Effect of protein disulfide isomerase on the regeneration of bovine ribonuclease A with dithiothreitol

Hang-Cheol Shin, Harold A. Scheraga*

Baker Laboratory of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853-1301, USA

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Abstract The role of protein disulfide isomerase (PDI) in the regeneration of ribonuclease A with dithiothreitol (DTT) was investigated at three different temperatures. The rates of formation of the native protein were markedly increased in the presence of PDI, 9-fold at 15°C, 6-fold at 25°C and 62-fold at 37°C, respectively. In the presence of PDI, major changes were found in the distribution of intermediates in the three-disulfide region at 25 and 15°C and also in the one-disulfide region at 15°C, with the fast accumulation of the two native-like species des-[65-72] and des-[40-95]. The present results indicate that PDI does not alter the two major parallel pathways involving des-[65-72] and des-[40-95] in the regeneration of ribonuclease A with DTT.

Key words: Protein disulfide isomerase; Ribonuclease A; Folding; Dithiothreitol; Distribution of intermediate

1. Introduction

The in vitro refolding of a protein is generally a spontaneous process driven by a difference in Gibbs free energy under native conditions with kinetic accessibility during refolding. All the necessary structural information required for correct folding is encoded in the amino acid sequence of the protein [1]. In many cases, however, refolding does not proceed efficiently, because of intermolecular aggregation and slow folding reactions such as disulfide formation or proline isomerization. Inside the cell, protein folding is assisted by cellular factors, such as protein disulfide isomerase (PDI) [2], peptidyl prolyl *cis-trans*-isomerase [3] and molecular chaperones [4]. The roles of these proteins in the in vivo folding mechanism need to be elucidated by examining their effects on well-characterized folding reactions in vitro.

PDI is an abundant protein found in the endoplasmic reticulum, where it catalyzes disulfide-coupled folding of proteins [2]. Earlier studies found that PDI increased the refolding rate of bovine pancreatic trypsin inhibitor in the presence of dithiothreitol (DTT) [5,6] or glutathione [7]. Similar results were found in the oxidative folding of ribonuclease A (RNAse A) in the presence of glutathione [8]. An earlier attempt to use PDI in the oxidative folding of RNAse A in the presence of DTT, however, was unsuccessful, with the formation of only one- (1S) and two-disulfide (2S) intermediates [5]. No formation of three- (3S) or four-disulfide species, including correctly refolded RNAse, was observed. The use of DTT as a redox couple is more advantageous in protein folding studies because, unlike glutathione, DTT does not form stable mixed disulfides with protein thiols. This drastically reduces the number of possible disulfide-bonded intermediates from 7191 to 762 in the case of RNAse A which has four disulfides and, therefore, significantly simplifies protein folding studies [9]. Much research has been carried out in our laboratory [9–16] and elsewhere [17,18] to investigate the folding mechanism of RNAse A in vitro. Regeneration of RNAse A from the reduced form by oxidized DTT (DTT^{ox}) at 25°C proceeds through two major parallel pathways [13,14] and two minor ones [15,16], involving the formation of two native-like 3S intermediates, des-[65-72] and des-[40-95], which are missing the 65-72 and 40-95 disulfide bonds, respectively.

In this paper, we report the first successful regeneration of native RNAse A with DTT in the presence of PDI. The effect of PDI on the rate of formation of the native protein and the distribution of intermediates was investigated at three different temperatures.

2. Materials and methods

2.1. Materials

Native and reduced RNAse A (type 1-A, Sigma) were prepared as described previously [9]. Reduced DTT (DTT^{red}) (ultrapure) was obtained from Sigma. DTT^{ox} (Sigma) was purified by the method of Creighton [19]. 2-aminoethyl methanethiosulfonate (AEMTS) was synthesized as described by Bruce and Kenyon [20]. Protein disulfide isomerase was purified from bovine liver according to the method of Hillson et al. [21]. All other reagents were of the highest grade commercially available.

2.2. Regeneration of RNAse A

Regeneration experiments with DTT^{ox}/DTT^{red} were carried out at a starting concentration of 100 mM DTT^{ox} and 28 μM reduced RNAse A in the presence and absence, respectively, of 4 μM PDI. After initiation of the regeneration process, aliquots were taken at various refolding times and quenched with AEMTS [20,22]. Blocking by AEMTS introduces one unit of positive charge into the regeneration intermediates, so that they can be separated in the subsequent ion-exchange HPLC analysis on the basis of the number of cysteines blocked by AEMTS. In addition to the usual blocking procedure, aliquots were subjected to a 60 s pulse of concentrated DTT^{red} (final concentration of 10 mM) prior to AEMTS blocking, as described previously [14].

3. Results and discussion

In the absence of PDI, oxidative folding of RNAse A is a slow process with a short lag period at the beginning. Regeneration is accelerated by adding PDI and essentially no lag period appears (Fig. 1). In all regeneration experiments, both in the absence and presence of PDI, the kinetics are well

*Corresponding author. Fax: (1) (607) 254-4700.
E-mail: has5@cornell.edu

Abbreviations: RNAse A, ribonuclease A; PDI, protein disulfide isomerase; DTT^{ox}, oxidized dithiothreitol; DTT^{red}, reduced dithiothreitol; AEMTS, 2-aminoethyl methanethiosulfonate

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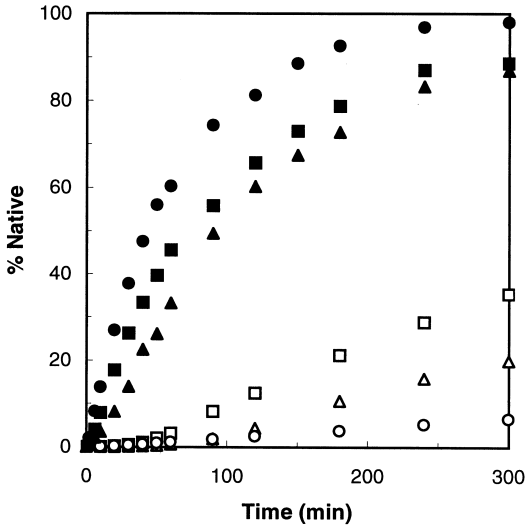

Fig. 1. Appearance of native protein as a function of the regeneration time. The conditions were 100 mM DTT^{ox}, 28 μM reduced RNAse A, 100 mM Tris-acetate/2 mM EDTA (pH 8.0) under an argon atmosphere. Regeneration is shown in the absence of PDI at 15°C (△), 25°C (□) and 37°C (○) and in the presence of 4 μM PDI at 15°C (▲), 25°C (■) and 37°C (●), respectively.

approximated by a single first-order reaction. The rate constants for regeneration were determined by a first-order rate of appearance of native protein, N, and integrating the rate equation: $\ln(1-N) = -kt$, where $(1-N)$ is the concentration fraction of all non-native species. In the absence of PDI, the rate constants for the formation of native RNAse A were calculated to be 8.4×10^{-4} min^{-1} (15°C), 15.8×10^{-4} min^{-1} (25°C) and 2.3×10^{-4} min^{-1} (37°C), respectively. The rates were markedly increased in the presence of PDI to 76.1×10^{-4} min^{-1} (15°C), 86.6×10^{-4} min^{-1} (25°C) and 143.2×10^{-4} min^{-1} (37°C), respectively. The rate accelerations by PDI were 9-fold at 15°C and 6-fold at 25°C, respectively. The most dramatic effect was found at 37°C where PDI increases the rate of the formation of native RNAse A by a factor of 62. The unusually low regeneration rate at 37°C in the absence of PDI has been attributed to the relative instability of two native-like 3S intermediates, des-[65-72] and des-[40-95], which are missing the 65-72 and 40-95 disulfide bonds, respectively, and their faster rearrangement rate back to the 3S ensemble [14]. The rate of formation of two native-like intermediates and their oxidation to the native protein can be increased significantly in the presence of PDI, because of the effect of PDI on disulfide formation and rearrangement by thiol/disulfide exchange [2]. Therefore, the contribution of the rearrangement of des-[65-72] and des-[40-95] back to the 3S species is less significant and the rate of formation of native protein is dominated by the formation of the two des-species in the presence of PDI.

In addition to the changes in the rate of formation of N, significant changes in the distribution of intermediates were also found. In the absence of PDI, essentially the same distributions were obtained as found previously [12], except for a small additional peak found between the two prominent peaks (indicated by * in Fig. 2) in the 3S region at 15°C. This small peak was identified by a reduction pulse experiment [14] as a

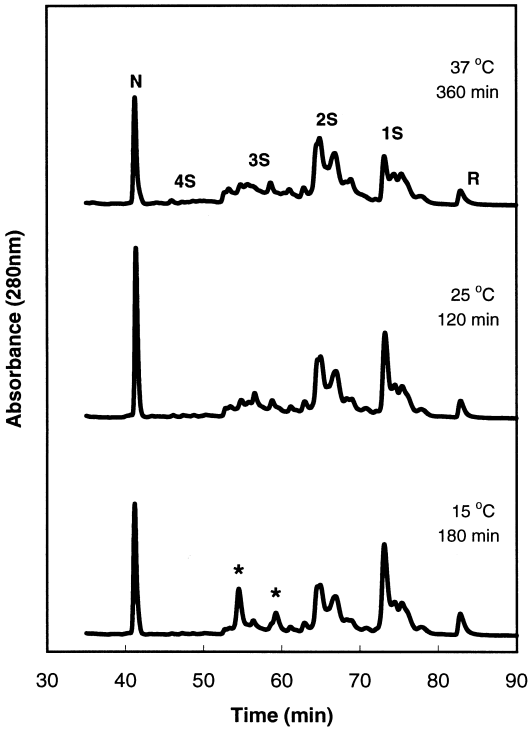

Fig. 2. Distribution of regeneration intermediates at three different temperatures in the absence of PDI. The reduced protein is designated R and the native protein is N. An Arabic numeral corresponding to the number of intramolecular disulfide bonds followed by S denotes the various groupings of disulfide-bonded intermediates. Initial regeneration conditions were 100 mM DTT^{ox}, 28 μM reduced RNAse A, pH 8.0. All regeneration studies were carried out in 100 mM Tris-acetate/2 mM EDTA under an argon atmosphere.

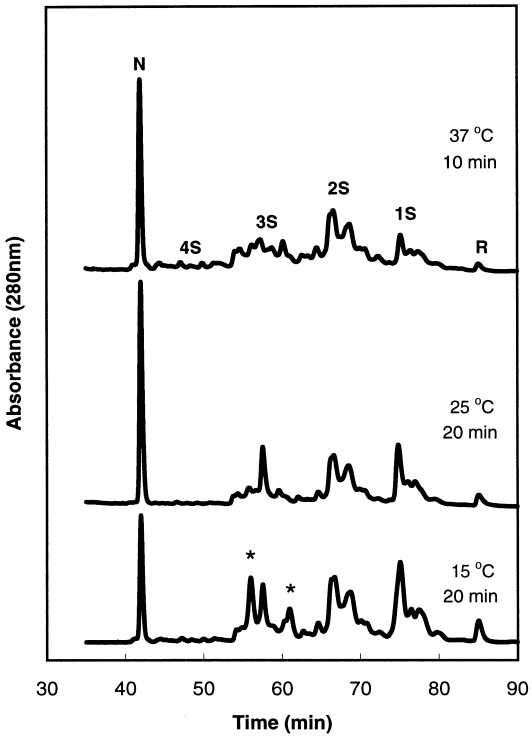

Fig. 3. Distribution of regeneration intermediates at three different temperatures in the presence of PDI. Conditions are the same as for Fig. 2 except for the addition of 4 μM PDI (final concentration).

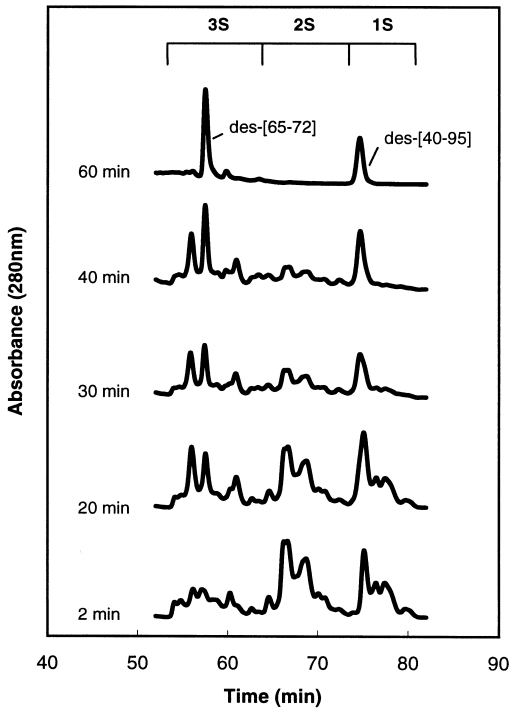

Fig. 4. Change in the distribution of 1S, 2S and 3S regions as a function of time in the presence of PDI at 15°C.

des-[65-72] intermediate. In the presence of PDI, major changes were found in the 3S regions at 25 and 15°C (compare Figs. 2 and 3) and also in the 1S region after 30 min regeneration at 15°C (Fig. 4).

At 25°C, the 3S region exhibits the same distribution in both the presence and absence of PDI, at the start of regeneration, but after 10 min, the relatively complicated 3S distribution in the absence of PDI starts to show a single major peak in the presence of PDI (Fig. 3), which was identified as a des-[65-72] species by a reduction pulse experiment. The population of the des-[65-72] keeps on accumulating in the presence of PDI at 25°C up to 60 min regeneration time where slightly less than half of the native structure was formed ($t_{1/2} = 80$ min). Afterwards, the population of the des-[65-72] species starts to decrease gradually.

At 15°C, the three major peaks (two large peaks and the des-[65-72] peak between them) in the 3S region keep on increasing up to 40 min and, afterwards, the two large peaks rapidly disappear with des-[65-72] continuing to increase in the presence of PDI (Fig. 4). The rates of accumulation of the three peaks up to 40 min at 15°C are also different, with des-[65-72] increasing faster than the other two species. The des-[65-72] peak remained relatively constant from 90 min up to 300 min when the sampling was terminated. At 15°C, the 1S distribution started to show a single dominant peak after 30 min, which became a single peak at 60 min (Fig. 4). This peak was identified by a reduction pulse experiment as the des-[40-95] intermediate. After 60 min, the des-[40-95] species started to decrease. The peak ratio of des-[65-72]/des-[40-95] was 1.7/1 at 60 min and 5/1 at 180 min regeneration time at 15°C, in contrast to the behavior in the absence of PDI where des-[40-95] accumulates to a greater extent at 25°C than does des-[65-72] [13,14].

The des-[65-72] and des-[40-95] are the two major 3S intermediates that follow after the transition states in the regeneration pathways of RNAse A [13,14]. The large number of theoretically possible 3S species (420 including des-[65-72] and des-[40-95]) rearranges to these two native-like des-species in the rate-determining steps. The relatively fast accumulation of two native-like des-species in the presence of PDI at 25 and 15°C indicates that PDI effectively accelerates the rearrangement step from 3S to des-[65-72] and des-[40-95], respectively. It is interesting to compare the rate of formation of N and the distribution of intermediates at 37°C in the presence of PDI with those at lower temperatures. In contrast to the results at 25 and 15°C, no dominant des-species accumulated at 37°C. This suggests that the oxidation of the des-species to N could be much faster at 37°C, off-setting the accumulation of the two des-species due to the increased rearrangement rates.

In conclusion, we have shown here the first successful regeneration of native RNAse A with DTT^{ox} in the presence of PDI and the fast accumulation of the des-species at 25 and 15°C due to the increased rate of rearrangements from 3S to the two native-like des-species. This indicates that PDI does not alter the two parallel pathways involving des-[65-72] and des-[40-95] in the regeneration of RNAse A with DTT.

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