Two new structured intermediates in the oxidative folding of RNase A

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Abstract Two new three-disulfide intermediates have been found to be populated in the oxidative folding pathway of bovine pancreatic ribonuclease A at a low temperature (15°C). These intermediates, des-[26–84] and des-[58–110], possess all but one of the four native disulfide bonds and have a stable tertiary structure, similar to the two previously observed intermediates, des-[65–72] and des-[40–95]. While the latter two des species each lack one surface-exposed disulfide bond, the newly discovered intermediates each lack one buried disulfide bond. The possible involvement of these species in the rate-determining steps during the oxidative folding of RNase A is discussed and a specific role for such species during oxidative folding is suggested.

Key words: Ribonuclease A; Folding; Dithiothreitol; Oxidative refolding; Disulfide bond; Intermediate

1. Introduction

The existence, nature, role and significance of structured folding intermediates have become a central issue in the field of protein folding [1–3]. Whether native or non-native interactions maintain the structures of the intermediates, whether folding proceeds in a sequential manner or follows side pathways with dead-end intermediates and whether the existence of the intermediates is obligatory on the folding pathways are questions that are still largely unanswered.

Studies of proteins whose structures are stabilized by disulfide bonds have provided considerable insight into the folding process [4]. Coupling of the covalent chemistry of disulfide bond formation to the non-covalent folding processes makes it more feasible to trap, isolate and characterize intermediates. Disulfide-coupled folding (oxidative refolding) can be carried out at physiological pH values and temperatures without applying denaturants such as guanidinium hydrochloride or urea, whose effects are still not very well understood. Additionally, the disulfide distribution of the unstructured species can reveal local interactions and the sequential introduction of native disulfide bonds (which lowers the entropy) can result in partially folded intermediates that provide detailed information about the pathways of protein folding.

The roles of native-like structured intermediates on the oxidative folding pathways of proteins have not yet been completely elucidated. Native-like, structured disulfide species are considered to be either productive and on-pathway intermediates or kinetic dead-ends, depending on the availability of their thiol groups [5,6]. Bovine pancreatic trypsin inhibitor (BPTI) and bovine pancreatic ribonuclease A (RNase A), two well-known examples of disulfide-containing proteins, have been investigated in great detail. Structured native-like species with buried thiols were found and thoroughly characterized in the oxidative folding pathway of BPTI. The buried thiols in these intermediates were not easily accessible to the oxidizing agent [5,7]. Consequently, these intermediates were reported to be kinetic dead-ends in the folding process.

Work in our laboratory has focussed on the elucidation of the interactions that determine the oxidative folding pathways of RNase A, which has four disulfide bonds (at positions 26-84, 40-95, 58-110 and 65-72) and for which on-pathway structured intermediates have been identified [8–10]. Kinetic studies [9] have led to the mechanism illustrated in Scheme 1. The species ensembles of disulfide species with n disulfide bonds (nS) are ensembles[2] of n disulfide bonds, there being 28, 210, 420 and 104 theoretically possible species, with $n = 1$, 2, 3 and 4, respectively, in addition to the fully reduced (R) and completely folded (N) forms. The ensembles R and nS attain a pre-equilibrium steady state, after which one or more species in the 3S ensemble undergo a disulfide exchange reaction to form des-[40-65] and des-[65-72] in rate-determining steps and then, these species convert rapidly to N [8,9,11,12]. Both des-[40-95] and des-[65-72] contain three native disulfide bonds. The kinetic data comparing the rates of formation of the intermediates as well as of the native protein at 25 and 15°C suggested that one or more additional species from the three-disulfide ensemble that is stabilized at lower temperatures are involved in rate-determining steps [11].

In this article, we report the identification of stable species that are populated in the 3S ensemble of RNase A at 15°C and discuss also their significance.

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; RNase A, bovine pancreatic ribonuclease A; des-[40-95], des-[65-72], des-[26-84], des-[58-110], disulfide species of RNase A containing three native disulfide bonds but lacking the disulfide bond in brackets; DTT^{ox}, oxidized dithiothreitol; DTT^{red}, D,L-dithiothreitol (reduced dithiothreitol); AEMTS, 2-aminoethylmethanethiosulfonate; HPLC, high performance liquid chromatography; nS, the ensemble of disulfide species with n disulfide bonds

[2] A disulfide species is defined as a protein with a particular set of disulfide bonds, while a disulfide ensemble is a collection of disulfide species. In particular, the nS ensemble consists of all disulfide species with n disulfide bonds. For example, des-[40-95] is a single disulfide species in the 3S ensemble. Thus, redox reactions are interconversions among such disulfide ensembles, e.g. 1S→2S, while disulfide reshuffling reactions are interconversions among disulfide species within an ensemble, e.g. [65-84]→[65-72] in the 1S ensemble.

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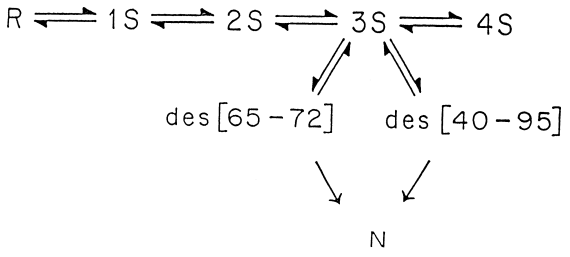

Scheme 1.

2. Materials and methods

2.1. Materials

RNase A (type 1-A, Sigma Chemical) and oxidized dithiothreitol (DTT^{ox}) (Sigma Chemical) were purified as described in [12,13]. 2-Aminoethylmethanethiosulfonate (AEMTS) and disodium 2-nitro-5-thiosulfobenzoate were prepared as described in [14,15]. Bovine pancreatic trypsin (type III) and α-chymotrypsin (type II) were obtained from Sigma Chemical. All other reagents were of the highest grade commercially available.

2.2. Methods

Fully reduced RNase A was regenerated with 100 mM DTT^{ox} at 15°C. At various regeneration times, aliquots were withdrawn and thiol-disulfide exchange reactions were quenched by blocking all free thiol groups with AEMTS at 15°C. The mixture was then fractionated by cation-exchange high performance liquid chromatography (HPLC). These experiments were carried out as described by Volles et al. [16]. In some experiments, the AEMTS-blocking step was preceded by a reduction pulse in which the aliquot was incubated for 30 s with 6 or 12 mM D,L-dithiothreitol (reduced dithiothreitol (DTT^{red})).

Peptide mapping, disulfide bond analysis, reduction and reblocking were carried out as in [16], with the following modification. After allowing the regeneration process to proceed for at least 9 h, the three major peaks in the 3S region of the chromatogram were collected. The isolated proteins were enzymatically digested and the resulting peptide fragments were separated by reverse-phase HPLC. The disulfide-containing peptide peaks were identified by the disulfide detection system and were collected. The peptide fragments were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry and re-analyzed by reduction and reblocking.

In another set of experiments, the AEMTS-blocking step following the reduction pulse was replaced by acid quenching. The resulting mixture of unblocked disulfide species was fractionated at 13°C at pH 5 on a cation-exchange column [9], the fractions were collected and immediately blocked with AEMTS. The blocked fractions were then re-applied to a cation-exchange column at pH 8.

3. Results

3.1. Reduction pulse

The chromatograms of the regeneration mixture at 15°C with and without a reduction pulse exhibit three prominent peaks in the 3S region (Fig. 1). The concentrations of DTT^{red} that were applied can reduce all the unstructured species except the native and quasi-native ones [9][3] The middle peak, the smallest one, co-eluted with purified des-[65-72] (data not shown). Additionally, the middle peak was later found to be resistant to tryptic and chymotryptic digestion at room temperature, indicating that the middle peak is des-[65-72], which is also populated at 25°C. The areas of the first and third peaks make up 30 and 15% (for a total of 45%) of the 3S ensemble without des-[65-72] and des-[40-95].

3.2. Mapping

The AEMTS-blocked peaks (species) were collected after 9 h regeneration, at which time they had accumulated to a considerable extent. The first peak was identified as des-[58-110], which has three native bonds (26-84, 40-95 and 65-72) but missing (58-110). The third peak was identified as des-[26-84], which has three native bonds (40-95, 65-72 and 58-110) and missing 26-84.

3.3. Separation of the unblocked species on cation-exchange chromatogram

The unblocked regeneration mixture was subjected to a reduction pulse following acid quenching. The resulting unblocked mixture was fractionated by HPLC as in Section 2 and the fractions were collected and blocked immediately with AEMTS below 15°C. All the blocked fractions were re-applied to a cation-exchange column. From the resulting chromatogram (at pH 8), it was possible to deduce that the new des species co-eluted with the native fraction under unblocked conditions (pH 5, 13°C). Therefore, attempts to isolate the newly discovered des species in the unblocked form failed because the chromatographic behavior of these unblocked species is very similar to that of the native protein.

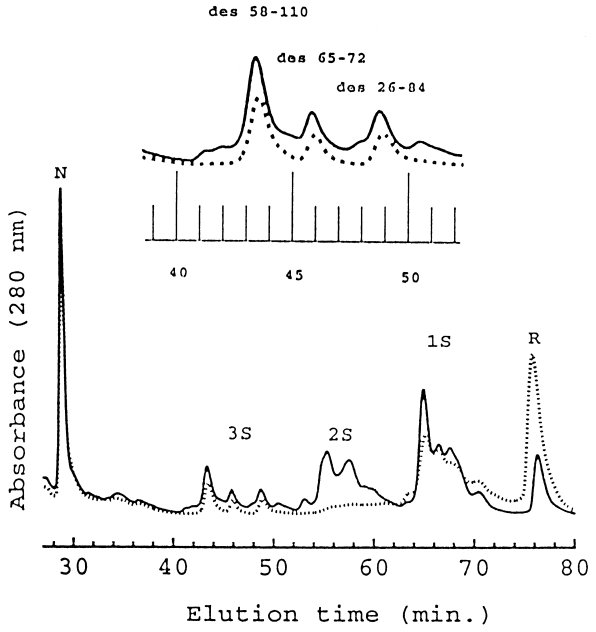

Fig. 1. Chromatograms illustrating the reduction pulse experiment. (—) A typical regeneration mixture: 100 mM DTT^{ox}, 32 μM RNase A, pH 8.0, 15°C, 270 min, blocked with AEMTS. (- - -) The same mixture as above that was subjected to a 30 s reduction pulse of DTT^{red} (final concentration 6 mM) and subsequently blocked with AEMTS. The reduced protein is designated R and the native protein N. An Arabic numeral corresponding to the number of intramolecular disulfide bonds followed by S denotes the various groupings of disulfide-bonded intermediates. Inset: 3S region of the chromatogram (—) and the results of the reduction pulse described above (- - -) showing the des species that can withstand such a pulse. Des-[58-110], des-[65-72] and des-[26-84] correspond to species that contain three native disulfide bonds but lack the 58-110, 65-72 and 26-84 disulfide bonds, respectively. Des-[40-95] co-elutes with the 1S ensemble on the chromatogram.

[3] The 1S region of the chromatogram is also populated under these conditions because a considerable amount of 1S species is in equilibrium with R and des-[40-95] elutes in the 1S region of the chromatogram.

4. Discussion

A reduction pulse (~6 mM DTT^{red}) can be used as a probe for the formation of the native-like structure among intermediates that are populated during the regeneration process. The two newly discovered des species (des-[26-84] and des-[58-110]) are structured enough to withstand a reduction pulse and their chromatographic behavior (they elute with native protein when unblocked) suggests that they have a quasi-native structure.

The relative populations of these new des species are probably determined by both kinetic and thermodynamic factors. The atomic interactions that stabilize these species can be elucidated by examining their cysteine to alanine or serine mutant analogs. However, preliminary experiments [17] indicated that these mutant analogs do not have much enzymatic activity at 25°C. They also do not fold at 25°C, which can explain why these intermediates were not detectable at 25°C in the regeneration mixture [9]. It would appear that these buried disulfide bonds ([26-84] and [58-110]) contribute more to the stability of the protein than the exposed [40-95] or [65-72] bonds.

The new des species (des-[26-84] and des-[58-110]) can be consumed in three different ways: reduction to 2S, oxidation to native and reshuffling back to the 3S ensemble. Considering the very low concentration of DTT^{red} present in the oxidative regeneration mixture and the fact that these species withstand 12 mM DTT^{red} during the reduction pulse, the first process seems improbable. Either oxidation or back-reshuffling can account for the observed rates of regeneration of the native protein and its intermediates at 15°C [11]. The direct oxidation of the new des species to form the native protein may occur at a rate that is comparable to that of the previously known pathways [9]. These new pathway(s) can then account for the enhanced rate of formation of native protein at 15°C [11]. Even if these pathway(s) do not contribute significantly, these species can still accelerate the rate of folding if their rate of back-reshuffling (to 3S) is fast enough.

The formation of the native protein must proceed through intermediates that have three native disulfide bonds. Because of the need to search through the large number (viz. 420) of possible three-disulfide species, the formation of three native bonds and the native-like structure that can protect them becomes rate-determining [9]. A back-reshuffling pathway from the new des species to des-[65-72] or des-[40-95] involves at least two rearrangement steps. The first reshuffling step requires either local or perhaps global conformational unfolding of the protein and yields (in principal) 12 species with two native and one non-native disulfide bonds. If global conformational unfolding took place, the back-reshuffled 3S species obtained in the first reshuffling step may not have any remaining structure that can protect their native bonds during the second rearrangement step. Thus, either the native or the non-native bonds can be broken essentially by chance. Because many of these species have the same two native bonds that des-[65-72] or des-[40-95] possess, this second reshuffling step can increase the population of des-[65-72] or des-[40-95], depending on the stability of the new des species.

If, on the other hand, a local unfolding step took place, any residual structure that can protect the two native bonds after the first back-reshuffling step can create a preferential pathway for the formation of des-[65-72] or des-[40-95] from these species with a considerable yield. The fact that these new des species make up 45% of the 3S ensemble without the des-[65-72] and des-[40-95] species supports the argument that they can contribute to the rate-determining step to form the old des species.

Obviously, we have to consider the possibility that neither the direct oxidation nor the back-reshuffling rate is great enough to accelerate the folding process significantly at 15°C. In this case, there may be one or more species, preceding the rate-determining step, that are stabilized at a lower temperature but not strongly enough to withstand a reduction pulse. Examination of mutants, with four cysteines converted to alanines which are analogs of the species with two native disulfide bonds, may answer this question.

In conclusion, we report here the existence of two new native-like intermediates on the oxidative regeneration pathways of RNase A (using DTT^{ox} at 15°C). The available kinetic data [11] are consistent with a productive role for these species in the oxidative folding process. We also suggest that structured native-like intermediates on the oxidative regeneration pathway of a protein, which can form the native structure in spite of having buried thiols and which heretofore were considered as useless dead-ends in the oxidative folding process [5,18], can actually accelerate the folding process depending on their stability.

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