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

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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*.

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

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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 Centracon-10 microconcentrator at 4000*g*. The protein concentration was determined spectrophotomically using the extinction coefficient of $1.06 \times 10^5\ M^{-1}\,cm^{-1}$ at 410 nm [32]. The acid titration was carried out by direct acid titration using concentrated (6 M) 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 H_2O.

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