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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

protein molecule, the appropriate pH function was introduced to describe the Gibbs free energy change between these two thermodynamic states.

In this study, the HCl-titration was measured with an isothermal titration calorimeter, the two ITC units of an MCS (MicroCal) with a cell volume of 1.368 and 1.344 ml. Titration was carried out with injections of 2, 5, and 10 μl of 20–400 mM HCl solution in 500 mM KCl using a 250 μl syringe. The control experiment was performed with control solution in the absence of protein.

We determined whether or not the equilibrium after the acid titration was reached by performing an experiment designed to elicit two different "pH scan rates". The slow pH scan rate was 10-fold slower than the normal rate, and was realized by making the duration between injections 10-fold that of the duration between normal injections. The results of the slow and the normal IATC experiments were in good agreement (data not shown). This good agreement between the IATC experimental results obtained using two different scan rates also demonstrated that the experimental system had reached equilibrium after each injection and that full reversibility of the transition was achieved, which was also confirmed by the CD measurements, as mentioned above.

The pH measurement of HCl-titration with cytochrome *c* was performed by using a glass electrode and an F23 pH meter (Horiba). The solution used for the pH measurements was identical to that used for the calorimetric measurements. For the pH measurements, the initial volume of the protein solution was 10 ml, and the injection volumes were determined as the ratios between the initial and the injection volumes became identical to those of each corresponding injection in the ITC measurements. Because the calorimetry cell has a fixed mixing volume, the concentrations of the protein and the ligand in the cell were slightly different from those in the vessel for pH measurement. However, we have confirmed that the pH difference does not exceed 0.02 pH unit in the present condition. The temperature of the titration vessel was kept constant in a hand-made glass-bath with circulating water from a VM-150 thermostat water bath (Advantec). Three-point calibration was carried out using standard pH solutions of pH 2, 4, and 7. The observed pH values of the protein solution were corrected with the second-order polynomial function, the coefficients of which were determined by using the pH values of three standard solutions at the experimental temperature.

2.5. Two-state global fitting analysis

In our previous paper [17], the enthalpy function of pH at one temperature was analyzed by using the two-state model. In this study, we used a two-state global fitting method based on the thermodynamic relationship between the temperature and the pH dependence of the thermodynamic functions; this approach was chosen in order to more accurately analyze the temperature dependence of the thermodynamic functions.

The derivative of the enthalpy change with temperature shows the heat capacity change in the following equation:

$$\left(\frac{\partial \Delta H}{\partial T}\right)_{\mathrm{pH}} = \Delta C_{\mathrm{p}}. \tag{1}$$

The derivatives of the Gibbs free energy with temperature and pH are shown by the following equations:

$$\left[\frac{\partial}{\partial T}\left(\frac{\Delta G}{T}\right)\right]_{\mathrm{pH}} = -\frac{\Delta H}{T^2} \tag{2}$$

$$\left(\frac{\partial \Delta G}{\partial \mathrm{pH}}\right)_{T} = \ln 10 RT \Delta v, \tag{3}$$

where R, T, and Δv indicate the gas constant, the absolute temperature, and the proton-binding number difference for the transition, respectively. The following Eq. (4), a Maxwell relation, can be derived from Eqs. (2) and (3).

$$\left(\frac{\partial \Delta H}{\partial \mathrm{pH}}\right)_{T} = -\ln 10 RT^2 \left(\frac{\partial \Delta v}{\partial T}\right)_{\mathrm{pH}} \tag{4}$$

The Gibbs free energy change is shown as a function of temperature and pH by Eqs. (1)–(4). The enthalpy of the total system is represented using the Gibbs free energy change in the following equations:

$$f_{\mathrm{MG}}(T, \mathrm{pH}) = \{1 + \exp[\Delta G(T, \mathrm{pH})/RT]\}^{-1} \tag{5}$$

$$H(T, \mathrm{pH}) = H_{\mathrm{N}}(T, \mathrm{pH})[1 - f_{\mathrm{MG}}(T, \mathrm{pH})] + H_{\mathrm{MG}}(T, \mathrm{pH}) f_{\mathrm{MG}}(T, \mathrm{pH}). \tag{6}$$

In this study, the enthalpy function of the native state at each temperature, H_{N}, was found to be well described by an exponential function of pH with three parameters; H_{N}', H_{N}^0, and A, as shown in Eq. (7); H_{MG} and the Gibbs free energy change were described by seven global fitting parameters, $\Delta C_{\mathrm{p}}'$, ΔC_{p}^0, $\Delta H'$, ΔH^0, n, k, and $\mathrm{pH_d}$, as shown in Eqs. (8)–(10).

$$H_{\mathrm{N}} = H_{\mathrm{N}}' \{\exp[A(\mathrm{pH} - \mathrm{pH}_0)] - 1\} + H_{\mathrm{N}}^0 \tag{7}$$

$$\Delta H(T, \mathrm{pH}) = [\Delta C_{\mathrm{p}}' (\mathrm{pH} - \mathrm{pH_d}) + \Delta C_{\mathrm{p}}^0](T - T_0) + \Delta H' (\mathrm{pH} - \mathrm{pH_d}) + \Delta H^0 \tag{8}$$

$$H_{\mathrm{MG}}(T, \mathrm{pH}) = H_{\mathrm{N}}(T, \mathrm{pH}) + \Delta H(T, \mathrm{pH}) \tag{9}$$

$$\Delta G(T, \mathrm{pH}) = -\left[\Delta C_{\mathrm{p}}' T \ln\left(\frac{T}{T_0}\right) + (\Delta C_{\mathrm{p}}' T_0 - \Delta H')\left(1 - \frac{T}{T_0}\right)\right](\mathrm{pH} - \mathrm{pH_d}) + \ln 10 RT \frac{n}{k}\{1 - \exp[-k(\mathrm{pH} - \mathrm{pH_d})]\} - \Delta C_{\mathrm{p}}^0 T \ln\frac{T}{T_0} - (\Delta C_{\mathrm{p}}^0 T_0 - \Delta H^0)\left(1 - \frac{T}{T_0}\right) \tag{10}$$

That is, the enthalpy of the MG state was expressed as a sum total of the native enthalpy and ΔH. Four global parameters, $\Delta C_p'$, ΔC_p^0, $\Delta H'$, and ΔH^0 were necessary to describe the temperature dependence of the Gibbs free energy change, where the temperature dependence of the heat capacity change was neglected and the pH dependence of both the heat capacity change and the enthalpy change was approximated as linear functions of pH. Three global parameters, n, k, and pH_d were necessary to describe the pH dependence of the Gibbs free energy change at $T=T_0$;

$$\left(\frac{\partial \Delta G}{\partial \mathrm{pH}}\right)_{T=T_0} = (\ln 10) RT_0 \Delta v(\mathrm{pH}, T_0)$$

$$= (\ln 10) RT_0 n \exp[-k(\mathrm{pH} - \mathrm{pH_d})] \qquad (11)$$

namely n is the proton-binding number difference at the mid-point pH, $T=T_0$, and k represents the pH dependence of the difference. T_0 and pH_0 were the standard temperature and pH, and were set to 303.15 K and 5, respectively, in this study. A fitting program was developed in-house in order to determine the adjustable parameters to fit the experimental data by Levenberg–Marquardt method with the nonlinear least-squares package SALS [20].

3. Results

Fig. 1 shows the UV-CD spectra at 30 °C. The far-UV CD spectrum of cytochrome c in the native state (500 mM KCl, pH 5.4) and in the MG state (500 mM KCl, pH 2.3) are nearly equal, but are clearly different from the spectra of the denatured state (20 mM KCl, pH 1.8 and 500 mM KCl, 4 M GnHCl, pH 1.7) (Fig. 1a). The near-UV CD spectrum of cytochrome c in the MG state (500 nM KCl, pH 2.3) differs from that of the native state (500 mM KCl, pH 5.4). The near-UV CD spectra of the acid-denatured state (20 mM KCl, pH 1.8) and the GnHCl-denatured state (500 mM KCl, 4 M GnHCl, pH 1.7) show that the tertiary structure was completely disordered. These CD spectra in 500 mM KCl, pH 2.3, at 30 °C revealed the typical features for the MG state, where considerable secondary structure remained, but the tertiary structure was disordered.

Fig. 2 shows the temperature dependence of the partial molar heat capacity of cytochrome c in 500 nM KCl at pH 4.5 (A) and 2.5 (B). The thermal transition at pH 4.5 and 500 mM KCl (N to D) and at pH 2.5 and 500 mM KCl (MG to D) were observed. In the temperature range from 20 to 35 °C, the thermal transition had not yet begun at either of the pH levels. The mole fraction of the native (A) and the MG (B) state evaluated by the two-state analysis was found to be consistently over 0.95 at a temperature range from 20 to 35 °C.

Based on the results of the CD and DSC experiments, it was considered possible to observe the transition of cytochrome c from the native to the MG state in 500 mM KCl, and in a pH range from pH 4.5 to 2.5, by IATC experiments in a temperature range from 20 to 35 °C.

Fig. 3A show typical raw calorimetry data of the titration with 30 mM HCl solution to the protein solution (A(1)) and

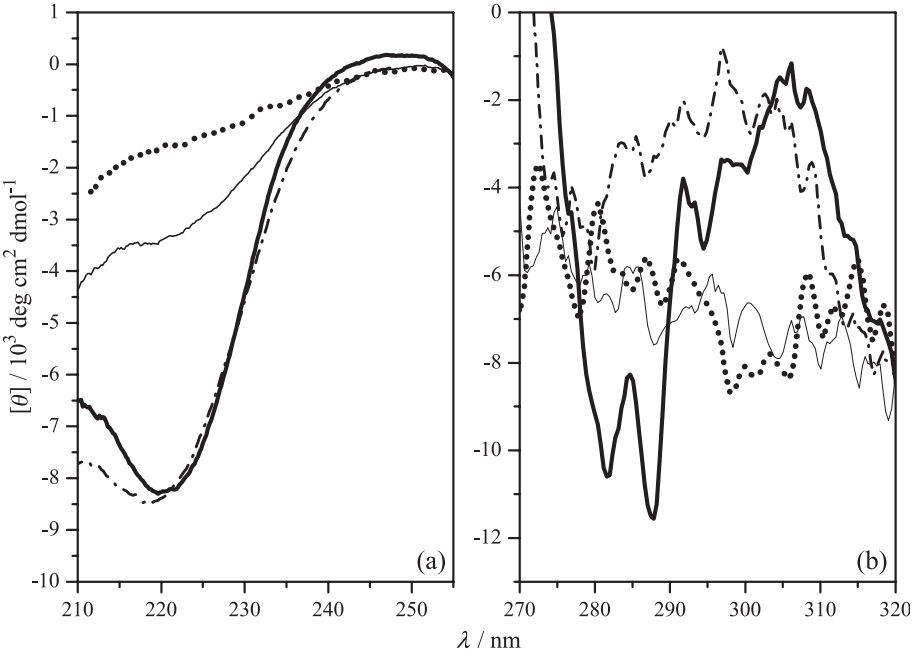

Fig. 1. Far- and near-UV CD spectra of bovine cytochrome c. All experiments were performed at 30 °C. In (a), the far-UV CD spectra are shown in the ellipticity per molecule of residue. In (b), the near-UV CD spectra are shown in the ellipticity per molecular of the protein molecule. The molecular ellipticities of cytochrome c in the native state (500 mM KCl, pH 5.4, thick solid lines), the MG state (500 mM KCl, pH 2.3, broken lines), the acid-denatured state (20 mM KCl, pH 1.8, thin solid lines), and the denatured state (denaturant: 500 mM KCl 4 M GnHCl, pH 1.7, dotted lines) are shown.

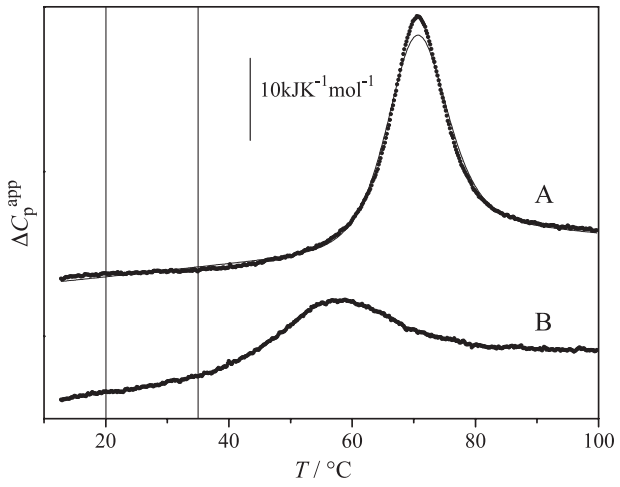

Fig. 2. The temperature dependence of the partial molar heat capacity of bovine cytochrome *c*. The experiments were performed by using 50 mM sodium acetate/HCl buffer in 500 mM KCl at pH 4.5 (A) and 50 mM glycine buffer in 500 mM KCl at pH 2.5 (B). The dotted lines show the experimental heat capacity profile. The solid lines show the theoretical curves determined by nonlinear least-squares fitting assuming a two-state model. The vertical lines denote a temperature range from 20 to 35 °C.

its control (A(2)). As shown in Fig. 3A(1), three injection volumes were used in these experiments. In the first stage, each 2 μl of the HCl solution was injected several times to decrease the pH to 3.5; in the second, each 5 μl aliquots were titrated to decrease to 3.1; and in the final, each 10 μl were titrated to pH 2.6. In the initial stage of the titrations, the exothermic reactions were clearly observed. After several injections of 2 μl HCl solution, the large and rather broad endothermic peak appeared after the sharp exothermic peak in each injection. These endothermic peaks derive from the pH-induced transition of the protein molecule. The endothermic peaks are appeared in the latter half part injections of 2 μl titrations and all injections of 5 and 10 μl titrations. It indicates that the pH-induced transition has not yet completed until pH 2.6.

Fig. 3B also show typical raw calorimetry data with 400 mM HCl solution to the protein solution (B(1)) and its control (B(2)). The 400 mM HCl solution was titrated at 2, 5, and 10 μl increments to decrease the pH from its initial value to 2.0. As shown in Fig. 3B(1), the pH-induced transition of protein molecule has appeared in first five injections. A small endothermic peak has appeared in second injection. The exothermic peaks of third and forth injection were smaller than those of the control titration (Fig. 3B(2)).

By combining the heat of 30 mM HCl titrations with that of 400 mM HCl titrations, the enthalpy change that derive from the structure change of the protein molecule was evaluated. In this experiment, the enthalpy in the pH range from its initial value to 2.7 was evaluated using the results of 30 mM HCl titrations, and that from 2.7 to 2.0 was from these of 400 mM HCl titrations.

Fig. 4 shows the enthalpy functions of cytochrome *c* under several conditions. Fig. 4A shows the transition of cytochrome *c* from the native to the denatured (N to D) state at a

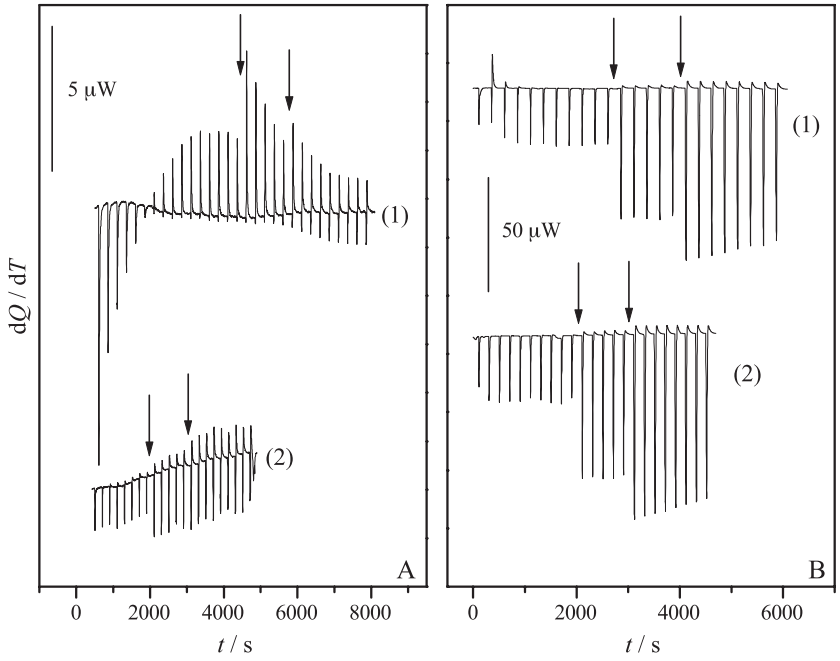

Fig. 3. Typical raw calorimetry data of acid titration to bovine cytochrome *c* in 500 mM KCl solution. 30 mM HCl in 500 mM KCl (A) and 400 mM HCl in 500 mM KCl (B) was titrated to the protein solution in the calorimeter cell at 30 °C. A(1) and B(1) show the titrations of the HCl solution to the protein solution. A(2) and B(2) show the control titration of the HCl solution to the 500 mM KCl solution. The titration was performed with injection of 2.0, 5.0, and 10.0 μl each of the HCl titrant with a syringe of 250 μl. The injection volumes were set to be increased stepwise in order to keep the pH change of the protein solution. The downward-pointing arrows show the time points at which the injection volume was changed.

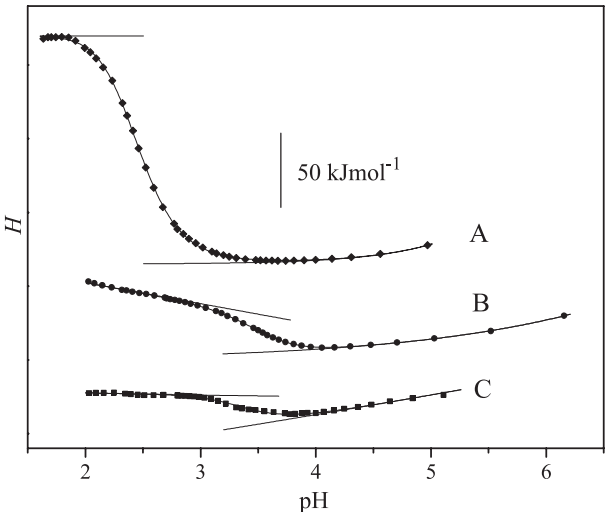

Fig. 4. The enthalpy of bovine cytochrome *c* as a function of pH. The enthalpy functions of cytochrome *c* in 20 mM KCl at 30 °C (A), 500 mM KCl at 30 °C (B), and 500 mM KCl at 20 °C (C). The solid lines show the theoretical fitting curves, the enthalpies of the native and denatured states, and the observed enthalpy, as calculated by a simple two-state transition model (A) and a two-state global analysis (B and C).

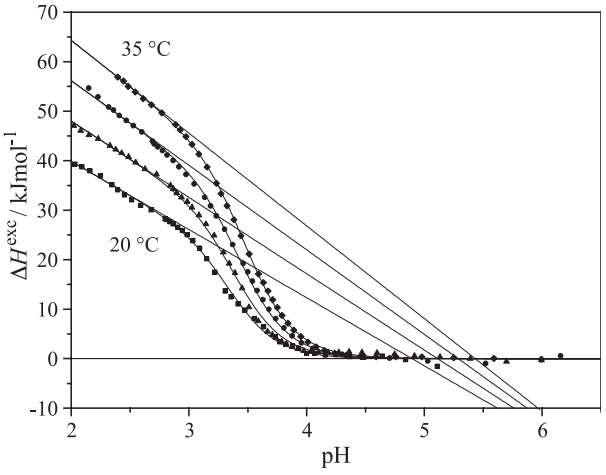

Fig. 5. The excess enthalpies, ΔH^{exc}, of bovine cytochrome *c* as a function of pH calculated using the results of the two-state global analysis. The solid lines show the theoretical curves of the excess enthalpies at 20–35 °C. The broken lines show the theoretical curves of the excess enthalpies of the MG state. The filled symbols show the experimental data for the excess enthalpies at 20 °C (filled squares), 25 °C (filled triangles), 30 °C (filled circles), and 35 °C (filled diamonds), respectively.

low salt concentration and at 30 °C. The transition of the enthalpy was observed within a pH range from 3 to 2. It was confirmed that this transition reflected the acid-denaturation of cytochrome *c* by CD experiments. The theoretical curve (solid line) was calculated assuming a two-state model, and this curve agreed well with the experimental data. The thermodynamic parameters of the N–D transition are shown in Table 1. Fig. 4B,C shows the pH-induced N–MG structural transition in 500 mM KCl at 30 °C (B) and 20 °C (C). Slight changes in enthalpy were observed in the transition that occurred within a pH range of 4 to 3. The enthalpy functions in the temperature range from 20 to 35 °C were analyzed together with the two-state global fitting method. The theoretical curves calculated by the two-state global fitting analysis agreed fairly well with the experimental data shown in Fig. 4B,C. The thermodynamic parameters determined by the global analysis are displayed in Table 1.

The thermodynamic parameters of the pH-induced transition of cytochrome *c* with IATC are shown in Table 1. The enthalpy function of the transition at 30 °C was analyzed according to two different procedures. One of these procedures was a normal two-state fitting model using only the data obtained at 30 °C, and the other procedure was the two-state global fitting method using all of the data in the temperature range from 20 to 35 °C. The two results agreed within the estimation error, and the error decreased when all of the experimental data were considered. The enthalpy change, ΔH, of the of transition of cytochrome *c* from the native to the MG state at 30 °C was 30 kJ mol^{-1}. The heat capacity change, ΔC_p, of the transition from the native to the MG state was 1.1 kJ K^{-1} mol^{-1}. This value was smaller than that of the transition from the native to the denatured state, i.e., 6.0 kJ K^{-1} mol^{-1} [14], thus indicating that the MG state of cytochrome *c* retains a considerable hydrophobic core in comparison with the denatured state.

Fig. 5 shows the excess enthalpies, ΔH^{exc}, of bovine cytochrome *c* as a function of pH calculated with the results of the two-state global fitting analysis. The excess enthalpy is defined by the following equation:

$$\Delta H^{exc}(T, \mathrm{pH}) = H^{obs}(T, \mathrm{pH}) - H_N(T, \mathrm{pH}), \quad (12)$$

where H^{obs} is the observed enthalpy, and H_N is the enthalpy of the native state calculated using the results of the two-

Table 1
The thermodynamic parameters, such as mid-point pH, pH_d, proton binding-number difference, Δv, enthalpy change, ΔH, and heat capacity change, ΔC_p, with pH-induced transition of bovine cytochrome *c* determined by isothermal acid-titration calorimetry at 20–35 °C

The transition	Solution condition, temperature	pH_d	Δv	ΔH/kJ mol^{-1}	ΔC_p/kJ K^{-1} mol^{-1}
N⇔D	20 mM KCl, 30 °C[a]	2.47±0.01	2.41±0.04	154±5	6.0[b]
N⇔MG	500 mM KCl, 30 °C[a]	3.54±0.09	2.2±0.3	27±6	–
N⇔MG	500 mM KCl, 30 °C[c]	3.54±0.01	2.1±.0.1	30±1	1.1±0.1
N⇔MG	500 mM KCl, 20 °C[c]	3.47±0.03	2.1±0.1	20±2	1.1±0.1

Δv, ΔH, and ΔC_p were evaluated at the mid-point pH, pH_d, of the given temperature.
[a] Evaluated with a simple two-state transition analysis.
[b] Data quoted from Ref. [14].
[c] Evaluated with a two-state global analysis.

state global fitting analysis. As shown in this figure, the temperature dependence of the excess enthalpy is well explained by the theoretical curves, indicating that the temperature dependence of the Gibbs free energy change can be explained by the directly observed enthalpy change. Thus, the present findings strongly suggest the validity of the assumed two-state model, and it can be concluded that there is no stable intermediate state in this transition.

4. Discussion

Kuroda et al. [6] demonstrated that the following four distinct thermodynamic states of horse heart cytochrome *c* could be observed in the thermal unfolding process under acidic conditions and with a high anion concentration: native, MG1 (they referred to this state as II_b), MG2 (they referred to this state as II_c), and the denatured state. They also reported the thermodynamic parameters at 40 °C, ΔH, and ΔC_p, for the transition between each state, except between the native and the MG1 state. Their thermodynamic parameters were as follows: ΔH: 243 kJ mol^{-1}, ΔC_p: 5.76 kJ K^{-1} mol^{-1} for the N to D transition; ΔH: 183 kJ mol^{-1}, ΔC_p: 4.57 kJ K^{-1} mol^{-1} for the MG1 to D transition; and ΔH: 129 kJ mol^{-1}, ΔC_p: 1.67 kJ K^{-1} mol^{-1} for the MG2 to D transition. Although the transition from the native to the MG1 state was not observed in their study, the thermodynamic parameters for this transition were predicted from the difference between the values of the parameters for the N to D transition and the MG1 to D transition. These parameters were as follows: ΔH: 60 kJ mol^{-1}, ΔC_p: 1.2 kJ K^{-1} mol^{-1} for the N to MG1 transition. The thermodynamic parameters for the transition from the native to the MG state of bovine heart cytochrome *c* determined by IATC in this study were ΔH: 44 kJ mol^{-1} (this value is extrapolated to 40 °C), ΔC_p: 1.1 kJ K^{-1} mol^{-1}. These parameters are in good agreement with those of the N to MG1 transition suggested from the spectroscopic measurements [6].

The transition from the native to the MG state was previously observed spectroscopically using weak salt denaturants, and the heat capacity change of 3.9 kJ K^{-1} mol^{-1} for the N to MG transition was reported based on data obtained at a rather high temperature range, i.e., from 25 to 55 °C [15]. This value deviated substantially from that of this study, 1.1 kJ K^{-1}mol^{-1}. The former value may correspond to the heat capacity change of the N to MG2 transition, considering the temperature range used in the former study. In addition, the previous study did not separate the two MG states; Fig. 2B indicates that the MG1 is no longer stable at temperatures exceeding 40 °C.

The present study identifies two properties of the MG state of cytochrome *c*. The first property is that the enthalpy of the MG state shows negative pH dependence. In Fig. 4B, the enthalpy reflects a substantial negative pH dependence of −13 kJ mol^{-1} pH^{-1} at 30 °C within a pH range from 2 to 3. This result indicates that the enthalpy of the MG state increases when the pH decreases. One possible reason for this pH dependence may be the protonation enthalpy in the MG state. With decreasing pH, the protein molecule becomes protonated. The proton acceptors in this case would be the carboxyl groups of Glu and Asp residues at low pH. However, the protonation enthalpies of glutamic acid and aspartic acid are very small and negative values such as −1.6 and −4.0 kJ mol^{-1} at 25 °C, respectively [21], indicating that the normal protonation enthalpy of these carboxyl groups cannot account for the observed increase in enthalpy accompanying protonation. On the other hand, it seems to be reasonable to assume that the enthalpy of the MG state increases by protonation if the electrostatic repulsion is also considered. With decreasing pH, the negative charges of the carboxyl group of Glu and Asp residues of the protein molecule disappeared by protonation, and the protein molecule will then have net positive charges, which will increase by protonation.

The second property of the molten-globule state of bovine cytochrome *c* is that the heat capacity of the MG state is pH-dependent. In Fig. 4B,C, the above-mentioned pH dependence of the enthalpy of the MG state at 30 °C is larger than that at 20 °C. In Fig. 5, the pH dependence of the excess enthalpies of the MG state clearly shows positive temperature dependence. The following equation indicates the existence of the pH dependence of the heat capacity of the MG state of cytochrome *c*:

$$\frac{\partial}{\partial T}\left(\frac{\partial H}{\partial \mathrm{pH}}\right)_T = \frac{\partial}{\partial \mathrm{pH}}\left(\frac{\partial H}{\partial T}\right)_{\mathrm{pH}} = \left(\frac{\partial C_p}{\partial \mathrm{pH}}\right). \tag{13}$$

The pH dependence of the heat capacity of the MG state deduced by the global fitting analysis was −1.0 kJ K^{-1} mol^{-1} pH^{-1}. This finding indicates that the heat capacity of the MG state increases when the pH decreases. This change in the heat capacity of the MG state may be explained by changes in the hydration of the protein molecule. With decreasing pH, the protein molecule loses the negative charges of the carboxyl groups on its surface by protonation. Because the negative charge on the surface of the protein molecule has been hydrated, the heat capacity of the MG state is estimated to increase by decreasing the hydrophilic hydration. Moreover, increasing hydrophobic hydration may be another possible explanation of the heat capacity change, provided the gradual structural change of the MG state occurs due to electrostatic repulsion of the positive charges on the surface of the molecule. If the structure of the MG state is extended, and when the electrostatic repulsion on the surface increases, the hydrophobic residues in the interior of the protein may be more exposed to the solvent.

In this study, the two-state transition of bovine cytochrome *c* from the native to the pH-induced MG state with only a slight change in enthalpy was directly observed by isothermal acid-titration calorimetry. It was also demonstrated that IATC can be used to evaluate transitions with small enthalpy changes that cannot typically be detected

even when using a highly sensitive scanning calorimeter. The pH dependence of both the enthalpy and the heat capacity of the MG state were clearly detected in the present study. The mechanism of pH dependence in this context should be clarified by further studies comparing the structure and thermodynamic properties of the MG state.

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