

Equilibrium titrations of acid-induced unfolding–refolding and salt-induced molten globule of cytochrome *c* by FT-IR spectroscopy

Aichun Dong*, Troy Lam

Department of Chemistry and Biochemistry, University of Northern Colorado, Greeley, CO 80639, USA

Received 27 November 2004, and in revised form 12 January 2005

Available online 25 January 2005

Abstract

Despite extensive investigations on the acid-unfolded and acid/salt-induced molten globule(-like) states of cytochrome *c* using variety of techniques, structural features of the acid-unfolded state in terms of residual secondary structures and the structural transition between the acid-unfolded and acid/salt-refolded states have not been fully characterized beyond the circular dichroism (CD) spectroscopy. It is unusual that secondary structure(s) of the unfolded state leading to the molten globule state, an important protein folding intermediate, as determined by CD was not fully corroborated by independent experimental method(s). In this study, we carried out an equilibrium titration of acid-induced unfolding and subsequent acid- and salt-induced refolding of cytochrome *c* using Fourier transform infrared spectroscopy. The spectral profiles of the equilibrium titration reveal new structural details about the acid-unfolded state and the structural transition associated with the acid/salt-refolded molten globule(-like) states of cytochrome *c*. © 2005 Elsevier Inc. All rights reserved.

Keywords: Cytochrome *c*; FT-IR spectroscopy; Secondary structure; A-state; Molten globule; Acid-induced unfolding; Acid-induced refolding; Salt-induced refolding

The molten globule, defined as a compact protein state with native-like secondary structures, but lacking well-defined tertiary structure [1], is one of the most widely studied folding intermediate states of proteins. It has been proposed that the molten globule state is an intermediate state common to many globular proteins [2–4]. As research data accumulate, it becomes apparent that the molten globule(-like) states often include a broad range of conformations and various degrees of partial folding–unfolding, subject to the solvent conditions employed. However, the molten globule is most frequently observed at low pH conditions [5]. The folding process leading to the compact state is postulated to be driven primarily by anions binding to the positively charged groups and minimizing the intramolecular charge repulsion, in turn favoring intrinsic hydrophobic

interactions [5,6]. The net charge of the anions employed is the key determining factor for the formation of the molten globule state; the higher the charge, the lower the anion concentration may be required to produce the compact state [6]. Recently, a different scenario has been proposed to play important role in the formation of the molten globule state: the intermolecular excluded volume (macromolecular crowding) [7,8]. The evidence indicated that non-specific solutes, such as sugars, alcohols, and uncharged polymers, could also trigger a compact molten globule conformation by steric repulsion.

Cytochrome *c* is one of the most extensively studied proteins with respect to its molten globule state. Equine cytochrome *c* is an α -helix predominant monomeric protein consisting of 104 amino acid residues. It has a heme group covalently bound to Cys14 and Cys17 and axially ligated with His18 and Met80 in its native state [9]. It contains three major helices (N-helix, 60's helix and

* Corresponding author. Fax: +1 970 351 1269.

E-mail address: Aichun.Dong@unco.edu (A. Dong).

C-helix) and two minor helical elements connected by loops [9]. Variety of experimental techniques have been used to characterize the structural, physical, and biochemical properties of the molten globule state of cytochrome *c*, including circular dichroism (CD) [5,6,10,11], Fourier transform infrared (FT-IR)¹ [12], Raman [13], fluorescence [14,15], NMR [1,16,17], UV-Vis [18], mass spectroscopy [19,20], isothermal titration calorimetry [21], differential scanning calorimetry [22], and small angle X-ray scattering [23,24], among the others.

Surprisingly, while diverse techniques including FT-IR spectroscopy were used to characterize the molten globule state, few investigators employed experimental techniques other than Far-UV CD in characterizing the acid-unfolded state and monitoring the course of acid/salt-induced structural transitions of the protein in terms of secondary structures. It is unusual that the key unfolding/refolding process leading to the formation of molten globule(-like) state has not been fully characterized by techniques other than the CD spectroscopy. With growing popularity of FT-IR spectroscopy in protein conformation and structural dynamics analysis [25], it is highly desirable that the acid-unfolded state and acid/salt-induced conformational changes as reported by CD spectroscopy be fully characterized by FT-IR spectroscopy, thus providing important reference for the application of FT-IR spectroscopy in studies of folding/unfolding of proteins in general, as well as providing new insight into the acid-unfolded state of cytochrome *c*.

FT-IR spectroscopy has proven to be a powerful tool for providing conformational and structural dynamics information of proteins in the native and/or denatured states under various physical conditions (e.g., aqueous, film or dry solid) that are not readily available from other methods [25]. The protein amide I band (1700–1600 cm⁻¹), arising primarily from the C=O stretching vibration of the peptide backbone with a small contribution from an out-of-phase C–N stretch and a C–C–N deformation [26], is known to be extremely sensitive to secondary structural composition and conformational changes induced by various factors, such as temperature [25], oxidation state [27], ligand-binding [28], and chemical denaturants [29]. With the aid of second-derivative or Fourier self-deconvolution analysis, 14 underlying amide I band components, arising from various secondary structural elements and variations within the same structural group, can be resolved [30,31]. The fine resolution of the protein amide I spectrum could provide detailed information about the secondary structural composition and structural transitions of proteins [25,31].

The acid-unfolded state of cytochrome *c* is known to be extremely sensitive to salt, with even small amounts of

the oxidizing agent potassium ferricyanide preventing reaching the maximum unfolded state [5]. However, it is not clear whether or not the difference in sampling concentrations between CD and FT-IR affects the degree of unfolding due to the effect of macromolecular crowding. The sampling concentrations used in Far-UV CD studies were generally in the range of 2–50 μM [5], whereas the cytochrome *c* concentration used in this study is 1.18 mM (~15 mg/mL), a typical sampling concentration for FT-IR spectroscopic analysis of proteins. Several questions have to be addressed. Could the acid-induced unfolding and refolding of cytochrome *c* as reported by CD studies also be observed by FT-IR under similar experimental conditions? To what extent does the difference in sampling concentration affect the protein conformation in the unfolded state? We report here the results of equilibrium titrations of HCl-induced unfolding and refolding (A-state) at low ionic strength and KCl-induced refolding (molten globule) of cytochrome *c* using FT-IR spectroscopy. The results provide new insight into the course of structural transitions of cytochrome *c* induced by hydrochloric acid at low ionic strength and induced by potassium chloride in acidic condition.

Materials and methods

Materials

Cytochrome *c* (Horse heart, C-7752), being essentially in the oxidized form, was purchased from Sigma (St. Louis, MO) and used without further purification. Reagent grade hydrochloric acid, potassium chloride, and phosphate-buffered saline (10 mM phosphate buffer, 120 mM NaCl, and 2.7 mM KCl) (PBS) are all products of Sigma-Aldrich (St. Louis, MO).

Sample preparation

Two different procedures were used to prepare cytochrome *c* solutions with similar results obtained from both procedures. The first procedure follows the sample preparation described previously by Goto et al. [6] with minor modification. Cytochrome *c* solution was first dialyzed extensively against double-distilled water at 4 °C and then concentrated by centrifugation using a Centricon-10 microconcentrator at 4000g. The protein concentration was determined spectrophotometrically using the extinction coefficient of 1.06 × 10⁵ M⁻¹ cm⁻¹ at 410 nm [32]. The acid titration was carried out by direct acid titration using concentrated (6M) HCl stock solution [6,33]. The final concentration of the protein was 1.18 mM (~15 mg/mL). In the second procedure cytochrome *c* solution was prepared by dissolving 30 mg of lyophilized protein powder in 1.0 mL of deionized H₂O.

¹ Abbreviations used: FT-IR, Fourier transform infrared; CD, circular dichroism; gdnHCl, guanidine hydrochloride.

For the acid titration, 0.2-mL aliquot protein samples were loaded into 0.5-mL Slide-A-Lyzer 7K dialysis cassettes (Pierce, Rockford, IL) and dialyzed against an HCl-titrated deionized H₂O solution at various pHs at 4°C for 24 h. For the salt titration, a 0.3 mL sample was loaded into 0.5-mL Slide-A-Lyzer 7K dialysis cassettes and dialyzed in an HCl-titrated H₂O solution at pH 2.0 at 4°C for 24 h. The pH of dialysis solutions was determined using an Accumet pH meter Model-910 (Fischer Scientific, Hampton, NH). Protein samples extracted from the dialysis cassettes were titrated with a 2.0 M KCl stock solution. The final concentration of the protein samples was 1.18 mM (~15 mg/mL).

FT-IR spectroscopy

Infrared spectra were measured using an ABB Bomem (Que., Canada) MB-104 Fourier transform infrared spectrometer. Protein samples were loaded in a heatable IR cell (P/H2500) with CaF₂ windows and a 7.5- μ m polyimide spacer (Chemplex). For each spectrum, a 128-scan interferogram was collected in a single beam mode with a 4 cm⁻¹ resolution. The reference spectrum was recorded under identical scan conditions with only the corresponding dialysis solution or dialysis solution/KCl in the cell. The protein spectra were obtained according to previously described criteria and water subtraction procedures [30,31]. The residual water vapor signals, if present, in the spectrum of protein were removed by subtracting the spectrum of gaseous water. Second-derivative spectra were obtained with a seven-point Savitsky–Golay derivative function. All second-derivative spectra were baseline corrected as previously described [25]. Final spectra were treated with a 2 \times FFT interpolate function and plotted with SigmaPlot 8.0 software (Systat Software Inc.). Secondary structural contents of selected cytochrome *c* samples were determined by the curve-fitting analysis of the inverted second-derivative spectrum as described previously [34].

Results

Fig. 1 shows the primary spectra of cytochrome *c* recorded at pHs 7.0, 3.0, 2.3, 2.1, and 2.0, respectively. The major peak between 1700 and 1600 cm⁻¹ is the so-called amide I band, composed of several underline components arising from various secondary structural elements, such as α -helix, β -sheet, β -turns, and random coil [26,35] and thus sensitive to the conformational changes of proteins [25,36]. For the spectra obtained at low pHs, the weak bands centered around 1720 cm⁻¹ arise from the protonated carboxyl side chain vibrations of glutamic and aspartic acid residues [26,37]. Equine cytochrome *c* contains nine glutamic acid and three

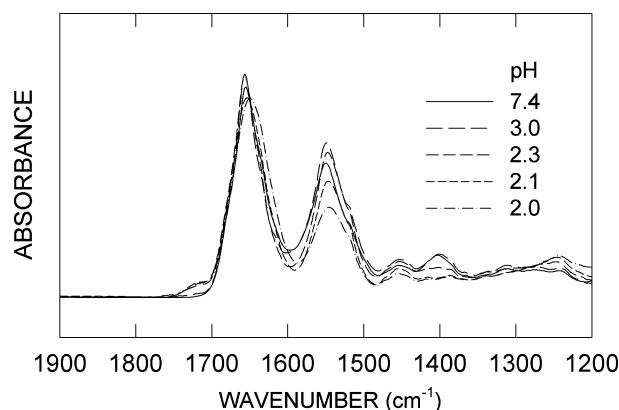


Fig. 1. The original infrared spectra of cytochrome *c* recorded at pHs 3.0, 2.2, 2.1, and 2.0 and low ionic strength. The spectrum of cytochrome *c* in PBS buffer (pH 7.4) is included for comparison. The spectra of corresponding solvent references were subtracted from the spectra of the protein as described under “Materials and methods.”

aspartic acid residues [9]. The bands corresponding to the deprotonated carboxyl side chains of those residues located near the 1580 cm⁻¹ region [26,37,38], overlapping with the amide II bands (1600–1500 cm⁻¹) arising from an out-of-phase combination of N–H in-plane bending and C–N stretching vibrations of peptide linkages [26]. As the pH lowered, the intensities of the amide I bands gradually decreased, accompanied by the frequency shifting from ~1657 cm⁻¹ to a slightly lower wavenumber, indicating the structural transition from an α -helix predominant to a random coil rich conformation [30,31]. More detailed information about the acid-induced structural transition (unfolding) can be provided by the second-derivative analysis of these spectra [30,31].

Acid-induced unfolding

Fig. 2 shows the second-derivative spectra of acid-induced unfolded cytochrome *c* between pH 3.0 and 2.0 in the amide I region. The second-derivative spectrum of cytochrome *c* obtained at pH 3.0 is similar to that obtained at pH 7.0 with minor differences (lower panel). The band components revealed by the second-derivative analysis can be assigned to α -helix (1657 cm⁻¹), β -extended structure (1633 cm⁻¹), β -turns (1675 and 1682 cm⁻¹), and random coil (1648 cm⁻¹), respectively [30,31]. Large spectral changes were observed at pH conditions below 3.0, especially near 2.0. The relative intensities of the 1657 cm⁻¹ band (α -helices) decreased as the pH lowered, accompanied by a band intensity increase near 1650 cm⁻¹ (random coil) and between 1690 and 1670 cm⁻¹ region (β -turns). The acid-induced shift in the absorbance maximum is most prominent near pH 2.0, indicating a major structural transition from an α -helix predominant to a random coil rich conformation [30,31]. The results are generally consistent with those reported by Far-UV CD studies [6–8,12].

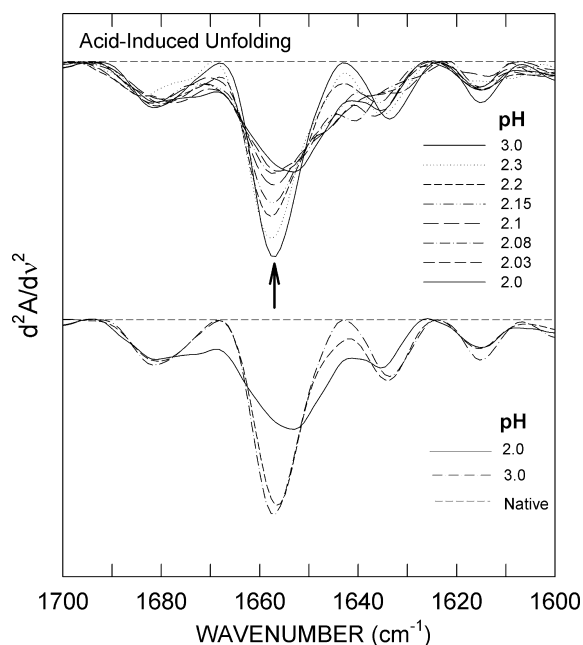


Fig. 2. The second-derivative amide I spectra of cytochrome *c* recorded at pHs 3.0, 2.3, 2.2, 2.15, 2.1, 2.08, 2.03, and 2.0. The arrow indicates the direction of spectral changes as a function of pH. The second-derivative spectrum of the native state (in PBS) is included for comparison (lower panel).

For more detailed structural information, the curve-fitting analysis was carried out on the spectra measured at pH 2.0 and 7.0 (Fig. 3). The parameters of the curve-fitting analysis are listed in Table 1. The results show that the α -helical structure content of cytochrome *c* decreased from 51.6% at pH 7.0 to 37.4% at pH 2.0, while the random coil content increased from 15.4% at pH 7.0 to 29.8% at pH 2.0. The remainder of the α -helices at pH 2.0 experienced a highly heterogeneous microenvironment, evidenced by a broad half-bandwidth for the 1658 cm^{-1} band, indicating the loss of contact/interaction among the peptide segments. This result is consistent with the loss of tertiary structure under this condition. However, despite a dramatic increase in the random coil structure content, all regular secondary structural elements (α -helix, β -extended and β -turns) were observed at pH 2.0, the maximum unfolding pH condition for cytochrome *c* [5]. These results suggest that even at pH 2.0, cytochrome is not fully unfolded.

Acid-induced refolding (the A-state)

Fig. 4 shows the second-derivative spectra of acid-titrated cytochrome *c* below pH 2.0 in the amide I region. As the pH value of the protein solution decreased below pH 2.0, the direction of intensity change in the α -helix band near 1657 cm^{-1} reversed, suggesting an anion-induced structural transition from an acid-unfolded state to a molten globule-like state (A-state) [1,6]. At pH 0.5, the α -helices were fully

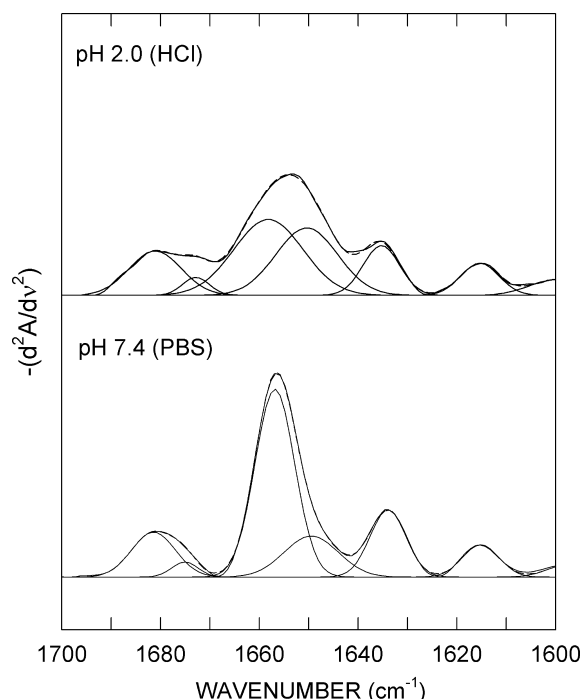


Fig. 3. Curve-fitted inverted second-derivative amide I spectra of cytochrome *c* recorded at pHs 2.0 and 7.4. The inversion of second-derivative spectra was done by factoring by -1 . The curve fitting was carried out as previously described [34].

Table 1

Comparison of the curve-fitted infrared second-derivative amide I spectra of cytochrome *c*: the acid-unfolded and native states^a

pH 2.0/HCl		pH 7.4/PBS		Assignment
ν (cm^{-1})	Area (%) ^b	ν (cm^{-1})	Area (%)	
1681	16.4	1682	13.5	β -turn
1673	3.5	1675	2.8	β -turn
1658	37.4	1657	51.6	α -helix
1650	29.8	1649	15.4	Random
1635	12.9	1633	16.7	β -strand (extended)
1615	n/a	1615	n/a	Side chains

^a The frequencies are rounded up to the nearest whole numbers.

^b The accuracy of the second-derivative method used to estimate the protein secondary structure is $\pm 5\%$ [25,30].

refolded, which is evidenced by the recovery of the 1657 cm^{-1} band (lower panel, Fig. 4). However, the band assigned to the β -strand structure remained near 1640 cm^{-1} , similar to that of the partially unfolded state, especially at pH 2.08 (Fig. 2).

Fig. 5 shows a plot of the relative intensity change at the 1657 cm^{-1} band (α -helix) of cytochrome *c* as a function of pH. The intensity of the α -helix band was calculated as a difference in the second-derivative spectra between the negative peak at maximum and the baseline. The values of relative intensities were normalized using the intensity value measured at pH 3.0 as 100%. It clearly shows that the acid-induced unfolding reaches its maximum at pH 2.0 as monitored by its predominant secondary structural component, the α -helix structure.

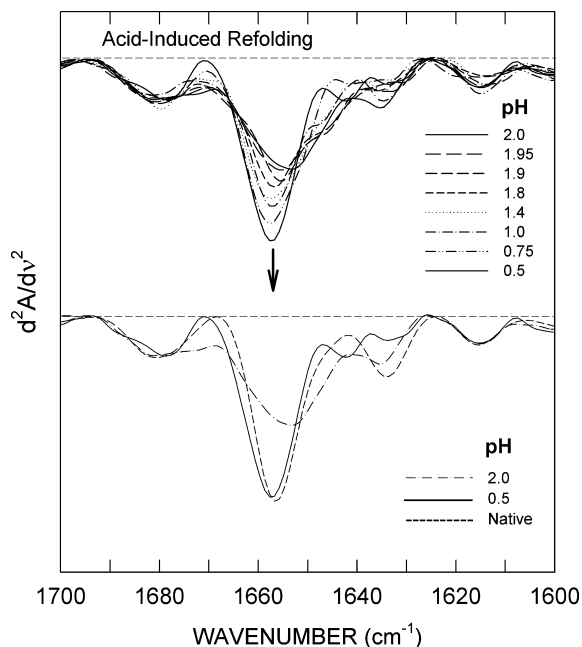


Fig. 4. The second-derivative amide I spectra of cytochrome *c* recorded at pHs 2.0, 1.95, 1.9, 1.8, 1.4, 1.0, 0.75, and 0.5. The arrow indicates the direction of spectral changes as a function of pH. The second-derivative spectrum of the native state (in PBS) is included for comparison (lower panel).

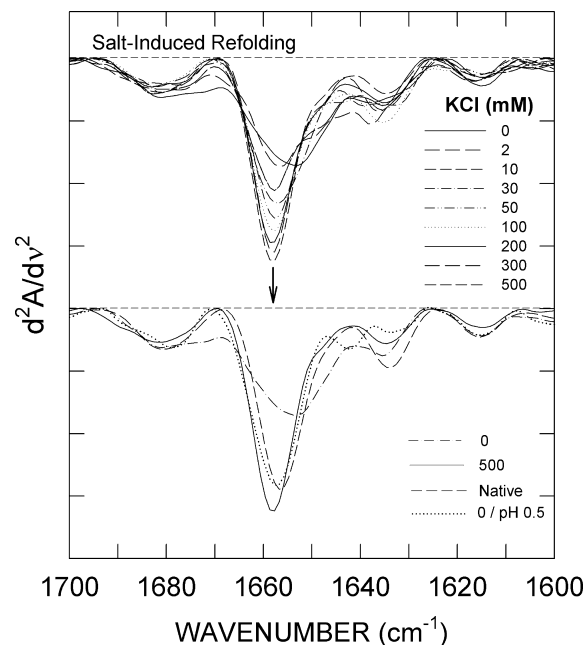


Fig. 6. The second-derivative amide I spectra of cytochrome *c* recorded at pH 2.0 as a function of KCl concentration (from 0 to 500 mM). The arrow indicates the direction of spectral changes. The spectra of the native state (in PBS) and the acid-refolded state at pH 0.5 without KCl are included for comparison (lower panel).

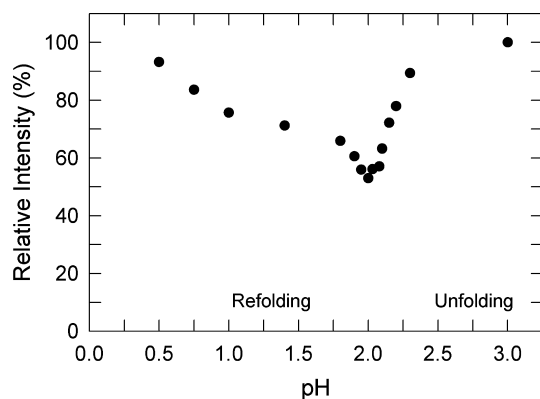


Fig. 5. Acid-induced structural transition associated with unfolding and refolding of cytochrome *c* as revealed by pH-dependent changes in the relative intensity of α -helix bands at 1657 cm^{-1} . The values of relative intensities were calculated using the α -helix band intensity measured at pH 3.0 as 100%.

Salt-induced refolding (the molten globule state)

Fig. 6 presents the second-derivative spectra of KCl-titrated cytochrome *c* at pH 2.0 in the amide I region. The conformation of cytochrome *c* in terms of secondary structures is extremely sensitive to changes in salt concentration of the solution at pH 2.0. The protein refolded quickly as the salt concentration increased and reached its maximum refolded conformation (molten globule state) at 0.5 M KCl. The α -helix band of the mol-

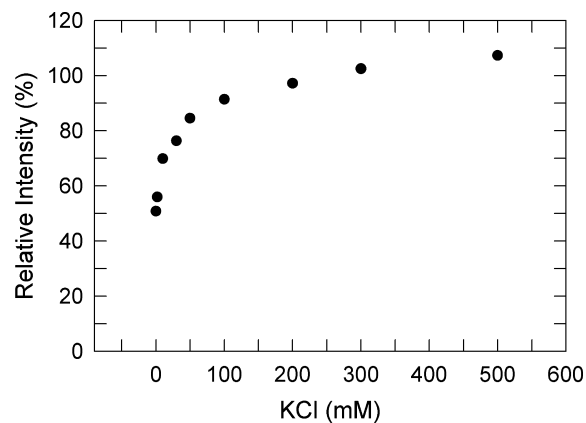


Fig. 7. Salt-induced refolding of cytochrome *c* at pH 2.0 as revealed by pH-dependent changes in the relative intensity of α -helix bands at 1658 cm^{-1} . The values of relative intensities were calculated using the α -helix band intensity at 1657 cm^{-1} measured at pH 3.0 as 100%.

ten globule state has a slightly higher frequency than that of the native state (1658 cm^{-1} vs 1657 cm^{-1}). The salt-induced refolding process can be further visualized by the relative intensity change at 1658 cm^{-1} as the recovery of the α -helical structure (Fig. 7).

Discussions

The acid-induced unfolding/refolding and salt-induced refolding of cytochrome *c* have been studied extensively in the past decade [6,12,33]. It is well known

that under low ionic strength conditions, the acid-induced unfolding of cytochrome *c* occurs in a single cooperative transition between pHs 3.0 and 2.0, accompanied by the loss of axial Met80-heme ligation [32] and reaches its maximum around pH 2.0 [33]. Further decreases in pH result in a compact, folded state referred to as the A-state [1]. A similar compact state, referred to as molten globule state, could also be formed by the addition of salts and/or uncharged molecules under acidic conditions [1,7,8,21].

It is well known that the extent of acid-induced unfolding of cytochrome *c* is extremely sensitive to the ionic strength of the protein solution [5]. Simple treatment by potassium ferricyanide for the purpose of fully oxidizing the protein could prevent achieving the maximum unfolded state, even after extensive dialysis, due to strong interaction between the ferricyanide ions and cytochrome *c* [5]. With all precautions to avoid any unwanted interference, detailed spectral analysis of the maximum unfolded cytochrome *c* at pH 2.0 reveals a much higher content of regular secondary structural elements (37.4% α -helix, 12.9% β -strand, and 19.9% β -turns) than those reported by Far-UV CD studies under comparable experimental conditions, which gave only 4% residual α -helix and no other regular secondary structure [6]. There are two most likely explanations for the apparent discrepancy between the two results. First, it is possible that a portion of the random coil/loop structure adapted a band frequency similar to that of the α -helical structure [27,39] as those found in Cu,Zn superoxide dismutase [27]. If this was the case, the percentage of random coil should be higher than 24.1% and the content of α -helix would be lowered. Second, the higher sampling concentration used in FT-IR spectroscopic analysis (1.18 mM for IR vs 2.0–50 μ M for CD) may result in some degree of self-stabilization in the protein conformation due to the effect of macromolecular crowding [8,40]. These two factors could affect, individually or collectively, the contents of α -helix and random coil in the maximum unfolded state. However, it is difficult to assess the extent of macromolecular crowding effect on the residual secondary structures of the acid-unfolded state. Dedmon et al. [41] reported that it takes ≥ 400 g/L of glucose, bovine serum albumin, or ovalbumin to produce conformational change from a disordered to ordered form in the intrinsically disordered FlgM protein.

It is noteworthy that significant amounts of β -strand (12.9%) and β -turn (19.9%) were also found in the acid-unfolded state, suggesting that the acid-induced unfolding of cytochrome *c* was incomplete and retained significant amounts of non-random coil structural elements. A similar conclusion has also been reached for CD studies when the spectral comparisons were made between the CD spectra of unfolded states induced by denaturing agents (gdnHCl and urea) and by acids

[42,43]. However, no further structural details could be provided by Far-UV CD spectroscopic analysis due to the interference of denaturing agents on the spectra. In an earlier study, Bowler et al. [29] monitored gdnHCl-induced unfolding of iso-1-cytochrome *c* in 20 mM Tris (pH 7.5) using FT-IR spectroscopy and reported that at ≥ 1.5 M gdnHCl, the spectra of fully unfolded iso-1-cytochrome *c* contain multiple bands in the amide I region assignable to β -turns (1687 and 1666 cm^{-1}) and 3_{10} -helix (1660 cm^{-1}). More importantly the second-derivative spectra of the protein continues to change after the unfolding transition, indicating that the structure of the unfolded state is dynamic and continue to change as the concentration of gdnHCl increases [29]. The spectral differences between the acid-unfolded equine cytochrome *c* (Fig. 3) and the gdnHCl-unfolded iso-1-cytochrome *c* are apparent. The former contains all secondary structural elements (α -helix, β -strand, β -turns, and random coil) and the latter contains only the β -turns and 3_{10} -helix [29].

Similar, but not identical, second-derivative amide I spectra for the acid-refolded state (A-state) and salt-refolded state (molten globule) were observed. The restoration of α -helical structure is concentration dependent in both the acid- and salt-refolded states. At the end of refolding, each state contains a large amount of α -helical structure, which is comparable to the native state (Figs. 4 and 5). These results are generally consistent with those reported by Far-UV CD studies [5].

The infrared protein amide I band, the most informative spectral region, provides mainly the conformational information on the protein secondary structures, such as α -helices, β -sheets, β -turns, and random coils [26,36]. However, information about the protein conformational changes at tertiary structural levels may also be derived from spectral changes in the β -turn region (1660–1688 cm^{-1}). Unlike other secondary structural elements, the β -turn structures are closely associated with the tertiary structure of proteins [44,45]. Conformational changes in the tertiary structural level are undoubtedly accompanied by structural changes at β -turns, which in turn will be reflected in the spectral changes at the region assignable to the β -turns. For example, the tertiary structural changes in hemoglobin [46] and myoglobin [47] associated with the changes in redox state and ligand-binding are clearly reflected in the spectral change at the β -turn region of the FT-IR spectra [44]. The spectral changes at the β -turn region associated with conformational changes at tertiary structural level may be revealed as an alteration in percentage content of β -turn structures, changes in band frequencies, half-bandwidth and/or numbers of band components [44,45]. It is clear from the present study that the unfolding and refolding of cytochrome *c* have affected the β -turn structures in both content and number of components (Figs. 3 and 5). A significant increase in the percentage content of β -turns

was observed in the spectrum of the acid-unfolded state (19.9% at pH 2.0 vs 16.3% at pH 7.4). The acid- or KCl salt-refolded states exhibited a β -turn band near 1686cm^{-1} , which is 4cm^{-1} higher than that of the native state, indicating that the refolding of the secondary structure is not accompanied by the restoration of the tertiary structure. This conclusion is in agreement with those arrived by near-UV CD [5,48], hydrogen exchange/2D NMR [49], and X-ray scattering [50] studies.

Conclusions

Although the molten globule state of cytochrome *c* has been extensively characterized, the results of this study may have many important implications. First, this study provides strong secondary structural evidence confirming the acid- and acid/salt-induced structural transition processes reported by CD spectroscopy. Second, using a well-characterized model protein, this study provides an important spectral reference for FT-IR spectroscopic studies of molten globule(-like) states of proteins in general. Third, this study also provides a substantial amount of detail about the residual structures of the acid-unfolded state of cytochrome *c* that cannot be easily obtained by other techniques due to the dynamic nature of the unfolded state.

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