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Volumetric Properties of the Molten Globule State of Cytochrome *c* in the Thermal Three-State Transition Evaluated by Pressure Perturbation Calorimetry

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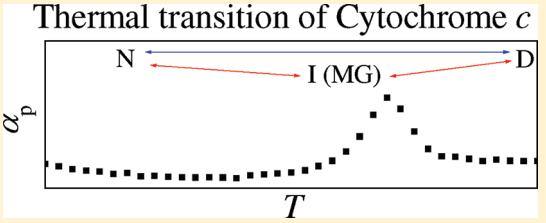
ABSTRACT: Thermodynamic properties, including volumetric properties, provide important information on protein stability and folding. Pressure perturbation calorimetry (PPC) is an effective technique for evaluating the temperature dependence of the thermal expansion coefficient, α_p, of biomaterials. Recently, the thermal N-to-D transition of cytochrome *c* at nearly pH 4 was found to be a three-state transition including a molten globulelike intermediate state. In this study, the thermal N-to-D transition of cytochrome *c* was examined by PPC measurements with a three-state model. As far as we know, this is the first report of a three-state analysis of PPC, since two-state analyses are traditionally applied. The V_p of the MG1-like intermediate state in the N-to-D transition at pH 4 was found to be the value between the partial volumes of the N and D states, suggesting an increase of the hydrophobic hydration in this intermediate state.



INTRODUCTION

Thermodynamic properties, including volumetric properties, provide important information on protein stability and folding.[1−3] In particular, volumetric parameters such as the partial volume, V_p, and the thermal expansion coefficient, α_p, are affected sensitively by the hydration state of protein surfaces. The volume change, ΔV_p, upon the conformational transition of proteins is composed of two contributions, i.e., the change in the internal void volume and the volume change accompanying the change of the hydration state of the molecular surface exposed to the solvent.[2]

Pressure perturbation calorimetry, PPC, is an effective technique for evaluating the temperature dependence of the thermal expansion coefficient, α_p, of biomaterials such as amino acids, proteins, nucleic acids, and lipids.[4−13] Previous studies have revealed that the ΔV_p in the conformational transition of proteins was very small, and the value of ΔV_p could be either positive or negative. Recently, the α_p functions of the thermal transitions of several small globular proteins by PPC experiments were analyzed by a two-state transition model using the transition parameters from DSC experiments.[6,14] They showed that the $\Delta V_p/V_p$ upon unfolding of the proteins, such as ribonuclease A, lysozyme, ubiquitin, and eglin c, appeared to converge to a common value at high temperature.[6] On the other hand, the $\Delta V_p/V_p$ upon unfolding of cytochrome *c* showed properties different from those of the above proteins, because cytochrome *c* had a loosely packed structure around the heme region.[6]

Several globular proteins are known to exhibit an equilibrium molten globule (MG) state, which is a compact denatured state with a nativelike secondary structure but a largely disordered tertiary structure.[15−17] The structure and thermodynamic properties of the MG state are shown to be important for understanding the folding, stabilization, and functional mechanisms of proteins.[16−23] The MG state of cytochrome *c* at low pH and high anion concentration was one of the first identified examples of the MG state (we designated it the MG1 state).[15] Most MG states of cytochrome *c* were reported under special solution conditions, such as an acidic pH and a high salt concentration;[15,24,25] an alkaline pH and a high salt concentration;[26−29] a moderate concentration of denaturant, alcohol, or surfactant;[30−34] and acetylation of the side chain of lysine residues at low pH.[35] While the MG state of α-lactoalbumin shows noncooperative thermal unfolding,[36] the MG state of cytochrome *c* shows a cooperative thermal transition.[37−40] Although the MG1-to-D transition of horse cytochrome *c* was initially described as a two-state transition based on DSC and isothermal titration calorimetry (ITC) data,[38,39] the thermal MG1-to-D transition under an acidic condition was confirmed with the three-state transition including an intermediate state (we designated it the MG2 state) by circular dichroism (CD) and calorimetric studies.[40,41] The volumetric properties of the MG state of cytochrome *c* were examined by ultrasonic velocimetry or the high pressure spectroscopic method.[42,43] A negative volume change has been reported in the thermal MG-to-D transition by the high pressure spectroscopic method assuming a two-state model.

Most PPC studies of proteins have been performed in the thermal N-to-D transition. PPC studies of the MG state of proteins have been limited: only the thermal expansion coefficient in the noncooperative thermal unfolding of the MG state of α-lactalbumin has been examined by PPC.[11] The volumetric properties of the cooperative MG-to-D transition have not yet been reported. Recently, the equilibrium MG-like intermediate state of authentic cytochrome *c* under a native solvent condition, in which the native state exists stably at low temperature, has

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been observed in the thermal N-to-D transition of cytochrome *c* by a precise calorimetry and CD measurement.[37] This intermediate (I) state observed in the thermal N-to-D transition of cytochrome *c* had the similar structural and thermodynamic properties as the MG1 state at low pH under a high salt condition.[40] In this study, the volumetric properties of the MG1-like intermediate state of cytochrome *c* under a native solvent condition were examined by the PPC method with the three-state analysis of the thermal N-to-D transition of cytochrome *c*.

MATERIALS AND METHODS

Lysozyme and Cytochrome *c* Solution. Lyophilized powder of hen egg white lysozyme (100940; Seikagaku Corporation, Tokyo, Japan) was dissolved in 50 mM glycine (171−09; Nacalai Tesque, Kyoto, Japan) at pH 2.5. Lyophilized powder of horse cytochrome *c* (c-2506; Sigma, St. Louis, MO) was dissolved in 50 mM sodium acetate/acetic acid buffer at pH 4.1−4.5. This protein solution was dialyzed with a Spectra/Por dialysis membrane (132660; Spectrum Lab., Rancho Dominguez, CA) using a cutoff molecular weight of 6000−8000 Da at 4 °C for overnight against 1 L buffer solution. The pH of all the experiments was measured with a glass electrode and a *F*23 pH meter (Horiba, Kyoto, Japan). The reading of the pH values was corrected by using the standard pH solutions of pH 2, 4, and 7 (Horiba, Kyoto, Japan). The protein solution was ultrafiltrated with a MolCut ultrafilter unit (USY-20; Advantec, Tokyo, Japan), with a cutoff molecular weight of 200 kDa to remove any aggregate that might have been produced during dialysis. The concentration of proteins was determined spectrophotometrically with a UB-35 spectrophotometer (Jasco, Tokyo, Japan) and by using an extinction coefficient of $\varepsilon_{280} = 3.85 \times 10^4\ M^{-1}\ cm^{-1}$ (hen egg white lysozyme) and $\varepsilon_{409} = 10.6 \times 10^4\ M^{-1}\ cm^{-1}$ (horse cytochrome *c*), respectively. Before all experiments, a procedure to degas 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).

Density Measurement. All densities of the solutions were measured at 20.000 °C by using a DMA5000 high-precision vibrating tube densimeter (Anton Paar, Graz, Austria). Air and water were used in the calibration of the densimeter as indicated by the manufacturer.

The partial specific volume of protein, v_p, was evaluated by using the following equations

$$v_s = \frac{1}{d_s} \tag{1}$$

$$v_s = (v_p - v_b)f_p + v_b \tag{2}$$

where d_s is the density of the protein solution, v_s and v_b are the specific volume of the protein solution and buffer, respectively, and f_p is the mass fraction of the protein in the protein solution. The density was measured with 0−20 mg/mL protein solution. The partial molecular volume of the protein, V_p, was deduced by using the following equation

$$V_p = \frac{v_p M_p}{N_A} \tag{3}$$

where M_p and N_A are the mole mass of the protein and Avogadro's number, respectively. The M_p of hen egg white lysozyme and horse cytochrome *c* are 14300 g/mol and 12384 g/mol, respectively.

Differential Scanning Calorimetry. Differential scanning calorimetry (DSC) experiments were performed with a highly sensitive differential scanning calorimeter, VP-DSC (Microcal, Northampton, MA). DSC measurements were performed with around 1 mg/mL solution of the protein as described previously.[40] The thermodynamic parameters of the thermal transition of lysozyme were determined by the double deconvolution method and refined by the nonlinear least-squares fitting method[44,45] using SALS libraries.[46] The DSC measurements of the thermal N-to-D of cytochrome *c* were reported previously.[37,40]

PPC. PPC experiments were performed by using a highly sensitive differential scanning calorimeter, VP-DSC, equipped with a PPC accessory pressurizing system (Microcal, Northampton, MA).[4] In short, the valuable thermodynamic information on protein hydration, expansibility, and relative volume changes upon thermal denaturation can be obtained by measuring the heat, ΔQ, which is released on small pressure changes, ΔP. The difference between the heat of the two cells (reference and sample cell) accompanying pressure changes is precisely measured by the PPC method. At every temperature step, an identical small pressure jump of ±0.5 M Pa was applied to both the sample and reference cells using pressurized N_2 gas.

The thermal expansion coefficient of the protein molecule, α_p, is calculated by the following equation

$$\alpha_p = \alpha_w - \frac{\Delta Q^{b-w}}{TV_1\Delta P} - \frac{v_s \Delta Q^{s-b}}{f_p v_p T V_1 \Delta P} \tag{4}$$

where f_p is the mass fraction of protein, V_1 is the cell volume of the calorimeter, and α_W is the thermal expansion coefficient of water. The parameters v_p and v_s are the partial specific volume of protein and the specific volume of the protein solution, respectively. The values of $\Delta Q^{b\text{-}w}$ and $\Delta Q^{s\text{-}b}$ indicate the heat change accompanying the small pressure change, ± 0.5 M Pa, during the water-buffer run and the sample-buffer run, respectively. Although α_p has been calculated without considering the temperature dependence of v_p in most previous studies, α_p was calculated with the temperature dependence of the v_p and v_s by the iteration method in this study. In the first step, the α_p at every temperature was calculated with the v_p value at 20 °C evaluated by density measurements. At the next step, the α_p and v_p at every temperature were evaluated by iterative calculation with the calculated α_p and v_p values using a program developed in house for iterative calculation. The difference of the α_p calculated with or without the iteration method was found to be small (within 1%) in all cases in this study. In this study, the temperature derivative of the partial molecular volume of protein, $(\partial V_p/\partial T)_p$, was used for the two-state and three-state analysis. The $(\partial V_p/\partial T)_p$ is deduced by the following equation

$$\left(\frac{\partial V_p}{\partial T}\right)_p = \alpha_p V_p = \frac{\alpha_p v_p M_p}{N_A} \tag{5}$$

The 14 mg/mL lysozyme and 15−17 mg/mL cytochrome *c* solutions were used for PPC experiments. The PPC runs of sample−buffer and buffer−buffer were repeated 3 times at every temperature to evaluate the $(\partial V_p/\partial T)_p$ precisely. The average values of the $(\partial V_p/\partial T)_p$ were used with the two- and three-state analysis. For the PPC experiments, in general, a relatively dilute protein solution (less than 5 mg/mL) was recommended[6,14]

because the increased intermolecular interaction will affect the α_p of the native state in the highly concentrated protein solution.[5] However, it was necessary to use a highly concentrated protein solution for PPC experiments to analyze and extract the sensitive volumetric parameters, such as ΔV and $\Delta\alpha_p$, precisely in the thermal three-state transition of proteins in this study. Recently, it was shown that the N and MG state of cytochrome *c* existed as a stable monomer even in highly concentrated (17 mg/mL) cytochrome *c* solution at low temperature (10−25 °C) by the solution X-ray scattering experiments.[40] We performed DSC experiments of the highly concentrated cytochrome *c* solution for checking the reversibility of the thermal transition. We confirmed that 11 mg/mL cytochrome *c* showed fully reversible transition and no concentration dependence was detected up to this concentration in the thermal N-to-D transition at pH 4.1 and low salt condition. We also confirmed that the volumetric parameters, such as ΔV and $\Delta\alpha_p$, in the N-to-D transition of lysozyme were consistent with the previous study performed with a low protein concentration.[4,6]

The Two- and Three-State Analysis of PPC Data for Thermal Transition of Small Globular Proteins. The temperature dependence of the partial molecular volume, V_p, in the thermal transition is described by the following equation

$$V_p(T) = \sum_i V_{p,i}(T) f_i(T) \tag{6}$$

where $V_{p,i}$ and f_i are the molecular volume and the mole fraction of each state. When both sides of eq 6 are differentiated by temperature, the following equation is derived

$$\left(\frac{\partial V_p}{\partial T}\right)_p = \sum_i \left(\frac{\partial V_{p,i}}{\partial T}\right)_p f_i + \frac{\sum_{i<j} \Delta V_{ij}(T)\Delta H_{ij}(T) f_j(T) f_i(T)}{RT^2} \tag{7}$$

The temperature function of the $(\partial V_p/\partial T)_p$ evaluated from PPC experiments was analyzed by the two-state or three-state model in eq 7.

In this study, the $(\partial V_p/\partial T)_p$ of each state is approximated by exponential functions as the following eq 8. As temperature increases, the $(\partial V_p/\partial T)_p$ of the native and denatured state of proteins monotonically decreases in the manner of an exponential function[4]

$$\left(\frac{\partial V_{p,i}}{\partial T}\right)_p = b_1\{\exp(-b_2 T) - 1\} + S_i \tag{8}$$

where b_1, b_2, and S_i, are fitting parameters. The b_1 and b_2 of each state are fixed to the same value in this study. The S_i shows the S of each state, and the fitting parameters related to the temperature dependence of the volume change are shown in the following equations

$$\left(\frac{\partial \Delta V_{p,ij}}{\partial T}\right)_p = (S_j - S_i) \tag{9}$$

$$\Delta V_{p,ij}(T) = \int_{T_{m,ij}}^{T} \frac{\partial \Delta V_{p,ij}}{\partial T} dT + \Delta V_{p,ij}(T_{m,ij}) = (S_j - S_i)(T - T_{m,ij}) + \Delta V_{p,ij}(T_m) \tag{10}$$

where $\Delta V_{p,ij}(T_{m,ij})$ is a fitting parameter, meaning as the $\Delta V_{p,ij}$ at $T_{m,ij}$. The fitting parameter of the $(\partial \Delta V_p/\partial T)_p$ in the N-to-I transition, $(\partial \Delta V_{p,N-I}/\partial T)_p$, of cytochrome *c* at pH 4.1−4.5 was fixed to suppress the degrees of freedom of the three-state fitting. We checked the residuals of the three-state analysis in the case with the $(\partial \Delta V_{p,N-I}/\partial T)_p$ fixed to the −100% to +200% of $(\partial \Delta V_{p,N\text{-}D}/\partial T)_p$. The residuals of the three-state fitting at pH 4.1−4.5 became within the experimental error with the $(\partial \Delta V_{p,N-I}/\partial T)_p$ fixed to the +60% to +80% of $(\partial \Delta V_{p,N\text{-}D}/\partial T)_p$. The change in the thermal expansion coefficient, $\Delta\alpha_{p,ij}$, of each state is determined using the following equation

$$\Delta\alpha_{p,ij}(T) = \alpha_{p,j}(T) - \alpha_{p,i}(T) = \frac{1}{V_{p,j}(T)}\left(\frac{\partial V_{p,j}}{\partial T}\right)_p - \frac{1}{V_{p,i}(T)}\left(\frac{\partial V_{p,i}}{\partial T}\right)_p \tag{11}$$

A fitting program was developed in house to determine the adjustable parameters to fit the experimental data by the Levenberg−Marquardt method with the nonlinear least-squares package SALS.[46] In this study of cytochrome *c*, all the parameters except for volumetric terms, such as $\Delta H_{ij}(T)$, $f_i(T)$, $f_j(T)$, and T_m, were determined by DSC, as previously reported.[37,40] It is useful to use the transition parameters from DSC to determine the volumetric parameters in PPC analysis.[6,14]

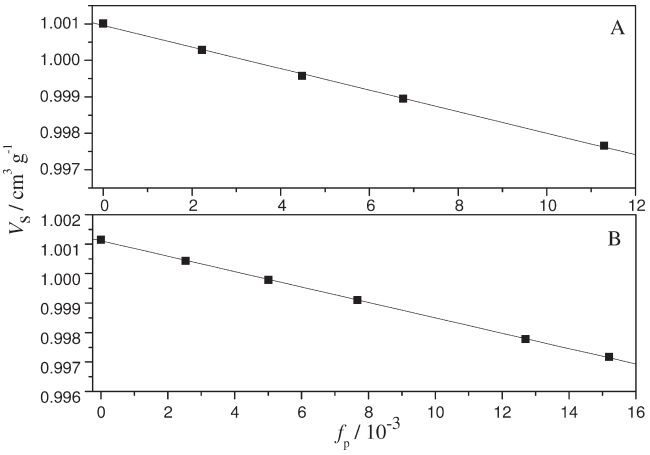

Figure 1. The mass fraction dependence of the specific volume of protein solution. (A) Lysozyme at pH 2.5 in 50 mM glycine, (B) cytochrome *c* at pH 4.1 in 50 mM sodium acetate/acetic acid. Filled squares show the experimental data. Solid lines show the linear fitting curves.

RESULTS

In Figure.1, we show the mass fraction dependence of the specific volume of protein solution determined by density measurements. The partial specific volumes of proteins at 20 °C evaluated by the linear least-squares fitting of the data were 0.714 ± 0.006 mL/g (native state, N, of lysozyme at pH 2.5), 0.740 ± 0.002 mL/g (N state of cytochrome *c* at pH 4.1), and 0.732 ± 0.004 mL/g (N state of cytochrome *c* at pH 4.5), respectively. The partial molecular volumes of proteins calculated by eq 3 were 17.0 ± 0.1 nm^3 (N state of lysozyme at pH 2.5), 15.22 ± 0.04 nm^3 (N state of cytochrome *c* at pH 4.1), and 15.05 ± 0.08 nm^3 (N state of cytochrome *c* at pH 4.5), respectively.

Figure 2 shows the temperature dependence of the heat capacity (2A), the mole fraction (2B), and $(\partial V_p/\partial T)_p$ (2C) in the thermal two-state transition of lysozyme at pH 2.5

evaluated by DSC and PPC. In Figure 2C, the thermal transition was observed in the temperature range from 50 to 70 °C. The theoretical fitting curve calculated with the two-state model using thermodynamic parameters determined by DSC agreed well with the temperature dependence of the $(\partial V_p/\partial T)_p$ evaluated by PPC. The volumetric parameters in the N-to-D transition of lysozyme at pH 2.5 are listed in Table 1. The volumetric parameters evaluated by using thermodynamic parameters from DSC (DSC parameters) and those evaluated without DSC parameters almost perfect agreement. However, the estimated error of these volumetric parameters evaluated with DSC parameters was smaller than those evaluated without DSC parameters, as previously reported.[6,14] The values of $\Delta V_{p,N-D}$ and $\Delta\alpha_{p,N-D}$ were -18.8×10^{-3} nm^3 and 1.28×10^{-4} K^{-1}, respectively. The $\Delta V_{p,N-D}/V_p$ of lysozyme was −0.1%. These volumetric parameters, $\Delta V_{p,N-D}$ and $\Delta\alpha_{p,N-D}$, were consistent with the volumetric parameters by PPC reported previously.[4,6] This indicates that the volumetric parameters under the condition of a high protein concentration in this study could be equal to those under the condition of a low protein concentration.

Figure 3 shows the temperature dependence of the heat capacity (3A), the mole fraction (3B), and $(\partial V_p/\partial T)_p$ (3C) in the thermal transition of cytochrome *c* at pH 4.1 evaluated by DSC and PPC. In Figure 3C, a large positive peak was observed in the temperature range from 60 to 80 °C. In this study, the $(\partial V_p/\partial T)_p$ in the thermal transition of cytochrome *c* at pH 4.1 was analyzed by a three-state transition model. The thermodynamic parameters in the thermal transition of cytochrome *c* at pH 4.1−4.5 are listed in Table 1. In the three-state analysis without DSC parameters, the thermodynamic parameters in the N-to-I transition could not be evaluated definitely because of too many fitting parameters. Therefore the three-state analysis was performed with fixed DSC parameters including $\Delta H_{ij}(T)$, $f_i(T)$, and $f_j(T)$. One of the fitting parameters, $(\partial\Delta V_{p,N-I}/\partial T)_p$, could not be determined

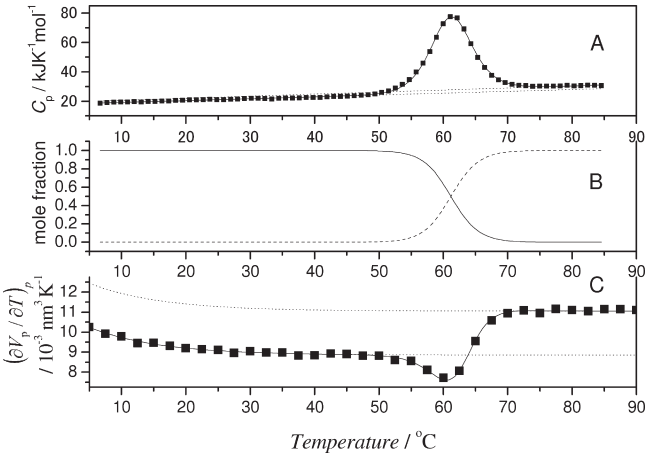

Figure 2. (A) The heat capacity function of 1 mg/mL lysozyme at pH 2.5 in 50 mM glycine evaluated by DSC measurement. Filled squares show the experimental data. Solid lines indicate the theoretical fitting curve calculated by two-state transition analysis. Dotted lines illustrate the baselines of the N and D state. (B) The temperature dependence of the mole fraction of each state, N (thin line) and D (broken line), in the thermal transition of 1 mg/mL lysozyme at pH 2.5 in 50 mM glycine evaluated by DSC measurement. (C) The temperature dependence of the $(\partial V_p/\partial T)_p$ of 14 mg/mL lysozyme at pH 2.5 in 50 mM glycine. Filled squares show the experimental data. Solid lines indicate the theoretical fitting curves calculated by two-state transition analysis using thermodynamic parameters by DSC experiments (DSC parameters). Dotted lines illustrate the baselines of the N and D state calculated by two-state transition analysis using DSC parameters.

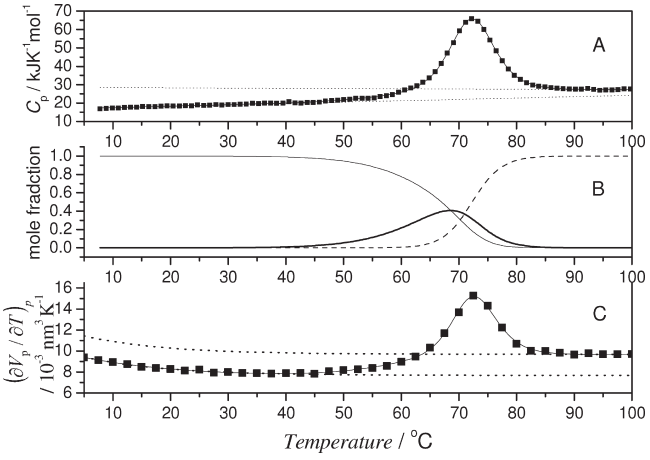

Figure 3. (A) The heat capacity function of 1 mg/mL cytochrome *c* at pH 4.1 in 50 mM sodium acetate/acetic acid buffer evaluated by DSC measurement.[37] Filled squares show the experimental data. Solid lines indicate the theoretical fitting curve calculated by three-state transition analysis. Dotted lines illustrate the baselines of the N and D states calculated by three-state transition analysis. (B) The temperature dependence of the mole fraction of each state, i.e., the N (thin line), I (thick line), and D (broken line) states, in the thermal transition of 1 mg/mL cytochrome *c* at pH 4.1.[37] (C) The temperature dependence of $(\partial V_p/\partial T)_p$ of 15 mg/mL cytochrome *c* at pH 4.1 in 50 mM sodium acetate/acetic acid buffer. Solid lines show the theoretical fitting curves calculated by three-state transition analysis using DSC parameters. Dotted lines illustrate the baselines of the N and D states calculated by three-state transition analysis using DSC parameters.

Table 1. Thermodynamic Parameters of the Thermal Transition of Proteins

	transition	T_m/°C	$\Delta V^c/10^{-3}$ nm^3	$(\partial\Delta V_p/\partial T)_p/10^{-3}$ nm^3 K^{-1}	$\Delta\alpha_p/10^{-4}$ K^{-1}
lysozyme at pH 2.5[a]	N-to-D	61.2 ± 0.2	−16 ± 1	2.22 ± 0.04	1.29 ± 0.03
lysozyme at pH 2.5[b]	N-to-D	61.18 ± 0.04	−18.8 ± 0.3	2.20 ± 0.03	1.28 ± 0.02
cytochrome *c* at pH 4.1[a]	N-to-I	75 ± 30	100 ± 200	−1 ± 20	−0.5 ± 10
	N-to-D	72 ± 4	90 ± 10	2.1 ± 0.2	1.3 ± 0.1
cytochrome *c* at pH 4.1[b]	N-to-I	69 ± 1	23.3−26.9	1.07−1.41	0.71−0.93
	N-to-D	70.2 ± 0.3	69.8−72.1	1.76−1.79	1.16−1.18
cytochrome *c* at pH 4.5[b]	N-to-I	73 ± 1	62.7−65.4	1.1−1.4	0.73−0.96
	N-to-D	74.1 ± 0.2	100.7−102.6	1.80−1.84	1.20−1.22

[a] Evaluated by an analysis where all parameters are free. [b] Evaluated by the analysis with transition parameters determined by DSC.[37,40] [c] Evaluated at T_m.

definitely in the three-state analysis even with DSC parameters because of the still large degree of freedom. Therefore, the fitting parameter of the $(\partial\Delta V_{p,N-I}/\partial T)_p$ of cytochrome *c* at pH 4.1−4.5 was fixed in order to suppress the degrees of freedom of the three-state fitting as described in the Materials and Methods. The residuals of the three-state fitting at pH 4.1−4.5 became within the experimental error by the analysis with the $(\partial\Delta V_{p,N-I}/\partial T)_p$ fixed to the range of 60−80% of the $(\partial\Delta V_{p,N-D}/\partial T)_p$. In Figure 3C, the theoretical fitting curve calculated by the three-state transition model with DSC parameters agreed very well with the experimental data. The $\Delta V_{p,N-D}/V_p$ values at T_m of cytochrome *c* at pH 4.1 and 4.5 were +0.5% and +0.7%, respectively. These results seemed to be consistent with the previous PPC study.[6] The $\Delta V_{p,N-I}$ values at pH 4.1−4.5 are listed in Table 1. The values of $\Delta V_{p,N-I}/V_p$ at pH 4.1 and 4.5 were +0.2% and +0.4%, respectively. Thus, the partial volume of the I state at pH 4.1−4.5 assumed a value between the partial volume of the N and D state.

DISCUSSION

Iteration Method and the Three-State Transition Analysis for PPC Data. In this study, the iteration method was performed to evaluate the α_p with consideration for the temperature dependence of the volume. The difference of the α_p with or without the iteration method was small (within 1%) in all cases in this study. As the temperature dependence of the v_p of the protein can be easily considered by the present method, it may be required to use the method for precise analysis.

The volumetric parameters could be determined precisely by the three-state analysis using the transition parameters, such as T_m, ΔH, and the ΔC_p, determined from DSC. As described previously, this combination of PPC and DSC is expected to be a powerful technique to extract the volumetric parameters definitely for complicated phenomena such as the thermal transition of molten globule state of other proteins and the thermal transition of proteins accompanied ligand dissociation process.

Volumetric Properties of the Molten Globule State Observed in the Thermal Three-State Transition of Cytochrome *c* at pH 4.1−4.5 and Low Salt Condition. Recently, an intermediate (I) state was observed in the thermal N-to-D transition of cytochrome *c* and was shown to have structural and thermodynamic properties similar to those of an acid molten globule state, MG1.[37,40] This intermediate state was the first observation of the MG state of cytochrome *c* under a native condition, i.e., the solvent condition under which the N state exists stably at low temperature.

The ΔV_p of the N-to-I and N-to-D transition at T_m are listed in Table 1. The ΔV_p of the N-to-I and the N-to-D transition at 70 °C were calculated for evaluating ΔV_p of the I-to-D transition at 70 °C. The volumetric parameters at 70 °C are listed in Table 2. The V_p of the I state was larger than that of the N state, suggesting that this positive volume change in the N-to-I transition was generated by the increase of the hydrophobic hydration, such as the hydration of the heme region of the I state. That the V_p of the I state was smaller than that of the D state also suggests that some part of the hydrophobic core was still retained in this I state. These findings were consistent with the previous results that the tight side chain packing of the heme region was lost, and the hydrophobic hydration was increased in the MG1 state of cytochrome *c*, as observed by structural and thermodynamic studies.[37,40]

Table 2. Volumetric Parameters of Cytochrome *c* at 70 °C in the N-to-D Transition at pH 4.1−4.5

pH	transition	ΔV at 70 °C/10^{-3} nm^3	$(\Delta V_p/\Delta V_{p,N-D})$ at 70 °C
4.1	N-to-I	25−28	0.36−0.39
	I-to-D	44−45	0.61−0.64
	N-to-D	69−72	
4.5	N-to-I	58−62	0.63−0.65
	I-to-D	33−34	0.35−0.37
	N-to-D	93−95	

CONCLUSIONS

In this study, the thermal N-to-D transition of cytochrome *c* was examined by PPC measurements with a three-state model. As far as we know, this is the first report of a three-state analysis of PPC. The volumetric parameters could be evaluated precisely by the three-state analysis using the transition parameters, such as T_m, ΔH, and ΔC_p, determined from DSC. The partial volume of the MG1-like intermediate state in the N-to-D transition at pH 4 has a value between the partial volume of the N and D state. The V_p of the I state was larger than that of the N state, suggesting that this positive volume change in the N-to-I transition was generated by the increase of the hydrophobic hydration, such as the hydration of the heme region of the I state. That the V_p of the I state was smaller than that of the D state also suggested that some part of the hydrophobic core was still retained in this I state.

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REFERENCES

(1) Privalov, P. L. *Adv. Protein Chem.* **1979**, *33*, 167–236.
(2) Chalikian, T. V.; Breslauer, K. J. *Biopolymers* **1996**, *39*, 619–629.
(3) Chalikian, T. V. *Annu. Rev. Biophys. Biomol. Struct.* **2003**, *32*, 207–235.
(4) Lin, L. N.; Brandts, J. F.; Brandts, J. M.; Plotnikov, V. *Anal. Biochem.* **2002**, *302*, 144–160.
(5) Mitra, L.; Smolin, N.; Ravindra, R.; Royer, C.; Winter, R. *Phys. Chem. Chem. Phys.* **2006**, *8*, 1249–1265.
(6) Schweiker, K. L.; Fitz, W.; Makhatadze, G. I. *Biochemistry* **2009**, *48*, 10846–10851.
(7) Rayan, G.; Tsamaloukas, A. D.; Macgregor, R. B., Jr.; Heerklotz, H. *J. Phys. Chem. B* **2009**, *113*, 1738–1742.
(8) Cameron, D. L.; Jakus, J.; Pauleta, S. R.; Pettigrew, G. W.; Cooper, A. *J. Phys. Chem. B* **2010**, *114*, 16228–16235.
(9) Lee, S.; Heerklotz, H.; Chalikian, T. V. *Biophys. Chem.* **2010**, *148*, 144–147.
(10) Fan, H. Y.; Shek, Y. L.; Amiri, A.; Dubins, D. N.; Heerklotz, H.; Macgregor, R. B., Jr.; Chalikian, T. V. *J. Am. Chem. Soc.* **2011**, *133*, 4518–4526.
(11) Zhai, Y.; Okoro, L.; Cooper, A.; Winter, R. *Biophys. Chem.* **2011**, *156*, 13–23.

(12) Tsamaloukas, A. D.; Pyzocha, N. K.; Makhatadze, G. *J. Phys. Chem. B* **2010**, *114*, 16166–16170.
(13) Jayaraman, S.; Jasuja, R.; Zakharov, M. N.; Gursky, O. *Biochemistry* **2011**, *50*, 3919–3927.
(14) Schweiker, K. L.; Makhatadze, G. I. *Methods Enzymol.* **2009**, *466*, 527–547.
(15) Ohgushi, M.; Wada, A. *FEBS Lett.* **1983**, *164*, 21–24.
(16) Arai, M.; Kuwajima, K. *Adv. Protein Chem.* **2000**, *53*, 209–282.
(17) Ptitsyn, O. B. *Adv. Protein Chem.* **1995**, *47*, 83–229.
(18) Ikeguchi, M.; Kuwajima, K.; Mitani, M.; Sugai, S. *Biochemistry* **1986**, *25*, 6965–6972.
(19) Colon, W. C.; Roder, H. *Nat. Struct. Biol.* **1996**, *3*, 1019–1025.
(20) Balbach, J.; Forge, V.; Lau, W. S.; Van Nuland, N. A.; Brew, K.; Dobson, C. M. *Science* **1996**, *274*, 1161–1163.
(21) Fink, A. L. *Curr. Opin. Struct. Biol.* **2005**, *15*, 35–41.
(22) Dunker, A. K.; Brown, C. J.; Lawson, J. D.; Iakoucheva, L. M. *Biochemistry* **2002**, *41*, 6573–6582.
(23) Dunker, A. K.; Cortese, M. S.; Romero, P.; Iakoucheva, L. M; Uversky, V. N. *FEBS J.* **2005**, *272*, 5129–5148.
(24) Jeng, M. F.; Englander, S. W.; Elove, G. A.; Wand, A. J.; Roder, H. *Biochemistry* **1990**, *29*, 10433–10437.
(25) Santucci, R.; Bongiovanni, C.; Mei, G.; Ferri, T.; Polizio, F.; Desideri, A. *Biochemistry* **2000**, *39*, 2632–12638.
(26) Kumar, R.; Prabhu, N. P.; Rao, D. K.; Bhuyan, A. K. *J. Mol. Biol.* **2006**, *364*, 438–495.
(27) Rao, D. K.; Kumar, R.; Yadaiah, M.; Bhuyan, A. K. *Biochemistry* **2006**, *45*, 3412–3420.
(28) Bhuyan, A. K. *Biochemistry* **2010**, *49*, 7764–7773.
(29) Bhuyan, A. K. *Biochemistry* **2010**, *49*, 7774–7782.
(30) Bai, Y.; Sosnick, T. R.; Mayne, L.; Englander, S. W. *Science* **1995**, *269*, 192–197.
(31) Ferri, T.; Poscia, A.; Ascoli, F.; Santucci, R. *Biochim. Biophys. Acta* **1996**, *1298*, 102–108.
(32) Kamatari, Y. O.; Konno, T.; Kataoka, M.; Akasaka, K. *J. Mol. Biol.* **1996**, *259*, 512–523.
(33) Chattopadhyay, K.; Mazumdar, S. *Biochemistry* **2003**, *42*, 14606–14613.
(34) Qureshi, S. H.; Moza, B.; Yadav, S.; Ahmad, F. *Biochemistry* **2003**, *42*, 1684–1695.
(35) Goto, Y.; Nishikiori, S. *J. Mol. Biol.* **1991**, *222*, 679–686.
(36) Dolgikh, D. A.; Abaturov, L. V.; Bolotina, I. A.; Brazhnikov, E. V.; Bychkova, V. E.; Gilmanshin, R. I.; Lebedev, Y. O.; Semisotnov, G. V.; Tiktopulo, E. I.; Ptitsyn, O. B. *Eur. Biophys. J.* **1985**, *13*, 109–121.
(37) Nakamura, S.; Baba, T.; Kidokoro, S. *Biophys. Chem.* **2007**, *127*, 103–112.
(38) Potekhin, S.; Pfeil, W. *Biophys. Chem.* **1989**, *34*, 55–62.
(39) Hamada, D.; Kidokoro, S.; Fukada, H.; Takahashi, K.; Goto, Y. *Proc. Natl. Acad. Sci. U S A* **1994**, *91*, 10325–10329.
(40) Nakamura, S.; Seki, Y.; Katoh, E.; Kidokoro, S. *Biochemistry* **2011**, *50*, 3116–3126.
(41) Kuroda, Y.; Kidokoro, S.; Wada, A. *J. Mol. Biol.* **1992**, *223*, 1139–1153.
(42) Chalikian, T. V.; Gindikin, V. S.; Breslauer, K. J. *J. Mol. Biol.* **1995**, *250*, 291–306.
(43) Dubins, D. N.; Filfil, R.; Macgregor, R. B., Jr.; Chalikian, T. V. *Biochemistry* **2003**, *42*, 8671–8678.
(44) Kidokoro, S.; Wada, A. *Biopolymers* **1987**, *26*, 213–229.
(45) Kidokoro, S.; Uedaira, H.; Wada, A. *Biopolymers* **1988**, *27*, 221–225.
(46) Nakagawa, T., Oyanagi, T. *Program system SALS for non-linear least-squares fitting in experimental sciences, Recent Developments in statistical inference and data analysis*; North Holland publishing company: Amsterdam, Holland, 1980; pp 221−225.