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Non-redox-active small-molecules can accelerate oxidative protein folding by novel mechanisms

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Abstract

Multi-disulfide-bond-containing proteins acquire their native structures through an oxidative folding reaction which involves formation of native disulfide bonds through thiol-disulfide exchange reactions between cysteines and disulfides coupled to a conformational folding event. Oxidative folding rates of the four-disulfide-bond-containing protein bovine pancreatic ribonuclease A (RNase A) in the presence of the synthetic redox-active molecule, (+/−)-trans-1,2-bis(2-mercaptoacetamido)cyclohexane (BMC), and in combination with non-redox-active trimethylamine-*N*-oxide (TMAO), and trifluorethanol were determined by HPLC analysis. The data indicate that regeneration of RNase A is enhanced 2-fold by BMC (50 μM) and 3-fold upon addition of TMAO (0.2 M) and TFE (3% v/v) relative to control experiments performed in the absence of small-molecules. Examination of the native tendency of the fully-reduced polypeptide and the stability of key folding intermediates suggests that the increased oxidative folding rate can be attributed to native-like elements induced within the fully-reduced polypeptide and the stabilization of native-like species by added non-redox-active molecules.

Keywords: Oxidative folding; Small molecule; Thiol-disulfide exchange; Native tendency

1. Introduction

Oxidative protein folding (regeneration) is a composite process involving 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 form of disulfide-bond-containing proteins (the native species) [1–3].

Oxidative folding is characteristic of many proteins that are secreted outside the cell, or are membrane-bound, and possess disulfide bonds [4]. It involves acquisition of the native complement (or a minimal native subset) of disulfide bonds from the fully-reduced polypeptide followed by a conformational folding event leading to the generation of stable native (or native-like) structure. Conformational folding is pivotal to the oxidative folding process because the ensuing tertiary structure protects the formed disulfide bonds from reduction and reshuffling reactions [5–7]. However, in a majority of disulfide-bond-containing proteins, the formation of the native set of disulfides is preceded by the formation of non-native-disulfide-bond-containing unstructured intermediates prior to formation of the native protein [1,3]. For example, during the oxidative folding of the four-disulfide-bond-containing protein bovine pancreatic ribonuclease A (RNase A), 28 one-disulfide (1S), 210 two-disulfide (2S), 418 three-disulfide (3S) and 104 non-native four-disulfide (4S) containing intermediates are formed (at 25 °C and in the presence of the redox reagent dithiothreitol). In addition, two native-like species, viz., des [40–95] and des [65–72] which possess native-like structure and lack the (40–95) and (65–72) disulfide bond, respectively, are formed. These structured intermediates are also referred to as the 3S* species. The native protein (N) regenerates by oxidation of the

Abbreviations and symbols: AEMTS, 2-aminoethanethiosulfonate; BMC (+/−)-trans-1,2-bis(2-mercaptoacetamido)cyclohexane; DTT, dithiothreitol; HPLC, high performance liquid chromatography.

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remaining two cysteines in each of these species. (Fig. 1) [8–10]. In the absence of stable structure, native and non-native disulfide bonds whose distribution within any given unstructured ensemble (*n*S) depends upon loop-entropic and enthalpic interactions are reshuffled until the formation of a minimal number of *native* disulfides in an unstructured molecule triggers conformational folding and protection of the native set of disulfides within stable tertiary structure [1,11,12].

The need for oxidation reactions (to form disulfide bonds from cysteines), isomerization reactions (which permit non-native disulfide bonds to be converted to native ones) and reduction reactions (which are necessary to convert fully-oxidized non-native isomers their native-disulfide-containing isomers) makes oxidative protein folding an inherently slow process.

In vivo, oxidative protein folding is relegated to the lumen of the endoplasmic reticulum (E.R.) where an oxidizing environment facilitates the formation of disulfide bonds [4]. The process is catalyzed by protein disulfide isomerase, a 56-kDa E.R. resident that possesses oxidase and "shufflase" activity [13–16]. *In vitro* studies have demonstrated that PDI is ability to accelerate oxidative folding reactions (oxidation and reshuffling). It has also been shown to facilitate the rate of formation of the native form of multi-disulfide-bond-containing proteins including RNase A [17]. Furthermore, it has recently been demonstrated that in brains manifesting sporadic Parkinson's or Alzheimer's disease, PDI is s-nitrosylated; a reaction affecting a cysteine critical to its function as an oxidoreductase chaperone [18,19]. Additonally, a large number of disulfide-bond-containing proteins are overexpressed in foreign hosts for purposes of research and for biotechnological applications [20]. When such proteins are overexpressed, they often tend to accumulate within the cell in the form of aggregates known as inclusion bodies. A lengthy and cumbersome folding process is required before the native fold of the protein is realized from such inclusion bodies. PDI has therefore served as a model for the development of synthetic small-molecule oxidoreductases that can facilitate oxidative protein folding and have chemotherapeutic use [20,21].

In this study we have employed small-molecules to expedite *in vitro* oxidative protein folding. Our results indicate that the traditional approach involving acceleration of thiol-disulfide exchange reactions to catalyze oxidative folding can significantly benefit from partial induction/stabilization of native-like elements/native fold in the polypeptide chain.

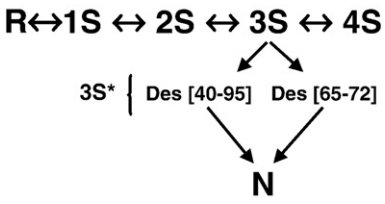

Fig. 1. Regeneration scheme of RNase A (pH 8, 25 °C). R, 1S, 2S, 3S and 4S are unstructured intermediates. Des [40–95] and des [65–72] are native-like three-disulfide-bond-containing intermediates and are also referred to as the 3S* species. N is the native protein.

2. Materials and methods

2.1. Materials

RNase A was purchased from Sigma and purified as previously described [8]. Oxidized (+/−)-*trans*-1,2-bis(2-mercaptoacetamido) cyclohexane (BMC) was purchased from TRC, Canada. Oxidized dithiothreitol (DTT^{ox}) and reduced dithiothreitol (DTT^{red}) were obtained from Sigma and used without further purification. All other chemicals were of the highest grade commercially available.

2.2. Methods

2.2.1. Preparation of fully-reduced RNase A

Fully-reduced RNase A was prepared by incubating native protein (10 mg/ml) in 6 M Gdn HCl and 100 mM DTT^{red} (pH 8, 100 mM Tris–HCl, 1 mM EDTA) for a period of 2 hours [8]. The mixture was then repeatedly dialyzed against 50 mM acetic acid at 4 °C prior to lyophilisation. Fully-reduced RNase A was then dissolved into 10 mM acetic acid to obtain a stock solution of 5 mg/ml. The stock solution was kept frozen at −20 °C.

2.2.2. Preparation of des [65–72] and des [40–95] RNase A

Native RNase A (5 mg/mL) was dissolved in a buffer containing 100 mM DTT^{red} (pH 8, 100 mM Tris–HCl, 1 mM EDTA, 15 °C). 20 hrs after initiation of reduction, glacial acetic acid was added to reduce the pH to 3. The sample was desalted using a G-25 column and all protein species were separated using strong cation-exchange chromatography as previously described [9].

2.2.3. Oxidative folding of RNase A

Aliquots of fully-reduced RNase A (33 μM final conc.) were incubated into solutions (pH 8, 100 mM DTT^{ox}, 20 mM Tris–HCl, 1 mM EDTA, 25 °C) containing BMC (50 μM), BMC (50 μM)+TMAO (0.2 M) and BMC (50 μM)+TMAO (0.2 M)+TFE (3% v/v). A control experiment was run in parallel and did not contain BMC, TMAO or TFE. Aliquots from the regeneration mixture were withdrawn at several times after the initiation of oxidative folding and subjected to a reduction pulse [(application of 2 mM DTT^{red} for a period of 2 min) [9]] before addition of glacial acetic acid which reduced the pH to 3. Samples were desalted on a G-25 column prior to application on a C-18 column for reversed-phase chromatographic analysis.

The rate of regeneration of the native protein was determined by integrating the areas of the peaks corresponding to the native protein, structured intermediates and the fully-reduced protein for each time point. The fractional increase in N was plotted as a function of time; the data were fitted to a single-exponential function to obtain the rate constant for the formation of N from R [22].

2.2.4. Measurement of native tendency

The native tendency of fully-reduced RNase A was determined by introducing 200 μl of the dissolved (10 mM acetic acid) polypeptide into a solution (pH 8, 100 mM Tris–HCl,

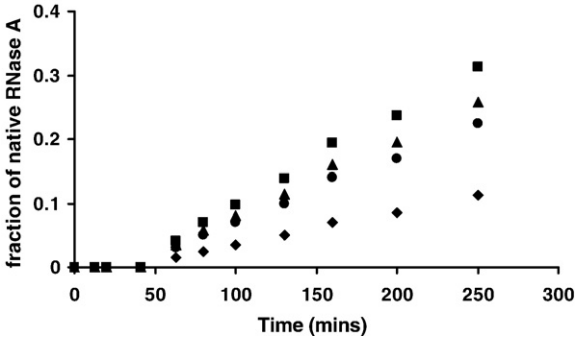

Fig. 2. Fraction of native RNase A formed as a function of added BMC (50 μM), ●; BMC (50 μM)+TFE (3% v/v), ▲; and BMC (50 μM)+TMAO (0.2 M), ■. The regeneration conditions were: 50 mM DTT^{ox}, pH 8, 20 mM Tris–HCl, 1 mM EDTA, 25 °C (♦).

1 mM EDTA) containing 1 mM *trans*-$[Pt(en)_2Cl_2]^{2+}$ [23,24]. Five minutes after addition of the Pt compound, each sample was subjected to a reduction pulse, as described above prior. All samples were then desalted and analyzed by reversed-phase HPLC (C18 column). In other experiments, the fully-reduced polypeptide was incubated with TMAO, TFE or both prior to addition of the solution containing *trans*-$[Pt(en)_2Cl_2]^{2+}$.

Native tendency of the fully-reduced protein was determined by measuring the peak areas corresponding to *N* and *R* in the HPLC chromatograms and applying the formula Native tendency $= N/(N+R)$ where *R* represents the quantity of fully-oxidized, scrambled (4S) species [24]. Native tendency is a measure of the degree of "native-like" structure present in the fully-reduced polypeptide chain. This is tested by rapid oxidation of the polypeptide cysteines to form the fully-oxidized polymer. Oxidation is more rapid than corrective thiol-disulfide isomerisation reactions (which eventually permit native disulfide bonds to form) and hence the method samples the degree of "nativeness" in the fully-reduced polypeptide. The value of "native tendency" can vary between 0 and 1. If the fully-reduced polypeptide were native-like then the native tendency would equal 1 because those pairs of cysteines than form the native disulfide bonds would be oriented in the "native-like" albeit, fully-reduced polypeptide. If the fully-reduced polypeptide was a statistical coil, then the native tendency value would approach 0.

2.2.5. Stability of RNase A intermediates

Des [40–95] and des [65–72] RNase A were separately incubated in solutions containing GdnHCl and different concentrations of TMAO and/or TFE (pH 8, 20 mM Tris–HCl, 25 °C) for a period of 5 min prior to the addition of 1 mM *trans*-$[Pt(en)_2Cl_2]^{2+}$. Two minutes after the addition of the Pt oxidant, the samples were subjected to a reduction pulse. Any free thiols were blocked by the addition of 2-aminoethanethiosulfonate (AEMTS) prior to being desalted and analyzed on a strong cation-exchange column (TOSOH TSK SP-5PW) using HPLC [9].

The peak areas corresponding to both native and fully-reduced species were determined from each chromatogram and converted to "survival of des species ($=N/[N+R]$)".

3. Experimental results

3.1. Regeneration of RNase B

Fig. 2 is a plot of formation of native RNase A in the presence of select small molecules [Oxidized (+/−)-*trans*-1,2-bis(2-mercaptoacetamido) cyclohexane (BMC); trimethylamine-*N*-oxide (TMAO) and trifluoroethanol (TFE)]. The sizes of the symbols indicate the standard deviations in repeat experiments. The oxidative folding rate of RNase A (50 mM DTT^{ox}, pH 8; ♦) is enhanced 2-fold in the presence of BMC (50 μM; ●), and further boosted by the presence of TFE (3%; ▲) and TMAO (0.2 M; ■).

The data were fitted to a first-order rate equation, $\ln(1-N) = -kt$, where $1-N$ is the fractional concentration of all non-native species, *k* represents the rate constant for the formation of the native protein, and *t* is the reaction time [22]. Table 1 summarizes the rate-constants for the formation of the native protein as a function of the small-molecule fold adjuvant. A 2-fold increase in the rate of formation of the native protein is observed in the presence of 50 μM BMC. Although TFE improves the regeneration rate, it is not as effective (at the maximum tested concentration of 3% v/v) as TMAO (at 0.1 M or 0.2 M). The rate is further accelerated in the combined presence of BMC, TMAO and TFE (50 μM BMC, 0.2 M TMAO and 3% v/v TFE) to obtain a maximum enhancement factor of 3.

Table 1
Rate constants for the regeneration of RNase A as a function of added small-molecule (pH 8, 50 mM DTT^{ox}, 20 mM Tris–HCl, 1 mM EDTA, 25 °C)

Folding condition	Rate constant for $*R \rightarrow N$ ($\times 10^4$ min^{-1})
R	6.2±0.3
R+50 μM BMC	12±0.2
R+50 μM BMC+0.1 M TMAO	14±0.3
R+50 μM BMC+1% TFE	12.6±0.8
R+50 μM BMC+0.2 M TMAO	16.01±0.4
R+50 μM BMC+3% TFE	13.5±0.3
R+50 μM BMC 0.2 M TMAO+1% TFE	16.5±0.5
R+50 μM BMC 0.2 M TMAO+3% TFE	18.5±0.8

$*R$ denotes fully-reduced RNase A and N is the native species.

Table 2
Native tendency of fully-reduced RNase A as a function of added fold adjuvant

Folding condition	Native tendency of $*R$ (%)
R	0.55±0.3
R+0.1 M TMAO	1.7±0.4
R+0.2 M TMAO	2.8±0.2
R+1% TFE	1±0.1
R+3% TFE	1.5±0.3
R+0.2 M TMAO+1% TFE	4.5±0.15
R+0.2 M TMAO+3% TFE	6±0.2

All experiments were performed at pH 8 (20 mM Tris–HCl, 1 mM EDTA) and 25 °C using 1 mM *trans*-$[Pt(en)_2Cl_2]^{2+}$ as the oxidizing agent. $*R$ denotes fully-reduced RNase A.

Table 3
Survival of des [40–95] and des [65–72] in 1 M and 1.5 M GdnHCl, respectively

Folding condition	%N	Folding condition	%N
Des [40–95]+1 M GdnHCl=A	20±2	Des [65–72]+1.5 M GdnHCl=B	50±1
A+0.1 M TMAO	35±2.5	*B*+0.1 M TMAO	59±1.6
A+0.2 M TMAO	45±1	*B*+0.2 M TMAO	66±2.2
A+1% TFE	22±1.5	*B*+1% TFE	52±3
A+3% TFE	26±3	*B*+3% TFE	56±1.1
A+0.2 M TMAO+1% TFE	38±2.2	*B*+0.2 M TMAO +1% TFE	61±1.7
A+0.2 M TMAO+3% TFE	54±2.5	*B*+0.2 M TMAO +3% TFE	73±0.9

%N indicates the fraction of des species that survived and hence could be oxidized to the native protein. All experiments were performed at 25 °C (pH 8, 20 mM Tris–HCl, 1 mM EDTA).

3.2. Native tendency of fully-reduced RNase A

Table 2 summarizes the native tendency value of fully-reduced RNase A in the presence of TMAO and TFE. The native tendency of the polypeptide chain increases from 0.55% in the absence of small-molecule adjuvants to 6% in the combined presence of TMAO (0.2 M) and TFE (3%).

Stability of des [40–95] and des [65–72]. Table 3 summarizes the "survival" of the des species as a function of denaturant (GdnHCl) and added combinations of TMAO and TFE. Both TMAO and TFE are able to exert stabilizing effects on both des species which is evident upon comparing the "% survival" of each des species (%N in Table 3) as a function of TFE and TMAO concentration.

4. Discussion

Oxidative folding is the process by which multi-disulfide-bond-containing proteins acquire their native disulfide bonds and undergo a conformational folding reaction in order to form the biological active, native fold.

In early stages of the oxidative folding process (i.e., in R–*n*S unstructured intermediates), thiol-disulfide exchange reactions are governed by loop-entropy and enthalpic interactions in the absence of structure [11]. However, in most multi-disulfide-containing proteins, these effects are not sufficiently strong to favor native disulfide bonds over non-native ones. For proteins containing two-or-more disulfide bonds, the probability of forming non-native disulfide bonds is greater than native disulfide bonds and the folding landscape is therefore replete with non-native isomers. The quasi-stochastic formation of a minimal native set of disulfide bonds, which is necessary to initiate conformational folding, ensures a slower time-frame for completion of oxidative folding compared to conformational folding reactions of proteins that lack disulfide bonds.

In vivo, oxidative protein folding takes place within the oxidizing environment of the E. R. lumen [4]. In the E. R. environs, nature has invested in an oxidoreductase chaperone, protein disulfide isomerase (PDI), among other oxidoreductases, which facilitates rapid exchange between thiols and disulfides and catalyzes oxidative folding of "substrate" proteins [15,25,26]. Thus, within a give temporal window, a native set of disulfide bonds may be sampled more frequently in the presence of the oxidoreductase than in its absence. A higher sampling frequency of native bonds simply translates into a greater opportunity to conformationally fold and protect the native bonds from exchange.

In vitro, in the absence of PDI, oxidative protein folding rates strongly depend upon the nature of the extrinsic redox reagent [(such as air, dithiothreitol or glutathione) [5]. Dithithothreitol is a weak oxidizing agent, resulting in slower oxidation reactions; nevertheless, its advantageous use lies in its inability to form mixed-disulfides with "substrate" protein which makes studies of oxidative protein folding processes easier. Glutathione is a more potent oxidant but forms mixed disulfides with protein thiols resulting in a very large number of folding intermediates; this complicates the analytical separation procedures necessary to study oxidative protein folding. A number of other synthetic redox reagents have been used to accelerate oxidative folding and these agents exhibit varying efficiencies [20,23,27].

Independent of the presence of PDI and/or extrinsic redox reagents is the process by which native disulfides and the native fold is acquired. The fully-reduced polypeptide acquires its full complement of disulfide bonds (native and non-native) sequentially (R→1S→2S→3S→4S) (Fig. 1). Late-stage reduction and/or isomerization reactions permit either the full complement or a minimal subset of native bonds to form from their unstructured isomers (Fig. 3; $3S_U \rightarrow 3S_N$; where $3S_U$ and $3S_N$ represent unstructured three-disulfide bond containing species containing one non-native disulfide bond and three native disulfide bonds, respectively). Such a species has a chance to fold conformationally and generate native or native-like structure ($3S_N \rightarrow 3S^*$) [28]. It is this stable structural envelope that protects the formed native disulfide bonds from further thiol-disulfide exchange reactions or reduction reactions and thereby effectively removes the native protein or the native like species from the quasi-equilibrium distribution of unstructured intermediates [6]. Conformational folding resulting in the formation of a stable structure is thus pivotal to effective regeneration.

PDI, dithiothreitol and glutathione facilitate oxidation, reduction and thiol-disulfide exchange and therefore, accelerate

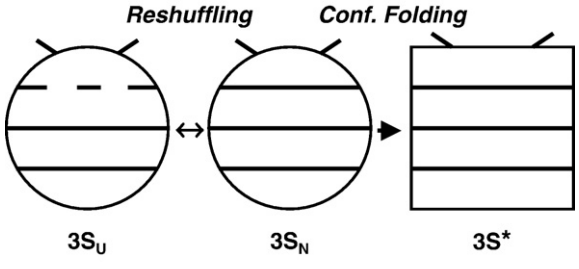

Fig. 3. Structure-coupled oxidative folding step in RNase A. An unstructured, non-native-disulfide-bond-containing three disulfide intermediate ($3S_U$) first reshuffles to its native-disulfide-bond-containing isomer ($3S_N$), followed by conformational folding of the latter to form a structured intermediate (3S*). Structure protects the native disulfide bonds in the 3S* species from reshuffling to non-native disulfide bonds.

oxidative folding to a certain extent (depending upon thiol pKa's and disulfide $E^{o'}$ values). The synthetic dithiol, BMC has been previously used in its reduced form to catalyze the reduction of scrambled RNase A (4S) and promote the formation of the native molecule through isomerization reactions [2,20]. In this study, we have employed it's oxidized form to significantly accelerate oxidative folding. While the microscopic mechanism of action was not investigated here, and is possible by an examination of the rate-constants for formation of the *n*S ensemble from its $(n-1)$S precursor, it is likely that the small-molecule oxidant is able to accelerate the acquisition of the native state by accelerating the formation of every unstructured ensemble in the folding pathway. Evidence for this exists in the rate of disappearance of R in the presence of DTT^{ox} and DTT^{ox}/BMC. In the latter instance, a 2.4-fold increase in the rate of disappearance of R was observed.

Interestingly, the addition of non-redox-site-containing molecules led to a further acceleration in the oxidative folding rate over that achieved by BMC^{ox}. The addition of TMAO and TFE (in the presence of 50 μM BMC) resulted in a maximal increase of 54% in the oxidative folding rate over that obtained using BMC and DTT^{ox}.

To determine whether TMAO and TFE impacted early-stages of oxidative folding, we examined the native tendency of RNase A in the presence of these two small molecules. The native tendency is a measure of the ability of the fully-reduced polypeptide to form the native species upon oxidation of its disulfide bonds (in the absence of thiol-disulfide exchange reactions) [24]. Our results indicate that both TMAO and TFE were able to increase the native tendency of RNase A. When combined, their effects were greater than when applied independently. TMAO has previously been shown in conformational folding studies to induce/stabilize the native fold in fully-reduced RNase T1, a two disulfide-bond-containing protein. The addition of 2.5 M TMAO resulted in acquisition of (100%) native structure in the absence of disulfide bonds [29]. Its mechanism of action is through the solvophobic effect where presence of the polar osmolyte leads to destabilization of the unfolded state [30]. TFE is known to induce helical content in polypeptides [30]. A study on induced-helicity in β-lactoglobulin revealed that addition of 80% v/v TFE to resulted in formation of 78% helical structure [31]. Twenty other proteins in the same study responded differently with induced helicities ranging between 10–26% at 40% v/v added TFE. Our studies were conducted at much lower concentrations of both reagents and were intended to determine the impact (and mechanism), if any, of these non-redox active molecules on *oxidative* protein folding. Our results indicate that these molecules are able to induce conformational-order in reduced ribonuclease at the concentrations tested in an oxidative folding model and thereby impact oxidative folding rates. This ordering favors the probability of native disulfide bonds over non-native ones as evidenced by the native tendency assay.

Des [40–95] and des [65–72] are two three-disulfide-bond-containing structured intermediates that are formed from their 3S unstructured isomers through isomerization reactions; a process that constitutes the rate-determining step in RNase A. Their formation is a pivotal event in the oxidative folding process because these species are able to protect their native disulfide bonds from reshuffling reactions. The protection is possible because of an envelope of stable structure that makes these bonds inaccessible to thiol-disulfide exchange reactions.

When placed under slightly denaturing conditions, these intermediates, depending on their stability, are able to reshuffle in part to their unstructured isomers (the 3S ensemble). The addition of an oxidizing agent results in the oxidation of the formed 3S ensemble to the 4S ensemble and of the remaining des species to the native protein (N). A reduction-pulse allows discrimination between the structured native molecule and its unstructured isomers (4S). The data in Table 3 indicate that at the applied concentration of denaturant, only 20% of des [40–95] survives whereas 50% of the more stable des [65–72] remains structured. The addition of TMAO results in the partial "rescue" of both intermediates from denaturation. The degree of rescue is proportional to the concentration of added TMAO. TFE is also able to impart stability to both des species, albeit to a lesser extent at the concentrations tested. The combined effects of TFE and TMAO provide further stability to both the des species resulting in their ability to withstand the denaturing effects of GdnHCl.

Oxidative protein folding is relatively slow compared to conformational folding. However, upon acquisition of structure by an intermediate, successive oxidation of its cysteines to form the native molecule are relatively rapid simply because the half-cystines are spatially aligned, resulting in their easy oxidation to form disulfide bonds. However, it is only late-stage isomerization reactions that are responsible for the structure-coupled oxidative folding step and are in part, a cause for the protracted nature of the regeneration process. This is because a minimal set of native disulfide bonds must be present before folding can take place (in RNase A, three of the four bonds are necessary to induce conformational folding).

If the requirement for the formation of a minimal set of disulfide bonds prior to conformational folding can be circumvented, then oxidative protein folding can be expedited. That is, if partial native structure can be formed, then oxidative protein folding can be radically accelerated because native conformational order in a polypeptide would result in a native alignment of half-cystines which can readily form disulfide bonds. Note that generation of complete structure can be detrimental to the formation of the native molecule because cysteine residue could become buried within tertiary structure and therefore, inaccessible to oxidation. Therefore, partial order which promotes the formation of native disulfide bonds and restricts the formation of non-native ones is desired.

Furthermore, if native-like intermediates can be stabilized, then the probability of their back-reshuffling to form unstructured intermediates decreases and oxidative folding rates can be accelerated. Both TMAO and TFE accelerate oxidative folding through at least two mechanisms: induction of native-like order in the fully-reduced polypeptide and stabilization of two native-like intermediates.

The inability of certain proteins to fold efficiently or the formation of off-pathway intermediates leads to a number of

misfolding-related diseases [32]. Acquisition of the native structure in a timely manner is the bedrock of every cell and living being. It is clear that the resident chaperones and the quality-control mechanism while effective, represent, but a snapshot in the evolutionary process, and are still not perfectly adapted to ensure a "misfolding free" cellular (and extracellular) milieu; hence the prevalence of disease. Ironically, in certain cases the quality control mechanism is so unforgiving that, relatively unstable, but otherwise functional mutants are fed into the proteasome machinery leading to diseased states [32]. The data presented here demonstrate that it is possible to accelerate oxidative protein folding through a host of small-molecules that are effective through multiple mechanisms which do not involve redox chemistry. Although TFE and TMAO may not be the physiologically relevant molecules of choice, this work demonstrates two different and novel mechanisms by which non-redox-active molecules can accelerate the oxidative folding of ER-processed proteins. Thus retention of these newly discovered non-redox-active mechanisms becomes an important criterion for the design of more bio-active molecules that are endowed with similar function and are physiologically more effective. The long-term goal of such research involves fine-tuning of small-molecule fold adjuvants to achieve target-protein specificity, leading to the development of small-molecule chemotherapeutics for the alleviation of misfolding-related diseases.

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References

[1] M. Narayan, E. Welker, W.J. Wedemeyer, H.A. Scheraga, Oxidative folding of proteins, Acc. Chem. Res. 33 (2000) 737–820.
[2] K.J. Woycechowsky, R.T. Raines, Native disulfide bond formation in proteins, Curr. Opin. Chem. Biol. 4 (2000) 533–539.
[3] J.L. Arolas, F.X. Aviles, J.Y. Chang, S. Ventura, Folding of small disulfide-rich proteins: clarifying the puzzle, Trends Biochem. Sci. 31 (2006) 292–301.
[4] B.P. Tu, J.S. Weissman, Oxidative protein folding in eukaryotes: mechanisms and consequences, J. Cell Biol. 164 (2004) 341–346.
[5] W.J. Wedemeyer, E. Welker, M. Narayan, H.A. Scheraga, Disulfide bonds and protein folding, Biochemistry 39 (2000) 4207–4216.
[6] E. Welker, M. Narayan, W.J. Wedemeyer, H.A. Scheraga, Structural determinants of oxidative folding in proteins, PNAS USA 98 (2001) 2312–2316.
[7] E. Welker, W.J. Wedemeyer, M. Narayan, H.A. Scheraga, Coupling of conformational folding and disulfide-bond reactions in oxidative folding of proteins, Biochemistry 40 (2001) 9059–9064.
[8] D.M. Rothwarf, H.A. Scheraga, Regeneration of bovine pancreatic ribonuclease A. 1. Steady-state distribution, Biochemistry 32 (1993) 2671–2679.
[9] D.M. Rothwarf, Y.J. Li, H.A. Scheraga, Regeneration of bovine pancreatic ribonuclease A: identification of two nativelike three-disulfide intermediates involved in separate pathways, Biochemistry 37 (1998) 3760–3766.
[10] M. Iwaoka, W.J. Wedemeyer, H.A. Scheraga, Conformational unfolding studies of three-disulfide mutants of bovine pancreatic ribonuclease A and the coupling of proline isomerization to disulfide redox reactions, Biochemistry 38 (1999) 2805–2815.
[11] X. Xu, D.M. Rothwarf, H.A. Scheraga, Nonrandom distribution of the one-disulfide intermediates in the regeneration of ribonuclease A, Biochemistry 35 (1996) 6406–6417.
[12] M.J. Volles, X. Xu, H.A. Scheraga, Distribution of disulfide bonds in the two-disulfide intermediates in the regeneration of bovine pancreatic ribonuclease A: further insights into the folding process, Biochemistry 38 (1999) 7284–7293.
[13] H.C. Hawkins, R.B. Freedman, The reactivities and ionization properties of the active-site dithiol groups of mammalian protein disulphide-isomerase, Biochem. J. 275 (1991) 335–339.
[14] J.S. Weissman, P.S. Kim, Efficient catalysis of disulphide bond rearrangements by protein disulphide isomerase, Nature 365 (1993) 185–188.
[15] B. Wilkinson, H.F. Gilbert, Protein disulfide isomerase, Biochim. Biophys. Acta 1699 (2004) 35–44.
[16] G. Tian, S. Xiang, R. Noiva, W.J. Lennarz, H. Schindelin, The crystal structure of yeast protein disulfide isomerase suggests cooperativity between its active sites, Cell 124 (2006) 61–73 (Erratum in: Cell 124 (2006) 1085–1088).
[17] H.C. Shin, H.A. Scheraga, Catalysis of the oxidative folding of bovine pancreatic ribonuclease A by protein disulfide isomerase, J. Mol. Biol. 300 (2004) 995–1003.
[18] M.P. Mattson, Nitro-PDI incites toxic waste accumulation, Nat. Neurosci. 9 (2006) 865–867.
[19] T. Uehara, T. Nakamura, D. Yao, Z.Q. Shi, Z. Gu, Y. Ma, E. Masliah, Y.S. Nomura, S.A. Lipton, S-nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration, Nature 441 (2006) 513–517.
[20] E.A. Kersteen, R.T. Raines, R.T. Catalysis of protein folding by protein disulfide isomerase and small-molecule mimics, Antioxid. Redox Signal. 5 (2003) 413–424.
[21] P.T. Chivers, M.C. Laboissiere, R.T. Raines, The CXXC motif: imperatives for the formation of native disulfide bonds in the cell, EMBO J. 15 (1996) 2659–2667.
[22] G. Xu, M. Narayan, H.A. Scheraga, The oxidative folding rate of bovine pancreatic ribonuclease is enhanced by a covalently attached oligosaccharide, Biochemistry 44 (2005) 9817–9823.
[23] T. Shi, D.L. Rabenstein, *trans*-Dichlorotetracyanoplatinate (IV) as a reagent for the rapid and quantitative formation of intramolecular disulfide bonds in peptides, J. Org. Chem. 64 (1999) 4590–4595.
[24] M. Narayan, E. Welker, H.A. Scheraga, Native conformational tendencies in unfolded polypeptides: development of a novel method to assess native conformational tendencies in the reduced forms of multiple disulfide-bonded proteins, J. Am. Chem. Soc. 123 (2003) 2909–2910.
[25] H.F. Gilbert, Protein disulfide isomerase, Methods Enzymol. 290 (1998) 26–50.
[26] S.W. Fewell, K.J. Travers, J.S. Weissman, J.L. Brodsky, The action of molecular chaperones in the early secretory pathway, Annu. Rev. Genet. 35 (2001) 149–191.
[27] J.D. Gough, E.J. Barrett, Y. Silva, W.J. Lees, *ortho*- and *meta*-substituted aromatic thiols are efficient redox buffers that increase the folding rate of a disulfide-containing protein, J. Biotechnol. 125 (2006) 39–47.
[28] K. Saito, E. Welker, H.A. Scheraga, Folding of a disulfide-bonded protein species with free thiol(s): competition between conformational folding and disulfide reshuffling in an intermediate of bovine pancreatic ribonuclease A, Biochemistry 40 (2001) 15002–15008.
[29] I. Baskakov, D.W. Bolen, Forcing thermodynamically unfolded proteins to fold, J. Biol. Chem. 273 (1998) 4831–4834.
[30] D.W. Bolen, Effects of naturally occurring osmolytes on protein stability and solubility: issues important in protein crystallization, Methods 34 (2004) 312–322;
M. Buck, Trifluoroethanol and colleagues: cosolvents come of age. Recent studies with peptides and proteins, Q. Rev. Biophys. 31 (1998) 297–355.
[31] K. Shiraki, K. Nishikawa, Y. Goto, Trifluoroethanol-induced stabilization of the alpha-helical structure of beta-lactoglobulin: implication for non-hierarchical protein folding, J. Mol. Biol. 245 (2) (Jan 13 1995) 180–194.
[32] K. Romisch, A cure for traffic jams: small molecule chaperones in the endoplasmic reticulum, Traffic 5 (2004) 815–820.