Denaturation of Thermophilic Ferricytochrome *c*-552 by Acid, Guanidine Hydrochloride, and Heat†

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ABSTRACT: The denaturation of *Thermus thermophilus* cytochrome *c*-552 by acid, guanidine hydrochloride, and heat was studied by measuring the changes in absorption and circular dichroism. Cytochrome *c*-552 was remarkably resistant to acid; the p*K* of the transition from the low- to the high-spin form was roughly 0.3. The effect of guanidine hydrochloride on the heme iron–methionine bond of *Thermus* and horse cytochromes *c* was also investigated; a comparison of the free-energy change for the displacement of the bond indicated that the coordination in cytochrome *c*-552 is highly stable. The spectra of guanidine hydrochloride unfolded cytochrome *c*-552 were dependent on the pH; the titration curve showed the presence of a cooperative single transition of p*K* = 4.7, with a one-proton dissociation, suggesting the ionization of a histidine residue. In the presence of guanidine hydrochloride, the influence of the heat on the ligand bond in cytochrome *c*-552 was studied. The van't Hoff plots of the reaction were biphasic. The enthalpy changes in the higher temperature range were independent of the guanidine hydrochloride concentration, while those in the lower range were not.

From X-ray crystallographic studies of several cytochromes *c* from eucaryotes and bacteria (Dickerson et al., 1971; Takano et al., 1973; Salemme et al., 1973; Tanaka et al., 1975; Timkovich & Dickerson, 1976; Swanson et al., 1977; Korszun & Salemme, 1977; Almassy & Dickerson, 1978), it has been shown that these proteins share a native tertiary structure known as the "cytochrome fold" and that the heme iron is coordinated with two strong ligands, the histidine and methionine residues. The methionine ligand bond to the heme iron produces a weak absorption band around 695 nm in the ferricytochromes *c* (Shechter & Saludjian, 1967; Sreenathan & Taylor, 1971). The presence of the 695-nm band being used as an indicator of molecular integrity has been shown to be characteristic of the physiologically active conformer of the cytochromes *c* in the ferric state (Lambeth et al., 1973; Yu et al., 1973; Dickerson & Timkovich, 1975).

Cytochrome *c*-552 from an extreme thermophile, *Thermus thermophilus* HB8, was readily extractable from the cell by means of an aqueous solution. It was shown that this cytochrome is a monoheme protein and that it has the molecular weight of 15000, the redox potential of +0.232 V, and the isoelectric point of 10.8 (Hon-nami & Oshima, 1977). Furthermore, cytochrome *c*-552 reacted rapidly with cytochrome oxidase (EC 1.9.3.1) isolated from *T. thermophilus* HB8 (Hon-nami and Oshima, unpublished results). Cytochrome *c*-552 in the ferric state has an absorption band at 690 nm, and the resonance Raman scattering and high-resolution proton magnetic resonance spectroscopy of cytochrome *c*-552 indicate that the heme iron of ferricytochrome *c*-552 is coordinated with the histidine and methionine residues, like other cytochromes *c* (Hon-nami & Oshima, 1977; Kihara et al., 1978; Hon-nami et al., unpublished results). It has been demonstrated that, in ferricytochrome *c*-552, the heme iron–methionine coordination bond is more stable to heat and to an alkaline solution than that of horse cytochrome *c* (Hon-nami & Oshima, 1977; Kihara et al., 1978), in addition to the higher stability of the overall protein conformation to Gdn·HCl[1] and to heat compared with that of horse cytochrome *c* (Nojima et al., 1978; Hon-nami et al., 1979).

To elucidate the basis for the unusual stability of ferricytochrome *c*-552, it is of interest to study the effects of acid and Gdn·HCl on the iron–methionine linkage of this cytochrome. This paper will deal with studies of the denaturation of ferricytochrome *c*-552 by acid, Gdn·HCl, and heat, as followed by absorption and CD changes, calling attention to the stability of the heme crevice structure.

Experimental Procedures

Materials. Cytochrome *c*-552 was prepared from *T. thermophilus* HB8 (Oshima & Imahori, 1971, 1974), as described in a previous paper (Hon-nami & Oshima, 1977). Horse heart cytochrome *c* (Type VI) was obtained from Sigma Chemical Co. and was oxidized by adding an excess of ferricyanide and then dialyzed. The protein concentrations were determined spectrophotometrically after reduction with sodium dithionite by using molar extinction coefficients of 27.7×10^3 M^{-1} cm^{-1} at 550 nm for horse cytochrome *c* (Margoliash & Frohwirt, 1959) and 21.1×10^3 M^{-1} cm^{-1} at 552 nm for cytochrome *c*-552 (Hon-nami & Oshima, 1977). Gdn·HCl of a special grade was purchased from Nakarai Pure Chemicals Co. The concentration of Gdn·HCl stock solutions was determined by refractive index measurements.

Methods. The spectrophotometric measurements were performed by using a Cary 17 spectrophotometer. The CD spectrum was measured with a JASCO J-40A spectropolarimeter. In heating experiments, absorbance changes at 690 nm were recorded by using a Gilford 2400 spectrophotometer. The temperature in a cuvette was monitored with a thermojunction. The temperature was continuously raised from approximately 15 to 80 °C over a period of 140 min by using a water-jacketed cell holder and a water bath.

The equilibrium constant for the displacement of methionine from the heme iron was calculated as

$$K = \frac{A_N - A_{obsd}}{A_{obsd} - A_D}$$

where A_{obsd} is the measured absorbance at 690 or 695 nm. In the experiment on the effect of Gdn·HCl at 25 °C, A_N and A_D are the absorbances in the native and denatured states, respectively. In the heating experiments, A_N is the absorbance

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[1] Abbreviations used: Gdn·HCl, guanidine hydrochloride; CD, circular dichroism.

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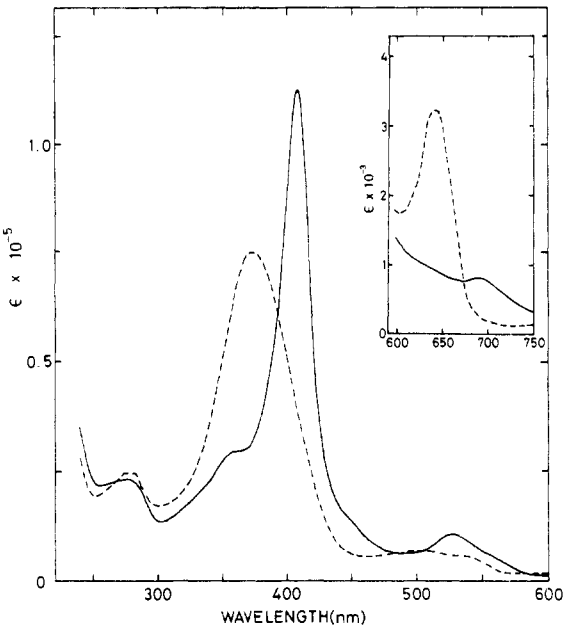

FIGURE 1: Absorption spectra of ferricytochrome *c*-552 at neutral and acid pH. (—) In pH 7.5; (---) in pH 0.1.

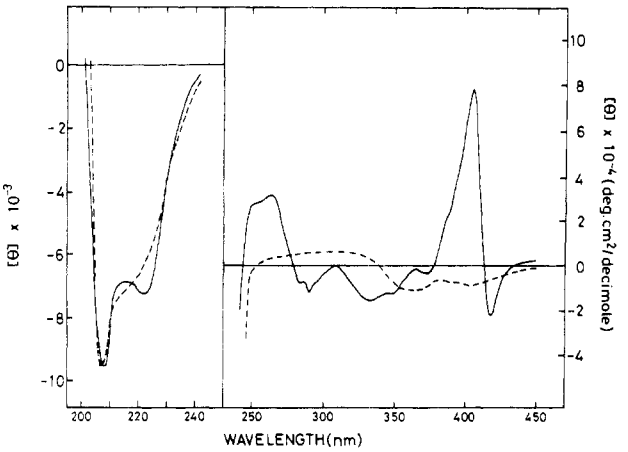

FIGURE 2: CD spectra of ferricytochrome *c*-552 at neutral and acid pH. (—) In pH 7.5; (---) in pH 0.1.

at 20 °C, while A_D is the minimum absorbance, which could not be decreased by a further increase in the temperature.

Results

Acid Denaturation. The effect of acid on the absorption spectrum of ferricytochrome *c*-552 is shown in Figure 1. The absorption spectrum at pH 1.25 was virtually indistinguishable with that at neutral pH. However, a remarkable change was observed by lowering the pH to a value of 0.1. The Soret band and a band at 528 nm became much broader, with maxima at 374 and 507 nm, respectively. The 690-nm band was replaced by a more hypochromic absorption band at 642 nm. Figure 2 shows the CD spectra of ferricytochrome *c*-552 in the range from 200 to 450 nm at neutral and acidic pH values. The spectrum of the native form is qualitatively similar to that of horse ferricytochrome *c*. In contrast, the spectrum at pH 0.1 significantly differed from that at neutral pH, indicating changes in the protein conformation.

The spectrophotometric titration of acid heme-linked ionizations is shown in Figure 3. The titration curve shows a transition with its midpoint at pH 0.3. Since the spectra at extreme acid, pH 0.5 to ~0.2, changed with time, the absorbances of the Soret band and at 374 nm in this pH region

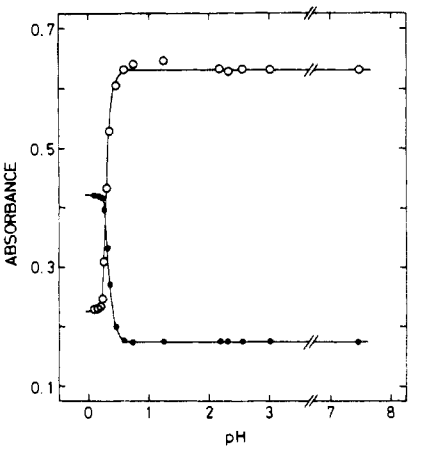

FIGURE 3: Spectrophotometric titration of ferricytochrome *c*-552 (5.8 μM) at 408 (○) and 376 nm (●).

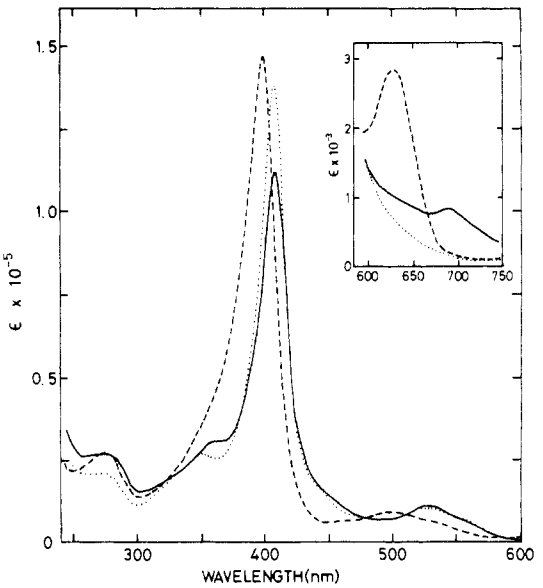

FIGURE 4: Absorption spectra of ferricytochrome *c*-552 in various solvents. (—) Native form or renatured form treated with 6.4 M Gdn·HCl and then dialyzed; (···) in 6.4 M Gdn·HCl, pH 7.0; (---) in 6.4 M Gdn·HCl, pH 2.4.

Table I: Comparison of the Spectral Properties of Native, Denatured, and Renatured Ferricytochrome *c*-552

absorption band	ε (mM^{-1} cm^{-1}) native	denatured[a]	renatured[b]
690 nm	0.83	0.14	0.83
Soret	112	138	111

[a] In 6.4 M Gdn·HCl. [b] Upon removal of 6.4 M Gdn·HCl by dialysis.

were measured immediately after preparing the samples; therefore, the p*K* value of 0.3 represents only a rough estimation. It was impossible to measure the spectral changes in the 690-nm region in strongly acidic solvents because of the formation of insoluble aggregates of the protein.

Gdn·HCl Denaturation. Upon the addition of 6.4 M Gdn·HCl to a solution of ferricytochrome *c*-552 at neutral pH and 25 °C, the absorption band at 690 nm disappeared, while the intensity of the Soret band was increased with a slight blue shift from 407.7 to 407.1 nm, as shown in Figure 4. Upon the removal of Gdn·HCl, the absorption spectrum was restored to that of the native protein, indicating that the Gdn·HCl denaturation of cytochrome *c*-552 is completely reversible. The

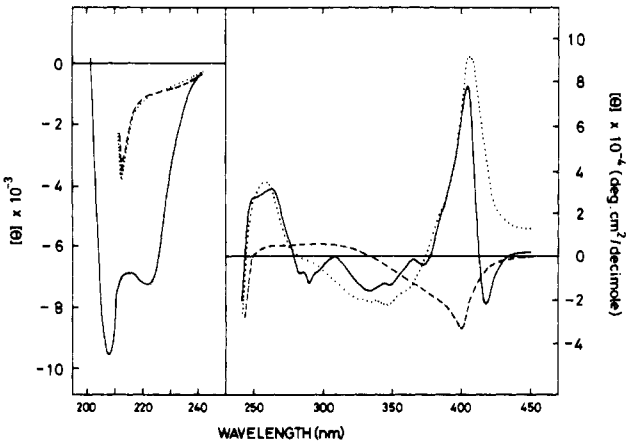

FIGURE 5: CD spectra of ferricytochrome *c*-552. (—) Native form or renatured form treated with 6.4 M Gdn·HCl and then dialyzed; (···) in 6.4 M Gdn·HCl, pH 7.0; (- - -) in 6.4 M Gdn·HCl, pH 2.4.

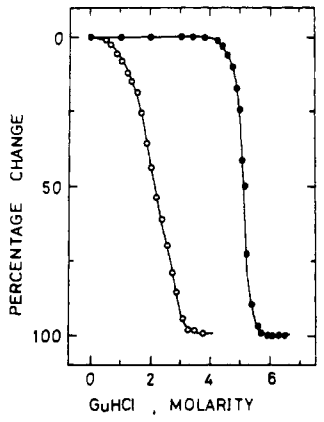

FIGURE 6: Changes in absorbance at the near-infrared band as a function of the concentration of Gdn·HCl. (●) *T. thermophilus* cytochrome *c*-552; (○) horse cytochrome *c*.

spectral properties of native, denatured, and renatured proteins are summarized in Table I. In 6.4 M Gdn·HCl at a neutral pH, the complex dichroic pattern of native ferricytochrome *c*-552 was remarkably simplified over the entire spectral region (Figure 5). The troughs at 222 and 207 nm were replaced by that at 212 nm with a significant decrease in magnitude. The dichroic peaks at 283 and 291 nm were obliterated, and an asymmetric pattern in the wavelength region 250–280 nm was replaced by a single positive band with a maximum at 260 nm of a larger magnitude than the 263-nm peak in the native form. The Soret region was reduced to a single large positive peak at 407 nm.

The decreases in the absorbance in the near-infrared band of *T. thermophilus* and horse ferricytochromes *c* at 25 °C as a function of the Gdn·HCl concentration are shown in Figure 6. The midpoints of the absorption change were 5.15 and 2.14 M Gdn·HCl for *T. thermophilus* and horse cytochromes *c*, respectively. The apparent free energy of ligand displacement was calculated according to the relation $\Delta G = -RT \ln K$. Aune & Tanford (1969) have proposed a method for the estimation of the free energy of the stabilization of native protein in the absence of a denaturant, $\Delta G°$, assuming that denaturation results from denaturant binding, which leads to the equation $\Delta G = \Delta G° - (\Delta n)RT \ln (1 - ka_{\pm})$ where k, $a_{\pm}$, and Δn represent the binding constant of the denaturant to the protein, the mean ion activity of the denaturant, and the difference in the number of denaturant molecules bound to the denatured and native states of the molecule, respectively. In the following calculation, we employed $a_{\pm}$ values from Aune & Tanford (1969) and $k = 1.20$, which was used in the evaluation of $\Delta G°$ for lysozyme (Aune & Tanford, 1969), mitochondrial cytochromes *c* (Knapp & Pace, 1974), and *T. thermophilus* cytochrome *c*-552 (Nojima et al., 1978). The values of ΔG were plotted against $\ln (1 + ka_{\pm})$, together with the best-fit curve calculated by the least-squares analysis (Figure 7). The resulting estimated $\Delta G°$ and Δn values are given in Table II, together with the data based on the disappearance of secondary structures.

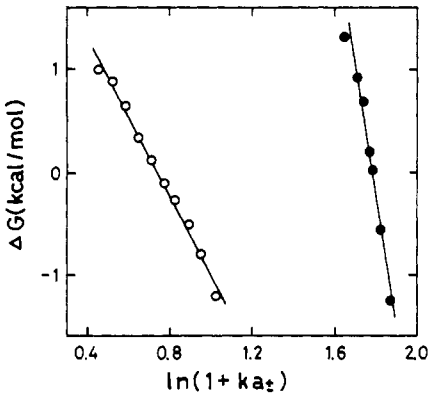

FIGURE 7: ΔG plots vs. $\ln (1 + ka_{\pm})$ at 25 °C. The activity ($a_{\pm}$) of Gdn·HCl was estimated from the following expression: $\log a = -0.5191 + 1.4839 \log c - 0.2562 (\log c)^2 + 0.5884 (\log c)^3$, where c is the Gdn·HCl molarity. ΔG values are calculated from the data in Figure 6. (●) *T. thermophilus* cytochrome *c*-552; (○) horse cytochrome *c*.

Table II: Analysis of the Gdn·HCl Denaturation of Ferricytochromes *c* at 25 °C

	$\Delta G°$ (kcal/mol)	Δn
Met ligation		
T. thermophilus	29.0	40.0
horse	5.2	11.9
secondary structure		
T. thermophilus[a]	28.5	36.5
horse[b]	12.7	26.4

[a] Nojima et al. (1978). [b] Knapp & Pace (1974).

Acidification of Gdn·HCl-Unfolded Cytochrome c-552. As shown in Figure 4, the Soret maximum was shifted from 407 to 398 nm, and a new band appeared at 628 nm upon the acidification of ferricytochrome *c*-552 to pH 2.5 in 6.4 M Gdn·HCl, indicating a transition from the low- to the high-spin form. The CD spectrum in the wavelength region from 250 to 450 nm was also remarkably changed by the treatment, while no change was observed in the far-UV region (Figure 5).

The spectrophotometric titrations at 398 and 628 nm in 6.4 M Gdn·HCl indicated a single-step transition with a pK value for the heme-linked ionization of 4.7, as shown in Figure 8. The plots of the logarithms of high spin–low spin ratios against the pH were linear, and the estimated slope was 1.22, suggesting a one-proton dissociation (Figure 9).

Effect of the Temperature on the 690-nm Band. Figure 10 shows the effects of temperature on the absorbance at 690 nm of ferricytochrome *c*-552. In the presence of Gdn·HCl (3.6–4.2 M), the denatured temperatures ($T_{1/2}$), as defined by the midpoint in the absorbance change shown in Figure 10, are listed in Table III. The van't Hoff plots of these results were biphasic for any concentrations of Gdn·HCl studied (Figure 11). The slopes of the higher temperature range were independent of the concentrations of the denaturant, and the enthalpy change for this temperature range was calculated as

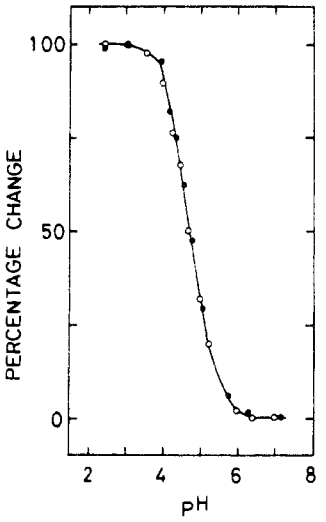

FIGURE 8: Spectrophotometric titration curves of the spin-state transformation of ferricytochrome *c*-552 in 6.4 M Gdn·HCl at 25 °C. Protein concentrations were 4.6 and 58 μM for the measurements at 398 (●) and 628 nm (○), respectively.

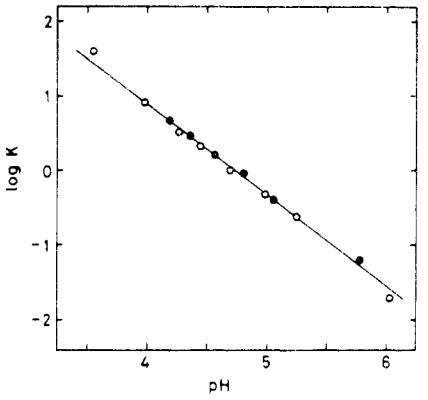

FIGURE 9: Hill plots of the titration data in Figure 8.

Table III: Thermodynamic Parameters for the Displacement of the Methionine Ligand of *T. thermophilus* Cytochrome *c*-552[a]

Gdn·HCl (M)	ΔH (kcal/mol)	ΔG (kcal/mol)	ΔS (eu)	$T_{1/2}$ (°C)
3.64	11.0	1.68	30.7	67
3.81	12.7	1.92	35.6	65
3.98	15.7	2.05	44.2	63
4.24	17.7	2.04	51.8	59

[a] Enthalpy values are calculated from equilibrium constants for the lower temperature range.

105.6 kcal/mol. On the other hand, the slopes at lower temperatures were dependent on the Gdn·HCl concentration. The thermodynamic parameters for the displacement of methionine from the heme iron in the lower temperature range are summarized in Table III.

Discussion

The visible absorption (Hon-nami & Oshima, 1977), resonance Raman (Kihara et al., 1978), and proton magnetic resonance (Hon-nami et al., unpublished results) spectra have suggested that the heme iron in the native ferricytochrome *c*-552 is coordinated with histidine and methionine residues, forming a low-spin coordination complex.

In the early studies, the pH dependence of the absorption spectrum of horse ferricytochrome *c* was demonstrated by Theorell & Åkesson (1941). They distinguished three different spectra in the acidic pH region: type I (high spin) below pH

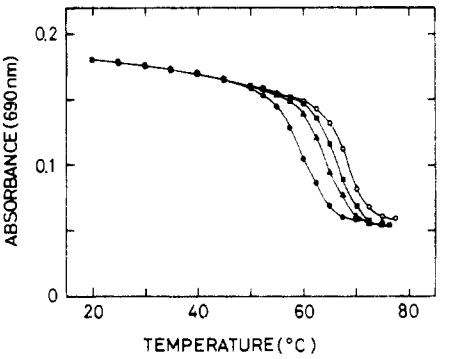

FIGURE 10: Effect of temperature on the absorbance at 690 nm of *T. thermophilus* cytochrome *c*-552, in various concentrations of Gdn·HCl: (○) 3.64; (■) 2.98; (●) 4.24 M. The concentration of the protein is 0.23 mM in 40 mM Tris-HCl buffer, pH 7.5.

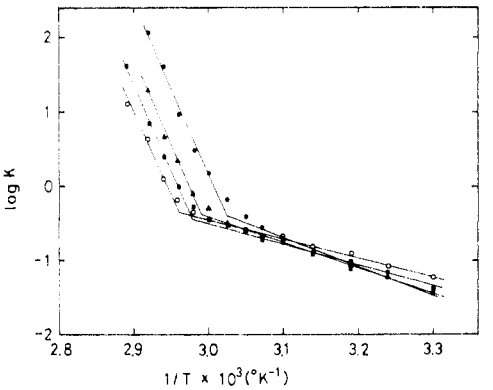

FIGURE 11: van't Hoff plots of changes at 690-nm absorption of *T. thermophilus* cytochrome *c*-552. Conditions are as described in Figure 10.

0.42, type II (high spin) between pH 0.42 and 2.5, and type III (low spin) above pH 2.5. Upon acidification, a conversion from the low- to the high-spin form below pH 2.5 accompanies the unfolding of the protein conformation (Knapp & Pace, 1974). *T. thermophilus* cytochrome *c*-552 is, however, much more stable in an acid solution. The p*K* of the transition from the low- to the high-spin form was roughly 0.3.

In our previous studies, the denaturation of cytochrome *c*-552 was analyzed by CD and fluorescence measurements in the presence of Gdn·HCl. Although the cytochrome is resistant to the denaturant, a loss of the secondary structure was observed at the high Gdn·HCl concentration (Nojima et al., 1978). Similarly, the absorption spectrum of cytochrome *c*-552 was affected by the addition of the denaturant (Figure 4); the 690-nm band disappeared, and the Soret band increased in intensity. However, the heme group of the denatured form is still in a low-spin state, with a Soret maximum at 407 nm and with no absorption maximum around 620 nm. It seems that the heme moiety of the Gdn·HCl-denatured cytochrome *c*-552 is still coordinated to a strong field ligand, probably the histidine residue in the molecule, as in the cases of both horse and *Euglena* cytochromes *c* (Babul & Stellwagen, 1971; Aviram & Weissman, 1978).

The absorption spectrum of unfolded cytochrome *c*-552 in 6.4 M Gdn·HCl depended on the pH; a p*K* of 4.7 was observed. The Hill coefficient calculated for the ionization is close to unity, suggesting a single-proton dissociation. These observations were similar to those for horse and *Euglena* cytochromes *c* (Tsong, 1975; Aviram & Weissman, 1978) and suggest the participation of the histidine residue in the low spin–high spin conversion in Gdn·HCl-denatured cytochrome *c*-552.

A comparison of the estimated $\Delta G°$ values (Table II) of the denaturation of cytochromes *c* leads to the following conclusions: first, the iron–methionine bond of cytochrome *c*-552 is more stable than that of horse cytochrome *c*; second, the stability of the iron–methionine coordination is close to that of the secondary structure in the cytochrome *c*-552. In horse cytochrome *c*, on the other hand, the iron–methionine bond is less stable than its secondary structure, which is consistent with the proposal that the replacement of one or both protein ligands precedes the gross unfolding of the polypeptide chain in urea (Stellwagen, 1968).

Our previous attempts to bleach completely the 690-nm band of ferricytochrome *c*-552 with an increase in the temperature were unsuccessful because of the formation of the protein precipitate (Hon-nami & Oshima, 1977). As is shown in Figure 10, by the addition of Gdn·HCl (3.6–4.2 M) the transition temperature was lowered. However, the solution became turbid after an excess heating. The biphasic nature of the van't Hoff plots in the presence of denaturants and the independence of ΔH from the denaturant concentration in the higher temperature range were observed, as is shown in Figure 11. A similar result was reported in a study of horse cytochrome *c* (Kaminsky et al., 1973). They concluded that the disruption of the iron–methionine bond proceeds via different mechanisms at high and low temperatures.

In summary, the heme iron–methionine bond of ferricytochrome *c*-552 is highly stable to acid and to Gdn·HCl, in addition to alkaline and to heat, as compared with that of horse cytochrome *c*. Since the heme is considered to be coordinated with the histidine and methionine residues in both cytochromes *c*, the different stability in the heme–ligand bond is most probably due to the difference in the contribution of amino acid residues in forming the closed heme crevice structure.

Acknowledgments

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References

Almassy, R. J., & Dickerson, R. E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2674–2678.

Aune, K. C., & Tanford, C. (1969) *Biochemistry 8*, 4586–4590.

Aviram, I., & Weissman, C. (1978) *Biochemistry 17*, 2020–2025.

Babul, J., & Stellwagen, E. (1971) *Biopolymers 10*, 2359–2361.

Dickerson, R. E., & Timkovich, R. (1975) *Enzymes, 3rd Ed. 11*, 397–547.

Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A., & Margoliash, E. (1971) *J. Biol. Chem. 246*, 1511–1535.

Hon-nami, K., & Oshima, T. (1977) *J. Biochem. (Tokyo) 82*, 769–776.

Hon-nami, K., Oshima, T., Kihara, H., & Kagawa, Y. (1979) *Biochem. Biophys. Res. Commun. 87*, 1066–1071.

Kaminsky, L. S., Miller, V. J., & Davison, A. J. (1973) *Biochemistry 12*, 2215–2221.

Kihara, H., Hon-nami, K., & Kitagawa, T. (1978) *Biochim. Biophys. Acta 532*, 337–346.

Knapp, J. A., & Pace, C. N. (1974) *Biochemistry 13*, 1289–1294.

Korszun, Z. R., & Salemme, F. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5244–5247.

Lambeth, D. O., Campbell, K. L., Zand, R., & Palmer, G. (1973) *J. Biol. Chem. 248*, 8130–8136.

Margoliash, E., & Frohwirt, N. (1959) *Biochem. J. 71*, 570–572.

Nojima, H., Hon-nami, K., Oshima, T., & Noda, H. (1978) *J. Mol. Biol. 122*, 33–42.

Oshima, T., & Imahori, K. (1971) *J. Gen. Appl. Microbiol. 17*, 513–517.

Oshima, T., & Imahori, K. (1974) *Int. J. Syst. Bacteriol. 24*, 102–112.

Salemme, F. R., Freer, S. R., Xuong, Ng. H., Alden, R. A., & Kraut, J. (1973) *J. Biol. Chem. 248*, 3910–3921.

Shechter, E., & Saludjian, P. (1967) *Biopolymers 5*, 788–790.

Sreenathan, B. R., & Taylor, C. P. S. (1971) *Biochem. Biophys. Res. Commun. 42*, 1122–1126.

Stellwagen, E. (1968) *Biochemistry 7*, 2893–2898.

Swanson, R., Trus, B. L., Mandel, N., Mandel, G., Kallai, O. B., & Dickerson, R. E. (1977) *J. Biol. Chem. 252*, 759–775.

Takano, T., Kallai, O. B., Swanson, R., & Dickerson, R. E. (1973) *J. Biol. Chem. 248*, 5234–5255.

Tanaka, N., Yamane, T., Tsukihara, T., Ashida, T., & Kakudo, M. (1975) *J. Biochem. (Tokyo) 77*, 147–162.

Theorell, H., & Åkesson, Å. (1941) *J. Am. Chem. Soc. 63*, 1812–1827.

Timkovich, R., & Dickerson, R. E. (1976) *J. Biol. Chem. 251*, 4033–4046.

Tsong, T. Y. (1975) *Biochemistry 14*, 1542–1547.

Yu, C. A., Yu, L., & King, T. E. (1973) *J. Biol. Chem. 248*, 528–533.