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Direct observation of the enthalpy change accompanying the native to molten-globule transition of cytochrome *c* by using isothermal acid-titration calorimetry

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Abstract

The enthalpy change accompanying the reversible acid-induced transition from the native (N) to the molten-globule (MG) state of bovine cytochrome *c* was directly evaluated by isothermal acid-titration calorimetry (IATC), a new method for evaluating the pH dependence of protein enthalpy. The enthalpy change was 30 kJ/mol at 30 °C, pH 3.54, with 500 mM KCl. The results of the global analysis of the temperature dependence of the excess enthalpy from 20 to 35 °C demonstrated that the N to MG transition is a two-state transition with a small heat capacity change of 1.1 kJ K^{-1} mol^{-1}. The present findings were also indicative of the pH dependence of the enthalpy and the heat capacity of the MG state, −13 kJ mol^{-1} pH^{-1} and −1.0 kJ K^{-1} mol^{-1} pH^{-1}, respectively, at 30 °C within a pH range from 2 to 3.

Keywords: Molten-globule state; Cytochrome *c*; Isothermal acid-titration calorimetry; Heat capacity change; Enthalpy change; pH transition

1. Introduction

Molten-globule (MG) state, which is structurally distinct from both the native (N) and denatured (D) states, was originally proposed as an intermediate state of some proteins with a compact conformation, a considerable native-like secondary structure, and a largely fluctuating tertiary structure [1]. The thermodynamically stable MG states have already been reported for cytochrome *c*, α-lactalbumin, and various kinds of proteins (see Ref. [2], for a review). Transient MG states, which have structural features resembling those of the stable MG state, have already been observed in the folding pathways of a number of proteins [3–5]. The MG state is an important target in order to gain a better thermodynamic understanding of the mechanisms associated with protein stability and folding.

Cytochrome *c* is a protein whose stable and transient MG states have been investigated to the greatest extent. The structural properties of the stable MG state have been evaluated primarily under acidic conditions and high salt concentrations. Moreover, a wide range of approaches have been used to investigate the MG states of cytochrome *c*, namely, absorbance, circular dichroism (CD), fluorescence, nuclear magnetic resonance (NMR), viscosity, small angle X-ray scattering (SAXS), as well as other methods [1,2,6–13]. The results from such studies have indicated that the MG state of cytochrome *c* is stabilized at low temperatures, acidic pH, and with a high anion concentration.

The best means of evaluating the thermodynamic stability of MG states is the direct observation of enthalpy change by highly sensitive scanning calorimetry (DSC). However, to date, no studies have investigated the thermal transition of cytochrome *c* from the N to the MG state using this method, most likely because the small enthalpy change between these states enables no clear thermal transition. Therefore, the basic thermodynamic properties of this transition remain to be

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described; for example, the cooperativity of the N to MG transition and the heat capacity change accompanying this transition are not yet well understood. Although the transition from the N to MG state using a weak salt denaturant was reported recently by using spectroscopic methods [15], the validity of the two-state model for describing the transition between the N and MG states, which has provided the basis for analysis of the spectroscopic data, remains equivocal. On the other hand, the transition from the MG to the denatured state was easily observed previously by DSC [8,16] because of the large enthalpy change between these states. Salt-induced formation from the acid-denatured state to the MG state was also easily evaluated by isothermal titration calorimetry (ITC) [16].

Isothermal acid-titration calorimetry (IATC) has been recently proposed as a new method for evaluating the enthalpy of protein molecules as a function of pH using ITC [17]. The pH-induced structural transition of bovine ribonuclease A was clearly observed by IATC, and both the van't Hoff enthalpy and calorimetric enthalpy were determined according to this method, which enabled identification of the two-state transition. The heat capacity change accompanying the transition of bovine ribonuclease A from the N state to the D state was also able to be determined using this method.

In the present study, the acid-induced transition of cytochrome *c* from the N to the MG state was monitored using this new method in order to clarify the thermodynamic properties.

2. Materials and methods

2.1. Cytochrome c solution

Lyophilized powder of bovine cytochrome *c* (c-3131; Sigma, St. Louis, MO, USA) was dissolved as a 0.5 mg/ml solution with 20 mM and 500 mM KCl. This protein solution was dialyzed with a dialysis membrane, Spectra/Por (132660; Spectrum Lab., Rancho Dominguez, CA, USA) the cutoff molecular weight of which was 6000–8000 at 4 °C for 4 days against 2 l of 20 and 500 mM KCl solution with several solution exchanges. Before acid titration was performed, 50 mM NaOH (Wako, Osaka, Japan) was added to the protein solution in order to adjust the pH to 6.5–7.5. The pH measurement was carried out with a glass electrode and a F23 pH meter (Horiba, Kyoto, Japan). The reading of the pH values was corrected by using standard pH solutions of pH 2, 4, and 7 (Horiba) at the temperature of the calorimetric measurement. The protein solution was ultra-filtrated with a MolCut ultra filter unit (USY-20; Advantec, Tokyo, Japan), the cutoff molecular weight of which was 200 kDa in order to remove any aggregate that might have been produced during dialysis. The concentration of bovine cytochrome *c* was determined spectrophotometrically with a UB-35 spectrophotometer (Jasco, Tokyo, Japan) and using an extinction coefficient of $\varepsilon_{409}=9.197\times10^4\ M^{-1}cm^{-1}$. Complete degassing of the solution was performed for several minutes by aspiration with a ULVAC membrane pump (Sinku Kiko, Kanagawa, Japan) and the solution was simultaneously sonicated with a small sonication device, Perl Clean (Fkk, Tokyo, Japan). The 20–400 mM HCl solution with 20 and 500 mM KCl was created by the dilution of 1 M HCl (Nacalai Tesque, Kyoto, Japan). Guanidine hydrochloride (GnHCl) (Nacalai Tesque) was used for the circular dichroism spectroscopy of the denatured state.

2.2. Circular dichroism spectroscopy

Circular dichroism (CD) spectra from 210 to 320 nm were measured with a J-600 spectropolarimeter (Jasco) by using 2-mm path-length quartz cells. The same protein solution was used for the CD measurements, as was the case for the isothermal acid-titration calorimetry. The temperature of the cell was controlled by circulating water from a RC6 thermostat water bath (Lauda, Germany) around the cell holder. The reversibility of the transition from the native to the MG state was checked by re-measuring the CD spectra and by returning the pH of the solution to 5 after the measurement of the MG state spectrum at pH 2.3. The spectrum agreed well with that of the native state, indicating the full reversibility of the transition (data not shown).

2.3. Differential scanning calorimetry

Differential scanning calorimetry (DSC) experiments of cytochrome *c* were performed by using a highly sensitive differential scanning calorimeter, the VP-DSC (Microcal, Northampton, MA, USA). The lyophilized powder of the protein was dissolved as a 1.0 mg/ml solution in either a pH 4.5, 50 mM sodium acetate/HCl buffer in 500 mM KCl, or in a pH 2.5, 50 mM glycine buffer in 500 mM KCl. The solution was dialyzed in the same manner as that described above. The apparent heat capacity was analyzed using a two-state model and a nonlinear least-squares method, as reported previously [18,19]. In the present analysis, the heat capacity functions for the native and the denatured states were approximated by linear functions of temperature.

2.4. Isothermal acid-titration calorimetry

Isothermal acid-titration calorimetry (IATC) is a method for evaluating the enthalpy of protein molecules as a function of pH using isothermal titration calorimetry [17]. The measurement and analysis of IATC are briefly summarized as follows. First, in order to determine the enthalpy function, the observed heat detected by ITC was accurately corrected for by using the observed pH dependence of the acid dilution heat. Second, the two respective enthalpy functions for before and after the transition were estimated by using the appropriate pH functions. Third, in order to determine the thermodynamic stability of the