Communications to the Editor

Characterization of Multiple Reduction Pathways of Proteins in the Presence of a Denaturant

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There are many possible disulfide-bonded intermediates in the pathways of reductive unfolding of proteins containing several disulfide bonds.[1,2] These intermediates lack some of the native disulfide bonds and usually do not have the stable structure that protects the disulfide bonds of the native protein from attack by a reducing agent. Consequently, the disulfide bonds of the intermediates are more accessible, and hence more reactive, than those of the native structure, which makes it difficult to observe these intermediates in reduction experiments because they are rapidly reduced. In this paper, we describe a new experimental method to observe such unstable reduction intermediates directly by using a combination of a quench-flow technique and a high-performance liquid chromatography (HPLC) fractionation.

Consider the reduction pathway of the mutant of bovine pancreatic ribonuclease A (RNase A) in which Cys65 and Cys72 have been replaced by two serines ([C65S,C72S]).[3] This mutant retains three native disulfide bonds (26-84, 40-95, and 58-110) and has a nativelike folded structure but less global stability than the wild-type protein. Figure 1 shows the possible multiple reduction pathways of the mutant, which consist of six intermediates, three with two native disulfide bonds ([26-84,40-95], [26-84,58-110], and [40-95,58-110]) and three with one native disulfide bond ([26-84], [40-95], and [58-110]), in addition to the native-like folded state (N) and the totally reduced one (R). This scheme is reasonable if mixed-disulfide intermediates between the protein and a reducing agent, and also disulfide-reshuffled intermediates, are not populated in the reduction pathways. Indeed, when dithiothreitol (DTT^{red}), which is known to form an unstable mixed disulfide bond with protein thiols,[4] is used at high concentrations as a reducing agent at 25 °C, pH 8.0, these conditions are met for the case of the mutant RNase A on the basis of recent regeneration experiments.[5] If a high concentration of DTT^{red} is used at 25 °C, pH 8.0, the reduction pathways of other proteins could be similar to those of the mutant, provided that the rates of disulfide reshuffling and reduction of a disulfide bond do not differ significantly from those for RNase A.

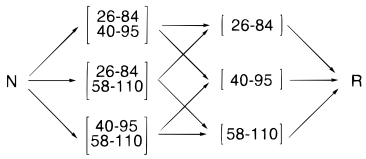

Figure 1. Multiple reduction pathways of [C65S,C72S] RNase A. N represents the native-like folded state, and R represents the totally reduced state of the mutant. Six possible partially reduced intermediates are shown in brackets with a pair of numbers that represent a cystine−cystine bond in the intermediate.

However, when [C65S,C72S] was reduced with DTT^{red} at 25 °C, pH 8.0 under a nitrogen atmosphere (normal conditions),[6] we could observe only N and R by cation-exchange HPLC[7] of the sample solutions that were removed from the reduction mixture after a certain reaction time when the reactions were quenched by addition of 2-aminoethyl methanethiosulfonate (AEMTS).[8] AEMTS blocks a protein thiol very rapidly,[4] and introduces a 2-aminoethylthio group. Since this protecting group has one unit of positive charge, the intermediates blocked by AEMTS could be separated on a cation-exchange column depending on the number of intramolecular protein disulfide bonds. Failure to observe partially reduced intermediates suggested that the rate-determining (slow) step is the first reduction, i.e., N → 2S (where 2S represents an ensemble of three possible intermediates with two native disulfide bonds).

To reduce the disulfide bond, N must first be unfolded so that the reducing agent can have access to the disulfide bond. This unfolded species (U) is unstable relative to N and will achieve a rapid equilibrium with N at 25 °C, pH 8.0 in the absence of a denaturant.[1] Therefore, an EX2 mechanism[9] can be applied to this reaction, i.e.,

$$N \overset{K}{\rightleftharpoons} U \xrightarrow{k[DTT^{red}]} 2S$$

where K is an equilibrium constant between N and U and k is a bimolecular rate constant for the reduction of an exposed disulfide bond with DTT^{red}. On the basis of the apparent rate constant for the reduction of the des-[65-72] intermediate, which is wild-type RNase A with the 65-72 disulfide bond reduced, Li et al.[1] estimated the value of the difference in free energy between U and N as 5.7 ± 0.1 kcal/mol. By analysis of a guanidine-induced denaturation curve of [C65S,C72S] at 25 °C, pH 8.0,[10] we found a value of 5.0 ± 0.5 kcal/mol for the denaturation free energy of

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(1) For a recent paper on reductive unfolding of RNase A, see: Li, Y.-J.; Rothwarf, D. M.; Scheraga, H. A. *Nat. Struct. Biol.* **1995**, *2*, 489−494.

(2) For recent papers on reductive unfolding of other proteins, see: Kuwajima, K.; Ikeguchi, M.; Sugawara, T.; Hiraoka, Y.; Sugai, S. *Biochemistry* **1990**, *29*, 8240−8249. Ewbank, J. J.; Creighton, T. E. *Biochemistry* **1993**, *32*, 3677−3693. Mendoza, J. A.; Jarstfer, M. B.; Goldenberg, D. P. *Biochemistry* **1994**, *33*, 1143−1148. Chang, J.-Y. *J. Biol. Chem.* **1997**, *272*, 69−75. Ma, L.-C.; Anderson, S. *Biochemistry* **1997**, *36*, 3728−3736.

(3) Shimotakahara, S.; Ríos, C. B.; Laity, J. H.; Zimmerman, D. E.; Scheraga, H. A.; Montelione, G. T. *Biochemistry* **1997**, *36*, 6915−6929.

(4) Rothwarf, D. M.; Scheraga, H. A. *J. Am. Chem. Soc.* **1991**, *113*, 6293−6294.

(5) The average time constant for reduction of an *exposed* disulfide bond has been estimated to be 1.3 s in the presence of 100 mM DTT^{red}, based on the average rate constant for reduction of an exposed disulfide bond (470 min^{-1} M^{-1}), while that for intramolecular disulfide reshuffling has been estimated to be 15 s, based on the average rate constant for intramolecular formation of a disulfide bond (4.0 min^{-1}) (see: Iwaoka, M.; Juminaga, D.; Scheraga, H. A. *Biochemistry* **1998**, *37*, 4490−4501). Since the regeneration intermediates of the mutant are mostly disordered, these time constants may not differ significantly from those in the presence of GdnCl.

(6) The same conditions were used in a study of the reduction of wild-type RNase A.[1]

(7) A ternary gradient was used. Buffer A contained 25 mM HEPES, 1 mM EDTA, pH 7.0. Buffer B contained 25 mM HEPES, 1 mM EDTA, 1M NaCl, pH 7.0. Buffer C contained 50 mM acetic acid, 1 mM EDTA, pH 5.0. The sample solution was injected on to the column equilibrated with buffer C. After changing the eluting buffer to buffer A (in 5 min), the system was equilibrated with 95% buffer A and 5% buffer B for 7 min. An NaCl gradient then was applied by changing the ratio of buffer B from 5% to 20% in 60 min.

(8) Bruice, T. W.; Kenyon, G. L. *J. Protein Chem.* **1982**, *1*, 47−58.

(9) Woodward, C.; Simon, I.; Tüchsen, E. *Mol. Cell. Biochem.* **1982**, *48*, 135−160. Bai, Y.; Milne, J. S.; Mayne, L.; Englander, S. W. *Proteins: Struct., Funct. Genet.* **1994**, *20*, 4−14.

(10) $\Delta G°_w$ = 5.0 ± 0.5 kcal/mol, m = 3.8 ± 0.4 kcal/mol/M, and $[GdnCl]_{half}$ = 1.3 ± 0.1 M for the guanidine-induced denaturation of [C65S,C72S] RNase A at 25 °C, pH 8.0. These values were determined by using UV absorbance at 287 nm.

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N in water. Therefore, the stability (and presumably the structure) of U appears to be similar to that of the denatured form of N.[11]

Our strategy to observe reduction intermediates that could not be observed under normal conditions is to speed up the reduction of N by reducing the protein in the presence of guanidine hydrochloride (GdnCl) as a denaturant so that U is stabilized relative to N. Under such denaturant conditions, N should be rapidly unfolded to U, with probable occurrence of partial proline isomerization,[11] at the beginning of the reaction, and then it will be reduced to R stepwise with DTT^{red}. In this case, the reduction will be completed on the order of seconds. We, therefore, have exploited a quench-flow technique to carry out the reaction.[12] *This approach is based on the assumption (justified by the fact that U appears to be similar to denatured N) that the reductive pathways of the mutant are similar in both the presence and absence of GdnCl.*[13]

The reduction reaction was initiated by mixing the protein solution with a solution containing DTT^{red} and GdnCl under a nitrogen atmosphere. The concentrations of [C65S,C72S], DTT^{red}, and GdnCl in the mixed solution were controlled to be 74 μM, 100 mM, and 3.26 M, respectively. The concentration of GdnCl was selected so that the mutant would be completely denatured, the time constant of the global unfolding (a fast phase) being 1.61 s,[14] and the time constant for reduction of the unfolded state being comparable, viz., 1.3 s. After 3 s, the solution was mixed with the quenching solution containing 1 M AEMTS. After standing for 5 min at room temperature, the collected solution was acidified to pH 4.7 by adding acetic acid and stored frozen at −20 °C. The sample solutions were later desalted and injected on to an HPLC system equipped with a cation-exchange column.[7] The resulting chromatogram is shown in Figure 2.

Many peaks were observed between those of N and R in the HPLC chromatogram of the mutant. We assigned the structure of each separated peak by application of a peptide-mapping method.[15] The assignments are shown in the legend of Figure 2. It should be noted that there was no strong evidence for the occurrence of disulfide reshuffling during the reduction time (3 s) within the detection limit of the mapping method. This observation supports the reduction pathways of Figure 1 and the validity of the new method for the study of protein reduction pathways. This is the first characterization of all intermediates in the multiple reduction pathways of a native-like protein in the presence of a denaturant.

(11) The observation that the kinetic intermediate U is slightly less stable than the equilibrated denatured form of N may be due partially to retention of the native conformations of the X-Pro peptide bonds in U; stopped-flow unfolding experiments of [C65S, C72S] (unpublished results) suggested that the time constant of X-Pro isomerization (a slow phase) is about 20 s at 25 °C, pH 8.0, and it does not change with the concentration of GdnCl.

(12) A schematic diagram of the quench-flow instrument used to carry out the reduction of proteins under denaturant conditions is given in the Supporting Information.

(13) By contrast, if U has some structure as in the first reduction steps of the native state of wild-type RNase A to three-disulfide intermediates,[1] the presence of a denaturant would alter the reduction pathways significantly.

(14) The time constant was determined by stopped-flow unfolding experiments under the same conditions.

(15) Xu, X.; Rothwarf, D. M.; Scheraga, H. A. *Biochemistry* **1996**, *35*, 6406−6417.

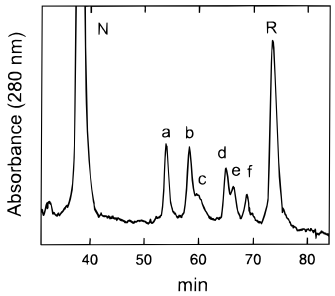

Figure 2. Cation-exchange HPLC chromatograms of the reduction mixture of [C65S,C72S] RNase A under the conditions of 74 μM [C65S,-C72S], 100 mM DTT^{red}, and 3.26 M GdnCl at 25 °C, pH 8.0 for a reduction time of 3 s. Assignments of the structure for each separated peak are **a**, [26-84,40-95]; **b**, [40-95,58-110]; **c**, [26-84,58-110]; **d**, [40-95]; **e**, [26-84]; **f**, [58-110]. The small peak to the left of N is due to deamidation of N.

It is interesting to compare the relative population of each intermediate within the 1S and 2S ensembles. Since disulfide reshuffling is slow, the relative populations are determined by the relative rates of formation and reduction of each intermediate. For the case of the 2S ensemble of [C65S,C72S], the population of [26-84,58-110] (peak **c**) is obviously smaller than those of the other two two-disulfide intermediates (peaks **a** and **b**). This indicates that the formation of [26-84,58-110] is slower and/or its reduction is faster. In other words, this means that the reactivity of the 40-95 disulfide bond is lower than those of the 26-84 and 58-110 disulfide bonds in the denatured state of N and/or that the reduction of [26-84,58-110], which does not have the 40-95 disulfide bond, is faster than those of the other two 2S intermediates ([26-84,40-95] and [40-95,58-110]), which do have the 40-95 disulfide bond. Thus, it is suggested that there is local structure in the vicinity of the 40-95 disulfide bond of the kinetic unfolded species (U) even in the presence of 3.26 M GdnCl.

Since conformational unfolding and reduction of disulfide bonds are coupled, this new approach will be useful for the isolation of intermediates (and their structural characterization) in the study of reductive *unfolding* of proteins. Such isolated intermediates will also be useful in *refolding* studies. More information about the kinetics of the reduction pathway and the structures of the intermediates can be obtained by changing the reaction time and the concentrations of GdnCl and DTT^{red}.

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Supporting Information Available: A schematic diagram of the quench-flow instrument (2 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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