Oxidative refolding of lysozyme in trifluoroethanol (TFE) and ethylene glycol: interfering role of preexisting α-helical structure and intermolecular hydrophobic interactions

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Abstract The oxidative refolding of equilibrium intermediates of lysozyme stabilized in trifluoroethanol (TFE) and ethylene glycol was monitored. Equilibrium intermediates of disulfide reduced lysozyme in TFE are known to contain considerable amounts of α-helical structure and resemble the early intermediate in the oxidative refolding of lysozyme. We find that the intermediates in TFE do not proceed to folding; they form aggregates. However, interestingly, intermediates in ethylene glycol refold to the native state with improved folding yield. Secondary structure of these intermediates was monitored by far-UV circular dichroism. Our results indicate that formation of α-helical structure prior to oxidative refolding does not help the process in the case of lysozyme. Interfering with intermolecular hydrophobic interactions in the unfolded state is more productive.

Key words: Lysozyme; Oxidative refolding; Secondary structure; Hydrophobic interaction

1. Introduction

For nearly half a century of our knowledge that the primary structure of a protein determines its native structure, very little advance has been made in the understanding of the actual pathways followed by proteins from their unfolded to native states and the underlying principles that determine the above course [1,2]. Kinetic studies with folding proteins provide some information about folding pathways; equilibrium intermediates resembling the kinetic intermediates are studied to complement the kinetic information. Equilibrium intermediates of many small proteins have been stabilized and studied to understand the folding pathways. Cosolvents such as alcohols are frequently employed to stabilize the equilibrium intermediates [3]. One of the alcohols, trifluoroethanol (TFE), stabilizes α-helical structures in proteins [4–10].

Lysozyme, being a small, single domain protein, has been a favorite system for protein folding studies. Refolding lysozyme studied using H-D exchange nuclear magnetic resonance revealed that the early intermediate of lysozyme is α-helical in structure [11]. Subsequently an equilibrium intermediate of lysozyme was stabilized in 40% TFE that resembles the kinetic intermediate in H-D exchange pattern [12]. However, intermediates formed by denatured lysozyme with intact disulfides do not reveal any information about the early folding intermediates, as the disulfides represent tertiary structure per se and also reduce the number of possible conformations. The disulfide reduced lysozyme serves as a better model to understand the intermediate states populated prior to formation of disulfide bridges for a complete picture of the folding pathway of lysozyme. In the recent past there have been a number of studies on disulfide reduced lysozyme [13–16] and its derivatives carboxymethyl-lysozyme [17] and TMAP-lysozyme [6] to model the intermediates in TFE and other alcohols. With TMAP-lysozyme the content and localization of α-helical structure observed in TFE are the same as in the case of disulfide intact lysozyme. Earlier studies showed that the intermediate of reduced lysozyme in 40% TFE resembled the intermediate of disulfide intact lysozyme in 40% TFE based on circular dichroism (CD) and fluorescence spectroscopic criteria [15]. In the present work, the question whether preformed secondary structure has any positive effect on the oxidative refolding of lysozyme has been addressed.

Protein aggregation, a major problem encountered during refolding of denatured proteins, results from intermolecular interactions by virtue of exposed hydrophobic surfaces. Ethylene glycol is known to interfere with hydrophobic interactions [18,19]. Here we studied the oxidative refolding of lysozyme in ethylene glycol to understand the parallel and non-productive contribution of intermolecular hydrophobic interactions to refolding.

2. Materials and methods

Hen egg white lysozyme, dithiothreitol (DTT), cystine dihydrochloride, guanidine hydrochloride were obtained from Sigma. Lysozyme was found to be pure by sodium dodecyl sulfate gel electrophoresis. 2,2,2-Trifluoroethanol and ethylene glycol were of analytical grade.

2.1. Preparation of native lysozyme and reduced and denatured lysozyme

Lysozyme (at 16 mg/ml) was dissolved in 80 mM Tris–acetate, pH 8.2. Separately, lysozyme was dissolved in a solution containing 6 M guanidine HCl, 80 mM Tris acetate, pH 8.2 and 100 mM DTT and incubated overnight at 25°C, to obtain reduced and denatured lysozyme.

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Table 1
Refolding of denatured lysozyme in the presence of TFE, TFE and 0.1 M NaCl and ethylene glycol

% Organic solvent in the sample	% Recovery of activity[a]		
	In TFE	In TFE+0.1 M NaCl	In ethylene glycol
0	25	14.8	25.0
10	1.1	1.1	28.4
20	1.1	1.1	29.5
30	1.1	1.1	33.0
40	1.1	1.1	36.4

[a]The percent recovery of activity is with respect to the activity of native enzyme of the same concentration.

2.2. Refolding of reduced and denatured lysozyme and its intermediates in TFE and ethylene glycol

The reduced and denatured lysozyme (10 μl) was introduced into refolding buffer with final concentrations of 100 mM Tris acetate, pH 8.2, 2 mM cystine dihydrochloride. The concentration of TFE or ethylene glycol was varied between 0 and 40% (v/v) in the refolding buffer. After introducing the reduced and denatured lysozyme in the refolding buffer, the enzyme was incubated for 15 min. Activity recovery in the presence and absence of the organic solvents was estimated by the enzyme assay [20]. 15 μl of the above solution containing the refolding enzyme was added to a suspension containing 0.22 mg/ml of *Micrococcus lysodeikticus* cells in 0.1 M phosphate buffer, pH 6.3. The change in optical density was followed at 450 nm, using a Hitachi U-2000 absorbance spectrophotometer, at 10 s intervals for 1 min. Blanks were prepared similarly without adding reduced and denatured lysozyme. Native lysozyme control was also prepared in 100 mM Tris acetate, pH 8.2. Percent recovery of activity was calculated in comparison with that of native lysozyme as described by Raman et al. [21].

2.3. CD studies of reduced and native lysozyme

CD spectra were recorded using a Jasco J-715 spectropolarimeter. cells of 1 mm path length were used. Spectra were recorded at 0.5 nm resolution. Five spectra were accumulated to reduce the noise. The samples were prepared by introducing a 10 μl sample of reduced and denatured lysozyme into a solution containing 50 mM glycine HCl, pH 2.5. TFE or ethylene glycol was added to a concentration of 0–40% (v/v). Proper blanks were prepared for all the samples and the spectra of the samples were blank corrected.

3. Results

3.1. Activity recovery after refolding the intermediates of reduced lysozyme in different organic solvents

When reduced lysozyme was incubated in a refolding buffer for 15 min, and the activity was measured by *M. lysodeikticus* lysis, there was 25% activity regain, compared to native lysozyme of the same concentration. But presence of TFE reduced the recovery of activity upon refolding to 1%. Addition of 0.1 M NaCl was found to be ineffective (Table 1). Reducing the protein concentration or temperature of refolding to 4°C gave only a marginal increase in the recovery of activity (results not shown). In contrast, the presence of ethylene glycol increased the recovery of activity. Activity regained in the presence of 40% ethylene glycol was 36.4%.

3.2. CD spectra of reduced lysozyme in TFE

The samples for CD spectroscopy were prepared in glycine HCl buffer, pH 2.5, since reduced lysozyme aggregates at pH values above 4.5. Moreover at pH values above 7.8 free sulfhydryl groups can undergo air oxidation. The CD spectra of reduced lysozyme in 40% TFE showed increase in negative ellipticity at 222 nm (Fig. 1). The correlation between predicted helix content and negative ellipticity measured at 222 nm was found to be significant [22]. This signal, corresponding to α-helicity, is more than 50% that of the native lysozyme, as observed earlier [15]. More importantly, addition of 0.1 M NaCl to reduced lysozyme in 40% TFE increased α-helical content further (Fig. 1). Aggregation was observed when reduced lysozyme was introduced directly into glycine HCl buffer, pH 2.5, with TFE in the concentration range of 10–30% (v/v).

3.3. CD studies with disulfide reduced lysozyme in ethylene glycol

As shown in Fig. 2, reduced lysozyme shows increase in negative ellipticity, around 214–218 nm, with increasing concentrations of ethylene glycol (from 0 to 40%). The minimum value in the CD spectrum is observed around 216.5 nm. Di-

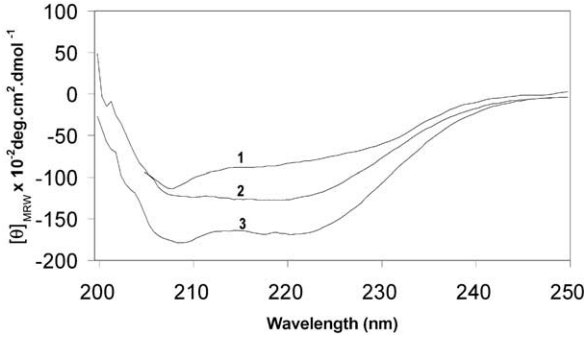

Fig. 1. Far-UV CD spectra of disulfide reduced lysozyme (2) in 40% TFE and disulfide reduced lysozyme in 40% TFE+0.1 M NaCl (3). The far-UV CD spectrum of native lysozyme (1) is included for comparison. With addition of salt the negative ellipticity increases around 220 nm, indicating an increase in α-helical structure. The spectra were recorded in glycine HCl buffer, pH 2.5. Protein concentration was 20.94 μM.

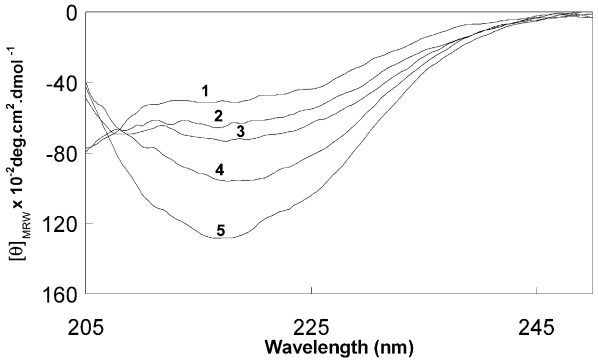

Fig. 2. CD spectrum of disulfide reduced lysozyme in increasing concentration of ethylene glycol. Spectra 1–5 correspond to 0–40% ethylene glycol at 10% intervals. With increase in ethylene glycol concentration the negative ellipticity increases around 218 nm. The spectra were recorded in glycine HCl buffer, pH 2.5. Protein concentration was 10 μM.

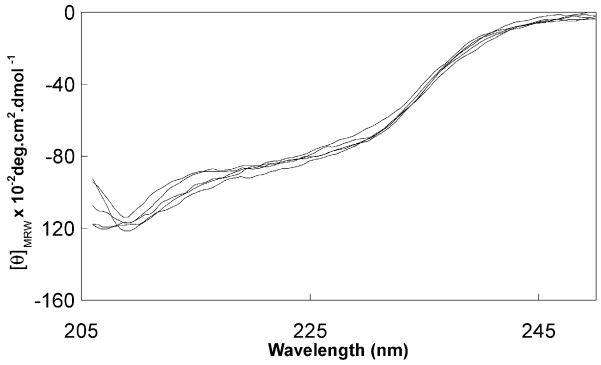

Fig. 3. Far-UV CD spectrum of disulfide intact lysozyme in increasing concentrations of ethylene glycol. Spectra correspond to 0–40% ethylene glycol at 10% intervals. With increase in ethylene glycol concentration there is a slight increase in negative ellipticity around 222 nm, indicating a marginal increase in α-helical structure. The spectra were recorded in glycine HCl buffer, pH 2.5. Protein concentration was 10 μM.

sulfide reduced lysozyme displays extended structure of a denatured protein. This increase in negative ellipticity indicates formation of β-sheet-like structure. Interestingly, in 40% ethylene glycol there is a flattened portion around 222 nm, which decreases further to form a trough later on around 216 nm. This feature suggests formation of some amount of α-helical structure along with β-sheet.

3.4. CD studies with disulfide intact lysozyme in ethylene glycol

CD spectra of disulfide intact lysozyme show a slight increase in α-helicity, although the increase is not as dramatic as with TFE (Fig. 3). Ethylene glycol like all other alcohols can change the polarity of the medium and also compete for hydrogen bonding with water, the effects in this case are not drastic enough to alter the structure of the protein at the concentrations of ethylene glycol used in our experiment.

4. Discussion

TFE is known to induce and stabilize α-helical structures in unfolded proteins. But this stabilization depends on the structural propensity of the amino acid sequences; it is not indiscriminate [5–10]. The induction of α-helical structures is dependent on the inherent structural preferences of the amino acid sequences. The TFE intermediate of lysozyme and its early folding kinetic intermediate are identical. But these studies pertain to the unfolded state where the disulfides are intact. Equilibrium intermediates of unfolded reduced lysozyme and its sulfhydryl derivatives are also known. The secondary structure, absence of tertiary structure and fluorescence properties of these equilibrium intermediates have been reported in detail [15]. The studies with peptide fragments of reduced lysozyme in 50% TFE showed that the amount of α-helical content is more than twice that of the native state [10]. This observation is in agreement with the amount of helicity observed with both disulfide reduced and intact forms of lysozyme in similar concentrations of TFE. Another interesting observation comes from studies with a derivative of disulfide reduced lysozyme, namely the TMAP-lysozyme. The equilibrium intermediate of TMAP-lysozyme was stabilized in TFE and was found to be similar to that of disulfide intact lysozyme [6]. Subsequently fragments of TMAP-lysozyme were generated and introduced into TFE. The total α-helical content of these fragments was the same as that of intact TMAP-lysozyme. Furthermore, the individual fragments that showed α-helical structure were found to be derived from α-helical regions of the protein. In the present work, we observed that although the disulfide reduced lysozyme goes into a helical state, resembling the early folding intermediate, this α-helicity does not help in its folding to native structure, under oxidizing conditions (Table 1). This might imply that the secondary structure does not help in holding the sulfhydryls in native-like proximity. On the other hand, arrangement of residues in amphipathic helices wherein the hydrophobic and hydrophilic surfaces are well defined, favor aggregation. In other words, the TFE induced secondary structure does not give any selective advantage for the formation of native disulfides over non-native disulfides. Decreasing the TFE concentration below 10% and carrying out refolding at lower temperatures did not increase the refolding yields (results not shown). From our data it appears that formation of native disulfides is an essential step and stabilization of secondary structures appears to be less important, as the enzyme can fold from the denatured state to the native state, provided the disulfides are intact. Based on refolding yields it can be concluded that the intermediates stabilized by TFE are not on the pathway of oxidative folding to the native state.

The refolding yields of lysozyme in neat organic solvents were found to be higher in the presence of salts. This was interpreted to be due to increased solubility of reduced unfolded lysozyme in the solvent system [23]. Moreover, in a non-polar environment, electrostatic interactions are known to play an important role. In view of the above, we have investigated the role of electrostatic interactions in the intermediate states of reduced lysozyme induced in TFE. Addition of 0.1 M NaCl to the intermediates in 0–30% TFE showed increased aggregation. In 40% TFE 0.1 M NaCl induced more α-helicity, compared to the TFE intermediate at the same concentration. Refolding these intermediates resulted in aggregation (Fig. 1).

TFE weakens hydrophobic–hydrophobic interactions [24]. Ethylene glycol is another solvent, which has a similar effect on the hydrophobic interactions. The effect of ethylene glycol on reduced lysozyme was studied previously at much higher concentrations of ethylene glycol, and a greater increase in α-helicity was reported [25]. But these intermediates did not form active enzyme upon refolding. Fourier transform infrared studies with disulfide intact lysozyme showed an increase in α-helical content with increasing ethylene glycol between 0 and 30% at pH 7.4 [26]. In the present study even at pH 2.5, the α-helicity of disulfide intact lysozyme increased with ethylene glycol concentration. However, reduced lysozyme showed a more extended and β-sheet-like structure (Fig. 2).

Interestingly, upon refolding the activity recovery of ethylene glycol intermediates is higher than that of reduced lysozyme refolded in an aqueous environment (Table 1), although these intermediates do not display α-helical structure (Fig. 2) and the extent of induction of secondary structure with native enzyme is less (Fig. 3). The ability of ethylene glycol to interfere with hydrophobic interactions [18,19] plays a major role in preventing aggregation. In addition, favorable contributions of viscosity effects of ethylene glycol to folding are also possible. The hydrophobic residues in these intermediates are exposed, since it was observed that these intermediates

bind ANS (C.R. Prabha and D. Khushwaha, unpublished observations). This intermediate can forge correct disulfide bridges more efficiently under oxidizing conditions. Alternatively, ethylene glycol by preventing aggregation allows the enzyme to fold on its own to go into the native state. Earlier, similar effects were reported with glycerol, but the study did not comment on the secondary structure of the intermediates [14]. In contrast the TFE intermediates are described to be extended and are of open nature, where inter-polypeptide disulfide bonding is favored over intra-polypeptide disulfide bonding [4]. Our results show that formation of α-helical structure in disulfide reduced lysozyme does not favor its oxidative refolding.

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