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The Structure-Forming Juncture in Oxidative Protein Folding: What happens in the ER?

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Abstract

The folding of disulfide bond containing proteins proceeds in a two-fold manner. Initially, cysteines are oxidized to form disulfide bonds. Structure is largely absent in this period. Next, when a minimally correct number of native linkages of disulfide bonds have been acquired, the biopolymer conformationally folds into the native, or a native-like, state. Thus, at the end of this “oxidative folding” process, a stable and native biologically active protein is formed. This review focuses on dissecting the “structure-forming step” in oxidative protein folding. The ability to follow this pivotal step in protein maturation in somewhat detail is uniquely facilitated in “oxidative” folding scenarios. We review this step using bovine pancreatic Ribonuclease A as a model while recognizing the impact that this step has in subcellular trafficking and protein aggregation.

Keywords

Oxidative folding; thiol-disulfide exchange; structure-forming step; cis-trans proline isomerization; rate-determining step; endoplasmic reticulum; protein trafficking

X. 1 Introduction

Disulfide bonds are the predominant covalent links that stabilize proteins over the usual non-covalent forces such as ionic interactions, Van der Waal’s forces, hydrogen bonding and hydrophobic associations [1, 2]. Disulfide bonds are found to exist in proteins that are secreted outside cells or stride the membranes of cells wherein a particularly harsh environment necessitates the existence of covalent bonds to support the tertiary structure.

Oxidative protein folding describes the procedure by which disulfide-bond-containing proteins mature to their biologically active form [3–8]. Oxidative protein folding involves the formation of disulfide bonds via oxidation of cysteine residues in the fully-reduced protein, followed by the conformational folding of the biopolymer to acquire the native state.

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It is a multi-step process involving chemical reactions such as oxidation, reduction, and thiol-disulfide exchange, and physical, non-covalent interactions as previously mentioned and takes place within the oxidizing environment of the endoplasmic reticulum (ER).

The study of oxidative protein folding via “classical” folders such as the four-disulfide bovine pancreatic Ribonuclease A (RNase A), leech carboxypeptidase inhibitor, hen egg white Lysozyme, the three-disulfide-containing Bovine Pancreatic Trypsin Inhibitor, the two-disulfide RNase T1 among others have provided a wealth of information about the mechanism by which the protein gradually acquires its tertiary structure [2, 5, 9–18]. The ability to better control elements of protein folding by studying oxidative protein folding pathways as opposed to studies focusing on conformational folding trajectories has been instrumental in revealing facets of protein folding that would otherwise elude inquiry [5–8]. These include understanding the origin of kinetic traps, rate-determining steps in protein folding, heterogeneity in folding pathways, dead-end species, among others [5–8, 17].

The use of a “reductionist” physical chemistry approach to dissect the mechanism by which proteins fold within the ER has been instrumental in unravelling the proceedings within this intracellular “black-box”. The application of experimental protocols and biophysical tools to understand oxidative protein folding has been thoroughly reviewed in a previous edition of this series and elsewhere [19, 20]. We initiate this review by recapitulating our current understanding of the “structure-forming step” which has been made possible through a series of disulfide-intact folding studies and some oxidative folding scenarios. Using the structure-forming step in RNase A as a template, we merge the interplay between a purely physical conformational process, viz. proline isomerization, with a chemical event, viz., thiol-disulfide exchange to examine the “chances” of successful acquisition of a native (biologically active) structure. The objective is to underscore the oxidative folding which often involves a competition between chemical reactions and physical structure-forming reactions. The ability of one to dominate in rate over the other often dictates the outcome. The “state” that the intermediate that is poised to fold, resides in, often simply per chance, attenuates its “fate”; i.e. whether it folds fruitfully, or recycles back to the unfolded ensemble.

X. 2 Oxidative folding of RNase A

In the following sections we will revisit the oxidative folding of RNase A to recapitulate features characteristic of oxidative protein folding. Particular emphasis will be paid to the structure-forming step in oxidative protein folding using that of RNase A as an example. The folding of ribonuclease has served as a paradigm for understanding the physicochemical processes governing the regeneration of disulfide-bond-containing proteins and the folding of those proteins that lack the covalent linkage (cytosolic proteins). There are several reasons for this: It possesses a sufficient number of disulfide bonds to introduce a degree of complexity in the folding trajectory while still remaining analytically tractable for biophysical studies. By contrast, proteins with three or fewer disulfide bonds may not adequately represent the complexity of proteins that are processed through the ER (and those with greater than four disulfide bonds make separation of folding intermediates extremely difficult). The oxidative folding trajectory of RNase A encompasses factors and features

including kinetic traps, local unfolding processes, temperature-dependent variations, and on-pathway intermediates that serve as a “one-pot prototype” for researchers studying oxidative protein folding [5–8]. The details of the formation of disulfide bonds as the protein gets sequentially oxidized has been exceptionally well-characterized in that we have details of both entropic and enthalpic tendencies that impact the formation of cysteines. The disulfide-intact folding of RNase A has also been studied in-depth; with these studies serving as an analog for studying conformational folding. Within this ambit, the conformational folding of this protein has been found to proline-isomerization dependent and limited. Together, the exhaustive characterization of RNase A folding (disulfide-coupled and conformational) and the variety of pathways it adopts to acquire the native state make RNase A folding pathways exemplar for researchers and students of protein folding.

RNase A is a four-disulfide-bond containing protein that has been used as a model to understand the mechanism by which proteins fold. The native disulfide bonds in this protein are located as follows: [65-72], [40-95], [26-84], and [58-110]. The process of oxidative folding is initiated by introducing the fully-reduced protein into an “oxidative” environment involving a suitable buffer and an appropriate redox couple such as GSSG/GSH or DTT^{ox}/DTT^{red} [21–24]. The concentrations of the elements within the couple are chosen such as to obtain a “net” oxidizing potential.

Oxidative folding of RNase A begins with the initial oxidation of fully-reduced RNase A (R) to form an ensemble of one-disulfide-bond-containing (1S) intermediates. The preparation of R and analysis of the oxidative folding intermediates and their kinetics of formation has been previously described [19, 21–24] and will be summarized in brief here:

Native RNase A, usually sourced commercially, is denatured and reduced by introduction into a buffer containing a denaturing agent and a strong reducing agent for a period of 1 hr (pH 8, 100 mM Tris-HCl, 6 M Gdn, 100 mM DTT^{red}). R is then desalted and introduced into a folding buffer (pH 8, 100 mM Tris-HCl, 100 mM DTT^{ox}). Note that small quantities of DTT^{red} are automatically generated from the reduction of DTT^{ox} by cysteines in reduced RNase A as a function of oxidative folding. Samples from the regeneration mixture are periodically withdrawn and blocked with a suitable thiol-blocking agent such as Aminomethylethanethiosulfonate (AEMTS) [21–24]. Blocking with this cationic reagent facilitates fractionation of the regeneration ensemble by their disulfide content and subsequent kinetics analysis [21–24]. Periodically, the presence of structured intermediates that may populate the regeneration pathway can be identified by the application of a brief reduction pulse [1 mM DTT^{red}, pH 8, 2 minutes, no AEMTS]. The pulse serves to reduce all unstructured species back to R. By contrast, any structured intermediates and N possess buried disulfides that are not impacted by the pulse. This serves to facilitate the separation and collection of these intermediates (and N). The intermediates can be easily characterized for enzymatic activity, disulfide mapping, thermal stability and structure by first blocking their cysteines with AEMTS to prevent reshuffling. Once the disulfide-bond-connectivity has been established, the remaining cysteines can be mutated (for eg, to Alanine) to facilitate further characterization of the intermediates [25, 26].

In RNase A, eight cysteines can form 28 distinct 1S isomers. The frequency by which individual isomers populate the 1S landscape depends upon both entropic and enthalpic contributions [1, 27–29]. For example, the probability of forming a disulfide bond between cysteines 26 and 110 of RNase A is entropically highly disfavored [28]. This is because these cysteines are the farthest away from each other in the chain and the resulting loop generated upon forming the [26-110] disulfide bond results in the largest loss of entropy to the peptide chain. In contrast, by purely entropic considerations, both [65-72] and [58-65] have the highest and equal probabilities of formation. Enthalpic interactions on the other hand can also influence the distribution of disulfide connectivities [28, 29]. Favorable enthalpic interactions bring the chain together and reduce the entropic penalty that would normally result upon disulfide-bond-induced loop closure. By contrast, unfavorable side-chain derived enthalpic interactions around the neighborhood of cysteines poised to form a disulfide bond would reduce the propensity to form that particular disulfide bond [28, 29].

A second feature of the 1S ensemble is the unstructured nature of the populace. Though the protein is no longer as “free” as R, there is no evidence of structure within this ensemble. Nevertheless, attempts to characterize this ensemble by intramolecular distance measurements using FRET revealed constraints within the chain [30]. The 1S ensemble, devoid of essential structure, is readily reduced back to R because it is unable to protect its disulfide bonds from the reducing agent [5]. Note that the reducing agent is present as part of the redox couple or is generated after the first cycle of disulfide bond formation in any protein). The 1S isomers are also able to freely isomerize intramolecularly with the equilibrium distribution of the 1S ensemble dictated by the entropic and enthalpic influences described above [5–8].

The oxidation of cysteines to disulfides continues with resulting 2S, 3S and 4S ensembles populating the folding landscape [21–24; Fig 1]. The 2S, 3S and 4S in RNase A are still unstructured and as a result their disulfides can be reduced easily or isomerize (with the exception of the 4S ensemble wherein intramolecular isomerization is not possible). Independent of the strength of the redox couple and folding conditions, a distribution of R-4S species emerges such that the species are in equilibrium with each other. The number of isomers within each ensemble in this equilibrium is governed by the number of cysteine residues in the protein. In RNase A, there are predicted to be 28 1S isomers, 210 2S isomers, 320 3S isomers and 104 4S isomers. Detailed experimental investigation of the 1S ensemble has confirmed the presence of all 28 1S isomers in the oxidative folding trajectory of RNase A in both the 1S ensemble and the 2S ensemble [28, 29].

X. 2. 1 The structure-forming step in the oxidative folding of RNase A

Two intermediates having three disulfide bonds each, viz. des [65-72] and des [40-95] (also called 3S* species; with the asterisk denoting structure), have been identified in the 3S ensemble to possess native-like structure [31, 32; Fig 2]. The terminology, des [x-y] reflects the missing disulfide bond in each species. Des [65-72] and des [40-95] possess melting point temperatures of 38 °C each and enzymatic activities of 95% and 5%, respectively, relative to native RNase A. These values perhaps reflect the “contribution” of the missing disulfide to the correct and stable orientation of the catalytic domain.

The native protein, N, is formed from these des species through the oxidation of their remaining cysteines (Fig 2). The native protein is stable ($T = 65\text{ }^{\circ}\text{C}$) and possesses 100% biological activity.

There are features pertaining to the formation of the 3S^* species from the 3S ensemble that are particularly relevant to the folding landscape. As long as the folding conditions are below the melting point of the 3S^* species, their formation from 3S is irreversible. This permits the 3S^* species to be removed from the equilibrium distribution of 3S species (and from the R-4S pool). It facilitates the rapid formation of N, given that there is no more intramolecular reshuffling and the remaining two cysteines are suitably spatially “positioned” to form a disulfide bond. It is notable that the formation of N from 3S^* is also irreversible under normal folding conditions for the aforementioned reasons. Thus the acquisition of structure (specifically via the 3S^* species, in RNase A) transitions the folding intermediates from an otherwise endless cycle of oxidation, reduction and reshuffling reactions to an intermediate that can acquire the final folded topology (N) at a much faster rate.

In RNase A, the formation of the 3S^* species from the 3S ensembles constitutes the rate-determining step in the process $\text{R} \rightarrow \text{N}$ [31, 32]. It is a composite step involving thiol-disulfide exchange reactions (reshuffling or isomerization reactions) between isomers within the 3S ensemble and, a conformational folding step resulting in native-like structure. The conformational folding step results in the burial of the existing disulfide bonds and insulates them for further reshuffling (back to the 3S ensemble of isomers).

Of interest is the fact that two other 3S^* species are also populated at lower temperatures. Des [58-110] and des [26-84] emerge onto the folding landscape when oxidative regeneration (refolding) is carried out at $15\text{ }^{\circ}\text{C}$; but not at room temperature [33; Fig. 2]. These observations suggest that their thermal transitions reside between 15 and $25\text{ }^{\circ}\text{C}$. Furthermore, both des [58-110] and des [26-84] persist in the oxidative folding landscape even after all other intermediates have been consumed to form N, suggesting that they are kinetically trapped species [7, 8]. Analysis of the tertiary structure of RNase A reveals that disulfides [58-110] and [26-84] are buried in the native structure. Therefore, there is a steric barrier to the oxidation of cysteines 26 and 84 and cysteines 58 and 110, making des [26-84] and des [58-110] kinetically trapped. Energetically, a local unfolding event is required to expose either pair of cysteines for oxidation to the respective disulfide [7, 8]. The free energy of the local unfolding process however is commensurate with the free energy of global unfolding. As a result both species are vulnerable to back-reshuffling to the 3S ensemble rather than oxidation to N.

X. 3 Deconvoluting the formation of 3S^* from 3S

There are a total of 420 3S isomeric species that, in principle, can populate the 3S ensemble. Of these, 416 species contain one or more non-native disulfide bonds and are termed 3S_{NNU} (to denote the presence of non-native disulfide bonds and that the species lack structure). Any 3S species that contains one or more non-native disulfide bonds prevents it from conformational folding [5–8]. Thus, there exists, a pool of potentially 416 species that are in

equilibrium with each other through thiol-disulfide exchange (isomerization) reactions [5]. These 416 species are also in equilibrium with the rest of the unstructured species that populate the RNase A folding landscape. As discussed earlier, this equilibrium is established through oxidation and reduction reactions. Four of the 3S isomers (among the 420 species) contain only native disulfide bonds. These species are unstructured and termed $3S_{NU}$ to distinguish them from other, non-native disulfide-bond-containing, unstructured 3S species. The formation of $3S_{NU}$ takes place from $3S_{NNU}$ via isomerization of the sole non-native disulfide bond in the $3S_{NNU}$ species (Fig. 3). Thus, only those $3S_{NNU}$ species that possess two native bonds and one non-native bond can directly isomerize to $3S_{NU}$.

All four possible $3S_{NU}$ have two possible “fates” (Fig 3). (1) The $3S_{NU}$ species can isomerize back (back-reshuffle) to a $3S_{NNU}$ species and eventually any of the 3S isomers. This process happens by thiol-disulfide chemical exchange reactions that are “chemical” in nature. (2) $3S_{NU}$ can conformationally fold to form a $3S^*$ species. The $3S^*$ species, as previously discussed, possess native-like structure (below their melting temperatures). As a result, they protect their native disulfide bonds from further intramolecular thiol-disulfide exchange (isomerization) reactions. The conformational folding process is physical in nature and involves the formation of (via secondary and tertiary interactions) near-native-like structure in RNase A. The native-like $3S^*$ species do not backreshuffle to $3S_{NU}$ and therefore, $3S_{NU} \rightarrow 3S$ is unidirectional.

The formation of N from $3S^*$ is also unidirectional via the oxidation of the remaining two cysteines in $3S^*$.

X. 4 The rate-determining step in RNase A oxidative folding

There are unimolecular and bimolecular steps in RNase A oxidative folding. The unimolecular processes are the intramolecular isomerization reactions within the 1S, 2S and 3S ensembles (R and 4 S cannot chemically isomerize). The unimolecular processes are pH dependent since they involve thiol-disulfide exchange reactions. The isomerization may also be bimolecular if it involves an external thiol-disulfide isomerase (such as Protein Disulfide Isomerase (PDI) [34]) which can facilitate reshuffling. In fact, at an intracellular pH of ~6.7, “intramolecular” thiol-disulfide exchange reactions are likely to be intermolecular, because of PDI involvement, and therefore bimolecular. This is because the thiols of PDI are highly acidic with a pKa of 5.7. They would then be deprotonated at pH 6.7 and can engage/ facilitate disulfide exchange in the substrate.

Bimolecular processes include, in addition to the above, oxidation and reduction reactions that help transition R towards N (via 1S, 2S, etc). These reactions are bimolecular because they either involve a small-molecule-based external redox couples, viz GSSG/GSH, or PDI which can also act as a reducing/oxidizing agent. Under laboratory settings, the choice and concentration of the small-molecule couple can be selected to attenuate the rate of the bimolecular steps and potentiate the regeneration landscape. For example, the use of DTT^{ox}/red facilitates the study of oxidative folding in the laboratory by virtue of DTT^{ox} being a relatively weak oxidizing agent (compared to GSSG) (Rothwarf 1993 a, b, c, d). Furthermore, since DTT^{ox} does not form mixed disulfides with the protein thiols, it

simplifies the folding landscape and facilitates the identification of intermediates that populate the folding trajectory [28, 29].

It is quite obvious that the rate-determining step in the oxidative folding of RNase A is dependent on the nature of the redox reagent, presence of oxidoreductase chaperones such as PDI, temperature, etc. For example, in the absence of any redox couple and under anaerobic conditions, the rate-determining step in RNase A oxidative folding would be the formation of 1S from R. However, under “standard” folding conditions, the rate-determining step involves the formation of two 3S* species, viz. des [65-92] and des [40-95], from the 3S ensemble.

The rate limiting-step in RNase folding is the 3S to 3S* step (Fig. 3). It is the structure-forming juncture in the oxidative folding of RNase A. It is also, very often, the rate-determining step in oxidative folding of other proteins.

Using RNase A folding as a model, we will discuss why this is so in the following section. Understanding this step provides a window into protein folding and as a result, subcellular trafficking, in the endoplasmic reticulum. Towards this, we will examine the formation of 3S* from 3S using the formation of N from disulfide-intact denatured protein (U), as an analog. We will also review data that have been gathered on the formation of 3S* from 3S as a function of pH.

It is to be noted that the statistical rate of regeneration of RNase A is likely to be commensurate with its disulfide complexity. I.e., a four-disulfide-bond-containing protein is likely to regenerate much more slowly than say, a one disulfide-bond-containing protein. This is because it is likely to populate an exponential greater number of non-native disulfide-bond-containing intermediates. Conversely, the statistical regeneration rate of a one-disulfide-bond-containing protein with only two cysteines in the sequence is likely to be higher than a corresponding one-disulfide-bond-containing protein with three cysteines in the sequence. The actual regeneration rate is compounded by a variety of folding factors that may impact disulfide-bond-acquisition and conformational folding of each protein. The thermodynamic stabilities of intermediates and the presence of kinetic traps also influence folding rates. For example, in RNase A both des [26-84] and des [58-110], the two kinetically trapped species, persist in the regeneration mixture for a period of days; similar to the trap found in BPTI. The thermodynamic stabilities of des [65-72] and des [40-95] are likely similar to those of other intermediates because they are found on the regeneration pathways at room temperature. In vivo, the thermodynamic stability may be somewhat compromised as the ambient temperature is 37 °C and likely closer to the melting points of some intermediates in proteins.

X. 5 The folding of disulfide-intact RNase A: Role of proline isomerization

RNase A has 4 Prolines at positions 42, 93, 114 and 117. In native RNase A (N), two of these prolines possess cis X-Pro peptide bonds (Tyr 92-Pro 93 and Asn 113-Pro 114), while the other two are trans X-Pro peptide bonds (Lys 41-Pro 42 and Val 116-Pro 117) [35]. When disulfide-intact bovine pancreatic ribonuclease A (RNase A) is unfolded (U), the

X-Pro peptide bonds undergo cis-trans isomerization and eventually equilibrate to form a distribution of cis and trans conformers around their respective X-Pro peptide bonds. Under unfolded conditions, the equilibrium distribution is skewed to favor the trans conformation (70:30). Even though almost iso-energetic, the kinetic barrier between the conformers is large due to the presence of the double bond. The barrier height significantly restricts the cis isomerization rate and necessitates the presence of a catalyst (such as peptidyl prolyl isomerase (PPI)). In the presence of tertiary structure, the isomerization is expected to be even slower or non-existent. Therefore, in the native (or native-like) state, the prolines are said to be either “cis” or “trans” locked. Under such conditions, it is likely that “local” fluctuations are necessary to permit isomerization (or access to PPI).

In sum and substance, multiple species having different conformations about these X-Pro peptide bonds are present in the unfolded state of the protein. For example, the statistical fraction of the unfolded biopolymer which contains “all-cis” X-Pro peptide bonds would equal $0.3 \times 0.3 \times 0.3 \times 0.3 = 0.0081$ or 0.81% of the unfolded molecules. By contrast, the fraction of unfolded molecules possessing all-trans linkages would equal $(0.7)^4 = 24\%$; and so on.

When fully-denatured, disulfide-intact, RNase A (U), is allowed to refold in a folding buffer, there are multiple phases associated with the refolding process [36–38]. These phases, termed U_{vf} , U_f , U_s^{II} , and U_s^I associate with refolding time constants on the millisecond, millisecond to second, second to tens of seconds, and hundreds of seconds time scales, respectively [36–38]. These phases have been attributed to proline isomerization that is a part of the trajectory to the native structure; and precede the formation of native structure. The differences in conformations at the X-Pro peptide bonds result in different refolding rates for these unfolded species. For example, those unfolded disulfide-intact RNase A molecules that per chance possess the X-Pro conformations that coincide with the native (locked) X-Pro conformations would be “primed” to fold to N. Therefore, their refolding time constants would be shortest. By contrast, those unfolded RNase A molecules with its X-pro bonds exactly out of phase with their dispositions in N would be the slowest to refold. The former scenario has been validated using a “double-jump” experiment in which native RNase A was introduced into unfolding conditions before being rapidly reintroduced into a refolding buffer (via dilution). The dwell-time of 1s in the “unfolding condition”, prior to the second “jump” into refolding conditions, is sufficient to result in the complete loss of tertiary structure. However, the time spent in the unfolded state (1s) is not sufficient for the prolines to isomerize to their equilibrium unfolded state. Thus, reintroduction into “folding conditions” immediately after the 1s “unfolding condition dwell-time” resulted in a rapid (milli-second time constant) and monophasic folding of RNase A to N. By varying the “dwell-time” in the unfolded state, other phases were populated as a function of increasing time “spent” in the unfolded state.

These data, along with other data involving sequential mutations of the prolines alanine (which favors a trans-locked state) indicate that three of the four prolines, viz. 93, 114, 117 are “essential” to RNase A disulfide-intact folding. I.e., they impact the rate of conformational folding of RNase A (the $U \rightarrow N$ process). No effect on the refolding kinetics of the protein is observed from the isomerization of the X-Pro 42 peptide bond.

Based on the individual contributions of the three essential prolines to the refolding kinetics of RNase A, a box model for the conformational folding of RNase A has been proposed (Fig 4). Given the reversible nature of the N to U transitions, for simplicity we will examine disulfide-intact conformational unfolding of RNase A using the box model.

Each vertex of the box represents one of the eight possible isomeric states that the three prolines could exist in. For example, U_{cct} indicates an unfolded state with proline 93, 114 and 117 in their native conformations [the subscripts refer to the cis or trans states and the order in which they appear relate to 93, 114 and 117]. In other words, it is the product formed from N (N_{cct}) when the native protein introduced into unfolding conditions with an “unfolded state dwell-time” of 1s. Thus U_{cct} lies closest to N since its prolines are already “aligned” with the native state conformation. Consequentially, it possesses the fastest refolding time (millisecond time constant) as proline isomerization is not involved when going from U_{cct} to N_{cct} . As the prolines start isomerizing (by permitting U_{cct} to remain in an unfolding milieu for longer time frames), the time to refold back to N increases and the disulfide-intact unfolded intermediates occupy vertices farther away from the U_{cct} vertex. It is to be noted that the box model considers “one isomerization” at a time even though the isomerization reactions are likely independent of one another. The intermediate U_{ctt} is two steps away from folding to N_{cct} because Pro 114 is in the trans state in U_{ctt} and need to isomerize to cis (U_{cct}) before becoming locked in the native state (N_{cct}). U_{ctt} must proceed via U_{cct} before it can become N_{cct} . The disulfide-intact intermediate U_{tcc} is the farthest from U_{cct} (and from N_{cct}) since its prolines are in exactly the opposite conformation as the native state. It must proceed via three steps (intermediates) before it can fold to N (N_{cct}).

Using disulfide-intact RNase A as an example, the box model elegantly illustrates rate-determining features that may be encountered steps in the conformational folding of a protein. In RNase A, conformational folding is particularly complicated by the need for three (of four) prolines to isomerize to their native dispositions prior to conformational folding (and “locking-in” of those prolines via tertiary interactions).

X. 6 The structure-forming step in oxidative protein folding

We now integrate our understanding of the 3S to 3S* transition in the oxidative folding of RNase A along with our knowledge of disulfide-intact RNase A conformational folding to delineate the structure-forming juncture in oxidative protein folding.

The four possible 3S_{NU} species are expected to be conformationally nearly identical to U. The only difference is the lack of a fourth native disulfide bond in 3S_{NU} relative to U. Therefore, each of the four 3S_{NU} species can exist in potentially eighty “locally” heterogeneous conformations via variations in the dispositions of the three essential prolines. For example, 3S_{NU} species lacking the [40-95] disulfide bond could exist as an ensemble of the following unfolded state intermediates : 3S_{NU cct}; 3S_{NU ctt}; 3S_{NU ctc}; 3S_{NU ccc}; 3S_{NU tct}; 3S_{NU tcc}; 3S_{NU ttc}; 3S_{NU ttt}.

From the discussion involving the time constants for the proline-isomerization-dependent/limited U → N processes, we can infer that similar time-constants and folding constraints

characterize the $3S_{\text{NU}}$ to $3S^*$ conformational transition. Therefore, we can reconsider the box model within the context of oxidative folding pathway (Fig. 5a). This is possible by incorporating both, chemical oxidative folding elements and physical (in this case proline isomerization and conformational folding) feature, in the box model. This is achieved by placing (each of the four) three-disulfide-containing $3S_{\text{NU}}$ species at the corresponding proline-conformation dependent vertex. An example involving a $3S_{\text{NU}}$ species lacking the [40-95] disulfide bond is shown within the context of the box model (Fig. 5a).

From prior reading, it is evident that a $3S_{\text{NU}}$ species that has its prolines in the native conformation ($3S_{\text{NU cct}}$) is poised to fold on a milli-second time-scale to $3S^*$ (or $3S^*_{\text{cct}}$) (Fig. 5b). Thus, such a species ($3S_{\text{NU cct}}$) can “outcompete” the competing chemical thiol-disulfide exchange reaction that would otherwise result in $3S_{\text{NU}}$ becoming reshuffled to $3S_{\text{NNU}}$ (Fig. 5b). Three other species ($3S_{\text{NU ctt}}$; $3S_{\text{NU ccc}}$; $3S_{\text{NU tct}}$) are also likely to outcompete and therefore, evade, the reshuffling “threat” (Fig. 5b). While each of these species is one step away from $3S_{\text{NU cct}}$ and likely to possess millisecond to second folding time-constants, they remain at a greater “risk” of becoming reshuffled to $3S_{\text{NNU}}$ than is $3S_{\text{NU cct}}$. The remainder of the [40-95] $3S_{\text{NU}}$ isomers are likely to become back-reshuffled to $3S_{\text{NNU}}$ considering their prolonged refolding times.

X. 6. 1 Factors impacting the competition between conformational folding and chemical reshuffling ($3S_{\text{NNU}} \rightleftharpoons 3S_{\text{NU}} \rightarrow 3S^*$)

It is to be noted that the chemical disulfide-reshuffling reaction is highly pH dependent and therefore slight changes in the pH within the ER can amplify (or diminish) reshuffling rates. An increase in pH will favor reshuffling rates and may tilt the competition towards chemical reshuffling and formation of $3S_{\text{NNU}}$ since moderate changes in pH do not impact proline isomerization rates. However, unlike pH changes (fluctuation in these are not very plausible) the presence of an oxidoreductase can more practically impact chemical reshuffling. Furthermore, the presence of PDI is also likely to tilt the outcome of the $3S_{\text{NNU}} \rightleftharpoons 3S_{\text{NU}} \rightarrow 3S^*$ process towards $3S_{\text{NNU}}$ formation. This is because though PDI has no outcome on proline isomerization, it is likely to enhance the reshuffling process; making it competitive even at pH 7.4 with conformational folding of fast folders. The presence of subcellular peptidyl prolyl isomerase can accelerate the isomerization of X-Pro peptide bonds and facilitate slower folding isomers to fold faster. Other factors that can increase thiol-disulfide exchange could include high density of substrates within the ER, thereby permitting intermolecular thiol-disulfide exchange. Overall, the scenarios is likely to be complicated by a variety of “folding” considerations.

Conclusion

The ability of a disulfide-bond containing protein to rapidly bury its disulfide bonds within native structure is key towards maturation. Thus conformational folding to a native or native-like state is critical. Any compromise in this process will result in “back-reshuffling” to the equilibrium pool of unstructured intermediates. The same principle holds true for purely conformational folders (cytosolic proteins). Conformational folding to form the native

structure in such proteins exists in competition with water, salts, other amino acids and other proteins. Delays in folding increase the chances of terminal misfolding and degradation.

Cargo trafficking through the ER has an uphill journey before it can get successfully secreted. We anticipate that the first principles of protein folding, gleaned from test-tube studies, are applicable to the more complex confines of the ER. This is because “ex-vitro” experiments have relied on conditions that somewhat mimic those found in the confines of cellular compartments. The choice of pH, redox couples, inclusion of relevant chaperones in folding studies of RNase A and other disulfide-bond-containing proteins have been designed bearing in mind the intracellular milieu found within the ER. Furthermore, across slight variations in folding conditions, many proteins only demonstrate changes in the “rates of regeneration”. Others adopt alternative pathways with the regenerative flux redistributed between the original and newfound pathways.

Yet some other variations in conditions can be expected that may impact regeneration rate but are unlikely to impact the folding trajectory. For example, a primary difference between test-tube experiments and folding within the ER is the presence of high protein concentrations in the latter space. This could increase the tendencies of the biopolymers to aggregate before they can fold; thereby reducing their “yield” and/or regeneration rate. Furthermore, the complexity of the ER is such that multiple chaperones and folding factors are likely to act in concert on the disulfide-bond-containing substrate trafficking through. Nevertheless, even under such conditions, the fundamental principles governing protein folding in the ER and in the cytoplasm still remain the same as has been derived from a robust choice of physico-chemical conditions applied by a many number of researchers in this field.

In the ER, the “commitment” to secretion is in competition with statistically higher tendencies or factors that bias the cargo towards retrotranslocation (Fig. 6). For example, the myriad number of non-native chemical and physical isomers increase the proteins’ “dwell-time” in the unfolded state. This leads to a risk of misfolding, aggregation and retrotranslocation. To personify, it is clearly in the best “interests” of the protein to fold- a discussion for a later review. The ability to fold however, is predicated upon the “per chance” occurrence of the correct “pre-folding conditions. I.e., by the chance occurrence of a sufficient number of native disulfide bonds that can trigger conformational folding; by the chance occurrence of the “native” complement of X-Pro peptide bonds. Extraneous factors such as biological catalysts that increase the frequency with which the per chance occurrence of intrinsic native factors are sampled simply provide yet another opportunity for the protein to fold and protect its key features (disulfide bonds, prolines, salt-bridges, van der Waal’s forces) from dismantling. They do not influence the functional structure of the protein which is simply encoded in its sequence.

A thorough understanding of protein folding pathways is essential for facilitating the expression and folding of proteins not only for basic research but also for commercial applications as well. These include peptides like insulin and immunotoxins such as conotoxin [39–44]. Such an understanding is also critical for the introduction of non-native motifs within proteins including catalytic cysteines. A number of papers have also reviewed

the techniques used to increase the rate of folding and the yield of the biologically active folded form in the aforementioned scenarios [45–48]. Some of the strategies include co-expression of chaperones or the addition of small molecule folding adjuvants, tagging constructs of either leader sequences that export disulfide bond containing proteins to the appropriate folding loci in the cell or include helper proteins that can assist in folding, high-throughput selection of suitable clones that eliminate redundant disulfide bonds, and tuning of redox conditions. The overarching objective in all these cases is to sway the competition between folding and aggregation to favor rapid folding and burial of hydrophobic residues. Inroads have also been made to introduce disulfide bonds and also replace cysteines with Selenocysteine as potential mechanisms to intramolecularly catalyze disulfide bond formation [49]. Other techniques have explored and exploited pro-domains in catalyzing disulfide-associated folding [50]. Whereas the results have met with success, here too, the principles gathered from experimentally determine protein folding trajectories apply. Cysteines that are buried have the potential to result in the formation of kinetically-trapped intermediates and reduce folding yields/slow down regeneration rates [51]. Thus, incorrectly introduced cysteines can be detrimental. Furthermore, the introduction of every additional cysteine in a protein adds to the total number of intermediates in the folding ensemble. Particularly, it specifically populates the nonnative ensemble and would therefore likely delay oxidative folding when considered from a statistical view-point.

In sum and substance, an intimate knowledge of oxidative folding mechanisms drives an understanding of subcellular trafficking, events that can promote misfolding and the emergence of diseases associated with improper folding such as Parkinson's, Alzheimer's, Huntington and mad-cow among others. Such a knowledge can also guide the efficient regeneration of proteins that have found biomedical and industrial use.

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Abbreviations

ER	Endoplasmic Reticulum
RNase A	bovine pancreatic ribonuclease A
DTT^{ox}	Oxidized dithiothreitol
DTT^{red}	Reduced Dithiothreitol
GSH	Reduced glutathione
GSSG	Oxidized glutathione
Des species	a folding intermediate lacking one disulfide bond

$3S_{\text{NNU}}$	three-disulfide-bond-containing intermediate with one or more non-native disulfide linkages
$3S_{\text{NU}}$	Unstructured three-disulfide-bond-containing intermediate with native disulfide linkages
$3S_{\text{x,y,z}}$	Unstructured disulfide-intact intermediate where x,y, and z refer to the cis- or trans- orientations of the X-Pro peptide bonds around prolines 93, 114 and 117, respectively
$3S_{\text{NUx,y,z}}$	Unstructured three-disulfide-bond-containing intermediate with native disulfide linkages where x,y and z, refer to the cis- or trans- orientations of the X-Pro peptide bonds around prolines 93, 114 and 117, respectively

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Fig 1.

Regeneration pathway of Ribonuclease A (pH 8, 25°C; DTT^{ox/red}) depicting key features of the oxidative folding trajectory (Narayan et al. 2000). The fully-reduced protein R becomes increasingly oxidized to form ensembles of 1S, 2S, 3S, and 4S species wherein the numerals refer to the numbers of disulfide bonds in a species belonging to an ensemble. The possible number of isomers in each ensemble is also listed below each *n*S species. R-4S are unstructured and prone to thiol-disulfide exchange reactions (oxidation, reduction, reshuffling). Therefore, they are shown in equilibrium with one another. Select 3S molecules with native disulfide bonds can fold to form des [65-72] and des [40-95] (or 3S* species) in which native disulfide bonds are protected from thiol-disulfide-exchange reactions. The native protein (N) emerges from the 3S* species via oxidation of the remaining cysteines. The inset is a typical HPLC chromatogram in which the folding mixture is successfully fractionated using a thiol-blocking agent.

Fig. 2.

Regeneration Pathways of RNase A at 15 °C *(Welker et al. 1999). In addition to the characteristic features observed in Fig 1, two kinetically trapped appear in the low-temperature regeneration pathway, These species, viz. des [26-84] and des [58-110] persist for over four days after initiating regeneration. They contain native, disulfide bonds, native-like structure but buried cysteines (which prevents their oxidation to N; making them kinetically trapped). With thermal transitions around 20 °C, they simply return to the pool of unstructured 3S isomers if regeneration is carried out at 25 °C. The inset is a typical HPLC chromatogram showing the products of the low-temperature refolding process when most of the unstructured intermediates had converted to N, leaving behind the two kinetically trapped species.

Fig. 3.

Structure-coupled oxidative folding steps involves competition between chemical thiol-disulfide exchange rxns and a physical conf. folding rxn. The $3S_{NU}$ species can either conformationally fold to $3S^*$ and structurally protect its disulfide bonds from further reshuffling or, it can reshuffle back to the $3S$ ensemble via $3S_{NNU}$.

Fig. 4.

Box Model. The Proline isomerization depending (un)folding of disulfide-intact isomerase is illustrated by a “box” model. The time taken to fold ($U \rightarrow N$) is dependent upon the disposition of X-Pro peptide bonds around the three essential prolines of RNase A. In the native state, the X-Pro peptide bonds around prolines 93, 114, 117 are locked in the cis, cis, and trans state. However, in the unfolded state, there are eight possible combinations the three X-Pro peptide bonds could adopt. Thus, the refolding time is fastest for U_{cct} ; an unfolded intermediate whose X-Pro peptide bonds, per chance, is in the same conformation as that in N.

Fig. 5a. Proline-isomerization and the $3S \rightarrow 3S^*$ transition. Superimposition of a typical $3S_{NU}$ species over the box model. For example, a 3_{NU} species lacking the [40-95] disulfide bond can have eight different conformations of the X-Pro peptide bond around the three essential prolines. There are three other 3_{NU} species, all of which are poised to fold; but proline-isomerization limited.

Fig. 5b.

Two $3NU$ species with different conformations around their X-Pro peptide bonds. $3NU_{cct}$ possesses the native conformation of X-pro peptide bonds and can rapidly (millisecond time-scale) fold to form $3S^*_{cct}$ while evading reshuffling to $3S_{NNU}$. By contrast $3S3NU_{tct}$ is two steps away from being able to form $3S^*_{cct}$. Its time to refold to $3S^*_{cct}$ is therefore longer and while likely to fold to $3S^*$, it “risks” being reshuffled to $3S_{NNU}$.

Fig. 6. Within the ER, a disulfide-bond-containing protein has two fates. It can either fold oxidatively fold successfully and get secreted outside the ER. Or, it can misfold. Terminal or successive misfolding would trigger retrotranslocatory apparatus and result in degradation. *In vivo*, there are several factors affecting the “fate” of a disulfide-bond-containing protein and traffic within the ER.