Complete Thermal-Unfolding Profiles of Oxidized and Reduced Cytochromes *c*

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An electron-transfer protein, soluble monoheme cytochrome *c* (cyt *c*) is among the essential redox proteins sustaining biological activity. The cyt *c* exhibits a redox potential between +200 and +400 mV.[1a−c] Thus far, three-dimensional structures of cyts *c* have been determined,[2] providing structural information around the heme active site where redox reaction occurs. Another fundamental way to elucidate the redox function of cyt *c* is to characterize its thermodynamic property of both oxidized and reduced forms because the redox potential is derived from the difference between these two redox states.[3a−c] The unfolding temperatures of cyt *c*, especially in the reduced state, at the physiological pH without denaturant are so high that it has been difficult to analyze their complete unfolding profiles and characterize their thermodynamic properties.

Conventionally, there are two protein thermal-unfolding experiments. First, differential scanning calorimetric (DSC) measurements can be carried out at more than 100 °C.[4a,b] However, the protein sample must be highly concentrated, which often causes irreversible aggregation around physiological pH. Therefore, the DSC measurement is only successful under some limited solution conditions. The other useful tool is circular dichroic (CD) spectrometry. This method is applicable to diluted samples at any pH, but thus far cannot approach temperatures greater than 100 °C because of bubbling of the aqueous solution.

Here we developed a new pressure-proof cell compartment installed in a CD spectrometer.[5] The new apparatus facilitates protein thermal-unfolding experiments up to 180 °C.[6] The complete thermal-unfolding profiles of both oxidized and reduced forms of cytochrome c_{551} (PA) from mesophilic *Pseudomonas aeruginosa* and cytochrome c_{552} (HT) from thermophilic *Hydrogenobacter thermophilus* were obtained by the apparatus,[7,8] providing insight into their redox functions through protein thermodynamic properties. HT and PA consist of 80 and 82 amino acid residues, respectively, and have 56% sequence identity.[9]

We have extensively studied the structure, stability, and redox function of these proteins.[10a−e] Because the stability of the two proteins has been assessed only under nonphysiological conditions thus far,[10b−d] the thermodynamic results obtained cannot be reconciled with their physiological redox functions.[4b,10c,d]

We first measured CD spectra (200−250 nm) of oxidized and reduced HT and PA from 40 to 150 °C at pH 7.0 (Figure 1A). The presence of an isodichroic point at around 200 nm indicated the two-state unfolding for the protein. The fraction of the unfolded proteins calculated from CD ellipticity at 222 nm was plotted against
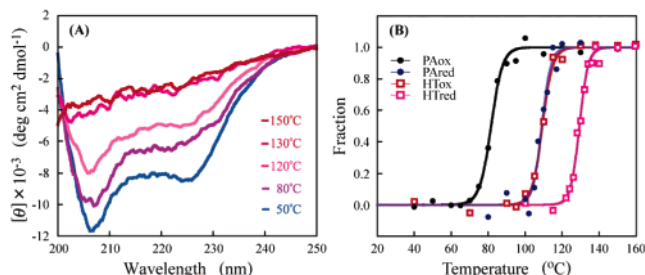
Figure 1. (A) CD spectra of HT in reduced states at 50, 80, 120, 130, and 150 °C. (B) The unfolded fraction at different temperatures of PA in oxidized states (○) and reduced states (●) and HT in oxidized states (□) and reduced states (■).

temperature, visualizing thermal unfolding profiles of the two proteins in the two redox states (Figure 1B).

We then obtained thermodynamic parameters upon the unfolding of oxidized and reduced PA and HT by nonlinear least-squares fitting employing the van't Hoff equation.[11] The procedures of fitting and the obtained thermodynamic parameters were indicated in the Supporting Information. Unfolding temperatures (T_m) of the oxidized and reduced PA and oxidized and reduced HT were 82.1, 109.0, 109.8, and 129.7 °C, respectively. Gibbs free energy changes of the unfolding (ΔG^{NU})[12] showed that the reduced forms of both proteins (ΔG_{red}^{NU} of PA and HT: 81 and 114 kJ mol^{-1}, respectively) were more stable than the respective oxidized forms (ΔG_{ox}^{NU} of PA and HT: 32 and 75 kJ mol^{-1}, respectively). These are consistent with the positive redox potential and the stability assessment using chemical denaturant.[13a,b] These values were obtained for the first time from both oxidized and reduced cyt *c* under the same physiological conditions. These data enabled us to construct a complete thermodynamic cycle, which relates the protein stability to the redox function (Figure 2).

In the thermodynamic cycle shown in Figure 2, the difference between ΔG_{red}^{NU} and ΔG_{ox}^{NU} is equal to the sum of ΔG between two redox forms in both unfolded (ΔG_{red-ox}^{U}) and native (ΔG_{red-ox}^{N}) states. ΔG_{red-ox}^{N} is calculated using the equation, $\Delta G_{red-ox}^{N} = -nFE^{0',N}$.[14] We carried out direct electrochemical analysis using cyclic voltammetry for native PA and HT at pH 7.0, which was the same pH as that for unfolding measurements, showing that the redox potential $E^{0',N}$ of the former (268 mV) was larger than that of the latter (227 mV). Thus, the ΔG_{red-ox}^{N} values of PA and HT were −26 and −22 kJ mol^{-1}, respectively.

ΔG_{red}^{NU} has maximum error values of 28% for PA and 16% for HT (see Supporting Information). By employing ΔG_{red-ox}^{N}, ΔG_{redNU}, and ΔG_{ox}^{NU} values with the error values, we estimated ΔG_{red-ox}^{U} values of unfolded PA and HT as 23.2 ± 6.5 and 17.7 ± 3.5 kJ mol^{-1}, respectively. These values were close to each other within error, as expected from the similarity in the heme environ-
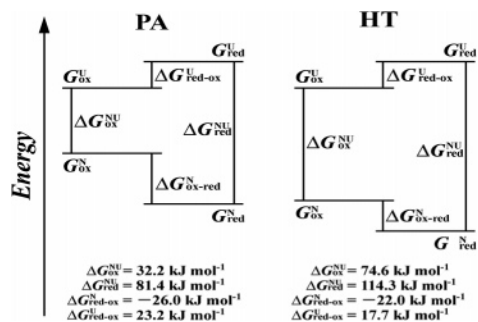
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Figure 2. Thermodynamic cycle for the oxidized and reduced forms of native and unfolded cyts c at standard condition (25 °C, 1 atm, and pH 7.0). N and U represent the native and unfolded states, ox and red represent the oxidized and reduced forms, ΔG_{ox}^{NU} and ΔG_{red}^{NU} are the Gibbs free energy changes for unfolding of oxidized and reduced forms, and ΔG_{red-ox}^{NU} and ΔG_{red-ox}^{U} are the redox potentials for the native and unfolded cyts c, respectively.

ment between the two proteins in the unfolded state. Furthermore, they were reasonably close to the corresponding value of 18.1, 19.3, and 15.9 kJ mol^{-1}, the former two values were obtained in the electrochemical studies on GdnHCl-unfolded cyt c[15a] and iso-1-cyt c,[15b] respectively, while the last one was determined by kinetic refolding experiment of cyt c.[15c] Now we are able to compare the thermodynamics of redox reaction and protein stability between PA and HT on the basis of the established cycle at standard condition, 25 °C, 1 atm, and pH 7.0 (Figure 2). HT was more stable than PA, in both oxidized and reduced forms, as judged from the T_m, ΔG_{ox}^{NU}, and ΔG_{red}^{NU} values. Furthermore, the values of 42 and 33 kJ mol^{-1} obtained for the differences in the ΔG_{ox}^{NU} and ΔG_{red}^{NU} values between HT and PA, respectively, indicate that HT is more stabilized in the oxidized form than in the reduced form, compared with the corresponding forms of PA. Therefore, the cycle shows the enhanced stabilization of the oxidized HT leads to the smaller ΔG_{red-ox}^{N} value that results in lowering $E^{0',N}$. This is why HT has the lower $E^{0',N}$ when compared with PA.

In the present study, we have shown that the novel pressure-proof cell compartment installed in the CD could be used to directly measure the stability of highly stable cyt c. Through the analysis for both redox states of the two proteins, we can conclude that the stability of oxidized cyt c influences its redox function more than the reduced form does. Considering that HT is one of the most stable naturally occurring proteins, the present technique can be used to obtain complete thermal-unfolding profiles of most proteins under desired solution conditions, which provide detailed thermodynamics of the protein stability.

Supporting Information Available: Thermodynamic parameters of PA, non-linear least squares fitting of thermal unfolding profile, and fitting results of thermal unfolding observed with CD. This material is available free of charge via the Internet at http://pubs.acs.org.

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(2) One hundred twenty structures of cyt c including their wild type and variants are registered in the Protein Data Bank.

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(5) The airtight pressure-proof cell compartment with quartz windows was designed to tolerate 10 atm where the boiling temperature of water is higher than 180 °C. The pressure in the compartment was kept by nitrogen gas, which was connected to a gas cylinder via a pressure-reducing valve. The temperature of the cell was controlled and monitored by an electric heater and a thermistor mounted in the cell compartment on the bottom of the compartment. Ordinary CD cells, with any path lengths, were able to be set in the compartment. They were free from distortion because there was no pressure difference between the outside and inside of the cell. The distortion of the window of the compartment was small so that the baseline correction was easily done by the subtraction of a blank.

(6) To verify reliability of the pressure cell compartment, we measured three CD spectra (200–250 nm) of PA at 25 °C: in the pressure-proof cell compartment at 10 atm, the same cell compartment at 1 atm, and the ordinary cell compartment at 1 atm. There was no difference in the spectra among them. Next, we tested thermal unfolding of PA in the ordinary cell compartment at 1 atm and in the pressure-proof cell compartment at 10 atm below 100 °C. The thermal unfolding profiles showed no difference between them. Together, these results prove that high pressure and the cell shape do not affect CD measurement.

(7) Our preliminary experiment showed that reduced cyts c were easily autoxidized in the absence of excess amounts of reductants (data not shown). Some reductants, however, often disturb CD or absorption spectra. Thus, first, the optimization of experimental conditions, where the proteins are reduced throughout the thermal unfolding experiment, was performed. A reductant ascorbic acid exhibits a temperature-dependent characteristic CD spectrum at 200–250 nm. Previously, dithionite was shown to be decomposed at high temperature.[4a] Thus, ascorbic acid and dithionite were not used in the present study. We tested dithiothreitol (DTT) during thermal unfolding. This reductant was previously used in DSC measurements greater than 100 °C.[4a] When PA and HT were reduced with 0.5 mM DTT at room temperature and then the measurements of the absorption spectra were performed at 100 °C, the proteins became completely oxidized, possibly because of oxidation by dissolved O_2 in the protein solutions. After these pilot experiments, we finally adopted the protein reduction method. Briefly, the proteins were first reduced with dithionite under nitrogen, and then the stability assay was carried out in the presence of DTT without dithionite and O_2. The method established here worked well, PA and HT being kept reduced in the temperature ranges to complete thermal unfolding.

(8) The oxidized and reduced cyts c (final 10 μM) in 50 mM phosphate buffer (pH 7.0) and the same buffer containing 0.5 mM DTT, respectively, were subjected to the CD and visible spectra measurements. The temperature-dependent CD ellipticity change at 200–250 nm and the visible absorption change at 500–600 nm were followed in cuvettes of 1-mm and 1-cm path lengths, respectively. The spectra were recorded from 40 to 160 °C with temperature intervals of 2–20 °C. The CD ellipticity change at 222 nm (for both proteins) and absorption changes at 551 nm (PA) and at 552 nm (HT) were followed versus temperature.

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(11) The two-state unfolding fitting was performed with MATHEMATICA 3.0. Here, changes in heat capacity, ΔC_Ps, previously derived values under an oxidized condition at pH 3.6[4b] were used. Detailed procedures were provided as Supporting Information. The thermodynamic parameters were determined by averaging those obtained at more than three different independent measurements.

(12) (a) We assumed that the estimated ΔG values of the unfolding at 10 atm and at 25 °C are the same as that at standard condition (1 atm and 25 °C) based on the published study. Hawley, S. A. *Biochemistry* **1971**, *10*, 2436–2442. (b) The redox potential of cyt c is scarcely affected by the pressure from 1 to 200 atm. Smith, E. G. *J. Am. Chem. Soc.* **1995**, *117*, 6717–6719.

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(14) In this equation, n is the number of electrons during redox reaction, F is the Faraday constant (9.648 × 10⁴ C mol^{-1}), and $E^{0'}$ is the redox potential.

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JA046667T