Formation of native structure by intermolecular thiol-disulfide exchange reactions without oxidants in the folding of bovine pancreatic ribonuclease A

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Received 23 February 2000

Edited by Maurice Montal

Abstract It has been shown previously that the oxidative folding of bovine pancreatic ribonuclease A proceeds through parallel pathways with two major native-like three-disulfide (3S) intermediates. We show here that, under some conditions, the native disulfide bonds can also be regenerated through disproportionation reactions; in other words, the protein can serve as its own redox reagent. The results also show that disulfide species of the unstructured 3S ensemble have a strong propensity to participate in intermolecular interactions. These interactions are favored at high protein concentration, temperature and pH, and lead to formation of the native structure during disulfide reshuffling in the rate-determining step.

Key words: Ribonuclease A; Intermolecular thiol-disulfide exchange reaction; Partially folded intermediate; Protein folding

1. Introduction

Protein folding is a highly cooperative process in which the nascent polypeptide chain condenses into a compact globular structure that rearranges slowly to the correctly folded native structure [1]. To understand how proteins fold into a functionally active, unique, native conformation, much interest is currently being focused on folding intermediates. Although there is much experimental evidence for the accumulation of intermediates, the role of intermediates in the protein folding process still remains unclear [2–4].

Previous studies of the regeneration of bovine pancreatic ribonuclease A (RNase A) have shown that unstructured ensembles of one-, two-, three- and four-disulfide-bonded intermediates form sequentially and, after an initial period, reach a steady-state distribution [5]. These ensembles are denoted as 1S, 2S, 3S and 4S, respectively. RNase A folds through two major parallel pathways involving the formation of two native-like 3S species, des-[65-72] and des-[40-95], which are missing the 65-72 and 40-95 disulfide bonds, respectively [5–10]. The rate-determining steps are the reshuffling of 3S disulfide species to form the native-like des species, both of which then oxidize rapidly to form the native structure. Since the reshuffling step is a slow process with a time constant of around 100 min at 25°C and pH 8.0 [10], intermolecular thiol-disulfide exchange reactions can take place between disulfide intermediates during this stage in the folding process of RNase A. Therefore, in order to clarify the disulfide-coupled folding process of RNase A, it is necessary to investigate the role of intermolecular thiol-disulfide exchange reactions.

In the present study, we have focused on the formation of the native structure through such intermolecular thiol-disulfide exchange reactions. To elucidate the relationship between the non-native disulfide bond ensembles and intermolecular thiol-disulfide exchange reactions, we describe the reshuffling of the native-like des species (viz. des-[65-72] and des-[40-95]), and discuss this process in terms of a plausible pathway for the regeneration of RNase A. Unfolded and reduced proteins can regenerate the native sets of disulfide bonds spontaneously under suitable conditions, and reformation of disulfide requires the presence of a redox partner, such as glutathione or dithiothreitol (DTT) [5]. Remarkably, we observe that intermolecular thiol-disulfide exchange reactions between the unstructured 3S ensemble and the two des species can also regenerate the native disulfide bonds and thus induce the formation of the native structure. Our results may contribute to a more detailed understanding of the formation of the native structure in the folding processes.

2. Materials and methods

2.1. Preparation of des-[40-95] and des-[65-72]

Two native-like des species, des-[40-95] and des-[65-72], were obtained in their unblocked form by a reduction experiment, followed by a multi-step purification involving cation-exchange chromatography and reverse-phase high performance liquid chromatography (HPLC) [6]. The reduction experiment was carried out as follows: native RNase A was dissolved in degassed 100 mM Tris–HCl buffer and equilibrated at 15°C and pH 8.0 prior to the addition of a concentrated solution of reduced DTT (DTT^{red}) to final conditions of 10 mg/ml of RNase A and 400 mM DTT^{red}. After 8 h, the reaction was stopped with 0.1 volume of concentrated acetic acid. The reduced species were then fractionated by cation-exchange HPLC on a Rainin Hydropore SCX column (21.4 mm ID×10 cm) using a NaCl gradient.

The final purification was carried out on a YMC C_{18} column (10 mm ID×15 cm) using a linear acetonitrile–H_2O gradient in the presence of 0.1% TFA, with absorbance monitored at 280 nm. The purified material was lyophilized, and the purity was confirmed by addition of 2-aminoethyl methanethiosulfonate (AEMTS) to a solu-

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Abbreviations: RNase A, bovine pancreatic ribonuclease A; des-[40-95], RNase A lacking the 40-95 disulfide bond; 3S ensemble, molecules with three disulfide bonds but no stable tertiary structure; DTT^{red}, reduced dithiothreitol; AEMTS, 2-aminoethyl methanethiosulfonate ($(NH_2)C_2H_4SSO_2CH_3$); des species (3S*), des-[40-95] and des-[65-72]; CD, circular dichroism; HPLC, high performance liquid chromatography

PII: S0014-5793(00)01386-7

tion of the lyophilized material and by analysis by cation-exchange HPLC. The AEMTS was synthesized as described by Bruice and Kenyon [11].

2.2. Reshuffling of des-[40-95] and des-[65-72]

After purifying the two native-like des species, the reshuffling reaction was initiated by rapidly mixing the dissolved des species (pH 3.0, 0°C) with reshuffling buffer (100 mM Tris–HCl, 2 mM EDTA, pH 8.0 at several temperatures). The reshuffling buffer was degassed with argon for several hours prior to the experiment. The reshuffling reactions were carried out at constant temperature, with the reaction buffer being sparged continuously with argon. The appearance and disappearance of intermediates were monitored as a function of time. At various times after initiating reshuffling, aliquots were removed and the reaction was stopped by the addition of AEMTS to block the unreacted sulfhydryl groups. Since AEMTS introduces one unit of positive charge for every free thiol group that is blocked, populations of intermediates can be separated by cation-exchange HPLC on the basis of the number of free thiol-containing groups present [5]. The quenched samples were immediately desalted on a Pharmacia HR 10/10 column packed with Sephadex G-25 superfine resin and immediately injected onto an analytical cation-exchange HPLC column (Rainin Hydropore SCX, 4.6 mm×10 cm).

3. Results and discussion

3.1. Formation of native structure through intermolecular thiol-disulfide exchange reactions

The 3S ensemble is a composite of 420 possible 3S-bonded species, each of which may have native and non-native proline isomers. As illustrated in Fig. 1, the 3S ensemble shows a circular dichroism (CD) spectrum that is intermediate between those of the native and denatured forms. Such a spectrum implies the existence of a partially folded structure that is different from the native and denatured states; based on the positive ellipticity in the region around 200 nm and the negative ellipticity in the region between 210 and 220 nm. Currently, there is no direct evidence that partially folded states exist in the 3S ensemble. However, the CD spectrum provides general information about the nature of conformational changes and the degree of structural organization of the 3S ensemble. In this state, a protein thiolate can attack a disulfide bond on a different protein molecule. In such a situation, a disulfide bond can be exchanged from one protein molecule to another easily. This process may be described by the following scheme:

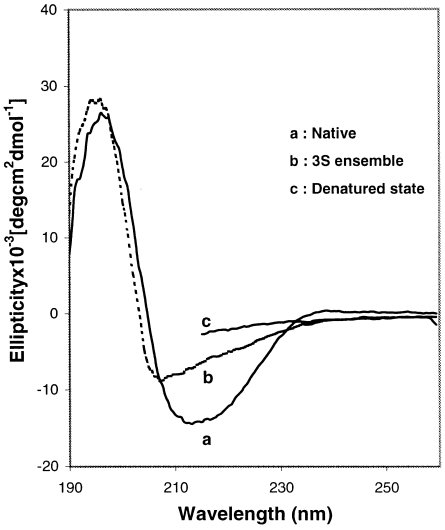

Fig. 1. CD spectra for (a) native RNase A, (b) the 3S ensemble, (c) RNase A denatured with 5 M GdnHCl at pH 5.0, 10 mM sodium acetate buffer. The spectra were obtained at 20°C. Each spectrum was the average of three scans. The protein concentration was 0.4 mg/ml.

$$3S + 3S \rightleftarrows 4S + 2S \quad (1)$$

$$3S + 3S^{*} \rightleftarrows N + 2S \text{ (or } 4S + 2S) \quad (2)$$

The presence of 2S and 4S species was identified by the chromatograms obtained from cation-exchange HPLC using AEMTS as the thiol blocking agent. The 2S and 4S species do not accumulate to high levels during reshuffling at 25°C (Fig. 2). However, these species are highly populated at 37°C (Fig. 3). Fig. 4 shows the appearance of the native, 2S and 4S ensembles, as a function of reshuffling time for des-[40-95] at 37°C. The formation of the 2S and 4S ensembles containing two and four disulfide bonds, respectively, occurs by intermo-

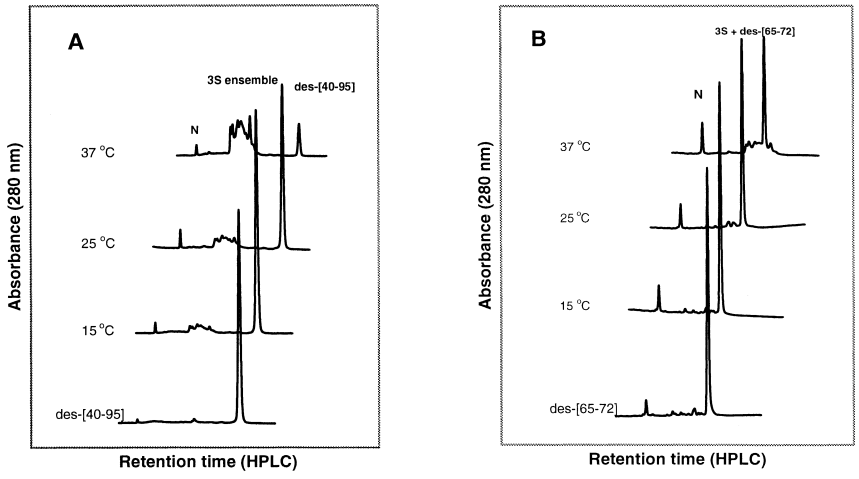

Fig. 2. Reshuffling of the des species, des-[40-95] and des-[65-72], to the 3S ensemble. Reshuffling started with purified native-like intermediates at pH 3 and 0°C, which were allowed to reshuffle by raising the pH to 8.0 in the absence of redox reagents under anaerobic conditions. At each temperature, the protein concentration was 10 μM and the reshuffling time was 5 min. The starting intermediate was (A) des-[40-95] or (B) des-[65-72], respectively. In (B), the initial amount of native was 3% of the total species which may arise from an oxygen contaminant. The curves at different temperatures are offset to the right for easy viewing.

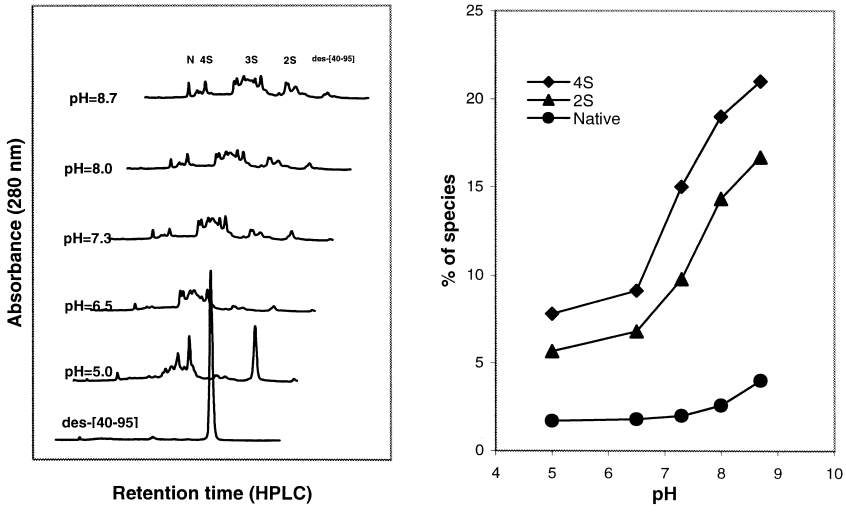

Fig. 3. Distribution of populated species during reshuffling of des-[40-95] to 3S at five different pHs. The reshuffling time was 30 min at 37°C. The protein concentration was 30 μM.

lecular thiol-disulfide exchange reactions during the reshuffling step. Furthermore, these reactions induce the formation of native structure. Fig. 4 also shows that the 4S ensemble is the dominant species at 37°C and pH 8.0. The population of the 4S ensemble is greater than that of the 2S ensembles predicted by Eq. 1 (viz. 4S = 2S). There are two possible explanations for the larger population of 4S: (1) formation of its fourth disulfide bond may occur by direct oxidation of the 3S ensemble (which contains unstable and mis-paired disulfide bonds) by stray oxidizing agent. (2) During reshuffling, some of the 3S ensemble may proceed through a transiently dimerized species arising from hydrophobic interactions involving the non-polar groups of the relatively unstructured 3S ensemble, and this dimerized species may have appeared at the same position as the 4S ensemble in the HPLC chromatogram. However, it is not possible to distinguish between 4S species that are formed by either of these two processes in the 4S ensemble in the HPLC chromatogram. There are several examples of associated states formed during the refolding of proteins [12–16].

3.2. *Reshuffling of des-[40-94] and des-[65-72] to the 3S ensemble*

Most native proteins possess a unique three-dimensional structure and a corresponding unique disulfide-bonding pattern. For the disulfide bonds of the intermediates to exchange intramolecularly, they must undergo rearrangements, depending on the conformational stability of the intermediates and the accessibility of the exchangeable sulfhydryl groups. Fig. 2 shows the reshuffling of the native-like des species to the unstructured 3S species, at three different temperatures. At each temperature, the reshuffling process was monitored for 5 min. The des-[40-95] species reshuffled preferentially to the 3S ensemble and then slowly rearranged to the more stable native-like 3S intermediate, des-[65-72] (data not shown). In the case of des-[65-72], the population of the 3S ensemble is small because the rate of conversion for the des-[65-72] to 3S step is about 21-fold slower than that for the des-[40-95] to 3S step at 25°C and pH 8.0 [6]. As shown in Fig. 2, reshuffling occurred most rapidly at 37°C. Disulfide reshuffling is expected to occur readily in the less stable intermediate, des-[40-95]. After 5 min reshuffling of des-[40-95] at 37°C, about 80% of des-[40-95] had reshuffled to the 3S ensemble. These results indicate that high temperature enhances the rate of reshuffling of the des species to the 3S ensemble, and suggest that reshuffling of des-[40-95] and des-[65-72] to the 3S ensemble is closely related to the conformational flexibility and stability of the two native-like intermediates.

3.3. *Effects of solvent conditions on the intermolecular thiol-disulfide exchange reactions*

In order to investigate the intermolecular thiol-disulfide exchange process further, we examined the effect of pH on the occurrence of the 2S, 4S and native species. The results are shown in Fig. 3. The rate of thiol-disulfide exchange depends on the extent of ionization of the thiol, and thus generally

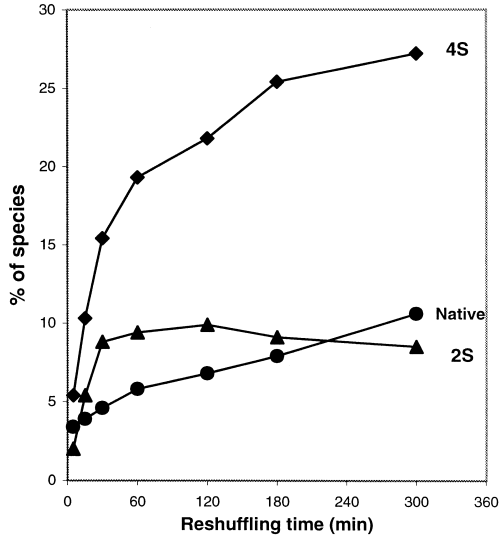

Fig. 4. Appearance of native and 2S and 4S species by intermolecular disulfide bond reshuffling as a function of reshuffling time, at 37°C and pH 8.0. The starting sample was des-[40-95] at a concentration of 10 μM. Aliquots of the reshuffling solution were blocked with AEMTS and injected onto an analytical cation-exchange HPLC column. The 4S ensemble is the dominant species.

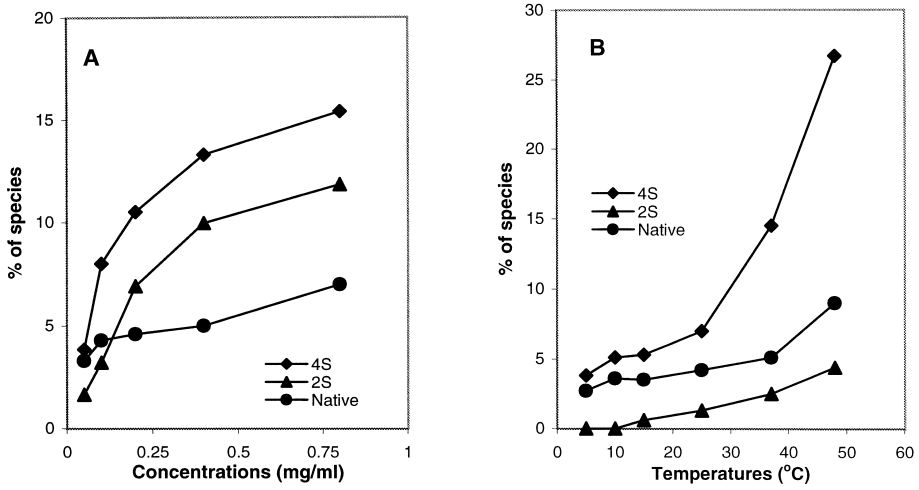

Fig. 5. Dependence of intermolecular disulfide bond reshuffled species on protein concentration at 37°C and on temperature at 0.14 mg/ml. The starting intermediate was des-[65-72] at 0.07 mg/ml and 5°C, at pH 8.0. The reshuffling time was 30 min.

increases as the pH increases. The HPLC chromatograms of Fig. 3 show that the populations of the 2S and 4S species can be increased by using a higher pH in the reshuffling experiments. At higher pH, the formation of native structure increases due to the faster intermolecular interactions. These observations suggest that ionization is coupled to the intermolecular interaction process in which intermediates are populated, and that free ionized SH groups and wrong disulfide pairing contribute to the intermolecular thiol-disulfide exchange reactions of partially folded intermediates.

As expected, the formation of native structure by intermolecular thiol-disulfide exchange reactions increases with increasing concentration during reshuffling of the des species to 3S (Fig. 5A). The formation of native structure also depends on the temperature, as shown in Fig. 5B. This result indicates that the 2S and 4S species are formed more rapidly at higher temperature in a highly temperature-dependent manner. In addition, the rate of conversion of the des species to 3S is very fast at physiological temperature (at 37°C) because the two des species are destabilized at elevated temperatures. As the temperature increases, the rate of interconversion back to the 3S ensemble increases significantly, particularly for des-[40-95] [10]. The mutant analog of des-[40-95], [C40A, C95A], exhibited a thermal transition with $T_m \sim 34$°C [8]. The pH and temperature dependences of the intermolecular thiol-disulfide exchange process suggest that it is promoted by intermolecular hydrophobic contacts and that the thiol-disulfide exchange must involve the thiolate anion. Since the 3S ensemble has non-native disulfide bonds and a mostly disordered structure, it is therefore likely that the 3S ensemble is prone to intermolecular interactions.

These results provide the first evidence for formation of the native structure of RNase A by intermolecular thiol-disulfide exchange reactions during the reshuffling of the native-like des species to 3S without any oxidants, and suggest that some partly folded states under physiological conditions undergo non-specific intermolecular disulfide bond interactions leading to the formation of native structure. The major driving force is likely to be the presence of non-native disulfide pairings and non-native contacts. These results also suggest that intermolecular thiol-disulfide exchange reactions may play an additional role in forming the native structure in the folding pathways. However, under the regeneration conditions used previously [10], viz. in the presence of an oxidizing agent, the kinetics were describable without considering intermolecular thiol-disulfide exchange.

3.4. Implication for the folding pathway of RNase A

The 3S ensemble has 420 theoretically possible 3S-bonded species including the free thiols and mis-matched 3S bond groupings. In addition, since the molecules are partially folded at the 3S ensemble stage, as shown in the CD experiment, with their thiol and disulfide groups still exposed to the solvent (but buried in the native state), there could be a high probability of intermolecular interactions. We propose the following mechanism to explain our experimental results. In the regeneration pathway of RNase A, the native structure is formed from the two native-like des species, each of which arises from the partially folded 3S ensemble on a separate pathway. In addition, under the conditions employed here, the native structure is also formed by intermolecular thiol-disulfide exchange reactions between the des species and the 3S species. As described above, this result implies that there are alternative folding pathways in the regeneration of RNase A, some of which lead rapidly and directly to the native structure, especially in the presence of an oxidizing agent, and another which involves intermolecular thiol-disulfide exchange reactions. These intermolecular thiol-disulfide exchange reactions can also induce native structure slowly without oxidants. They could also be involved in the reversible interconversion between 2S and 4S species to reach the native structure, in the absence of oxidants, since the wrong 4S species is unable to convert to the native protein because of the lack of free thiols. Thus, the formed non-native 4S species have to go over a high energy barrier to reach the native state, because two steps are required: the first step involves interactions with 2S and the second step involves interactions with the des species again to form the native structure. Thus, it is most likely that the occurrence of non-native 4S and transiently dimerized species, through intermolecular thiol-disulfide

exchange reactions and mis-paired disulfide bonds, resulted in slow regeneration of RNase A in in vitro experiments, particularly at physiological temperature (37°C) [5].

In the cellular environment, the high density of proteins results in a high probability of intermolecular interactions, particularly in the case of incompletely folded proteins. Although little is known about the molecular mechanism of intermolecular interactions, this process is assumed to occur through contacts between hydrophobic regions, exposed in unfolded or partially folded states of the polypeptide chain [16]. Such aggregation in vivo would be prevented by chaperones. The existence of specific intermolecular interactions between partially folded kinetic intermediates has been discussed for such proteins as bovine growth hormone [12], carbonic anhydrase B [13], myoglobin [14] and cytochrome *c* [15]. Therefore, the high density of proteins suggests that the unstructured *n*S species of many proteins will undergo intermolecular thiol-disulfide exchange in vivo, leading to the formation of the native structure, as well as other disulfide species such as the 'dead-end' 4S species of RNase A. On the other hand, the presence of the non-native 4S species and transiently dimerized species during the folding process, particularly at 37°C, will reduce the rate of regeneration of RNase A because they are not directly on the pathway to the native conformation. The accumulation of non-native 4S species provides evidence for the significance of protein disulfide isomerase as a catalyst in the folding process in the cell [17,18]. Because the basic role of this accessory molecule is assumed to prevent off-pathway processes and maintain the correct folding intermediates, it is likely that the probability of occurrence of incorrect disulfide bonds and the extent of accumulation of intermolecular interaction species will be reduced significantly in the presence of this thiol catalyst.

Acknowledgements: We thank Dr. H.-C. Shin for valuable comments and discussions, and Drs. D. Juminaga, K. Saito and W.J. Wedemeyer for comments on the manuscript.

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