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Articles

Thermostability of Cytochrome *c*-552 from the Thermophilic Hydrogen-Oxidizing Bacterium *Hydrogenobacter thermophilus*†

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ABSTRACT: The denaturation of the *c*-type cytochrome of the thermophilic bacterium *Hydrogenobacter thermophilus* cytochrome *c*-552 by heat and guanidine hydrochloride was studied by measuring the change in circular dichroic spectra. The melting temperature ($T_{1/2}$) of cytochrome *c*-552 in the presence of 1.5 M guanidine hydrochloride was 34 °C higher than that of the *c*-type cytochrome of *Pseudomonas aeruginosa* cytochrome *c*-551. *Hydrogenobacter* cytochrome *c*-552 is a much more stable protein than cytochrome *c*-551 of the mesophilic bacterium *P. aeruginosa*, even though their amino acid sequences are 56% identical and they have numerous other similarities. However, notwithstanding these similarities between the sequences of the cytochromes *c*-552 and *c*-551 that were compared, it is very likely that these differences in stability could be due to some heretofore undefined differences in their spatial structures. It has been suggested that α-helix structure and electrostatic interaction could be the source of the stable spatial structure of cytochrome *c*-552.

Hydrogenobacter thermophilus TK-6 (IAM 12695) produces a large amount of cytochrome *c*-552 within its cell structure and also in a culture broth (Ishii et al., 1987a). Cytochrome *c*-552 reacts with hydrogenase isolated from *H. thermophilus* TK-6, which is considered to be the first reaction in the electron flow in this hydrogen-oxidizing microorganism (Ishii et al., 1987b). Also, cytochrome *c*-552 has a molecular weight of 7600, which is believed to be the lowest molecular weight among bacterial cytochromes *c* ever reported (Ishii et al., 1987a).

We determined the whole amino acid sequence of cytochrome *c*-552 derived from *H. thermophilus* in a previous paper (Sanbongi et al., 1989). *Hydrogenobacter* cytochrome *c*-552 consists of 80 amino acid residues, and its amino acid sequence closely resembles that of cytochrome *c*-551 from *Pseudomonas aeruginosa*, which consists of 82 amino acid residues. However, cytochrome *c*-552 from an extreme thermophilic *H. thermophilus* is believed to be more stable in heat than cytochrome *c*-551 from a mesophilic bacterium, *P. aeruginosa*. Perutz and Raidt (1975) attempted to specify the amino acid residues contributing to thermostability. They

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compared the amino acid sequences of homologous ferredoxins which have a high degree of similarity in sequences but differ in thermostability. As for cytochrome *c*, the differences in stabilities against a denaturant of cytochromes *c* from *Thermus thermophilus*, *Candida krusei*, cow, and horse heart have already been discussed (Nojima et al., 1978; Knapp & Pace, 1974). Cytochromes *c* from *H. thermophilus* and *P. aeruginosa* will be two of the most appropriate materials for examining the structure–thermostability relationship of proteins because the molecular weights of these proteins are very small and the tertiary structure of cytochrome *c*-551 from *P. aeruginosa* has been already determined by X-ray analysis (Almassy & Dickerson, 1978).

In the present research, we examine the thermostabilities of cytochromes *c* from *H. thermophilus*, *P. aeruginosa*, and horse heart by following changes in circular dichroic (CD) spectra. Also, possible amino acid residues or intramolecular interactions contributing to the extreme thermostability of cytochrome *c*-552 are discussed.

Materials and Methods

Preparation of Cytocromes c. Cytochrome *c*-552 from *H. thermophilus* TK-6 was prepared as described in a previous paper (Ishii et al., 1987a). Cytochrome *c*-551 was extracted from *Pseudomonas aeruginosa* PAO 1161 cells grown on a nitrate medium (Ambler, 1963) and was prepared on DE 52 (Whatman Paper Ltd.) and subsequently Q-Sepharose (Pharmacia) column chromatographies which had been previously equilibrated with 50 mM Tris-HCl (pH 8.6) and eluted with a linear gradient of NaCl (0–0.5 M). Horse heart cytochrome *c* (type III) was obtained from Sigma Chemical Co., and an oxidized fraction was prepared on a column chromatograph of CM-TOYOPEARL 650 S (Toso Corp., Tokyo, Japan) which had been previously equilibrated with 10 mM KH_2PO_4–NaOH (pH 7.0) and eluted with a linear gradient of NaCl (0–0.5 M). The protein concentrations were determined by the Bio-Rad protein assay method with horse heart cytochrome *c* as a standard.

Circular Dichroism. Circular dichroic (CD) spectra were obtained with a JASCO automatic recording spectropolarimeter, Model J 20, which was also employed for the melting profile analysis. The melting profile analysis was a modified version of Fujita et al. (1979). In this study, the experiments were carried out in the presence of 10 mM KH_2PO_4–NaOH (pH 7.0), and the ellipticity at 222 nm was employed as the structural parameter. The sample (0.6 mL) was placed in a quartz cell with a 1-cm light path embedded in a brass jacket, through which ethylene glycol circulated. The temperature of the ethylene glycol was raised by the use of a Haake bath, and the temperature of the sample was directly monitored by a handmade thermister sensor which penetrated a silicone rubber cap to the sample cell. The temperature was continuously raised from approximately 20 to 100 °C over a period of 15 min. The temperature signal was fed into the *x* axis of an XY recorder, while the CD signal output from the spectropolarimeter was fed into the *y* axis. The whole system was operated in a room maintained at 25 °C. The protein concentrations for CD measurements were about 70 μg/mL for all experiments.

Denaturation with Guanidine Hydrochloride. The ellipticity at 222 nm was measured in the presence of each concentration of guanidine hydrochloride (Gdn-HCl, special grade purchased from Nakarai Pure Chemicals, Kyoto, Japan) at 25 °C. In order to reach the equilibrium of denaturation with Gdn-HCl, protein solutions were incubated at 4 °C for more than 48 h after the addition of Gdn-HCl to each sample solution and then left at 25 °C for at least 2 h before the measurements.

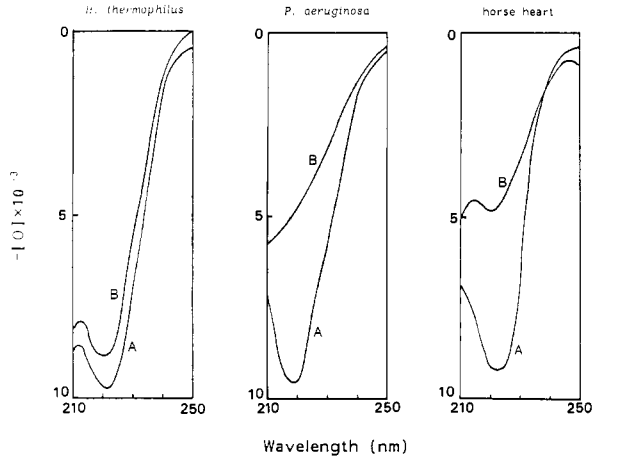

FIGURE 1: Circular dichroic spectra of cytochromes *c*. Curve A, spectra at 25 °C; curve B, spectra at 25 °C after autoclaving at 120 °C for 10 min.

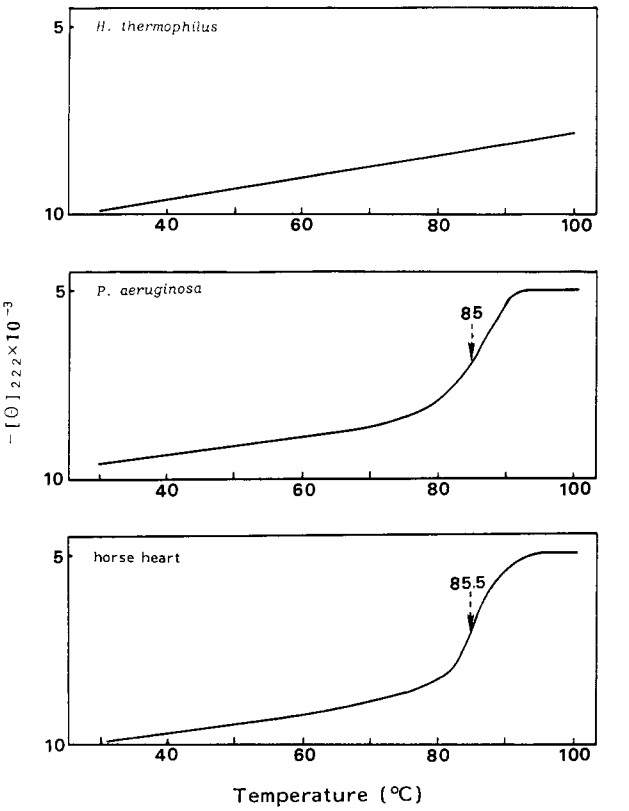

FIGURE 2: Melting profiles of cytochromes *c* in the absence of Gdn-HCl.

Results

CD Spectra. The CD spectra of native cytochrome *c* from each organism are shown in Figure 1. Each cytochrome *c* had a single minimum at 222 nm with $[\theta] = -10 \times 10^{-3}$ in the 210–250-nm region. The CD spectra of each sample, autoclaved at 120 °C for 10 min and cooled slowly to 25 °C, were also measured. The minimum peak at 222 nm remained only in the CD spectrum of *Hydrogenobacter* cytochrome *c*-552. In the cases of *Pseudomonas* and horse heart cytochromes *c*, the peaks at 222 nm disappeared.

Melting Profiles. Figure 2 shows the resulting melting profiles in the absence of Gdn-HCl as a function of temperature. A melting temperature ($T_{1/2}$) is defined as the temperature at which the melting profile crosses the midpoint

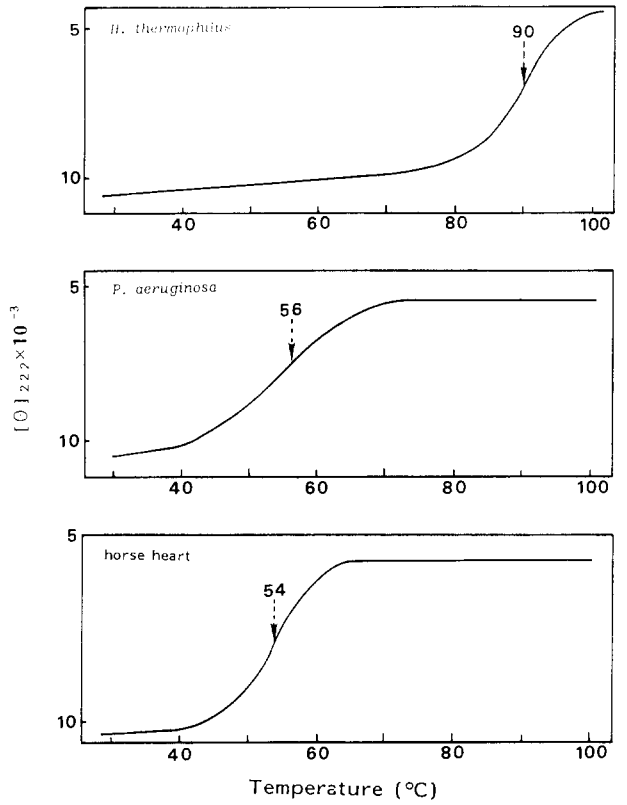

FIGURE 3: Melting profiles of cytochromes *c* in the presence of 1.5 M Gdn-HCl.

Table I: Thermodynamic Values for Thermal Unfolding in the Presence of 1.5 M Gdn-HCl

cytochrome *c*	ΔH (kcal/mol)	ΔS (units)	$T_{1/2}$ (°C)
H. thermophilus	83.5	230	90
P. aeruginosa	39.5	120	56
horse heart	62.1	190	54

between the two values extrapolated from the native and denatured regions. Melting profiles in the presence of 1.5 M Gdn-HCl were also measured (Figure 3). The $T_{1/2}$ values were as follows: *Hydrogenobacter*, 90 °C; *Pseudomonas*, 56 °C; horse heart, 54 °C. All the denaturation events shown in Figures 2 and 3 consisted of a single cooperative transition in this experimental system. The results in the presence of 1.5 M Gdn-HCl were analyzed by plotting ΔG as a function of temperature. The equilibrium constant (K) for the thermal denaturation was calculated by using the equation $K = (Y_n - Y)/(Y - Y_d)$, where Y denotes the molar ellipticity at a given temperature and Y_n and Y_d represent values for Y for the native and the denatured state, respectively. The apparent free energy change was calculated according to the relation $\Delta G = -RT \ln K$. The results provided estimates of the enthalpy and entropy at $T_{1/2}$, ΔH and ΔS (Table I).

Denaturation with Gdn-HCl. The denaturation curves obtained by the CD spectra of *Hydrogenobacter* and horse heart cytochromes *c* at 25 °C as a function of the Gdn-HCl concentration are shown in Figure 4. The primary unfolding events for each cytochrome *c* occurred in a single cooperative transition. The midpoints of the ellipticity at 222 nm were about 2.5 and 4.5 M Gdn-HCl for horse heart and *H. thermophilus* cytochromes *c*, respectively. The apparent free energy change was calculated by using the relation $\Delta G = -RT \ln K$. The free energy change of the stabilization of the native protein in the absence of a denaturant, $\Delta G°$, was calculated by the method of Aune and Tanford (1969) using the equation $\Delta G = \Delta G° - (\Delta n)RT \ln (1 + ka_{\pm})$ where k, $a_{\pm}$, and Δn

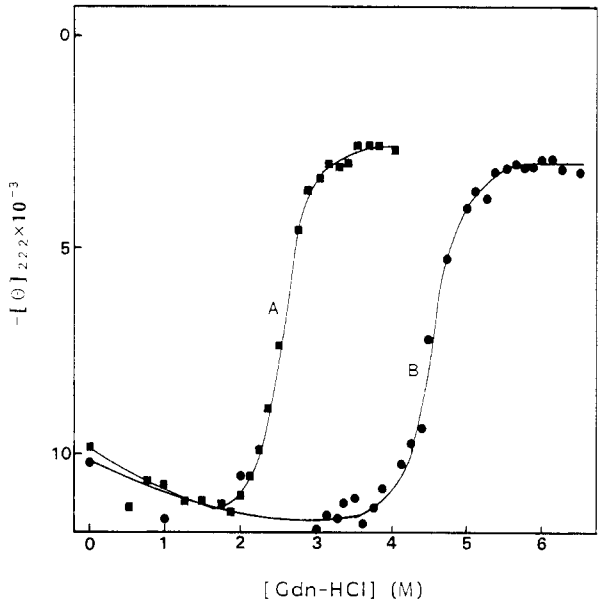

FIGURE 4: Isothermal denaturation profiles at 25 °C. Curve A, cytochrome *c* of horse heart; curve B, cytochrome *c*-552 of *H. thermophilus*.

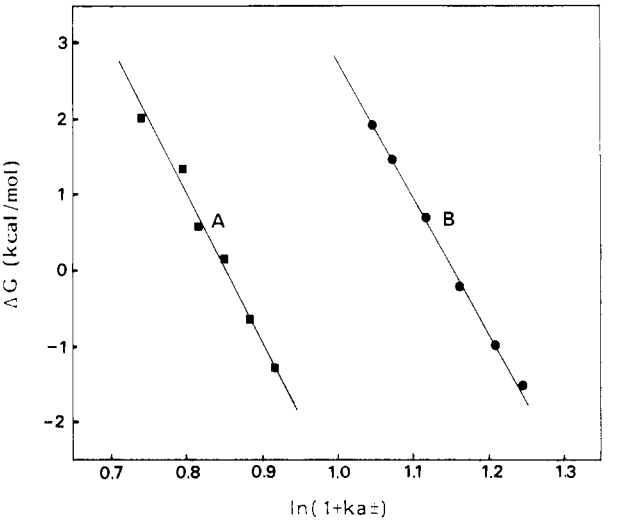

FIGURE 5: ΔG plots versus ln $(1 + ka_{\pm})$ at 25 °C. Curve A, cytochrome *c* of horse heart; curve B, cytochrome *c*-552 of *H. thermophilus*.

Table II: $\Delta G°$ Values in the Absence of Gdn-HCl at 25 °C (pH 7.0)

cytochrome *c*	$\Delta G°$ (kcal/mol)	Δn
H. thermophilus[a]	21.9	35.3
T. thermophilus[b]	28.5	36.5
Candida krusei[c]	14.0	32.9
cow[c]	15.4	30.4
horse[a]	15.1	29.6
horse[c]	12.7	26.4

[a] This work. [b] Nojima et al. (1978). [c] Knap & Pace (1974).

represent the binding constant of a denaturant to a protein, the mean ion activity of the denaturant, and the difference in the number of denaturant molecules bound to the denaturant and native states of the molecule, respectively. The values of ΔG were plotted against ln $(1 + ka_{\pm})$, together with a best-fit curve calculated by the least-squares analysis (Figure 5). We determined the values of $\Delta G°$ and Δn by using $a_{\pm}$ values from Aune and Tanford (1969) and $k = 1.20$. The resulting estimated $\Delta G°$ and Δn values are given in Table II, together with those of the cytochromes *c* of *Thermus thermophilus*

FIGURE 6: Comparison of amino acid sequences between cytochrome *c*-552 of *H. thermophilus* (top) and cytochrome *c*-551 of *P. aeruginosa* (bottom). The α-helical regions in *P. aeruginosa* cytochrome *c*-551 are indicated by underlines. (Ovals) Non-α-helix former; (▲) substitution giving more stable α helix in cytochrome *c*-552; (▼) substitution giving more stable α helix in cytochrome *c*-551; (asterisks) identical amino acid residues between two cytochromes *c*.

(Nojima et al., 1978), horse, cow, and *Candida krusei* (Knapp & Pace, 1974).

Discussion

Cytochrome *c*-552 from *Hydrogenobacter thermophilus* can return to its native structure even after being autoclaved at 120 °C for 10 min (Figure 1), which shows that the structure of cytochrome *c*-552 is stable or reversible over temperatures ranging up to 120 °C. Cytochrome *c*-552 has been proved to be more stable than cytochrome *c*-551 from *P. aeruginosa* and horse heart cytochrome *c* by the following results: (1) *Hydrogenobacter* cytochrome *c*-552 remains native up to 100 °C, but *Pseudomonas* cytochrome *c*-551 and horse heart cytochrome *c* are denatured. $T_{1/2}$ values of the latter two cytochromes *c* were both about 85 °C (Figure 2). (2) The $T_{1/2}$ value of cytochrome *c*-552 is higher than those of the other cytochromes *c* in the presence of 1.5 M Gdn-HCl (Figure 3). (3) The estimated $\Delta G°$ value of the denaturation of cytochrome *c*-552 is higher than those of the cytochromes *c* from other mesophilic organisms (Table II). The difference between our data and those of Knapp and Pace (1974) regarding the $\Delta G°$ values of horse heart cytochrome *c* is presumably due to the difference in techniques to follow unfolding. The thermostability of cytochrome *c*-552 is mainly the result of an increase in ΔH, partly mitigated by an accompanying increase in ΔS compared to the other cytochrome *c* (Table I).

It has been suggested that the thermostability of *T. thermophilus* cytochrome *c* was dependent on amino acid substitutions in the protein structure, and not on the existence of some additional factor which enhanced the thermostability of the proteins (Nojima et al., 1978). The amino acid sequence of cytochrome *c* from *T. thermophilus* has been determined (Titani et al., 1985), but the sequence did not have high similarity to any of the cytochrome *c* sequences ever reported. The amino acid sequence of *Hydrogenobacter* cytochrome *c*-552 closely resembles that of cytochrome *c*-551 from *P. aeruginosa* (Sanbongi et al., 1989). The sequence alignments of the cytochromes *c* of *H. thermophilus* and *P. aeruginosa* are compared in Figure 6. They have a sequential identity in 56% of the amino acid residues. Moreover, there are many identical substitutions of amino acid residues such as the substitution between glutamine and asparagine. Although a large degree of similarity can be seen between the sequences of the cytochromes *c*-552 and *c*-551 being compared, heretofore undefined differences in spatial structure could very likely be the basis for the differences in stability.

Factors which affect the thermostability of a protein have been discussed (Matthews, 1987). Some of them are a disulfide bond, hydrophobicity, secondary structures such as an α helix or a β sheet, a hydrogen bond, and ion pairs. Cytochromes *c* in this research have no S–S bonds or β sheets in their structures. We can adduce possible factors that influence the spatial structure in overall protein stability from a simple comparison of the amino acid sequences of cytochromes *c*-552 and *c*-551. From the study of the tertiary structure of cytochrome *c*-551 of *P. aeruginosa*, this protein has been proved to be composed of four α-helical regions in its structure (Almassy & Dickerson, 1978). The distribution of α-helix-breaking residues is very similar for cytochromes *c*-552 and *c*-551 (Figure 6). This fact suggests that they have essentially the same α-helical regions in their secondary structures. When the values of the conformational parameter of the α-helical structure of amino acid residues postulated by Chou and Fasman (1978) are compared, cytochrome *c*-552 tends to substitute a few more α-helix-forming residues as compared to cytochrome *c*-551 in the α-helical regions. This can be said especially in regard to the first and fourth α-helical regions from the N terminus (Figure 6). Such a tendency cannot be seen in the non-α-helical regions. The enhanced thermostabilities attributed to the replacement within an α helix of a poor helix-forming residue with a good helix former have been previously reported in a λ repressor (Hecht et al., 1986) and in a neutral protease from *Bacillus stearothermophilus* (Imanaka et al., 1986).

Another type of helix stabilization is made possible by electrostatic interaction between a negatively charged side chain and a positive charge attributed to the α-helix dipole near the N terminus of an α helix (Hol et al., 1978). Lys-28

in cytochrome *c*-551 is located near the N terminus of the second α helix (Figure 6). So, Lys-28 probably interrupts such an electrostatic interaction, which may result in the destabilization of cytochrome *c*-551. In cytochrome *c*-552, the corresponding residue is an uncharged Ala-26. On the contrary, Lys-48 in cytochrome *c*-552 located near the C terminus of the third α helix may contribute to the stabilization of this protein by its enhanced electrostatic interaction. In cytochrome *c*-551, the corresponding residue is Asn-50. Measurement of the pH dependence of the thermostabilities of the two proteins will give us conclusive information on the contribution of the electrostatic interactions mentioned above to the stabilization of cytochrome *c*-552.

Recently, the increase of thermostability of the yeast iso-1-cytochrome *c* mutant has been reported (Das et al., 1989). The mutation was that a helix-breaking residue, Asn-57, located within a short helical region was replaced by a helix-forming residue, Ile. The thermostability of this mutant has since been shown to depend on a major change of structure resulting from the expulsion of an internal molecule of water (Luntz et al., 1989). Analysis of the spatial structure of *Hydrogenobacter* cytochrome *c*-552 is necessary to determine the basis for the large differences in thermostability observed here. Also, changing the amino acid residues of cytochrome *c*-551 of *P. aeruginosa* to form a more stable structure by site-directed mutagenesis is the best way to prove or examine the hypotheses mentioned above. The amino acid sequence of cytochrome *c*-552 will give us very good examples for the design of stabilizing replacements.

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Registry No. Cytochrome *c*-552, 9048-78-6; cytochrome *c*-551, 9048-77-5; cytochrome *c*, 9007-43-6.

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