Effect of protein disulfide isomerase on the rate-determining steps of the folding of bovine pancreatic ribonuclease A

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Abstract The effects of protein disulfide isomerase (PDI) on the four structured des species that accumulate in the rate-determining steps of ribonuclease A folding were investigated at pH 8.0 and 15°C. The results indicate that PDI catalyzes the conversion of the kinetically trapped intermediates, des-[26–84] and des-[58–110], by reshuffling them into the on-pathway intermediate, des-[40–95], and the formation of native protein, by acting as both a chaperone and an oxidase on this on-pathway intermediate. These results provide the first strong evidence for the mechanism of PDI in the rate-determining steps of the oxidative folding pathways of ribonuclease A. Our approach, using PDI and blocked PDI, combined with the fast-blocking 2-aminoethyl methanethiosulfonate method, may be generally applicable to the clarification of the effect of PDI on folding intermediates. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Protein disulfide isomerase; Ribonuclease A; Protein folding; Rate-determining steps; Intermediates

1. Introduction

Protein disulfide isomerase (PDI) is a 55-kDa protein found in the endoplasmic reticulum where it plays a central role in the folding of newly synthesized secretory pathway proteins [1,2]. The function of PDI as a foldase is the combined outcome of its catalytic role as an oxidase and isomerase, as well as its chaperone-like activity [3–5]. PDI is largely non-specific, interacting with a variety of unstructured peptides and proteins as well as structured disulfide intermediates [6–10]. In particular, several experiments have suggested that PDI is able to reverse the accumulation of kinetically trapped disulfide species, and thus accelerate the regeneration of the native protein [8,10]. However, it has not been determined whether PDI acts on these kinetically trapped species primarily by reshuffling them, or by directly oxidizing/reducing them. It is also not clear whether conformational changes of substrates induced by non-catalytic binding with PDI influence the oxidative folding. To address these questions, we have investigated the effects of PDI on the rate-determining steps of the oxidative folding pathways of bovine pancreatic ribonuclease A (RNase A) that involve the formation of structured intermediates.

RNase A is a good model system for such investigations because its oxidative folding has been well studied [11–17]. The protein consists of 124 amino acid residues and contains four intra-molecular disulfide bonds (26–84, 40–95, 58–110 and 65–72). In the presence of oxidized and reduced dithiothreitol (DTT^{ox}/DTT^{red}) at pH 8.0 and 25 °C, RNase A folds through pathways involving a rapid pre-equilibrium resulting in an ensemble of three-disulfide (3S) species, followed by two dominant major and minor rate-determining steps in which two native-like three-disulfide intermediates, des-[40–95] and des-[65–72], respectively, are formed by disulfide rearrangement [11]. The two des species then oxidize to native RNase A. At lower temperature, 15°C, two other des species, des-[58–110] and des-[26–84], also accumulate [12] (Fig. 1). Recent data [18] suggest that these two additional des species are long-lived kinetic traps that slowly reshuffle back to the 3S ensemble and thence to the productive des species, des-[40–95] and des-[65–72] [13–18]. Therefore, we focus our attention on the effects of PDI on these four des species at 15°C, which are formed during the rate-determining steps. In particular, we seek to discern how PDI acts on the on-pathway intermediates, des-[65–72] and des-[40–95], and on the kinetic traps, des-[26–84] and des-[58–110].

2. Materials and methods

2.1. Materials

Native and reduced RNase A (type 1-A, Sigma) were prepared as described previously [19]. DTT^{ox} was obtained from Sigma and purified by the method of Creighton [20]. 2-Aminoethyl methanethiosulfonate (AEMTS) was synthesized as described by Bruice and Kenyon [21]. PDI was purified from bovine liver according to the method of Hillson et al. [22]. All other reagents were of the highest grade commercially available. The concentration of reduced RNase A was determined by using a molar extinction coefficient of 8160 M^{-1} cm^{-1} at 275 nm [19]. The concentration of PDI was determined by using a molar extinction coefficient at 280 nm of 47 300 M^{-1} cm^{-1} [23].

2.2. Preparation of 3S ensemble and blocked PDI

The 3S ensemble was prepared from the des-[40–95] or des-[65–72] species by incubating the purified des species [11,18] for 10 min in 100 mM Tris-acetate, 2 mM EDTA, pH 8.0 containing 6 M GdnHCl at 50°C. The disulfide reshuffling was stopped by the addition of acetic acid. The 3S ensemble was purified by reverse-phase HPLC, and the

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Abbreviations: RNase A, bovine pancreatic ribonuclease A; PDI, protein disulfide isomerase; DTT^{ox}, oxidized dithiothreitol; DTT^{red}, reduced dithiothreitol; AEMTS, 2-aminoethyl methanethiosulfonate; des-[ ], three-disulfide intermediate having three native disulfide pairings but lacking a disulfide bond specified within the brackets

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purity was checked by cation-exchange HPLC after blocking the 3S ensemble with AEMTS [21,24].

To prepare blocked PDI, reduced PDI was first prepared by incubating native PDI with 50 mM DTT^{red} for 5 h and then quickly removing DTT^{red} through a desalting column in 100 mM acetic acid under argon atmosphere. The total sulfhydryl content of PDI was determined by Ellman's reaction [25]. Blocked PDI was prepared by reacting reduced PDI with 500 mM iodoacetamide for 3 h and then removing iodoacetamide through a desalting column in 100 mM acetic acid. Complete blocking was confirmed by Ellman's reaction.

2.3. Reshuffling of 3S ensemble

Reshuffling was initiated by adding the purified 3S ensemble to a degassed buffer solution (100 mM Tris–HCl, 2 mM EDTA, pH 8.0, 15°C) containing 100 mM DTT^{ox}, 2 μM PDI+100 mM DTT^{ox} or 2 μM $PDI^{blocked}$+100 mM DTT^{ox}. The solution was kept under an argon atmosphere and immersed in a water bath at 15 °C. After selected time intervals, aliquots of 0.5 ml were collected from the regeneration mixture, and unreacted thiol groups were blocked by the addition of AEMTS (5 mg solid AEMTS per 0.5 ml reaction sample). The blocking reaction results in the rapid conversion of all free thiol groups in the sample to mixed disulfide molecules with cysteamine (2-aminoethanethiol). After 1 min, the pH of the sample was lowered below 5 by adding 5 μl of acetic acid, and the sample was rapidly desalted on an HR 10/10 column packed with Sephadex G-25 superfine resin (into 50 mM sodium acetate/1 mM EDTA, pH 5.0, 25°C). 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 [19]. The aliquots obtained during regeneration were analyzed by cation-exchange chromatography running on a Hewlett-Packard HPLC system 1100 series equipped with a Hydrophore 5-SCX from Rainin.

3. Results and discussion

3.1. Reshuffling of 3S ensemble in the absence of PDI and DTT^{ox}

In the absence of PDI and DTT^{ox}, the 3S ensemble reshuffled to the four des species, with preferential formation of des-[40–95]; about 43%, 6%, 8% and 17% of the starting 3S ensemble reshuffled to des-[40–95], des-[65–72], des-[26–84] and des-[58–110], respectively, within 3 h (Fig. 2). Under these conditions, about 9% of the 3S ensemble was converted into the native form in 3 h of reaction time. The occurrence of native protein could be due to residual oxygen in the buffer that may be acting as the oxidizing agent. Also intermolecular thiol/disulfide exchange between 3S molecules may contribute to the oxidation process as 2S species also accumulate [26] (Fig. 2).

3.2. Reshuffling of 3S ensemble in the presence of PDI and DTT^{ox}

In the presence of 2 μM PDI and 100 mM DTT^{ox}, the initially populated des-[40–95], des-[26–84] and des-[58–110] rapidly decrease with reaction time (Fig. 3). A large increase in the amount of native protein in the presence of 2 μM PDI and 100 mM DTT^{ox} (Fig. 4) is presumably due to the oxidation of des-[40–95] because des-[40–95] is known to be an easily oxidized species [11]. It is not clear whether des-[26–84] and des-[58–110] oxidize directly to native protein or reshuffle to 3S, which then reshuffles to the more stable des-[40–95] to oxidize to native protein. The rapid decrease in the amount of des-[40–95], des-[26–84] and des-[58–110] results in the more slowly oxidizing des-[65–72] becoming the preferred des species at 40 min. This is consistent with the increase in the rate of formation of des-[65–72] and des-[40–95] in the presence of PDI at 25°C, where the rate of conversion of 3S to des-[40–95] is roughly three times faster than that of 3S to des-[65–72] [16]. Under the same conditions, the oxidation rate of des-[40–95] to the native protein is roughly 23 times faster than that of des-[65–72] to the native protein [16].

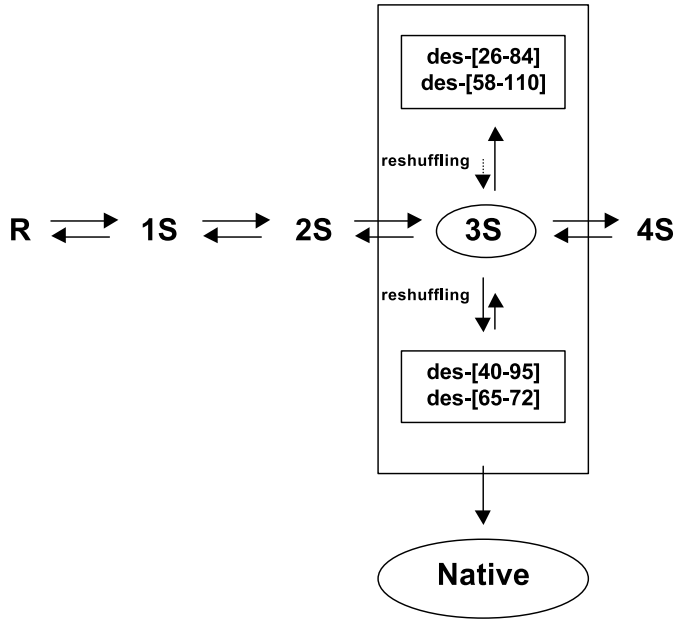

Fig. 1. The folding pathways of RNase A using DTT^{ox}/DTT^{red} as redox reagents at pH 8.0, 15°C. R represents the fully reduced protein, nS respresents an ensemble of species with n disulfide bonds, and des-[ ] respresents a 3S species with three native disulfide bonds lacking the disulfide bond in the brackets. In the absence of PDI, reshuffling of kinetically trapped intermediates, des-[26–84] and des-[58–110], back to 3S is very slow (short arrow), resulting in the accumulation of these des species. In the presence of PDI, however, the two des species disappear rapidly, due to the reshuffling of these two des species to 3S, which is catalyzed by PDI. In the presence of PDI, the reshuffling of 3S to the on-pathway intermediates, des-[40–95] and des-[65–72], is also catalyzed by PDI.

3.3. Reshuffling of 3S ensemble in the presence of $PDI^{blocked}$ and DTT^{ox}

To clarify whether des-[26–84] and des-[58–110] oxidize directly to native protein or reshuffle to 3S, and then to the easily oxidizing des-[40–95], blocked PDI in which the thiol groups of PDI were blocked by iodoacetamide was prepared. In the presence of $PDI^{blocked}$ and DTT^{ox}, where the action of PDI as an oxidoreductase and isomerase is blocked, the three des species, des-[26–84], des-[65–72] and des-[58–110] accumulated in a manner similar to that observed in the presence of DTT^{ox} alone or in the absence of PDI and DTT^{ox}. However, the population of des-[40–95] was strongly reduced, with a corresponding increase in the native protein, in the presence of $PDI^{blocked}$ and DTT^{ox} (Fig. 3). The rate of formation of native protein in the presence of $PDI^{blocked}$ and DTT^{ox} increased to a level considerably higher than that with DTT^{ox} alone (Fig. 4). This strongly indicates that the non-catalytic binding of PDI to its substrates contributes to the formation of native protein. Since des-[40–95] is the only species whose concentration is strongly reduced, it is very likely that formation of the fourth disulfide due to a conformational change induced by non-catalytic binding of PDI occurs mainly with des-[40–95].

Both disulfide bonds, 26–84 and 58–110, are buried in the native protein, and therefore the 26 and 84 thiol groups in des-[26–84], and the 58 and 110 thiol groups in des-[58–110], must be exposed before direct oxidation takes place; the exposure is induced by non-catalytic binding of PDI to the substrates. From the fact that des-[26–84] and des-[58–110] in the presence of $PDI^{blocked}$+DTT^{ox} accumulated similar to that in the presence of DTT^{ox} alone or in the absence of PDI and DTT^{ox}, the direct oxidation is highly unlikely. In conjunction with the results in the presence of PDI+DTT^{ox}, our data indicate that des-[26–84] and des-[58–110] convert to des-[40–95] via 3S by PDI-induced rearrangement.

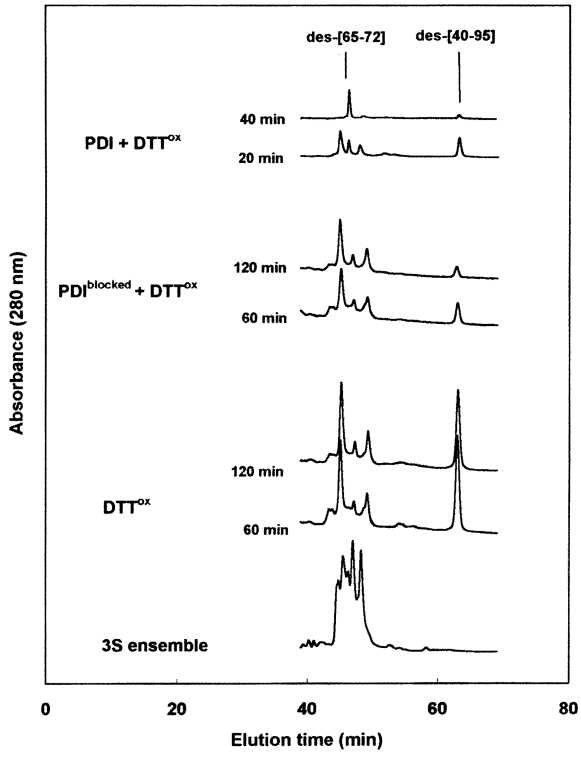

Fig. 3. Time course of the reshuffling reaction (at pH 8.0, 15°C) of 20 μM 3S ensemble with 2 μM PDI+100 mM DTT^{ox}, 2 μM $PDI^{blocked}$+100 mM DTT^{ox} or 100 mM DTT^{ox}. The effect of PDI during reshuffling of the 3S ensemble to form the native-like des species was determined by using the AEMTS thiol-blocking method and cation-exchange HPLC analysis.

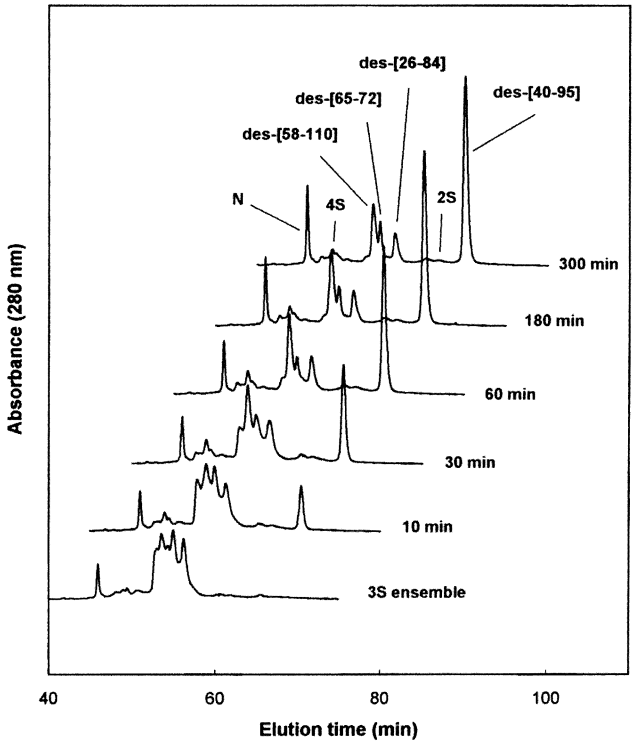

Fig. 2. Time course of the reshuffling reaction (at pH 8.0, 15°C) of 20 μM 3S ensemble in the absence of PDI and DTT^{ox}. All four des species accumulate with preferential formation of des-[40–95].

The rate constant for formation of native RNase A in the presence of PDI+DTT^{ox} is ca. eight-fold higher than that in the presence of $PDI^{blocked}$+DTT^{ox} (Fig. 4), indicating that most of the native RNase A is regenerated through the action of PDI as an oxidase/isomerase with the remainder affected by the non-catalytic chaperone-like activity of PDI.

3.4. Implication for the role of PDI in the rate-determining steps of the folding of RNase A

Some of the largely unstructured intermediates can evolve into misfolded species during protein refolding [27]. During reshuffling of the 3S ensemble, 4S (non-native four-disulfide-bonded species) and 2S (two-disulfide-bonded species) are formed. These species are generated through intermolecular thiol/disulfide exchange reactions between 3S species [26], and still exist after 5 h reshuffling time in the absence of PDI. In the presence of PDI, however, 4S and 2S species are observable only at early reshuffling times (data not shown), indicating that PDI assists the oxidative folding by decreasing the lifetime of misfolded species.

It has been reported that PDI increases the rates of folding of kinetically trapped BPTI folding intermediates by a factor of 3000–6000 [8]. Since blocked PDI was not used as a control for reshuffling, there was no evidence to exclude the possibility that the trapped intermediates oxidize directly to the native form even though the rate increase was attributed to disulfide-

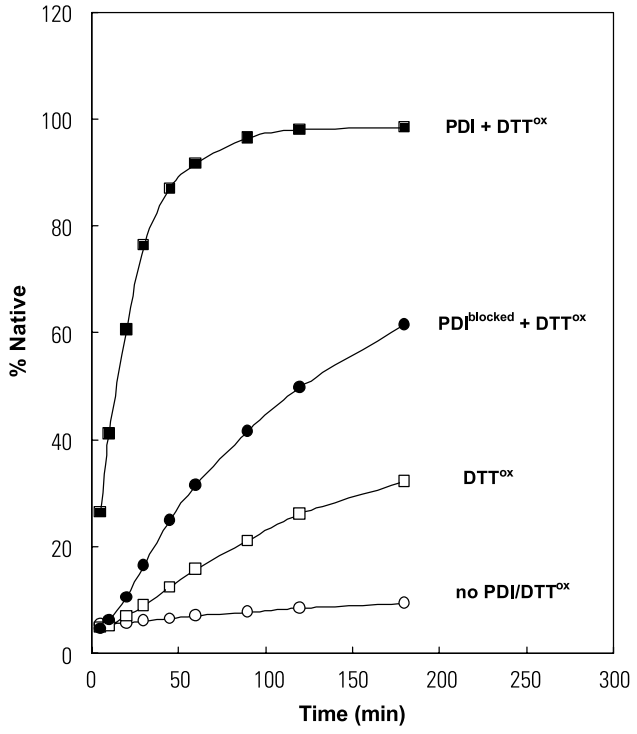

Fig. 4. Plot of appearance of native protein as a function of time at 15°C, pH 8.0. The rate constants for formation of native RNase A were calculated to be 399.5×10^{-4} min^{-1} ($PDI+DTT^{ox}$), 50.9×10^{-4} min^{-1} ($PDI^{blocked}+DTT^{ox}$), 23.1×10^{-4} min^{-1} (DTT^{ox}), respectively. The conditions are the same as in Figs. 2 and 3.

bond rearrangements by PDI. Only by comparing the effect of PDI and blocked PDI were we able to clarify the effect of PDI on the trapped intermediates as well as on on-pathway intermediates.

In summary, our results indicate that PDI catalyzes the conversion of the kinetically trapped intermediates, des-[26–84] and des-[58–110] by reshuffling them into the on-pathway intermediate, des-[40–95], via 3S and the formation of native protein, by acting as both a chaperone and an oxidase on this on-pathway intermediate (Fig. 1). Our approach using PDI and blocked PDI combined with the fast-blocking AEMTS method may be generally applicable to the clarification of the effect of PDI on folding intermediates.

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