

## Entrapping Intermediates of Thermal Aggregation in $\alpha$ -Helical Proteins with Low Concentration of Guanidine Hydrochloride\*

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Aggregation of proteins is a problem with serious medical implications and economic importance. To develop strategies for preventing aggregation, the mechanism(s) and pathways by which proteins aggregate must be characterized. In this study, the thermally induced aggregation processes of three  $\alpha$ -helix proteins (myoglobin, cytochrome *c*, and lysozyme) in the presence and absence of 1.0 M guanidine hydrochloride (GdnHCl) were investigated by means of infrared spectroscopy. In the absence of GdnHCl, intensities of the  $\alpha$ -helix bands ( $\sim 1656\text{ cm}^{-1}$ ) decrease as a function of temperature at above 50 °C. With myoglobin and cytochrome *c*, the loss of helix bands was accompanied by the appearance of two new bands at 1694 and 1623  $\text{cm}^{-1}$ , indicative of the formation of intermolecular  $\beta$ -sheet aggregates. For lysozyme, bands indicative of intermolecular  $\beta$ -sheet aggregates did not appear in any significant intensity. In the presence of 1.0 M GdnHCl, two major intermediate states rich in  $3_{10}$ -helix (represented by the band at 1663  $\text{cm}^{-1}$ ) and  $\beta$ -turn structure (represented by the band at 1667  $\text{cm}^{-1}$ ), respectively, were observed. These findings demonstrated that IR spectroscopic studies of protein aggregation using a combination of thermal and chemical denaturing factors could provide a means to populate and characterize aggregation intermediates.

Protein folding is arguably the most important process studied in biophysics and structural biology because it converts linear polypeptide chains into three-dimensional structures that endow proteins with all their vital activities (1–3). Studies of protein folding are often plagued competing, off-pathway aggregation processes. Aggregation of proteins is also a problem with serious medical implications, *e.g.* in human disease states like Alzheimer's disease (4), Parkinson's disease (5, 6), and monoclonal immunoglobulin amyloidosis (7, 8). Furthermore, protein aggregation during production, shipping, storage, and delivery of therapeutic proteins is a problem of significant economic importance (9–11). To develop strategies for preventing protein aggregation, the mechanism(s) and pathways by which proteins aggregate must be characterized. Such characterization is complicated because light scattering interferes with many of the optical techniques that are now standard for examining protein folding pathways, *e.g.* fluorescence and circular dichroism spectroscopies.

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In contrast, infrared (IR)<sup>1</sup> spectroscopy is insensitive to light scattering and thus provides a valuable method for studying protein aggregation. IR spectroscopy can be used to study not only the secondary structure of proteins in the soluble, native state (12–15) but also in precipitated states, both native (*e.g.* salted out) and denatured (*e.g.* thermally, chemically, or mechanically induced aggregates) (16–20). However, a shortcoming of IR spectroscopy is that relatively high protein concentrations (*e.g.* 20 mg/ml in H<sub>2</sub>O) are needed to obtain high quality spectra. With such high concentrations of protein, perturbations used to induce structural transitions leading to aggregation (*e.g.* high temperature) cause a rapid conversion of native protein to insoluble aggregates that are rich in intermolecular  $\beta$ -sheet (16–18, 21, 22). This conversion is so rapid with respect to typical IR spectral acquisition (*e.g.* 5 min) that intermediates in the aggregation pathway usually cannot be detected. Yet, current theoretical and experimental mechanisms for protein aggregation suggest that aggregates are formed from partially folded intermediates (23). Thus, what is needed to capitalize on the advantages of IR spectroscopy for the study of protein aggregation is a means of populating these folding intermediates sufficiently for IR spectroscopic characterization.

To address this issue, in the current study we combined thermal and chemical approaches to allow population of aggregation pathway intermediates. With this approach, the model protein pool for the unfolding studies may be expanded to include proteins with a wide range of secondary structural compositions and various degree of resistance to chemical denaturants. Here, we present the results of studies in which we compared unfolding and aggregation of three  $\alpha$ -helix predominant proteins (myoglobin, cytochrome *c*, and lysozyme) in the presence or absence of 1.0 M GdnHCl using IR spectroscopy. A 1.0 M GdnHCl concentration is chosen because earlier studies have shown that at this concentration of GdnHCl and room temperature, both myoglobin and cytochrome *c* are not unfolded (24, 25). Here we will show that elevation of the temperature above 25 °C allows partial unfolding to occur, whereas the presence of GdnHCl both lowers the temperature at which transitions occur and inhibits the conversion of these partially folded intermediates to aggregates.

### MATERIALS AND METHODS

**Protein Sources and Preparations**—Cytochrome *c* (type VI, horse heart), myoglobin (horse heart), and lysozyme (chicken egg white) were purchased from Sigma and used without further purification. Guanidine hydrochloride was SigmaUltra grade from Sigma. The stock solutions of proteins were prepared by dissolving lyophilized protein powder in 50 mM potassium phosphate (pH 7.2) at concentration of 40 mg/ml and followed by filtration with a 0.20- $\mu\text{m}$  syringe filter. The stock

<sup>1</sup> The abbreviations used are: IR, infrared; GdnHCl, guanidine hydrochloride.

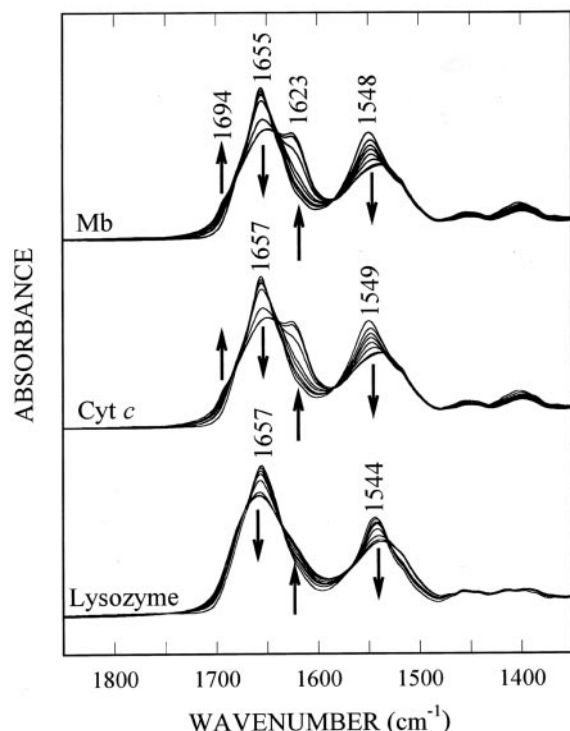


FIG. 1. The original infrared spectra of myoglobin (*Mb*), cytochrome *c* (*Cyt c*), and lysozyme in 50 mM potassium phosphate, pH 7.2, measured at temperatures range from 25 to 85 °C. The spectra of aqueous and gaseous water were subtracted from the spectra of proteins as described under "Methods and Materials."

solution of GdnHCl was prepared in 50 mM potassium phosphate at concentration of 2.0 M, and pH was adjusted with KOH solution. GdnHCl concentration was determined using a refractometer and following equation (26).

$$[\text{GdnHCl}] = 57.147(\Delta n)^3 + 38.68(\Delta n)^2 - 91.60(\Delta n) \quad (\text{Eq. 1})$$

where  $\Delta n$  is the difference between the refractive index of a GdnHCl solution and that of water. Samples for infrared analysis were prepared by mixing together the stock solutions of protein and GdnHCl at 1:1 ratio and equilibrated for at least 30 min before measurement. The final concentrations of the proteins were 20 mg/ml, and the GdnHCl was 1.0 M.

**Infrared Spectroscopy**—IR spectra were measured with a Bomem IR spectrometer equipped with a dTGS detector. Protein samples were placed in a P/N 20500 heatable cell with  $\text{CaF}_2$  windows and a 6- $\mu\text{m}$  spacer. For each spectrum, a 128-scan interferogram was collected in single beam mode with a 4  $\text{cm}^{-1}$  resolution. Reference spectra were recorded under identical scan conditions with only the buffer or 1.0 M GdnHCl buffer in the cell. The chosen temperature at which a spectrum was acquired was controlled within 0.5 °C using a custom built Peltier IR cell temperature controller. Spectral acquisition at a given temperature required approximately 6 min (*i.e.* dwell time at the given temperature). The average heating rate between spectral acquisition temperatures was 1.5 °C/min. Protein spectra were obtained according to previously established criteria and double subtraction procedure (15, 27). For the best result the spectra of buffer and 1.0 M GdnHCl buffer were subtracted from the spectrum of protein separately. The second derivative spectra were obtained with a 7-point Savitsky-Golay derivative function and then base line corrected as described previously (17).

## RESULTS

**Thermally Induced Structural Transitions**—Fig. 1 shows the infrared absorbance spectra of myoglobin, cytochrome *c*, and lysozyme in 50 mM potassium phosphate buffer (pH 7.2) as a function of temperature. The two strong bands centered near the 1656 and 1548  $\text{cm}^{-1}$  are the so-called the amide I and II bands, respectively. The amide I band arises primarily from the C=O stretching vibration of the peptide linkages that constitute the backbone structure of proteins and is known to be

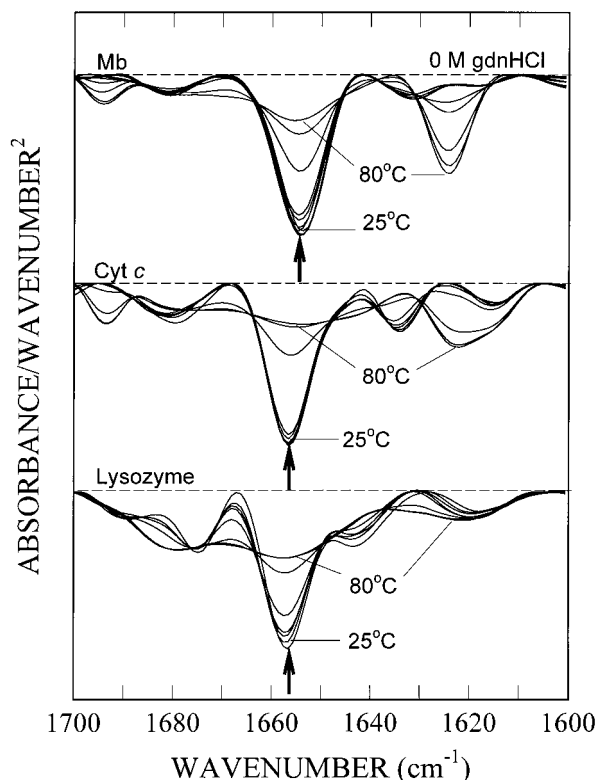


FIG. 2. The second derivative amide I spectra of myoglobin (*Mb*), cytochrome *c* (*Cyt c*), and lysozyme in 50 mM potassium phosphate, pH 7.2, recorded at 25, 35, 45, 55, 60, 65, 70, 75, and 80 °C. The arrows indicate the directions of spectral changes as a function of temperature.

sensitive to the secondary structural composition and conformational changes of proteins (12, 15, 28). The amide II band arises mainly from an out-of-phase combination of N-H in-plane bending and C-N stretching vibrations of peptide linkages (28) and is less useful in protein structural analysis. At 25 °C all three proteins exhibited the amide I band maximum near the 1656  $\text{cm}^{-1}$ , a frequency characteristic to proteins containing predominantly  $\alpha$ -helical structures (27). Elevation of temperature to  $\sim 80$  °C resulted similar spectral changes in all three proteins. The amide I absorbance maximum near 1656  $\text{cm}^{-1}$  decreased as a function of temperature, accompanied by intensity increase at 1623 and 1694  $\text{cm}^{-1}$ . In addition, a temperature-dependent intensity decrease and frequency red shift at the amide II bands were observed for all three proteins. The changes at the amide II region seem to be nonspecific to thermally induced protein unfolding and aggregation, because similar changes were also reported for the recombinant human factor XIII, a predominantly  $\beta$ -sheet protein (29).

Fig. 2 shows the second derivative amide I spectra of the three proteins in the absence of GdnHCl. Assignments of the amide I components can be made on the basis of previous infrared studies of over 50 proteins in  $\text{H}_2\text{O}$ -based solution (15, 27).<sup>2</sup> The bands near 1656  $\text{cm}^{-1}$  are assigned to  $\alpha$ -helix structure. The bands between 1670 and 1685  $\text{cm}^{-1}$  are due to  $\beta$ -turn structure. At 25 °C all three proteins exhibit a strong amide I band near 1656  $\text{cm}^{-1}$ . At temperatures between 35 and 50 °C, the intensities of the 1656  $\text{cm}^{-1}$  bands increases slightly from that of the native state at 25 °C. As temperature increases to above 55 °C, the intensities of the 1656- $\text{cm}^{-1}$  bands decrease dramatically. For myoglobin and cytochrome *c*, there is a con-

<sup>2</sup> A. Dong, J. F. Carpenter, and W. S. Caughey, unpublished observation.

comitant appearance of two new bands at  $1623\text{ cm}^{-1}$  (the low wave number  $\beta$ -sheet component) and the  $1694\text{ cm}^{-1}$  (the high wave number  $\beta$ -sheet component), indicative of intermolecular  $\beta$ -sheet (see "Discussion") in protein aggregates (17, 31, 32). The latter become a predominant spectral feature of thermally aggregated states of myoglobin and cytochrome *c*. When cooled from 80 to  $25\text{ }^{\circ}\text{C}$ , the two  $\beta$ -sheet aggregate bands remain unchanged in their intensity but shifted  $\sim 2\text{ cm}^{-1}$  to a lower wave number (data not shown). In addition to the appearance of IR bands assigned to intermolecular  $\beta$ -sheet, the presence of aggregated protein in the samples was documented by the observation that the cooled samples formed gels, which are characteristic extensive intermolecular interactions in protein samples.

For lysozyme, bands indicative of intermolecular  $\beta$ -sheet do not appear in any significant intensity, even though the pronounced loss of native  $\alpha$ -helix is irreversible (data not shown). The lack of well resolved bands suggest that the structure of thermally perturbed lysozyme is comprised of a heterogeneous ensemble of proteins with non-native  $\beta$ -turns, extended strands, and residual  $\alpha$ -helix.

An important feature of the IR spectra of all three thermally treated proteins in the absence of GdnHCl is the isosbestic points at  $1688$ ,  $1664$ ,  $1648$ , and  $1639\text{ cm}^{-1}$ . This observation suggests that heat treatment of the three proteins results in a transition between only two readily detectable states: the native and aggregated.

**Thermochemically Induced Structural Transitions**—To foster structural transitions and permit detection of unfolding/aggregation intermediate(s) during heating, we added a non-denaturing concentration of chemical denaturant GdnHCl (1.0 M) into the protein solution. In contrast to the aggregation induced by elevated temperature alone, the amide I absorbance maxima of the three proteins in the presence of 1.0 M GdnHCl shift to a higher wave number as a function of temperature (data not shown). Fig. 3 shows the second derivative spectra of the three proteins in the amide I region as a function of temperature in the presence of 1.0 M GdnHCl. Comparison of the spectra recorded at  $25\text{ }^{\circ}\text{C}$  with (Fig. 3) or without GdnHCl (Fig. 2) reveals that the presence of a non-denaturing concentration of GdnHCl does not alter the native structures of myoglobin and cytochrome *c* and causes only a small loss of native  $\alpha$ -helix ( $\sim 5\%$ ) in lysozyme. As the temperature increases, however, the proteins exhibit unfolding patterns distinctly different from those noted in the absence of GdnHCl. As intensities decrease, the maximum absorbance of predominant amide I components shift from near  $1656\text{ cm}^{-1}$  to  $1663\text{ cm}^{-1}$  and eventually to near  $1667\text{ cm}^{-1}$ . Intermolecular  $\beta$ -sheet bands ( $1623$  and  $1694\text{ cm}^{-1}$ ) in myoglobin spectra appear at temperatures above  $55\text{ }^{\circ}\text{C}$ , and the band at  $1666\text{ cm}^{-1}$  becomes more intense. However, the intermolecular  $\beta$ -sheet bands are less intense than those noted at the same temperatures in the absence of GdnHCl. With cytochrome *c* and lysozyme, even at elevated temperatures, no intermolecular  $\beta$ -sheet bands appear.

Fig. 4 shows a plot of the frequency of the most prominent amide I component as a function of temperature. It clearly shows that, within the temperature range of the experiment, the major secondary structural components of the thermally perturbed state are represented by the bands near  $1667\text{ cm}^{-1}$  for myoglobin and lysozyme and the band at  $1663\text{ cm}^{-1}$  for cytochrome *c*. On the basis of theoretical (28) and infrared spectroscopic studies (15, 33), the band component at  $1662 \pm 3\text{ cm}^{-1}$  can be assigned to the  $3_{10}$ -helix structure, and the band near  $1667\text{ cm}^{-1}$  can be assigned to the  $\beta$ -turn structures (27). Differing from the  $\alpha$ -helix in that the hydrogen bond is of the 4

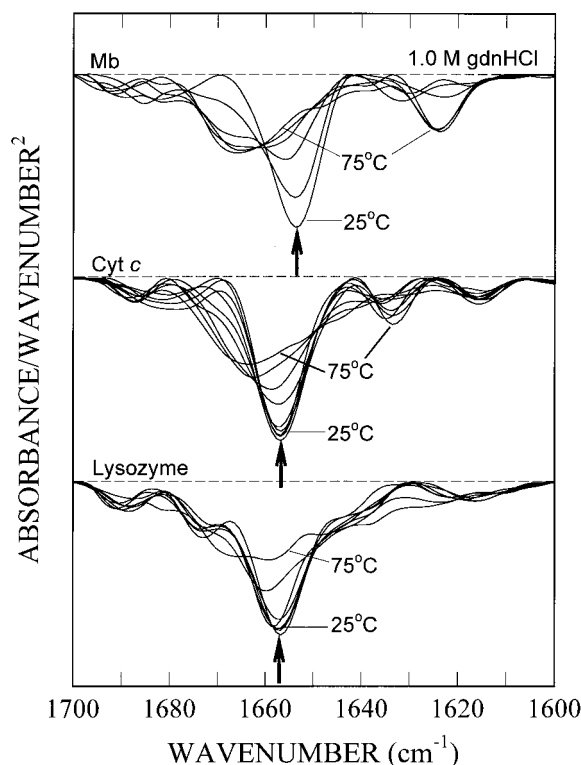


FIG. 3. Second derivative amide I spectra of myoglobin (*Mb*), cytochrome *c* (*Cyt c*), and lysozyme in 1.0 M GdnHCl, 50 mM potassium phosphate, pH 7.2, recorded at 25, 35, 45, 55, 60, 65, 70, and  $75\text{ }^{\circ}\text{C}$ . The arrows indicate the directions of spectral changes as a function of temperature.

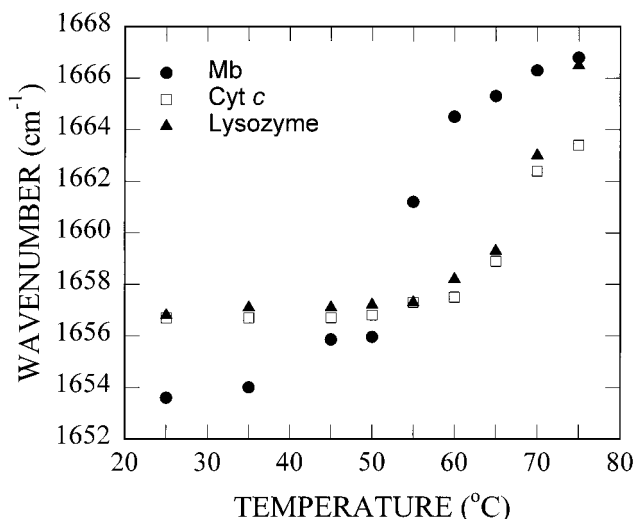


FIG. 4. Frequency change at the major amide I component as a function of temperature for the proteins in 1.0 M GdnHCl, 50 mM potassium phosphate. *Mb*, myoglobin; *Cyt c*, cytochrome *c*.

$\rightarrow 1$  type rather than the  $5 \rightarrow 1$  type,  $3_{10}$ -helix is also less common in proteins than  $\alpha$ -helix (34). Nevertheless,  $3_{10}$ -helix structure has been found by x-ray crystallographic analysis, for example, in peptides containing  $\alpha$ -aminoisobutyric acid residues (35, 36) and in globular proteins such as  $\alpha$ -lactalbumin (31%  $\alpha$ -helix and 20%  $3_{10}$ -helix) (37). By examining the IR spectra of synthetic peptides containing  $\alpha$ -aminoisobutyric acid residues, Kennedy and colleagues (33) showed that the peptides have an amide I band maximum between  $1666$  and  $1662\text{ cm}^{-1}$ . Prestrelski and co-workers (38) reported that the deconvoluted spectrum of  $\alpha$ -lactalbumin in  $\text{H}_2\text{O}$  solution exhibited a

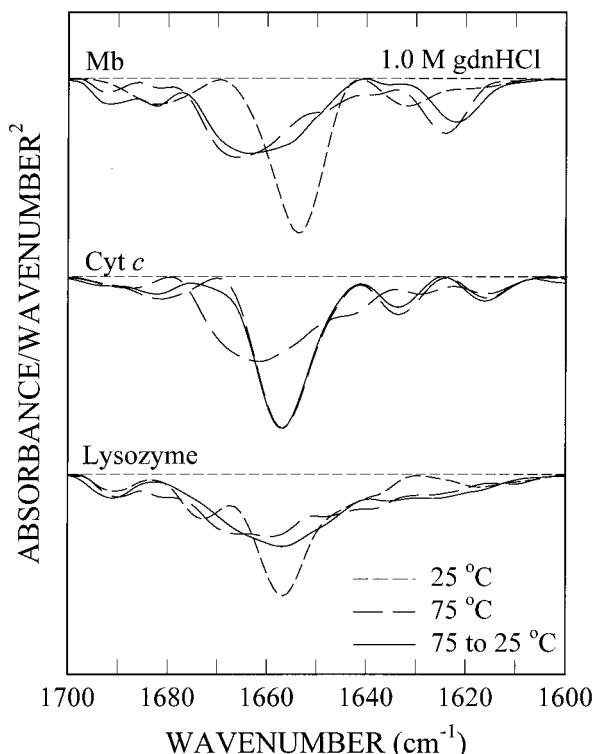


FIG. 5. Comparison of the second derivative amide I spectra of myoglobin (*Mb*), cytochrome *c* (*Cyt c*), and lysozyme in 1.0 M GdnHCl buffer measured at 25 and 75 °C and at 25 °C after being cooled from 75 °C.

strong amide I band component near  $1661\text{ cm}^{-1}$ . Our spectral analysis of  $\alpha$ -lactalbumin in  $\text{H}_2\text{O}$  agreed with the band assignment of  $1662 \pm 3\text{ cm}^{-1}$  to  $3_{10}$ -helix (data not shown). Based on this supporting evidence, we conclude that our current data document that in the presence of 1.0 M GdnHCl the conformations of the myoglobin, cytochrome *c*, and lysozyme undergo major structural changes from  $\alpha$ -helices to  $3_{10}$ -helices and then to a  $\beta$ -turn structure as temperature increases.

Fig. 5 shows the overlay of second derivative spectra of the three proteins in the presence of 1.0 M GdnHCl at 25 and 75 °C and after cooling from 75 to 25 °C. The result shows that the reversibility of the thermally induced transition depends on the conformation of the thermally perturbed state. Myoglobin structure at 75 °C contains intermolecular  $\beta$ -sheet and upon cooling does not revert to the native  $\alpha$ -helix conformation with or without 1.0 M GdnHCl. Cytochrome *c* in 1.0 M GdnHCl at 75 °C contains  $3_{10}$ -helices, which revert to native  $\alpha$ -helix upon cooling. When lysozyme is heated to 75 °C in 1.0 M GdnHCl, there is a conformational transition to a  $\beta$ -turn structure that is partially reversible upon cooling.

Fig. 6 shows a plot of the relative intensity of the  $\alpha$ -helix bands of three proteins with and without GdnHCl as a function of temperature. The intensities of  $\alpha$ -helix bands in the presence of GdnHCl were calculated at fixed frequency to discount effects of frequency shift. The result shows that, for all three proteins in the absence of GdnHCl, thermally induced loss of  $\alpha$ -helix occurs at temperatures between 65 and 80 °C with a midpoint around 72 °C. In the presence of GdnHCl, however, the structural transition starts at much lower temperature for all three proteins, especially for myoglobin. The midpoints of transition as monitored by the intensity changes at the  $\alpha$ -helix bands are about 65 °C for cytochrome *c* and lysozyme and about 40 °C for myoglobin.

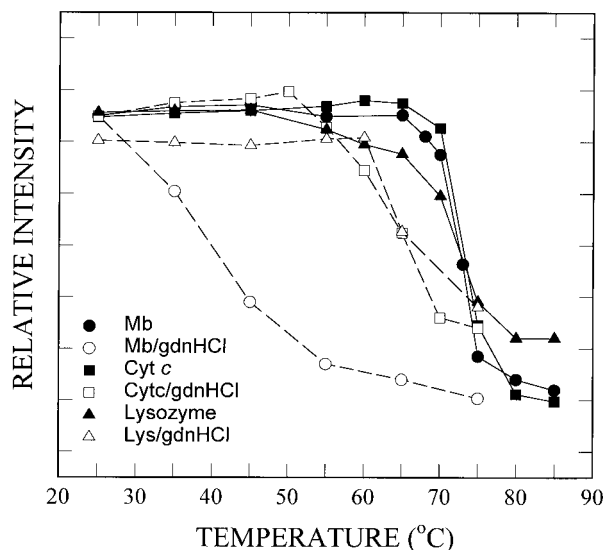


FIG. 6. Relative intensity changes at the amide I component assigned to the  $\alpha$ -helical structure as a function of temperature in the absence and presence of 1.0 M GdnHCl buffer. *Mb*, myoglobin; *Cyt c*, cytochrome *c*.

#### DISCUSSION

The thermally induced aggregation processes of the majority of the proteins studied by Fourier transform IR spectroscopy can be described with a two-state model. The predominant secondary structural element ( $\alpha$ -helix or  $\beta$ -sheet) decreases as a function of temperature and is concomitantly replaced by intermolecular  $\beta$ -sheet because of protein aggregation (16–18, 22, 39). The latter is evident by the appearance of a strong band near  $1624 \pm 8\text{ cm}^{-1}$  (low wave number  $\beta$ -sheet component) accompanied by a weak band near  $1693 \pm 5\text{ cm}^{-1}$  (high wave number  $\beta$ -sheet component). Intermolecular  $\beta$ -sheet structure is a common secondary structural element in the aggregated state of proteins (16–18, 22, 31, 32, 39). The formation of intermolecular  $\beta$ -sheet structure in thermally induced protein aggregates was clearly demonstrated by Clark and colleagues (40) using small angle x-ray scattering. Later, they reported a close relationship between the thermally induced intermolecular  $\beta$ -sheet aggregates and appearance of a new, well defined amide I band component near  $1620\text{ cm}^{-1}$  (31). More recently, Damaschun *et al.* (32) studied fibrils of phosphoglycerate kinase with small angle x-ray scattering and provided similar corroboration for assignment to the high and low wave number IR bands noted above to intermolecular  $\beta$ -sheet. Our data for the thermally induced structural transitions in myoglobin and cytochrome *c* in the absence of GdnHCl suggest a direct transition from the native conformation to intermolecular  $\beta$ -sheet aggregates, evidenced by the isosbestic points visible for both proteins (Figs. 1 and 2).

Lysozyme in the absence of GdnHCl also shows isosbestic evidence of a two-state transition (Figs. 1 and 2). The amount of  $\beta$ -sheet aggregate present in the thermally perturbed state of lysozyme is negligible, however, judging by the weak band near  $1625\text{ cm}^{-1}$  (low wave number  $\beta$ -sheet component). The major secondary structural elements in the thermally perturbed state of lysozyme are represented by two broad bands at 1657 and  $1685\text{ cm}^{-1}$ , assignable to the residual  $\alpha$ -helix and  $\beta$ -turn structure, respectively (15).

It is noteworthy that the random (unordered) structure observed in the thermally aggregated state of other proteins (16–18, 22, 39) is not a major element in the thermally perturbed state of the three  $\alpha$ -helix proteins studied here. Unordered structure is generally associated with an amide I com-

ponent at  $1645 \pm 4 \text{ cm}^{-1}$  for proteins in  $\text{D}_2\text{O}$  solution (12) and  $1648 \pm 2 \text{ cm}^{-1}$  for proteins in  $\text{H}_2\text{O}$  solution (15). The latter assignment is supported by the IR spectrum of the model compound poly-L-lysine in  $\text{H}_2\text{O}$  solution at neutral pH (38), in which the polypeptide is known to be a random coil (41–43). The lack of random coil as a major structural element in the thermally aggregated state has also been reported for the  $\beta$ -sheet predominant protein, recombinant human Factor XIII (29). We should point out that the so-called loop structure, which may be classified as part of random structure, could also contribute to the band intensity near  $1658 \text{ cm}^{-1}$  (14, 44, 45). Previous IR spectroscopic studies on superoxide dismutase have also shown that the random/loop structure is represented by a part of the  $1658 \text{ cm}^{-1}$  band (accounts for  $1/3$  of random/loop), in combination with a more prominent band at  $1647 \text{ cm}^{-1}$  (accounts for  $2/3$  of random/loop) (44). Furthermore, with existing IR spectroscopic data on a large number of proteins in  $\text{H}_2\text{O}$  solution,<sup>2</sup> a protein with a significant amount of random/loop structure that does not exhibit a major band in the  $1648 \pm 2 \text{ cm}^{-1}$  has not been observed. The spectra of the thermally perturbed states of proteins in the current study do not have a band in  $1648 \pm 2 \text{ cm}^{-1}$  region of their IR spectra. Thus, the remaining bands near  $1656 \text{ cm}^{-1}$  in their spectra are more likely associated with the residual  $\alpha$ -helix structure than with newly formed unordered/loop structure.

Addition of 1.0 M GdnHCl in conjunction with elevated temperatures results in significant accumulation of non-native species rich in  $3_{10}$ -helix and  $\beta$ -turn structures, which we suggest are intermediates between the native protein and aggregated states for myoglobin and cytochrome *c*. Similar structures are seen in lysozyme, although intermolecular  $\beta$ -sheet aggregates are not formed in the temperature range we tested. It is worth noting that similar spectral features have been previously observed in GdnHCl-induced unfolded state of iso-1-cytochrome *c* (46). Bowler and co-workers (46) reported that as the concentration of GdnHCl increased, the intensity of the  $1657 \text{ cm}^{-1}$  band of iso-1-cytochrome *c* because of  $\alpha$ -helix structure was lost gradually and replaced by three main features at 1687, 1666, and  $1660 \text{ cm}^{-1}$ , ascribable to  $\beta$ -turn,  $\beta$ -turn, and  $3_{10}$ -helix, respectively. Furthermore, Millhauser (47) has proposed a thermodynamic folding pathway for helical peptide: random coil  $\rightarrow$  nascent helix  $\rightarrow 3_{10}$ -helix  $\rightarrow \alpha$ -helix, after examining the equilibrium of  $3_{10}$ -helix/ $\alpha$ -helix from the perspective of crystallographic studies and spectroscopic data from double label electron spin resonance, nuclear magnetic resonance, and circular dichroism spectroscopies. In addition, molecular dynamics simulations have suggested that  $3_{10}$ -helices exist as kinetic folding intermediates of analogous  $\alpha$ -helical proteins (30, 48). Aggregation pathway intermediates containing  $3_{10}$ -helix and  $\beta$ -turn structures that we observe may also be the intermediates on a folding pathway. For many proteins, it has been found that aggregates are formed from intermediates on the folding pathway (reviewed in Ref. 23).

We have demonstrated in the present study that IR spectroscopic investigations of protein aggregation using a combination of thermal and chemical denaturing factors can provide a means to populate and characterize aggregation intermediates. This method should be valuable for studying the aggregation processes of a wide range of proteins. We speculate that identification and characterization of aggregation intermediates

may lead to new interdiction strategies for amyloidogenic human diseases, as well as to improvements in industrial processing, storage, and delivery of therapeutic proteins.

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