

Cyclization of the N-Terminal Glutamine. Nonenzymatic cyclization reactions of the protein and the model peptide were examined under various pH (7–8), salt concentration (10–200 mM phosphate), and temperature (4–37 °C) conditions, and monitored by cation-exchange (Rainin-Hyporepore strong cation-exchange column, absorbance at 280 nm) or reversed-phase (C18 column, absorbance at 222 nm) chromatography, respectively. The amount of the cyclized and uncyclized protein or peptide was established quantitatively by measuring the areas of the corresponding peaks on the chromatogram.

QC-catalyzed cyclization of the protein and the model peptide was carried out at pH 7 and 37 °C and analyzed as above. Human pituitary glutaminyl cyclase was kindly provided by Professor Robert C. Bateman, Jr. (13).

RESULTS

Frog onconase is expressed in *E. coli* as a mixture of the cyclized and uncyclized forms with about 50% being cyclized. Because cyclization of the N-terminal glutamine results in a change in the pI of the protein, the retention times on a cation-exchange column of the native (as well as of the intermediate and reduced) forms of the cyclized and uncyclized protein species differ, facilitating their isolation. The cyclized and uncyclized forms of the expressed native protein, found in the earlier and later eluting peaks, respectively, from a strong cation-exchange column, were identified by mass spectrometry. Cyclization of the N-terminal glutamine of ONC was further verified by N-terminal sequencing of both protein species as follows. The sequence (QD-LT FQKKHITNT, where the dash stands for W, which is modified under the conditions of Edman degradation and thus cannot be identified), found in the later-eluting peak, agrees with the expected sequence of expressed unONC. By contrast, the protein that is eluted earlier on the cation-exchange column was resistant to Edman degradation, which is consistent with an N-terminal cyclization of the glutamine (19).

Reductive unfolding of unONC was carried out on uncyclized onconase containing 10% cyclized protein that was used as an internal control for direct comparison. The (30–75) disulfide bond of ONC is susceptible to reduction under mildly reducing conditions resulting in the full conversion of the native protein to des [30–75] without detectable accumulation of any other species (54, 56). In the second stage, des [30–75] is converted to the fully reduced protein without significant accumulation of any other intermediates (54, 56). The two stages of the reduction of native ONC have to be examined in separate experiments. If examined in one experiment, under mildly reducing conditions, the reduction of des [30–75] (the second stage) to R-ONC takes a very long time, whereas, if strongly reducing conditions are applied, the reduction of the (30–75) disulfide bond in the native protein (the first stage) takes place very rapidly, not enabling one to make precise measurements of reduction rates under either reducing condition. Hence, the first stage in the reduction process of native ONC/unONC is studied under *mild* reducing conditions, and the second stage is carried out on isolated des [30–75] under *strongly* reducing conditions. The cyclization of N-terminal glutamine did not significantly alter the reduction rate of either stage

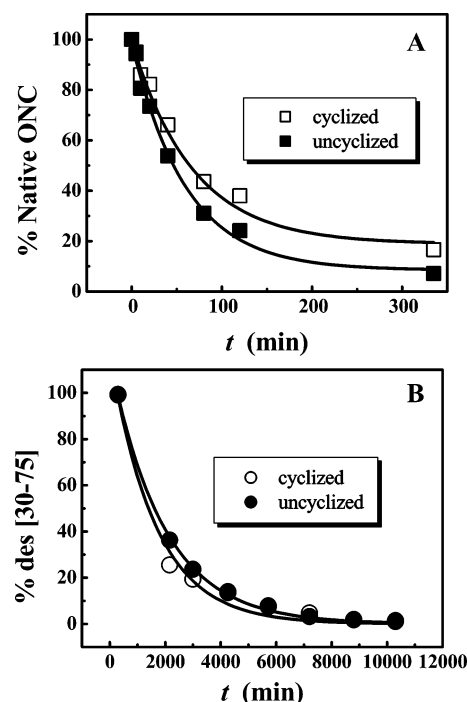


FIGURE 1: Reductive unfolding of cyclized and uncyclized onconase. (A) Percentage of native ONC/unONC in the presence of 10 mM DTT^{red}, starting from the native protein (first stage). (B) Percentage of des [30–75] in the presence of 100 mM DTT^{red}, starting the reduction from isolated des [30–75] (second stage).

of the reduction, although in the first stage, a slight increase of the reduction rate of unONC was observed (Figure 1A and B).

The oxidative folding of ONC/unONC was carried out at pH 8, 25 mM DTT^{ox} and 25 °C on a mixture containing 10% cyclized and 90% uncyclized protein, facilitating the direct comparison of the rates. Aliquots were taken at different times, and any further rearrangements of the disulfides were prevented by adding excess AEMTS as described in the Experimental Procedures section. No significant difference in the overall oxidative folding rates of the two forms was observed in these experiments.

However, further examination revealed some differences in the *distribution* of the oxidative folding intermediates in ONC versus unONC. A reduction pulse (a short, mild reduction), which is sufficient to reduce unstructured intermediates (in which the disulfide bonds are exposed) to the fully reduced protein without affecting the structured ones (in which the 3D structure protects the disulfide bonds from easy reduction), is a useful tool to distinguish structured intermediates from unstructured ones (42, 55, 58). We applied a reduction pulse to one-half of the aliquots of the regeneration mixture for both ONC and unONC, but no reduction pulse to the other half of the aliquots, before processing all of the samples for HPLC analysis. Figure 2A shows the chromatogram for the 2-h oxidation of R-unONC (containing 10% R-ONC) without a reduction pulse. A reduction pulse revealed the presence of three peaks for ONC corresponding to structured intermediates (I₁, I₂, and I₃) in an earlier study (55). I₁ includes two-disulfide-containing species, whereas I₂ and I₃ correspond to three-disulfide-containing intermediates (as concluded from their masses after AEMTS blocking). The corresponding three peaks for unONC are apparent, after the reduction pulse, in the

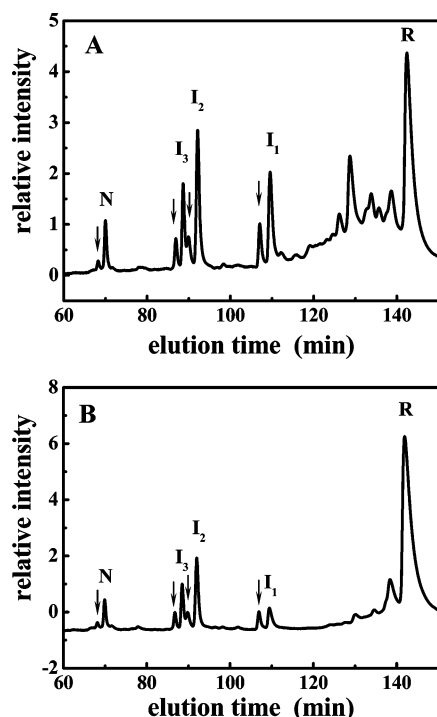


FIGURE 2: Chromatogram of the oxidative folding of R-unONC (containing 10% cyclized R-ONC) at pH 8, 25 mM DTT^{ox}, and 25 °C at 2 h. (A) Without and (B) with a reduction pulse. The earlier-eluting smaller peaks (indicated by arrows) corresponding to N, I₃, I₂, and I₁ are for the cyclized protein. All free thiols in each sample were blocked with AEMTS; the samples were then desalted, and analyzed using cation-exchange HPLC analysis.

chromatogram in Figure 2B. (The earlier-eluting smaller peaks are indicated by arrows in the Figure and correspond to the N, I₃, I₂, and I₁ species of ONC; unpublished data.) However, the relative proportion of these structured intermediates is different for the oxidative folding of ONC and unONC. Relative to I₂, the I₁ and I₃ species, respectively, are more abundant in the oxidative folding of the cyclized protein.

We also examined the effect of the three-dimensional structure on the nonenzymatic cyclization of the N-terminal glutamine of the protein. Native uncyclized ONC and its six-residue N-terminal peptide fragment were incubated under the various conditions described under Experimental Procedures. The two forms (cyclized and uncyclized) were separated from one another using reversed-phase HPLC and cation-exchange HPLC for the peptide and the protein, respectively. It takes less than 2 days for the nonenzymatic cyclization to be completed at 37 °C for both the peptide and the protein, in agreement with the literature (10, 59).

The cyclization of N-terminal glutamine in peptides and proteins is accelerated by an enzyme, glutaminyl cyclase (QC), *in vivo* (9–13). We examined the effect of QC on the cyclization of native unONC and of the N-terminal six-residue peptide under identical conditions of enzyme and substrate concentration (pH 7; 37 °C). Whereas QC dramatically accelerated the cyclization of the peptide (the cyclization is basically completed in 1 min, as evident from the different elution times of the peaks for 1-min incubation with and without QC in Figure 3A), it had no effect on the cyclization of the native, folded unONC (Figure 3B). In Figure 3B, there is one dominant peak at 1-h reaction times

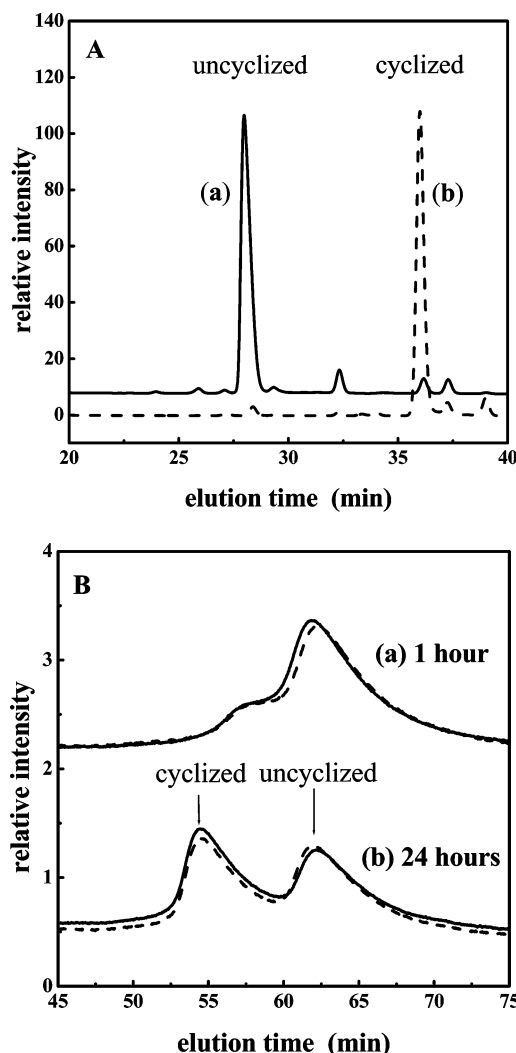


FIGURE 3: (A) Overlaid reversed-phase HPLC chromatograms of (a) the uncyclized model peptide (—) and (b) the QC-cyclized model peptide (---) at 1 min. (B) Strong cation-exchange chromatograms of native unONC after incubating with (—) and without (---) QC at (a) 1 h and (b) 24 h. The experiments were carried out at pH 7 and 37 °C.

(corresponding to the uncyclized protein) with or without QC, indicating that very little (enzymatic or nonenzymatic) cyclization of native unONC occurs in 1 h. In 24 h, equal fractions of cyclized and uncyclized proteins appear. The cyclized material resulted from slow nonenzymatic cyclization of unONC, and the solid and dashed curves overlap because no QC-catalyzed cyclization appeared in 24 h under these conditions.

Subsequently, we examined the reduced form and the oxidative folding intermediates of unONC to determine whether they were also resistant to QC catalysis. Aliquots of the oxidative folding mixture of unONC (at 4 h) were withdrawn, and the thiols were blocked with AEMTS, followed by desalting and incubation with and without QC before HPLC analyses. A comparison of the chromatograms in Figure 4 reveals that, whereas the structured species, N, I₁, I₂, and I₃, were resistant to QC catalyses (shown as the same elution pattern between 40 and 75 min in Figure 4A and B), the unstructured ensemble of unONC including the reduced protein (indicated by the peaks under the braces in Figure 4A and B) shifts to earlier elution times, indicating

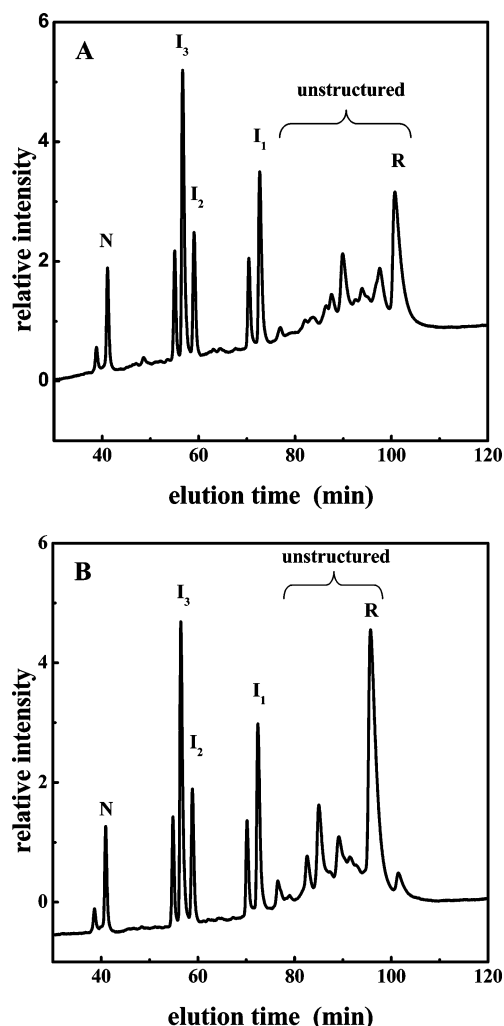


FIGURE 4: Chromatogram of an oxidative folding mixture of unONC (containing 10% cyclized ONC) at 4 h at pH 8 and 25 mM DTT^{ox}. All free thiols in each sample were blocked with AEMTS, and the samples were desalted and incubated without QC (A) and with QC (B) at pH 7.2 and room temperature prior to analysis using cation-exchange HPLC. The unstructured species are indicated with braces.

that QC is effective in catalyzing cyclization in unstructured species.

DISCUSSION

Although the nonenzymatic cyclization cannot be stopped/quenched, the cyclization rate is significantly slowed down at low temperatures (and is in fact relatively slow even at room temperature); thus, nonenzymatic cyclization did not cause detectable error during the processing of the samples to determine the rates of oxidative folding and reductive unfolding. However, this nonenzymatic cyclization of the intermediates of reductive unfolding and oxidative folding, could be exploited for the identification of the HPLC peaks of the corresponding cyclized and uncyclized intermediate pairs, when they are kept above 10 °C. This could be achieved by the isolation of peaks on a chromatogram of unONC intermediates and by re-injecting the isolated peaks after allowing cyclization to proceed.

Effect of Local Unfolding on the Reduction of Disulfide Bonds during Reductive Unfolding. The X-ray structure of native ONC in Figure 5 reveals a hydrogen bond between

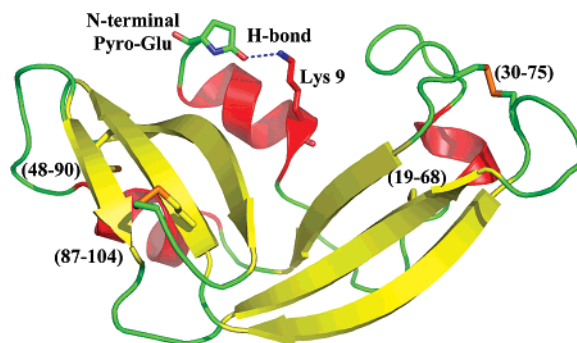


FIGURE 5: X-ray structure of native ONC (20). The four disulfide bonds and the hydrogen bond between the γ -carboxyl oxygen of the N-terminal pyroglutamic acid and the ϵ -amino hydrogen of Lys 9 are indicated.

the N-terminal pyroglutamic acid and Lys 9 (20). The absence of this hydrogen bond decreases the stability of the protein (22). This decreased stability is not evident in the reductive unfolding experiments (Figure 1) because the reduction rates are not significantly accelerated in unONC. This supports a previous contention that both the reduction of the first disulfide bond (30–75) and that of the second disulfide bond in des [30–75] are mediated by a local unfolding event (54, 56). This explanation is plausible because a similar phenomenon is observed in a mutant variant of RNase A, a homologue of ONC. The Pro93Ala mutation in RNase A, which greatly destabilizes the protein and increases the rate of reduction of the first, nearby (40–95) disulfide bond 120-fold, had apparently little effect on the reduction of the remote (65–72) disulfide bond; both disulfide bonds in RNase A are exposed for reduction by a local unfolding event (35, 60).

N-terminus of unONC Is Not Accessible in the Folded Protein. Application of the reduction-pulse technique led to the detection of structured intermediates corresponding to peaks I₁, I₂, and I₃ in the oxidative folding of both the cyclized and uncyclized forms. This is because the reduction-pulse technique effectively screens for stable 3D folds that provide protection to the disulfide bonds against the reducing agent (42). Such protection has been demonstrated only in structured intermediate species that have a native-like fold (42, 61, 62). It is known that disulfide-bond-containing proteins frequently retain their native structure in the absence of either one or even more disulfide bonds (57, 63–72). Considering the extremely high stability of ONC ($T_m = 85.4$ °C at pH 8) (28), it is expected that reduction of one or even two disulfide bonds in the protein might not cause unfolding at room temperature (27, 54, 72). Thus, it is plausible to propose that I₂ and I₃ may have the overall native fold of the protein because they possess three of the four native disulfide bonds and survive a reduction pulse (Figure 2). I₁, which also survives a reduction pulse, may also have the overall native fold but further structural characterization is necessary to determine the effect of the absence of two disulfide bonds on the native 3D structure of ONC in this species. Interestingly, the stability of I₁ of unONC is lower than that of I₁ of the cyclized species; this is evident from the effect of a reduction pulse (Figure 2B). The same phenomenon was observed at 37 °C with I₁ of ONC in an earlier study (55), suggesting that the effect of heat destabilization is similar

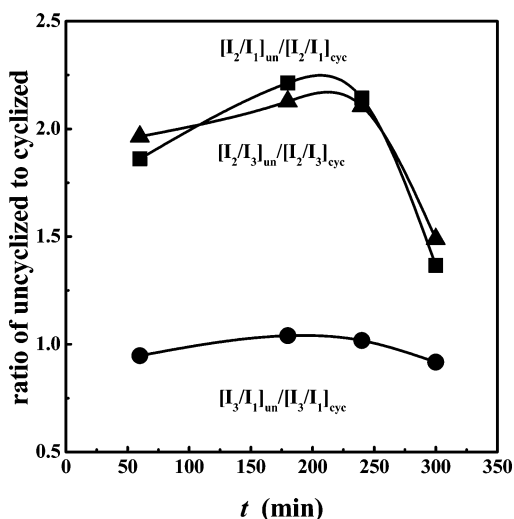


FIGURE 6: Ratios of fractions of the intermediates during the time course of oxidative folding of the uncyclized form relative to the cyclized form of onconase. \blacktriangle , $[I_2/I_3]_{un}/[I_2/I_3]_{cyc}$; \blacksquare , $[I_2/I_1]_{un}/[I_2/I_1]_{cyc}$; and \bullet , $[I_3/I_1]_{un}/[I_3/I_1]_{cyc}$.

to that due to the absence of the native-like interactions of the pyroglutamyl moiety in this intermediate.

We found that QC does not catalyze cyclization of the native uncyclized protein and its structured intermediates, although it effectively catalyzes cyclization of the fully reduced uncyclized protein, the unstructured uncyclized intermediates, and the N-terminal peptide. These results indicate that the folded structure hinders the catalysis by making the N-terminus of the protein non-accessible for the enzymatic action of QC. A similar hindrance was revealed in an earlier study of a bacterially expressed variant of onconase (73). In that study, the *in vitro* use of methionine amino peptidase (MAP) was attempted for the removal of the N-terminal methionine, but the results indicated that the enzyme had no access to the N-terminus of the protein; the first methionine residue could be removed from the polypeptide chain by the specific action of MAP only after denaturing the structure of the protein (73). In our bacterial expression system, used for both RNase A and ONC, the starting methionine is cleaved off together with the signal peptide.

Cyclization Changes the Rate of Formation of I_3 . A comparison of the oxidative folding of ONC and unONC further revealed important features of the oxidative folding process of this protein. Both the cyclized and uncyclized proteins seem to follow the same oxidative folding pathways, through I_2 or I_3 . However, from chromatograms (such as Figure 2), it is apparent that the ratios between the intermediates are different for the oxidative folding of the cyclized and uncyclized forms (Figure 6). Specifically, the concentrations of I_3 (and I_1) relative to I_2 are consistently lower in the uncyclized form than in the cyclized one throughout the course of the experiments. By contrast, the ratio of I_3 to I_1 is nearly identical in both forms of onconase at all times examined (Figure 6). Thus, cyclization altered the relative flux of the protein in these pathways by increasing the concentration of I_3 , a kinetically trapped intermediate (55).

In theory, the concentration of I_3 could be changed by decreasing the rate of its unfolding or by increasing the rate of its formation, or by both. We argue here that it is less likely that its unfolding rate (i.e., by reduction and/or reshuffling) is substantially changed, on the basis of the

following facts. ONC is an extremely stable protein, with a T_m of 85 °C (pH 8) under the conditions of the experiments (28). I_3 , which has three disulfide bonds, is plausibly a des species, as we have shown above. The absence of a disulfide bond, as in I_3 , destabilizes the protein, primarily, by increasing the entropy of the unfolded state compared to that of the four-disulfide native protein. The distances between the cysteines of the native disulfide bonds in the amino acid sequence of ONC are similar to or less than those in RNase A that has a fold analogous to that of ONC. Thus, it is not expected that the stability of ONC would decrease by ≥ 40 °C because of the absence of one disulfide bond. This argument is supported by the melting temperatures of two des species of ONC. (T_m of des [30–75] is decreased by ~ 30 °C, and T_m of mutant analogues of des [87–104] is decreased by ~ 20 °C (27, 28, 74)). Therefore, I_3 is plausibly stable at 37 °C, where its reshuffling rate or reduction remains very slow. Because the effect of the absence of the native-like interaction of the pyroglutamic moiety on the oxidative folding intermediates of unONC is very similar to that of increased temperature (37 °C) for both intermediates of ONC, the stability of I_3 -unONC at 25 °C should not be altered too much by the absence of the pyroglutamic moiety. The resistance of I_3 to a reduction pulse under these conditions (in contrast to I_1 in Figure 2 and in ref 55) further supports these arguments.

Presence of Kinetically Trapped Species in the Oxidative Folding of Onconase. The destabilization (melting) of disulfide-secure intermediates in oxidative folding was shown to alter the folding rate by 2 orders of magnitude (55). By contrast, the presence of a kinetically trapped species such as I_3 usually has little effect on the overall oxidative folding rate in the first stage of the oxidative folding process, in general, when folding can proceed by other pathways (55). The rate will drop more significantly only in the second stage of the process when most of the precursor intermediates are already converted to either the native or the kinetically trapped species. Because we measured the oxidative folding rates during the first 5 h of the process, it is understandable that a change in the concentrations of I_3 did not change the overall rate. The same consideration applies to I_1 , and thus, I_1 must also lie on a kinetically trapped pathway. The alternative explanation, that the effect of the cyclization on I_1 , which lies on a direct pathway, is compensated exactly by an opposite effect on this or on another direct pathway, is not very likely.

Support for an I_1 -to- I_3 Pathway. If we consider the parallel effects of both the absence of cyclization and an increase in temperature (to 37 °C) on I_1 and I_3 during oxidative folding (Figure 6 (55)), the most plausible explanation is that I_1 and I_3 lie on a common pathway where I_3 is a kinetically trapped species. A des species of ONC could form either by reshuffling from the 3S ensemble or by oxidation of precursor species of the 2S ensemble. Because an earlier study showed no formation of I_3 by reshuffling from the 3S species (54), I_3 must form by oxidation. There are three possible 2S precursor species of I_3 ; of them, I_1 is the only one that accumulates to a considerable extent, supporting the hypothesis of an I_1 -to- I_3 pathway. This interpretation is consistent with data found by the disulfide mapping of these two species (Gahl, R., and Scheraga, H. A., unpublished results). However, the alternative explanation cannot be excluded with

certainty, namely, that I_1 is also a kinetically trapped species, and the real 2S precursor of I_3 does not accumulate to a detectable level because its oxidation (to I_3) is extremely rapid.

Subcellular Localization of QC Catalysis. Our results provide further insight into the subcellular localization of *in vivo* QC catalysis. QC catalysis has been localized mainly in the secretory granules (18). By contrast, the resistance of folded unONC against QC-catalyzed cyclization that we revealed here suggests that *in vivo* QC catalysis of proteins may occur much earlier on the secretory pathways than what is observed with some hormone peptides. The activity of ONC depends on cyclization of the N-terminal glutamine (21). Because nonenzymatic cyclization is very slow, catalysis by QC is necessary to produce fully functional ONC *in vivo*. Given that the native but uncyclized onconase is resistant to QC catalysis as demonstrated by our studies, cyclization plausibly occurs in the ER, before the native 3D structure is formed. Because early forming structured intermediates of unONC are also resistant to QC catalysis, cyclization seems to take place very early during the oxidative folding process, probably co-translationally.

ACKNOWLEDGMENT

We are grateful to Dr. Vaclav Čerovský for helping with the synthesis of the model peptide and to Professor Robert C. Bateman, University of Southern Mississippi, for kindly providing a sample of QC and for helpful discussions.

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BI602495A