J|A|C|S
COMMUNICATIONS

Published on Web 03/06/2004

A New Method for Rapid Characterization of the Folding Pathways of Multidisulfide-Containing Proteins

Ervin Welker, Laura Hathaway, and Harold A. Scheraga*

Baker Laboratory of Chemistry and Chemical Biology, Cornell University, Ithaca, New York 14853-1301

Received December 11, 2003; E-mail: has5@cornell.edu

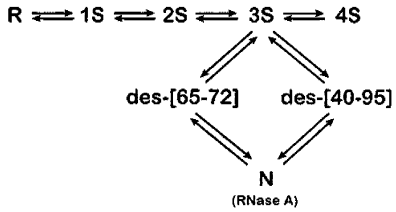
Oxidative folding is a composite process that consists of both the conformational folding to the native three-dimensional structure and the regeneration of the native disulfide bonds of a protein, frequently involving over 100 disulfide intermediate species. Understanding the oxidative folding pathways of a multiple-disulfide-containing protein is a very difficult task that often requires years of devoted research due to the high complexity of the process and the very similar features of the large number of intermediates.[1−7] Here we developed a method for overcoming these difficulties and rapidly delineating the major features of the oxidative folding pathways of a protein. The method examines the temperature dependence of the oxidative folding rate of the protein in combination with reduction pulses. Reduction pulses expose the presence of structured intermediates along the pathways. The correlation between the regeneration rate at different temperatures and the stability of the structured intermediates reveals the role that the intermediates play in determining the pathway. The method was first tested with bovine pancreatic ribonuclease A (RNase A) whose folding pathways were defined earlier.[8,9] Then, it was explored to discern some of the major features of the folding pathways of its homologue, frog onconase (ONC).[10] The results suggest that the stability of the three-dimensional structure of the native protein is a major determinant of the folding rate in oxidative folding.

RNase A and ONC share a low (30%) sequence identity but have a similar native structure.[10] Three out of their four disulfide bonds are in analogous positions in the native structure. A disulfide bond between Cys 87 and Cys 104 at the C-terminus of ONC replaces the (65−72) disulfide bond of RNase A.[11] Although the two proteins have very similar three-dimensional structure, they have remarkable differences in stability, catalytic activity, and toxicity.[12] The midpoint of the thermal transition of ONC is 90 °C, which is 25 °C higher than that of RNase A.[13] The oxidative folding pathway of RNase A is well studied and understood, but this is not so for its ONC homologue.

The oxidative folding pathways of proteins can be characterized in the terms of the existence and nature of the structured intermediates.[6] Two types of structured intermediates have been distinguished; in disulfide-secure intermediates, the disulfide bonds remain buried while the thiols are exposed. By contrast, in disulfide-insecure intermediates, the disulfide bonds and the thiols are simultaneously exposed by the breathing of the three-dimensional structure.[9] Disulfide-secure intermediates, in which the thiols are accessible for further reaction, have been suggested[9] as critical for accelerating the oxidative folding process. Our results here underline the relevance of this classification of the diverse oxidative folding pathways.

In RNase A, the 1S−4S species are populated by successive oxidation until a pre-equilibrium (quasi-steady-state condition) is established among the unstructured intermediates.[14,15] (nS denotes an ensemble of disulfide isomers with the same number of disulfide bonds.) No stable structure has been detected in these ensembles. The rate-determining steps in the regeneration of RNase A are the formation of the two des species, des [65−72] and des [40−95] species with nativelike structure at 25 °C.[6,14]

These species are formed largely by reshuffling from the 3S ensemble, although a small fraction (no more than 5%) may be formed by oxidation from the 2S ensemble.[6] Upon formation and folding of des [40−95] or des [65−72], their three native disulfide bonds become protected from reduction and reshuffling ("locked in"), causing these species to accumulate to much higher levels. However, their thiol groups remain accessible to the solvent and, hence, these species oxidize relatively rapidly to the native protein. These two pathways, of which des [40−95] is the dominant one, through which about 80% of the protein regenerates, appear to account for nearly all of the native RNase A regenerated under pH 8, 25 °C oxidative folding conditions.[8] At lower temperature (15 °C), two other nativelike structured species, des [26−84] and des [58−110], are stabilized and, thus, accumulate.[9] However, these two species are kinetic traps that reshuffle to other intermediates instead of oxidizing to the native protein.[9]



At present, the oxidative folding mechanism of ONC is not known. To obtain an understanding of the folding pathways of ONC in the absence of such information, the method developed here, and described below, was first tested with RNase A under conditions in which its folding had not been studied; then, the oxidative folding of ONC was studied under similar conditions. Reduced RNase A (36 μM) or ONC (22 μM) was incubated in 100 mM tris, 2 mM EDTA, 25 mM DTT^{ox}, pH 8 buffer at 15, 25, or 37 °C. Aliquots were taken at different times, and the unreacted thiols were blocked with 30 mM 2-aminoethyl methanethiosulfonate (AEMTS). The samples were desalted and applied to a cation exchange HPLC column. AEMTS blocking introduces a positive charge to each blocked cysteine that facilitates the separation of the intermediates on the cation exchange column. To identify the stable structured intermediates, a reduction pulse (5 mM DTT^{red} for 2 min) was applied to some of the samples before the AEMTS blocking. Such a reduction pulse (usually between 5 and 10 mM DTT^{red} for 0.5−2 min depending on the pH and temperature) is sufficient for the reduction of the unstructured intermediates whose disulfide bonds are exposed to the solvent; however, intermediates possessing a stable conformation that buries their disulfide bonds are not reduced under these conditions. Examples of such a reduction pulse are shown in Figure 1.

10.1021/ja031658q CCC: $27.50

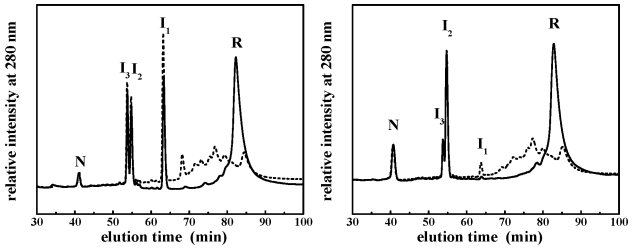


Figure 1. Cation exchange HPLC profiles of populated species during ONC regeneration after 1 h, both with (solid line) and without (dashed line) a reduction pulse. The conditions were pH 8 and 25 mM DTT^{ox} (A) at 25 °C and (B) at 37 °C. The samples were blocked with AEMTS and were injected, after desalting, onto the analytical cation exchange HPLC column. R, reduced protein; N, native protein; I_1, I_2, I_3, the peaks that correspond to the intermediates that withstand the reduction pulse.

Table 1. Observed Rate Constants for Regeneration of Native Species with DTT^{ox}/DTT^{red} at pH 8.0 ($\times 10^{-4}$ min^{-1})

	15 °C	25 °C	37 °C
RNase A	2.32 ± 0.4	5.50 ± 0.1	0.21 ± 0.06
ONC	3.72 ± 1.0	9.77 ± 0.3	23.9 ± 5.1

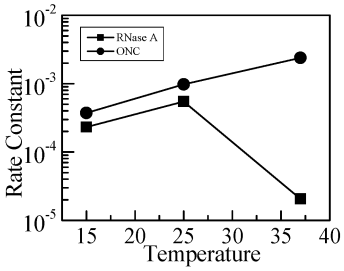


Figure 2. Temperature dependence of the regeneration rates of ONC and RNase A.

Results from these regeneration experiments showed that the rate of the formation of the native protein, N, is well approximated by a first-order rate equation, $\ln[1 - N] = -kt$, where $[1 - N]$ is the fractional concentration of all non-native species, k represents the rate constant for the formation of native protein, and t is the regeneration time (Table 1). Data were collected from the first period of the regeneration after the initial lag time where the pre-equilibrium among the unstructured species was already established but before they were fully consumed by conversion to the structured (intermediates and native) species.

In RNase A, the temperature dependence of the rate of regeneration leads to a bell-shaped curve, in good agreement with previous studies[8,14] (Figure 2). Furthermore, confirming previous results,[14] reduction-pulse experiments show that the stable des species of RNase A that accumulate at 25 °C do not accumulate at 37 °C (data not shown). These data are easily explained by examining the stability of these des species. The melting temperature of the mutant analogues of des [40−95] and des [65−72] is about 37 °C. Thus, the lack of the accumulation of these species is a result of the decreased stability of the three-dimensional structure that does not provide sufficient protection against the reshuffling of the disulfide bonds at 37 °C. Consequently, at 37 °C, a considerably large fraction of these des species that are formed during the regeneration reshuffle faster than the remaining two thiols can be oxidized to the native protein under the given redox conditions (25 mM DTT^{ox}). Interestingly, the presence of the kinetically trapped intermediates of RNase A apparently does not have much effect on the regeneration rate at 15 °C. During the regeneration at 15 °C, approximately 30% of the intermediates are found in this kinetically trapped state, thus preventing about 30% of the protein from converting to N but having no effect on the other 70%. This means that their presence can decrease the rate at most by 30%. By contrast, the destabilization of the disulfide secure intermediates between 25 and 37 °C results in a ∼20-fold decrease in the rate, indicating the critical role that these intermediates play in fast regeneration.

In ONC, the regeneration rate increases monotonically with the temperature (Figure 2), and the reduction pulses reveal the presence of three peaks corresponding to structured intermediates at all three temperatures (Figure 1), although peak I_1 apparently melted out at 37 °C. It should be noted that the peaks do not necessarily correspond to only one intermediate. Comparison of the regeneration rates of RNase A and ONC shows striking results. While the rates are comparable in magnitude at 15 and 25 °C, the regeneration rate of ONC at 37 °C is ∼100-fold faster than that of RNase A. These results strongly suggest the existence of a disulfide-secure structured intermediate(s) that is stable at 37 °C and plays a critical role in the fast regeneration of ONC. The melting of peak I_1 at 37 °C does not cause a decrease in the regeneration rate, indicating that this intermediate is not critical for the efficient regeneration of ONC. Peak I_3 is a kinetic trap since it is still present in the regeneration mixture at 15 and 25 °C, when all other intermediates have been converted to the native protein (data not shown). Thus, peak I_2 corresponds to a critical intermediate(s) that is responsible for the 100-fold faster regeneration rate of ONC at 37 °C.

In conclusion, this method revealed some basic features of the oxidative folding of ONC and suggests that the degree of stability of structure of the native protein that, in turn, affects the stability of the des intermediates is an important factor in determining the regeneration rates of multiple-disulfide-containing proteins.

Acknowledgment. This research was supported by NIH Grant No. 24893. We thank Mahesh Narayan for careful reading of the manuscript.

References

(1) Goldberg, M. E.; Rudolph, R.; Jaenicke, R. *Biochemistry* **1991**, *30*, 2790−2997.
(2) Weissman, J. S.; Kim, P. S. *Science* **1991**, *253*, 1386−1393.
(3) Goldenberg, D. P. *Trends Biochem. Sci*. **1992**, *17*, 257−261.
(4) Creighton, T. E. *Biol. Chem. Hoppe-Seyler* **1997**, *378*, 731−744.
(5) Roux, P.; Ruoppolo, M.; Chaffotte, A.-F.; Goldberg, M. E. *Protein Sci.* **1999**, *8*, 2751−2760.
(6) Narayan, M.; Welker, E.; Wedemeyer, W. J.; Scheraga, H. A. *Acc. Chem. Res.* **2000**, *33*, 805−812.
(7) Jarrett, N. M.; Djavadi-Ohaniance, L.; Willson, R. C.; Tachibana, H.; Goldberg, M. E. *Protein Sci.* **2002**, *11*, 2584−2595.
(8) Rothwarf, D. M.; Li, Y.-J.; Scheraga, H. A. *Biochemistry* **1998**, *37*, 3760−3776.
(9) Welker, E.; Narayan, M.; Wedemeyer, W. J.; Scheraga, H. A. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 2312−2316.
(10) Ardelt, W.; Mikulski, S. M.; Shogen, K. *J. Biol. Chem.* **1991**, *266*, 245−251.
(11) Mosimann, S. C.; Ardelt, W.; James, M. N. *J. Mol. Biol.* **1994**, *236*, 1141−1153.
(12) Boix, E.; Wu, Y.; Vasandani, V. M.; Saxena, S. K.; Ardelt, W.; Ladner, J.; Youle, R. J. *J. Mol. Biol.* **1996**, *257*, 992−1007.
(13) Newton, D. L.; Boque, L.; Wlodawer, A.; Huang, C. Y.; Rybak, S. M. *Biochemistry* **1998**, *37*, 5173−5183.
(14) Rothwarf, D. M.; Scheraga, H. A. *Biochemistry* **1993**, *32*, 2671−2703.
(15) Torella, C.; Ruoppolo, M.; Marino, G.; Pucci, P. *FEBS Lett.* **1994**, *352*, 301−306.

JA031658Q