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Reshuffling Activity of Protein Disulfide Isomerase Reduces Refolding Yield in the Structure-forming Step of the Oxidative Protein Folding Reaction

Rituraj Pal, Veronica Gonzalez, and Mahesh Narayan*
Department of Chemistry, University of Texas at El Paso, El Paso, Texas 79968, USA

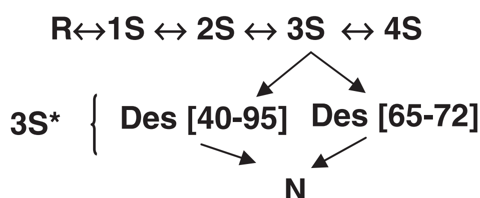
(Received December 24, 2009; CL-091137; E-mail: mnarayan@utep.edu)

We have determined the impact of the oxidoreductase chaperone protein disulfide isomerase (PDI) on the critical structure-forming step during the oxidative maturation of model disulfide-bond-containing proteins. This is achieved by using a novel tool to trap and populate native-disulfide-containing intermediates in unstructured forms that are poised to fold. Our data reveals that PDI inhibits the conformational folding step of oxidative fold maturation and, therefore, has limited overall catalytic efficiency as an oxidoreductase chaperone. Such an anomalous behavior of PDI during a key step in oxidative regeneration may contribute to misfolding in the endoplasmic reticulum, aggregation, and neurodegenerative disease.

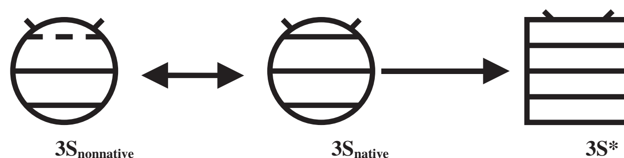
Disulfide-bond-containing proteins mature (regenerate) by the process of oxidative protein folding which involves the formation of the native set of disulfide bonds from the fully reduced polypeptide, coupled with a conformational folding reaction to obtain the native, biologically active structure.[1–3] Oxidative protein folding takes place in the endoplasmic reticulum (ER) and is characteristic of most proteins that are secreted outside the cell or are membrane-bound.[4]

The conformational folding reaction is pivotal to the oxidative folding process because the resulting tertiary structure protects the newly formed native disulfide bonds from reduction by extrinsic redox reagents and from intramolecular reshuffling reactions.[5–10]

However, the process of conformational folding is not straightforward. This physical reaction competes with thiol–disulfide (chemical) exchange reactions in typical oxidative folding landscapes.[7] For example, during the oxidative folding of the four-disulfide-bond-containing protein bovine pancreatic ribonuclease A (RNase A; Scheme 1), a competition between thiol–disulfide exchange reactions and a conformational folding step ensues prior to the formation of structured native-like intermediates (formation of native-like des [40–95] and des [65–72] from unstructured 3S).[7,11] Here, thiol–disulfide exchange



Scheme 1. Regeneration of RNase A (pH 8, 25 °C). R, 1S, 2S, 3S, and 4S are unstructured intermediates. Des [40–95] and des [65–72] are native-like three-disulfide-bond-containing intermediates and are also referred to as the 3S* species. N is the native protein.



Scheme 2. The structure-forming step in the oxidative folding pathway of RNase A. Dashed and solid horizontal lines are nonnative and native disulfide bonds, respectively. A competition exists between the formation of $3S_{nonnative}$ and 3S* from the unstructured intermediate that contains only native disulfide bonds ($3S_{native}$). Vertical lines are thiols capable of intramolecular attack.

reactions between an unstructured intermediate that contains native disulfide bonds ($3S_{native}$) and its nonnative-disulfide-bond-containing isomers ($3S_{nonnative}$) compete with the conformational folding of $3S_{native}$ to the native-like structured species (3S*) in the pivotal structure-coupled folding step ($3S_{native} \rightarrow 3S^*$) (Scheme 2).[7,11] In RNase A, proline isomerization slows conformational folding (i.e., the $3S_{native} \rightarrow 3S^*$ step) and a fraction of the $3S_{native}$ species reshuffle to their nonnative-disulfide-bond-containing isomers ($3S_{nonnative}$).[11] In α-lactalbumin, conformational folding of the molten-globular four native-disulfide-bond-containing intermediate is Ca^{2+}-dependent;[12] however, the calcium-free molten globule form is susceptible to reduction of disulfide bonds by extrinsic redox reagents in a chemical event that competes with Ca^{2+}-dependent conformational folding.[1] Such competitions between "chemical" (thiol–disulfide exchange) and "physical" (conformational folding) reactions are unique to disulfide-bond-containing proteins.[7]

In vivo, oxidative protein folding is catalyzed by the ER-resident oxidoreductase chaperone protein disulfide isomerase (PDI). PDI is a five-domain enzyme which possesses both oxidase and "shufflase" activity (Figure 1).[13–18] Of these, the a and a′ domains are redox-active whereas the b and b′ domains are hydrophobic and possess surface-exposed Ile residues that facilitate binding to peptides and proteins.[16]

Although in vitro studies have demonstrated that PDI is able to accelerate oxidative folding reactions and catalyze the regeneration of multi-disulfide-bond-containing proteins including RNase A,[17] the impact of PDI and its domains on the pivotal structure-coupled step that immediately precedes maturation is yet unknown. Furthermore, even under surveillance by PDI, a large fraction of ER-processed proteins misfold and are trafficked to the proteosome.[4]

Given this scenario, it is of interest to examine the catalytic efficiency of the principal machinery charged with maturation of ER-processed proteins.

3S* species of RNase A was isolated as previously described.[6] It was then incubated at pH 2 (50 mM acetic acid)

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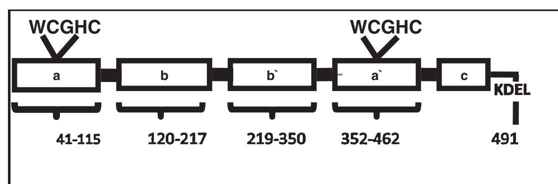

Figure 1. Schematic of PDI showing the active site.

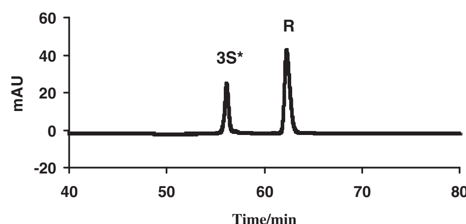

Figure 2. Typical reversed-phase HPLC chromatogram showing results of the competition between the structure forming step ($3S_{native} \rightarrow 3S^*$) and thiol–disulfide exchange reactions ($3S_{native} \rightarrow 3S_{nonnative}$). Any $3S_{nonnative}$ that is formed is transformed into the fully-reduced protein (R) because of reduction pulse.

before introduction into a pH 8.2 folding buffer (30 µM protein; 100 mM Tris-HCl, 1 mM EDTA) such that the final pH was 8. One minute later, the sample was subjected to a reduction pulse, as previously described which converts unstructured intermediates (such as $3S_{nonnative}$) to the fully reduced protein.[9] Samples were then desalted and analyzed by reversed-phase HPLC (Supelco Discovery® BIO Wide Pore C18, 5 µm, 15 cm × 100.0 mm) using an acetonitrile gradient ($1\%\ min^{-1}$).[21] In other experiments, reduced rat WT PDI (rWTPDI), null PDI or each domain (4 µM each) was expressed (see Supporting Information)[22] and introduced into the pH 8.2 buffer, prior to mixing with the 3S species.

Measurement of the impact of rat PDI, null PDI and domains on the structure-forming step was determined by measuring the areas under the HPLC peaks corresponding to fully reduced protein (R) and the structured 3S* species.[19–21]

% of $3S_{nonnative}$ was calculated using the formula:

$$3S_{nonnative} = 100 \times [\text{peak area (R)}] / [\text{peak area (R)} + \text{peak area } (3S^*)] \quad (1)$$

$$3S^*(\%) = 100 - 3S_{nonnative} \text{ (ref 21)} \quad (2)$$

Similarly, Ca^{2+}-depleted α-lactalbumin (ALAC; pH 8, 100 mM Tris-HCl, 5 mM EDTA) was introduced into a refolding buffer containing 20 mM Ca^{2+} and 1 mM DTT^{red} or 4 µM rWTPDI and the samples analyzed as above.

Figure 2 shows a typical HPLC chromatogram showing the formation of 3S* species and $3S_{nonnative}$ ensemble from the $3S_{native}$ intermediate. The peak labeled R corresponds to $3S_{nonnative}$. This is because the applied reduction pulse converts the unstructured $3S_{nonnative}$ intermediate to R while leaving undisturbed the structured 3S* species.[21] Table 1 summarizes the quantity of $3S_{native}$ species that conformationally folds to a stable intermediate (3S* species). In the absence of any added PDI or PDI subdomain, 32% of the $3S_{native}$ species is able to conformationally fold to the stable 3S* intermediate, in agreement with previously reported literature.[11] The addition of rWTPDI results in a large decrease in the amount of 3S* formed (8 × over control). Similarly, the rate of formation of 3S* by conformational folding of $3S_{native}$ is reduced by addition of either ab or b′a′ although their ability to reshuffle $3S_{native}$ to $3S_{nonnative}$ is diminished relative to WTPDI. In contrast, the addition of null PDI or the hydrophobic bb′ domain results in an enhanced conformational folding rate of the $3S_{native}$ species to 3S* relative to the control that lacks PDI (40 and 38% respectively, relative to 32%).

Table 1. Measurement of oxidoreductase impact on structure-forming step of RNase A (pH 8, 100 mM Tris-HCl, 25 °C)[a]

Species	$3S_{nonnative}$/%	3S*/%
—	68 ± 1.7	32 ± 1.4
WT reduced PDI	96 ± 2.2	4 ± 1.2
Null PDI	60 ± 0.7	40 ± 0.34
ab	81 ± 0.8	19 ± 1.9
b′a′	79 ± 0.67	21 ± 0.89
bb′	62 ± 2.3	38 ± 1.22

[a]Each experiment was performed in triplicate.

Table 2. Measurement of oxidoreductase impact on structure-forming step in the regeneration of ALAC (pH 8, 100 mM Tris-HCl, 20 mM Ca^{2+}, 25 °C)[a]

Protein	Control	+1 mM DTT^{red}	+4 µM rWTPDI
ALAC	0.99 ± 0.01	0.97 ± 0.09	0.67 ± 0.04

[a]Each experiment was performed in triplicate.

Table 2 shows the refolding yield of ALAC in the presence of 1 mM DTT^{red} or 4 µM rWTPDI, one minute after introduction of the molten globule to the Ca^{2+}-containing buffer. Data from the control experiment is also tabulated. The data indicate that the acquisition of the native fold (of ALAC) from its molten globule state is more effective in the presence of reducing conditions that are stronger than found in the ER milieu (1 mM DTT^{red})[4] than in the presence of rWTPDI. It is also noteworthy that the DTT^{red} concentration exceeds the oxidoreductase concentration by a factor of 250. Furthermore, a reduction of 30% in the formation of native ALAC by PDI is observed relative to the control.

The role of PDI in the pivotal conformational folding step of oxidative regeneration which inherently competes with thiol–disulfide exchange has heretofore not been explored. In this study, we have examined the impact of PDI and its domains in conformational folding of RNase A and ALAC during their oxidative regeneration. This was achieved by populating native disulfide-bond-containing unstructured intermediates of both proteins in states that are poised to fold. For example, in RNase A, the acid-trapped and denatured $3S_{native}$ species is poised to fold to the structured 3S* species but is at risk of becoming reshuffled to its nonnative-disulfide-bond-containing isomers ($3S_{nonnative}$) when introduce into folding conditions (pH 8).

Our results indicated that the presence of reduced WT PDI results in drastic decrease in the efficiency of conformational folding of both proteins. This is because PDI cysteines accelerate the thiol–disulfide exchange between exposed native

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disulfides bonds and convert native bonds to nonnative bonds before conformational folding can protect those exposed disulfide bonds within stable tertiary structure. That is, PDI-facilitated thiol–disulfide exchange outcompetes conformational folding in both proteins. The addition of null PDI or the hydrophobic bb′ domain to $3S_{native}$ results in a slight facilitation of conformational folding of $3S_{native}$ RNase A (40 to 32%). This may be due to binding of the hydrophobic bb′ domain to the unstructured protein and its stabilization in agreement with previous model peptide binding studies.[17] In conjunction with results obtained upon the addition of WT and null PDI, the addition of composite ab and b′a′ domains confirmed that the redox-active domains have a higher impact on back-reshuffling ($3S_{native} \rightarrow 3S_{nonnative}$) than the hydrophobic domain has on the forward rate ($3S_{native} \rightarrow 3S^*$) resulting in a net loss in formation of structured intermediates in the presence of WT PDI.

While it can be argued that a catalyst accelerates both the forward and reverse reaction, our experimental system was designed to evaluate the contribution of the back reaction to the overall folding step. Importantly, our results demonstrate the highly evolved redox-activity of PDI, which is fine-tuned to catalyze the highly redox dependent oxidative folding process becomes self-limiting in the pivotal and mandatory conformational folding event in oxidative regeneration. Previous data from our lab had demonstrated that PDI accelerates the overall $3S_{nonnative} \rightarrow 3S^*$ step by a factor of 11.[21] However, considering the observed eightfold inhibition in the formation of $3S^*$ due to back-reshuffling of species that are poised to fold, the full potential effectiveness of PDI could equal $11 \times 8 = 88$-fold, rather than only 11-fold. These data about the maximum catalytic potential of PDI cannot be obtained without a) being able to deconvolute the physical conformational folding step from the chemical thiol–disulfide isomerization step and b) without the ability to isolate an intermediate that is poised to fold. That is, a species that lacks structure but possesses native disulfide bonds.

In the ER, $3S_{native}$ is populated a) very sparsely and b) very transiently. Thus, the in vitro experimental tools advanced in this paper are pivotal to stabilizing and populating such a transient intermediate which is in turn necessary for furthering our understanding of PDI's impact on structure-coupled oxidative folding steps. Even within the ER, it is likely that PDI remains associated with the unstructured $3S_{native}$ until the substrate can fold, bury its hydrophobic residues, and expose a charged surface to PDI, resulting in its dissociation.[16] If the substrate ($3S_{native}$) can fold faster than PDI backreshuffles it to $3S_{nonnative}$, then it can protect its native disulfide bonds from PDI and regeneration yields improve.

Under conditions where proteins need to be regenerated quickly, the inhibitory activity of PDI may contribute to misfolding even under homeostasis. In vivo, other factors that may temper the detrimental impact of the oxidoreductase activity PDI during conformational folding include peptidyl prolyl isomerase which facilitates cis–trans isomerization of prolines. Even though detailed studies need to be carried out to deconvolute the impact of individual factors before a general picture of the process of in vivo fold maturation becomes clear, our in vitro studies may have implications for understanding factors contributing to misfolding in vivo.

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