Role of partial protein unfolding in alcohol-induced protein aggregation

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ABSTRACT

Proteins aggregate in response to various stresses including changes in solvent conditions. Addition of alcohols has been recently shown to induce aggregation of disease-related as well as nondisease-related proteins. Here we probed the biophysical mechanisms underlying alcohol-induced protein aggregation, in particular the role of partial protein unfolding in aggregation. We have studied aggregation mechanisms due to benzyl alcohol which is used in numerous biochemical and biotechnological applications. We chose cytochrome c as a model protein, for the reason that various optical and structural probes are available to monitor its global and partial unfolding reactions. Benzyl alcohol induced the aggregation of cytochrome c in isothermal conditions and decreased the temperature at which the protein aggregates. However, benzyl alcohol did not perturb the overall native conformation of cytochrome c. Instead, it caused partial unfolding of a local protein region around the methionine residue at position 80. Site-specific optical probes, two-dimensional NMR titrations, and hydrogen exchange all support this conclusion. The protein aggregation temperature varied linearly with the melting temperature of the Met80 region. Stabilizing the Met80 region by heme iron reduction drastically decreased protein aggregation, which confirmed that the local unfolding of this region causes protein aggregation. These results indicate that a possible mechanism by which alcohols induce protein aggregation is through partial rather than complete unfolding of native proteins.


Key words: stability; foldon; structure; cytochrome c; benzyl alcohol.

INTRODUCTION

Protein aggregates are involved in the pathogenesis of various diseases including neurodegenerative diseases1,2 and sickle cell anemia.3,4 Protein aggregates in therapeutic formulations trigger adverse effects in patients such as immunogenicity to the protein.5–7 Proteins aggregate in response to several stresses including changes in solvent conditions. Recently, addition of alcohols has been shown to aggregate proteins. For example, trifluoroethanol (TFE) induces aggregation of superoxide dismutase,8 ethanol induces insulin aggregation,9 whereas benzyl alcohol (BA) induces the aggregation of human interleukin-1 receptor antagonist.10 However, the mechanisms by which alcohols induce protein aggregation are not known. In this article, we probe the role of protein unfolding in alcohol-induced protein aggregation.

To study alcohol effects, we chose benzyl alcohol (BA) because of its wide range of biochemical and biotechnological applications compared to any other alcohol. BA has been used as an antimicrobial agent in protein formulations,11 as an anesthetic,12 as a membrane fluidizer,13 as a heat shock protein inducer,14 as a fragrance component and preservative in cosmetics such as hair shampoos, as a food additive, and in other applications.15 However, BA has been shown to induce aggregation of three proteins: interferon-γ,16 interleukin-1 receptor antagonist,17 and human granulocyte colony stimulating factor17 even at the extremely low levels of 1% v/v. So far, these studies have not provided any mechanistic insight into structural changes that induce protein aggregation, which is critical for the rational development of approaches to inhibit protein aggregation. Earlier studies indicate that BA does not affect global protein stability or structure,10,18,19 raising the question of whether protein stability plays any role in BA-induced protein aggregation. In addition to global unfolding, partial protein unfolding has...
been proposed to play an important role in general protein aggregation.\(^{20,21}\) We examined here whether alcohol-induced aggregation is caused by unfolding of local protein regions, previously referred to as foldons\(^{22}\) rather than global unfolding of the entire protein.

For studying the role of partial protein unfolding in protein aggregation, we chose cytochrome \(c\) [Cyt \(c\); Fig. 1(A)] as a model protein, for the reason that various optical and structural probes are available to monitor its global unfolding and especially its distinct partial unfolding reactions.\(^{23-25}\) Cyt \(c\) is predominantly an \(\alpha\)-helical protein [Fig. 1(A)], and hence circular dichroism (CD) at 222 nm is a good probe for measuring its global stability. Tryptophan fluorescence is a second probe for Cyt \(c\) global stability. Cyt \(c\) contains a single tryptophan at position 59 that is not fluorescent in the native protein due to Förster resonance energy transfer to the heme group (Förster distance \(R_0 = 34 \text{ Å}^{26}\)), but becomes highly fluorescent upon protein unfolding. An absorption band at 695 nm due to charge transfer from the Met80-S to the ferric iron provides a probe for unfolding of the local protein region around Met80. In addition, NMR and hydrogen exchange (HX) provide structural probes that can measure changes in protein structure and stability at an individual amino acid level. These various probes have been earlier used to characterize the partial unfolding of Cyt \(c\) under various solution conditions.\(^{22,27-31}\) Here we examine the role of such partial unfolding in protein aggregation.

Although Cyt \(c\) has been a well-studied model protein for five decades to understand mechanisms of protein folding and stability, its aggregation mechanisms have not been examined. Our results show that BA induces Cyt \(c\) aggregation. Increasing BA concentration accelerates aggregation kinetics and decreases the temperature at which Cyt \(c\) aggregates. However, BA did not significantly alter Cyt \(c\)’s global structure. Instead, it caused partial unfolding of a local protein region around Met80. Selective stabilization of this region decreased the aggregation. These results demonstrate that partial unfolding, rather than global unfolding, of Cyt \(c\) results in BA-induced aggregation. This study, for the first time, provides a structural insight into the role of unfolding of local protein regions in alcohol-induced protein aggregation. This work opens a new direction in understanding the effects of alcohols on partially unfolded states (foldons) and their role in protein aggregation. The newly emerging foldon dimension of protein molecules seems to control many other protein behaviors including protein folding and protein function.\(^{30}\)

**MATERIALS AND METHODS**

**Materials**

Equine Cyt \(c\) (type VI) was obtained from Sigma Chemical. Prior to experiments, protein was oxidized using potassium ferricyanide to remove any trace amounts of the

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**Figure 1**

BA induces Cyt \(c\) aggregation. A: Structure of Cyt \(c\). Cyt \(c\) contains three \(\alpha\)-helices and three \(\Omega\)-loops. Individual cooperatively unfolding regions (foldons) detected in the oxidized Cyt \(c\), at neutral pH and room temperature are colored in terms of their increasing stability: Red, Yellow, Green, and Blue. The structure also shows the residues Met80 and His18, the two axial ligands of the heme, and the single tryptophan residue at position 59. B: Fraction of soluble monomer remaining in solution after incubation at 37°C with shaking, as determined by the SEC gel filtration. The black and grey bars represent the values without and with 3% v/v BA. C: Aggregation kinetics of Cyt \(c\) at 60°C in the absence (Black curve) and presence of 3% v/v BA (Yellow symbols).
Mechanisms of Alcohol-Induced Protein Unfolding and Aggregation

Reduced form, dialyzed extensively against the desired buffer, and filtered through 0.22-μm filter. For experiments with the reduced form, Cyt c was reduced with ascorbate. BA was obtained from Merck (Germany) and deuterated BA was from Isotec (Sigma-Aldrich). Optical grade GdmCl was from MP Biomedicals. All other reagents used in this study were of the highest grade available from Fisher Scientific or Sigma Chemicals.

Size exclusion chromatography (SEC)

Cyt c (2 mM in 0.1M sodium phosphate, 0.15M sodium chloride, pH 7) was incubated at 37°C on a rotator (Thermo Scientific Labquake Shaker Rotisserie) and samples were taken at desired intervals. Concentration of the monomer was estimated by injecting 5 μL onto a TSKgel 5 μm G3000SWxl column on an Agilent 1100 HPLC. The mobile phase used for this column was 0.1M sodium phosphate, 0.1M sodium sulfate, pH 6.7 at a flow rate of 0.7 mL min⁻¹. Absorbance at 280 nm was used to estimate the protein concentration. The average of 0% v/v BA triplicates on Day 0 was used to normalize the peak area of subsequent sample sets.

Thermal scanning method

Changes in the optical density of Cyt c solutions at 695 nm was monitored as a function of increasing temperature using an Agilent 8453 UV-Visible spectrophotometer. Cyt c (300 μM) in 0.1M sodium phosphate, 0.15M NaCl, pH 7 was used for these experiments. Solution temperature was raised at a steady rate of 1°C min⁻¹ followed by 1-min equilibration before collecting the signal, and the signal was integrated for 0.5 s. First derivative of the optical density spectra was used for calculating the midpoint aggregation temperature (TmAg⁰⁸), using the software provided with the instrument. The melting temperature of the 695-nm absorbance (Tm⁶⁹⁵) at a particular BA concentration was determined as the temperature at which the absorbance is half of the native state value in the absence of BA.

Isothermal incubation experiments

Cyt c solution was incubated at the desired temperature, and the changes in optical density at 695 nm were measured as a function of the incubation time. For these experiments, the cuvette of buffer solution was initially equilibrated at the desired temperature, and Cyt c (300 μM final concentration) was added to the cuvette. The aggregation kinetics was monitored until the signal reached a plateau. At longer incubation times, the aggregates started settling down to the bottom of the cuvette, resulting in decreased optical density. At that point, the experiment was stopped.

Denaturant melts

Guanidinium Chloride (GdmCl) was used as the denaturant. Protein solutions at varying GdmCl concentra-

FT-IR experiments

IR spectra of Cyt c solutions (1 mM in 0.1M sodium phosphate, 0.15M NaCl, H₂O, pH 7, room temperature) were recorded using a Biomem MB-series FT-IR spectrometer (ABB Biocem Canada) and CaF₂ cell (BioTools). For each sample, 128 scans were acquired in single beam mode with 4 cm⁻¹ resolution. IR absorbance spectra were processed using GRAMS/Al 7.00 software (Thermo Galactic, Thermo Electron).

NMR titration experiments

The 2D gradient COSY experiments were run to monitor changes in Cyt c amide crosspeaks as a function of BA concentration using a Varian Inova 500 MHz NMR instrument. For these experiments, 3 mM Cyt c (0.1M sodium phosphate, 0.15M NaCl, pH 7) and deuterated BA (1–3% v/v) were used. NMR spectra were collected with 8000 Hz spectral width and 512 points in each direction. The spectra were processed in magnitude mode using the nmrPipe software (Delaglio) with zero filling to twice their real points, apodization with non-shifted sine multiplication, exponential broadening, and Gaussian transformation. The spectra were base line corrected in both dimensions. Changes in the peak positions (chemical shifts) and peak volumes were calculated using the nmrDraw package. Cyt c residue assignments available in literature were used for this purpose. The 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was used as a standard to reference the chemical shifts as well as to normalize the measured changes in crosspeak volumes with the addition of BA.

HX

Cyt c (3 mM) whose amides were protons was lyophilized in deionized water. It was dissolved in the appropriate buffer in D₂O just before running the HX experiment and filtered through 0.22-μm filter. A series of 2D gradient COSY was recorded back to back using a Varian Inova 600 MHz NMR instrument equipped with a cryprobe to monitor the HX. NMR spectra were processed
using nmrPipe software and the changes in peak volumes due to exchange were measured using nmrDraw software. These were normalized with the intensities of five nonexchangeable crosspeaks (C6H—C7H and C4H—C5H of Trp59, C6H—C7H of Tyr74, CyH—C8H of Leu64, and CyH—C9H of Leu98). The changes in peak intensities were fitted to a single exponential equation to obtain the exchange rate constants \(k_{ex}\). Using the calibrated HX rate constants in the unfolded state \(k_{chem}\) calculated using Excel spreadsheets available at http://hx2.med.upenn.edu, the stabilities of the protecting structures were determined using the equation

\[ \Delta G = -RT \ln \left( \frac{k_{ex}}{k_{chem}} \right) \]

where \(R\) is the universal gas constant and \(T\) is the absolute temperature.25 For this calculation, we assumed that the HX rates in the protein unfolded state were unaffected by the minimal concentrations of BA (1–3% v/v) used in this study.

**RESULTS**

**BA aggregates Cyt c**

We first tested whether BA induces Cyt c aggregation. Using size-exclusion chromatography (SEC) in combination with HPLC, we measured Cyt c monomer concentration as a function of the time the protein was kept shaking at 37 °C in isothermal incubation experiments [Fig. 1(B)]. This temperature was chosen because it is physiologically relevant and also to accelerate the aggregation kinetics compared to room temperature. There was no change in the monomer concentration over a time period of 4 days in the absence of BA whereas the monomer concentration sharply decreased in the presence of 3% v/v BA, indicating that BA induces the aggregation of Cyt c.

Since BA aggregates Cyt c on a time scale of days, we accelerated the aggregation kinetics by increasing the temperature so that the aggregation mechanisms can be probed on a convenient laboratory timescale. We measured protein aggregation kinetics at a fixed temperature (60 °C) in terms of the increase in solution turbidity as a function of the incubation time [Fig. 1(C)]. For these measurements, 695-nm wavelength was used such that we can simultaneously measure unfolding of the local protein region around Met80 and protein aggregation. The 695-nm absorbance initially decreased as the temperature increased, indicating unfolding of the Met80 region. This was followed by an increase in optical density at temperatures above 75 °C due to increase in solution turbidity because of protein aggregation.

**BA decreases the temperature at which Cyt c aggregates**

We followed the effect of BA on the temperature at which Cyt c aggregates using a thermal scanning method. The temperature of the solution was increased at a steady rate of 1 °C min⁻¹, and protein aggregation was estimated by measuring the increase in solution turbidity [Fig. 2(A)]. As before, 695-nm wavelength was used for these measurements to simultaneously measure unfolding of the local protein region around Met80 and protein aggregation. In buffer without BA, the 695-nm absorbance initially decreased as the temperature increased, indicating unfolding of the Met80 region. This was followed by an increase in the optical density at temperatures above 75 °C due to increase in solution turbidity because of protein aggregation. When the same thermal transition was monitored
using 800-nm wavelength where the protein does not absorb, we observed only the later part of the transition, indicating that the increase in optical density at 695 nm at higher temperatures [Fig. 2(A)] is because of protein aggregation and not due to increase in the 695-nm absorbance. With the increase in BA concentration, there was a significant decrease in the 695-nm absorbance even at 30°C. Also, 695-nm absorbance melted at lower temperatures in the presence of BA, indicating that BA decreased stability or enhanced unfolding of the local protein region around Met80. The proceeding thermal aggregation followed the same trend, that is, Cyt c aggregated at lower temperatures in the presence of BA. The midpoint temperature of Cyt c aggregation (Tm_Agg) decreased from 81°C in 0% v/v BA to 57°C in 3% v/v BA with a linear slope of 8°C/% v/v BA [Fig. 2(B) and Table I]. The aggregation temperature decreased linearly with the decrease in the melting temperature of the 695-nm absorbance (Tm_695 nm) upon BA addition [Fig. 2(C)]. These results indicate that the unfolding of the local protein region around Met80 by BA promotes protein aggregation, and thus this region acts as a “hot-spot” for triggering protein aggregation.

Table I
Aggregation Midpoint Temperatures (Tm_Agg) of Oxidized and Reduced Cyt c at Different BA Concentrations

<table>
<thead>
<tr>
<th>Protein</th>
<th>[BA] (v/v)</th>
<th>Tm_Agg (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyt c oxidized</td>
<td>0%</td>
<td>81.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>75.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>67.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>57.3 ± 0.1</td>
</tr>
<tr>
<td>Cyt c reduced</td>
<td>0%</td>
<td>&gt;95</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>94.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>87.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>69.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>78.3 ± 0.1</td>
</tr>
</tbody>
</table>

Tm_Agg was determined from the peak position in the first derivative spectra of the optical density increase with temperature shown in Figures 2A and 7A. For reduced Cyt c, complete transition was not observed for 0% v/v and 1% v/v BA, and for 3% v/v BA, a minor transition was observed in addition to the major transition. Tm_Agg for reduced Cyt c at 1% v/v BA was determined assuming that the increase in optical density reaches the same plateau value as that of 2% v/v and 3% v/v BA. The error bar on the Tm_Agg value was estimated from the three curves shown for each BA concentration.

We were unable to use CD signal at 222 nm to measure changes in the protein secondary structure with the addition of BA, because BA strongly absorbs in this wavelength region.

**BA unfolds the local protein region around Met80**

Since 695-nm absorbance was clearly affected by BA even at 30°C [Fig. 2(A)], we examined changes in the 695-nm absorbance as a function of BA concentration at room temperature [Fig. 3(A)]. Consistent with the results in Figure 2(A), the 695-nm absorbance decreased with increasing BA concentration indicating unfolding of the local protein region around Met80. Under the same conditions, no change in the native state fluorescence was observed indicating that the addition of BA does not increase the Trp59 to heme distance to an extent that results in increased fluorescence, implying that BA does not perturb the overall global protein structure.
Alternatively, we monitored the secondary structure change using vibrational bands in the amide I region of the FT-IR spectrum [Fig. 3(B,C)]. Although individual secondary structures absorb over multiple wavelengths, they have characteristic absorption bands in the second derivative absorption spectra. The band at 1654 cm$^{-1}$ is a signature of $\alpha$-helices. Cyt c, being an $\alpha$-helical protein, showed an intense band at 1654 cm$^{-1}$ [Fig. 3(C)]. With the addition of BA, the $\alpha$-helical content (1654 cm$^{-1}$) increased significantly.

We examined the BA effects on the global and local stability using denaturant melts with GdmCl as the denaturant. As mentioned above, we could not use CD at 222 nm because BA strongly absorbs in this wavelength region. Alternatively, we used tryptophan fluorescence to measure the changes in global stability. The equilibrium denaturant melts did not show significant variation at different BA concentrations [Fig. 4(A)].

Cyt c retained its overall global structure as there was no change in the native fluorescence signal with the addition of BA at zero denaturant concentration. Fluorescence of the unfolded state decreased at higher BA concentrations [Fig. 4(A)], probably due to quenching by BA or due to the formation of residual structure in the unfolded state upon adding BA leading to tryptophan fluorescence quenching by the heme. The normalized melting curves [Fig. 4(B)] showed a slight shift in the midpoint of the transition to lower denaturant values and a decrease in the steepness of the melt with increasing BA concentration, indicating a decrease in global stability with the addition of BA. Assuming that only native and unfolded states are present at equilibrium with no intermediates at all denaturant concentrations (two-state folding), the change in global stability was determined by fitting the melting curves to a two-state model.\(^3\)\(^3\) The Gibbs’ free energy $\Delta G$ varied linearly with the BA concentration with a slope of 1.3 kcal/mol/% v/v BA [Fig. 4(B) inset]. Importantly, since there was no change in the native fluorescence signal after the addition of BA in the absence of denaturant, no global structural change occurred, consistent with the results in Figure 3(A). In contrast to fluorescence, 695-nm absorbance showed a drastic variation with the addition of BA [Fig. 4(C)]. In buffer without denaturant, a clear decrease in 695-nm absorbance was seen with the addition of BA [similar to Figs. 2(A) and 3(A)], indicating decreased stability or enhanced unfolding of the local region around Met80. With increasing BA concentration, the 695-nm absorbance melted at lower denaturant concentrations, again indicating decreased stability of the local protein region around Met80. Assuming that the 695-nm absorbance has the same value in the native state in the absence and presence of BA, fitting the melting curves to a two-state equation resulted in a sharp decrease in $\Delta G$, even with just 1% v/v BA concentration [Fig. 4(C) inset]. It is interesting to note that at higher denaturant concentrations in the presence of 2 or 3% v/v BA, 695-nm absorbance seems to reappear at 2M GdmCl concentration and disappear at concentrations above 3.5M [Fig. 4(C)]. This artifact is because of the increased solution turbidity (measured by light scattering) in overnight equilibrated protein samples at these particular BA and denaturant concentrations.

$\text{BA increases dynamics of the local protein region around Met80}$

We monitored changes in the global protein structure using 2D NMR COSY spectra recorded as a function of
increasing BA concentration [Fig. 5(A)]. No significant changes were observed in the positions of individual amide crosspeaks, indicating that BA did not perturb the overall global protein structure. The chemical shifts of mainchain amide protons and Cα protons of all residues were within 0.1 ppm [Fig. 5(B)]. Although there was no change in the native state chemical shifts, some new weak peaks appeared, indicating an altered conformation in the presence of BA. When we mapped the locations of the residues for which we can identify the appearance of new peaks based on the disappearance of the adjacent old peak, we did not see any clustering of residues in the protein 3D structure, indicating that no specific binding pockets exist for BA. In relation, isothermal titration calorimetry experiments showed that the enthalpy changes observed as a function of the molar ratio of BA to Cyt c were minimal (below 0.07 kcal mol⁻¹) (data not shown), similar to that observed for other proteins, indicating that BA does not have any strong binding sites on Cyt c and the structural perturbation is small.

Although chemical shifts did not show significant variation, native state crosspeak volumes decreased with increasing BA concentration [Fig. 5(C)]. At 1% v/v BA, maximum volume changes were observed for residues Met80, Phe82, and Ile85. Decreased crosspeak volumes with no change in chemical shifts indicate that the native state is in exchange with a partially unfolded state in the presence of BA (slow to intermediate NMR exchange regime). The increased dynamics of the Met80 region appeared to be propagated to the other side of the heme group. In Cyt c, Met80 is ligated to the heme on one side whereas His18 is ligated on the other side of the heme. Some significant crosspeak volume changes were seen at residues around His18 and around Asn31. With increased BA concentration, the protein regions that showed large changes in crosspeak volumes expanded. For example, at 3% v/v BA concentration, all residues close to Met80 became more dynamic so that no crosspeaks were seen for many residues in this region, whereas at 1% v/v BA, only the three residues Met80, Phe82, and Ile85 showed significant volume changes compared to other residues in the Met80 region. However, since there was no change in the native state chemical shifts [Fig. 5(A, B)] or in the native state fluorescence signal [Fig. 5(A)] even at 3% v/v BA, the protein retained its overall global structure, except that it became more dynamic with the addition of BA.

**BA effects on partial unfolding and foldon substructure of Cyt c**

Since BA increased the α-helix content [Fig. 3(B, C)] and at the same time unfolded the protein region around Met80 [Figs. 1(C), 2(A), 3(A), and 4(C)], we probed how BA affects the stabilities of various local protein regions using HX methods. If the induced α-helix is stable over the native structure, the stabilities of those...
regions where the α-helix is formed should increase. Similarly, the stability of those regions that unfold with BA should decrease. Such study on alcohol effects on the stability of local protein regions or foldon substructure was not performed earlier on any protein; most earlier studies were on monitoring the alcohol effects on global protein structure and stability.

HX in combination with 2D NMR measures the local stability of structures around individual amino acids. For a proton to be exchanged with the solvent, it needs to be exposed to the solvent, and the exchange rate is proportional to the stability of the structure that is protecting the amide against exchange. Earlier HX experiments showed that the exchange rates for individual amides change considerably with the addition of denaturant. These experiments indicate that amide hydrogens in Cyt c exchange through three types of unfolding reactions: (1) local unfolding that involves unfolding of only the structure or hydrogen bond protecting that amide, (2) subglobal unfolding where individual protein regions unfold cooperatively, and (3) global unfolding where the entire protein is unfolded. Since local unfolding exposes little or no surface area, their ΔG is invariant with the denaturant concentration, because the slope or m-value is proportional to the surface area exposed upon unfolding. At low denaturant (GdmCl) concentrations, many residues exchange through local unfolding reactions because of their lower stability compared to subglobal and global unfolding reactions. Increase in the denaturant concentration promotes larger unfoldings, such as subglobal and global unfolding reactions. This is because these unfoldings expose significant

Figure 6
Effect of BA on partial unfolding and foldon substructure of Cyt c. A: ΔGs of various residues determined using HX methods as a function of GdmCl concentration (Fig. from Ref. 22). B: ΔGs determined at various BA concentrations. Within a foldon, residues that exchange through local unfolding reactions with denaturant retained their local unfolding character with BA, and residues that exchange through larger unfolding reactions such as unfolding of the entire foldon (subglobal unfolding) with the addition of denaturant showed decreased stability with BA.
surface area upon unfolding and hence have larger \( m \)-values resulting in a larger decrease in the \( \Delta G \) with increase in denaturant concentration. After a certain denaturant concentration, \( \Delta G \) of subglobal and global unfoldings become lower than that of the local unfolding and the amides exchange through these less stable, larger unfolding reactions. Such HX experiments performed as a function of the denaturant concentration have identified individual subglobal cooperatively unfolding regions in Cyt c, known as foldons, where the amides in a single foldon exchanged with the same \( \Delta G \) as the denaturant concentration is increased [Fig. 6(A)]. In the oxidized state, at neutral pH and room temperature, four foldons were seen, colored in terms of their increasing stability: Red, Yellow, Green, and Blue foldons\(^{30,36,37} \) [Fig. 1(A)]. Individual foldons contain particular residues, known as stability markers that exchange only through complete unfolding of the entire foldon. For example, residue Ile75 in the Red foldon exchanges only when the entire Red foldon unfolds. Hence, the stability measured by the residue Ile75 unfolding represents the stability of the Red foldon.\(^{36,37} \) Similarly, Lys60 and Leu64 are stability markers for the unfolding of the Yellow foldon. Leu68 is stability marker for the Green foldon, whereas Ala96, Tyr97, and Leu98 are stability markers for the Blue foldon. Here, we examined the effects of BA on such partial unfolding and foldon substructure of Cyt c.

Exchange rates for individual residues were measured as a function of BA concentration over a period of 3 months, and converted to \( \Delta G \)s using standard equations [see Methods; Fig. 6(B)]. Note that a decrease in \( \Delta G \) by 0.6 kcal mol\(^{-1} \) corresponds to an increase in the exchange rate by 2.7 times. Within a given foldon, the residues which exchange through local unfolding reactions at low denaturant (GdmCl) concentrations retained their local unfolding character with BA. For example, residues Lys7, Lys8, Ile9, Phe10, Val11, Gln12, Lys13, Cys14, Ala15, and His18 in the Blue N-terminal helix exchanged through local unfolding reactions at low GdmCl concentrations as well as at low BA concentrations [cf. Fig. 6(A,B)]. Their \( \Delta G \)s of unfolding did not change with the addition of BA, indicating that their unfolding with BA did not expose significant surface area. Similar results were obtained for residues in other foldons, except a few residues like Met65 in the Green helix, which exchanges through local unfolding at low GdmCl (zero \( m \)-value), but exchanges through an unfolding reaction that exposes significant surface area at low BA concentrations as evident from its nonzero slope of \( \Delta G \) variation with BA concentration. Similar to denaturant experiments [Fig. 6(A)], the stability markers for the individual foldons, Ile75 for the Red foldon, Lys60, and Lys64 for the Yellow foldon, and Lys68 for the Green foldon, exchanged with the highest \( \Delta G \) and with a steeper slope within that foldon with BA addition. This indicates that these residues unfold through larger unfolding reactions in the presence of BA, possibly through the unfolding of the individual foldons. The observation that individual amides exchange through similar unfolding reactions in the presence of denaturant as well as in the presence of BA indicates that at low concentrations, BA behaves like a denaturant. However, a closer examination of the results indicates the differences between an alcohol and a denaturant. \( \Delta G \) of the Yellow and Red foldon stability markers (Lys60, Leu64, and Ile75), instead of showing a continuous linear decrease with BA, seemed to show a slight upward curvature at higher BA concentrations [Fig. 6(B)], which might be due to the formation of stable structure in these loop regions. HX experiments with BA could not be performed at higher BA concentrations because of its limited solubility in water (<4% v/v). In addition, \( m \) values for individual foldons with BA do not follow the trend seen with denaturant. With GdmCl, the \( m \) values (kcal/mol/M [GdmCl]) of individual foldons increase in the order Red (1.5) < Yellow (2.3) < Green (3.1) < Blue (4.5), which lead to the hypothesis that these foldons unfold sequentially with the addition of denaturant\(^{36} \) that was later confirmed by “stability labeling” experiments.\(^{39} \) These earlier results indicate that unfolding of the Green foldon with denaturant includes unfolding of the Yellow and Red foldons. In contrary to GdmCl, \( m \) values with BA (kcal/mol/% v/v BA) of individual foldons vary in the order Red (0.8) < Green (0.9) < Yellow (1.2), (we were unable to measure \( m \)-value of the Blue foldon because its stability markers did not exchange within 3 months of exchange time). That means, unfolding of the Green foldon with BA does not include unfolding of the Yellow foldon, since \( m \)-value, which is proportional to the amount of surface area exposed upon unfolding, of Yellow foldon is higher than that of the Green foldon. The above two observations indicate that BA does not behave exactly like a denaturant. Further examination of these HX results and the effects of alcohols on foldon substructure are needed and are in progress. It is possible that the foldon whose unfolding causes Cyt c aggregation is much smaller than the Red foldon, because the stability measured by Ile75 unfolding did not change with the addition of BA, indicating that their unfolding with BA did not expose significant surface area.

Stabilizing the Met80 region decreases Cyt c aggregation

Our results indicate that BA induces local unfolding of the protein region around Met80 that leads to Cyt c aggregation. Therefore, stabilizing this local region should decrease the protein aggregation, or increase the temperature at which the protein aggregates. The best method that has been dem-
onstrated in literature to very specifically stabilize the Met80 region is by reducing the heme group (Fe$^{3+}$ to Fe$^{2+}$), which increases the stability of the Met80-heme bond by an exceptionally large amount of 3.2 kcal mol$^{-1}$ with no change in Cyt c’s structure.40 As before, thermal scanning experiments were used to measure the solution turbidity changes due to protein aggregation of reduced Cyt c [Fig. 7(A)]. When compared to the oxidized form, reduced Cyt c aggregated at higher temperatures at all BA concentrations [cf. Fig. 7(A) with Fig. 2(A)]. In 0% v/v BA, no melting transition was observed, hence only the lower limit of $T_m$ was shown. For 1% v/v BA, $T_m$ was determined assuming that it reaches the same plateau value as that of 2% and 3% v/v BA. B: Variation in the $T_m$ of oxidized and reduced Cyt c. Both decrease at the same rate with the addition of BA. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

**DISCUSSION**

**Alcohol-induced protein unfolding**

Alcohols have been used as “cosolvents” to study the effects of various physical parameters of solvents, such as hydrophobicity, on the structure and stability of proteins.41–43 Addition of alcohols weakens nonlocal hydrophobic interactions and enhances local polar interactions.42–44 This results in destabilization of the protein’s native hydrophobic core and increased formation of local hydrogen bonds resulting in extended secondary structures. However, none of these studies have examined the alcohol effects on partial protein unfolding or foldon substructure of proteins. Our results on Cyt c show that increasing concentrations of BA have minimal effect on the global structure of Cyt c [Figs. 3(A), 4(A), 5(A,B)], except an increase in the $\alpha$-helical content [Fig. 3(B,C)]. However, BA specifically unfolds a local protein region around Met80 [Figs. 1(C), 2(A), 3(A), and 4(C)]. It is important to note that the BA concentrations employed in this study range from 1% to 3% v/v, much lower than other alcohols used in previous studies (typical concentrations of TFE were ca. 15%–70% v/v). For example, in a recent study on alcohol effects on Cyt c’s native structure,45 methanol concentrations of up to 80% v/v were used that resulted in a slight increase in the native $\alpha$-helical structure with a magnitude similar to that observed here for 3% v/v BA (see Fig. 3). A possible reason for the significant effects of BA at such low concentrations may be its hydrophobicity (dielectric constants of BA and water are 13.546 and 80.1 respectively). Increase in hydrophobicity is a major factor in enhancing the denaturing efficiency of alcohols,42,47–49 besides other factors. In relation, it is notable that BA is not miscible in water at concentrations above 4% v/v.

We have also probed the effect of alcohols on foldon substructure of Cyt c [Fig. 6(B)]. This is particularly important because of the demonstrated role of foldons in controlling many protein behaviors which include protein folding and protein function22,28–30,50–52, and under-
Alcohol-induced protein aggregation

Recent studies indicate that alcohols induce aggregation of both disease and nondisease proteins. Experimental results in literature so far indicate that TFE accelerates the aggregation of Alzheimer’s β-protein53 and superoxide dismutase,8 ethanol accelerates the aggregation of insulin,9 whereas in the case of nondisease proteins, TFE induces the aggregation of α-chymotrypsin54,55 and barstar.56 In the case of BA, it induces the aggregation of interferon-γ,16 interleukin-1 receptor antagonist,10 and human granulocyte colony stimulating factor.17 The molecular mechanisms underlying such alcohol-induced protein aggregation were not understood. In particular, it was not clear whether there exits any direct correlation between alcohol-induced protein unfolding and aggregation.

We have used Cyt c to understand the alcohol-induced protein aggregation mechanisms, because of the reason that various optical and structural probes are available to monitor its global and partial unfolding. BA aggregates Cyt c (Figs. 1 and 2), similar to other proteins. BA decreased the temperature at which Cyt c aggregates. Proteins aggregate at higher temperatures because of the enhancement of hydrophobic effect due to increase in the disorder of solvent molecules. It is therefore likely that BA accelerates the aggregation kinetics or makes the protein to aggregate at lower temperatures because it increases the hydrophobicity of the solvent.

To establish the relationship between BA-induced protein unfolding and aggregation, we first studied the effects of BA on Cyt c partial unfolding. BA did not cause global unfolding of the protein, but unfolded a local protein region around Met80 (Figs. 3 and 4). The temperature at which Cyt c aggregates decreased linearly with the decrease in the melting temperature of the Met80 region [Fig. 2(C)]. When this region is stabilized, the aggregation temperature shifted to higher values (see Fig. 7). This clearly demonstrates that a mechanism by which BA induces Cyt c aggregation is by partially unfolding the native protein rather than global unfolding. However, since the aggregation is much slower at room temperature even when this partially unfolded state is half populated at equilibrium at higher BA concentrations [Figs. 1(B), 2(A), and 4(C)], the aggregation is possibly limited by a free energy barrier or the formation of a transient species. It will be interesting to probe the role of such partial unfolding in alcohol-induced protein aggregation with other alcohols and proteins to arrive at general principles of how alcohols induce protein aggregation.

Role of intra-protein stability variation and foldon substructure in protein aggregation

The results presented here show that the decrease in the stability of local protein regions (foldons) in a native protein increases protein aggregation. Increasing concentrations of BA populates a partially unfolded form where protein region around Met80 is unfolded, which increases the protein’s propensity to aggregate. That means, if we determine the stability of local regions in a native protein under aggregating conditions, it might be possible to identify the potential “hot-spots” for protein aggregation. It is interesting to note that the same region around Met80 plays an important role in Cyt c’s folding and function. For example, it is one of the first units to unfold52,57 and is linked to Cyt c’s alkaline transition.29,58

Our experimental results can be used to validate computational methods that predict the aggregation “hot-spots” in proteins. Aggregation of proteins is controlled by a few physical properties,59–61 mainly hydrophobicity, secondary structure propensity, electrostatics and hydrogen bonding. Prediction methods developed based on these physical properties, in particular, TANGO60 (http://tango.crg.es/) and AGGRESCAN62 (http://bioinf.uab.es/aggrescan/) point to Met80 region as one of the possible aggregation “hot-spots” (Supporting Information Figure). In addition, the 3D profile method ZipperDB (http://services.mbi.ucla.edu/zipperdb/)63 that scans the crystal structure of the cross-β spine of the hexapeptide NNQQNY against the protein sequence indicates Met80 region as one of the regions that can form intermolecular cross-β structure. In relation, FT-IR spectra of Cyt c aggregates do indicate that these aggregates contain significant intermolecular β-sheet (data not shown). Comparing the results from these computational methods, all the three point to a single region, the hexapeptide Met80-Ile81-Phe82-Ala83-Gly84-Ile85, as the common aggregation “hot-spot.” It is interesting to note that the three residues Met80, Phe82, and Ile85 that are part of this hexapeptide became more dynamic with the addition of 1% v/v BA compared to all other residues in Cyt c [Fig. 5(C)].

Methods to inhibit alcohol-induced protein aggregation

Alcohols such as BA are often used at low levels in multi-dose protein formulations and drug delivery systems to prevent microbial growth.11,64 However, these alcohols have been shown to induce protein aggregation.65 Here, we show that a fundamental physical mech-
anism underlying such alcohol-induced protein aggregation is partial unfolding of local protein regions. We also show that stabilizing such local regions by site-specific mutations reduces protein aggregation. This might be a useful strategy to adopt in the case of pharmaceutical proteins to counteract alcohol-induced protein aggregation or, in general, any aggregation, that is, identify the aggregation “hot-spots” and stabilize them using protein engineering methods.

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REFERENCES

Mechanisms of Alcohol-Induced Protein Unfolding and Aggregation