10840 *Inorg. Chem.* **2010**, *49*, 10840–10846
DOI: 10.1021/ic1005924

Inorganic Chemistry
Article

Control of the Stability of *Hydrogenobacter Thermophilus* Cytochrome c_{552} through Alteration of the Basicity of the N-Terminal Amino Group of the Polypeptide Chain

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Received March 30, 2010

In the denatured state of *Hydrogenobacter thermophilus* cytochrome c_{552} (HT), the N-terminal amino group of the polypeptide chain is coordinated to the heme Fe in place of the axial Met, the His$-N_{term}$ form being formed [Tai, H., Munegumi, T., Yamamoto, Y. *Inorg. Chem.* **2009**, *48*, 331−338]. Since the His$-N_{term}$ form can be considered as an ordered residual structure in the denatured protein, its stability significantly influences the energy of the denatured state. In this study, the His$-N_{term}$ forms of the wild-type HT and its mutants possessing a series of amino acid residues at the N-terminal, such as N1D, N1E, and N1G, have been characterized to elucidate the physicochemical properties of the N-terminal residue responsible for the control of the thermodynamic stability of the His$-N_{term}$ form. The study revealed that the thermodynamic stability of the His$-N_{term}$ form depends highly on the basicity of the N-terminal amino group of the polypeptide chain in such a manner that an increase in the pK_a value of the N-terminal amino group by 1 unit results in stabilization of the bond between heme Fe and the N-terminal amino group (Fe$-N_{term}$ bond) in the His$-N_{term}$ form by ∼4 kJ mol^{-1}. The empirical hard and soft acid and base principle could account for the observed relationship between the pK_a value of the N-terminal amino group and the stability of the Fe$-N_{term}$ bond in the His$-N_{term}$ form. In addition, the study demonstrated that the overall stability of the protein can be manipulated through the energy of the denatured protein by changing the thermodynamic stability of the His$-N_{term}$ form. Consequently, the overall stability of the protein has been shown to be controlled through alteration of the basicity of the N-terminal amino group of the polypeptide chain. These findings provide new insights into the stabilizing interactions in the denatured protein, which are relevant as to characterization of the protein stability and folding.

Introduction

There is considerable interest in enhancement of the stability of proteins.[1,2] A great number of studies have been carried out to delineate the structural determinants underlying the stability of proteins. Although the enhancement of protein stability is not an easy task in practice, the strategy for accomplishing it is quite clear in principle. Since protein stability is determined by the difference in the energy between the native and denatured states (ΔG_{ND}), further stabilization of the native state and/or further destabilization of the denatured one should result in enhancement of the protein stability. Much work on protein stability has focused on the native state rather than the denatured one, because the energetics of the well-ordered compact native states of proteins can be more straightforwardly analyzed on the basis of detailed structural data on the proteins readily available than in the case of the structurally ill-defined denatured states.[3−9] However, as pointed out by Bowler et al.,[10−12] recent advances in structural and thermodynamic studies on denatured states of proteins allow us to manipulate the ΔG_{ND} value through the properties of the denatured states.

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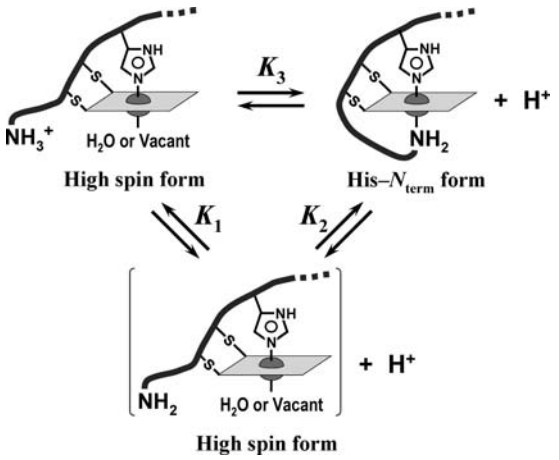
 Published on Web 11/08/2010

Scheme 1. pH Dependence of the Heme Coordination Structure in the Denatured HT and Its Mutants Considered in the Study



It is now widely accepted that the denatured states of proteins can contain somewhat ordered structures known as residual structures.[10−19] Such residual structures can significantly influence the energy of a denatured state.[12] We have shown that a residual structure is formed upon guanidine hydrochloric acid (GdnHCl)-induced unfolding of the oxidized form of *Hydrogenobacter thermophilus* cytochrome c_{552} (HT).[20] HT is a class I cytochrome *c* (cyt *c*) that contains a single heme covalently bound to a polypeptide chain composed of 80 amino acid residues.[21,22] In HT, His and Met residues are coordinated to the heme Fe as axial ligands in the native state.[23,24] However, upon unfolding of the protein, the N-terminal amino group of the polypeptide chain acts as an axial ligand for the heme Fe in place of the native axial Met, the His−N_{term} form being formed.[20,25] Since the formation of the His−N_{term} form has been shown to significantly influence the energy of the denatured state,[20] detailed knowledge of the properties of the His−N_{term} form is useful for manipulation of the ΔG_{ND} value through the energy of the denatured state.

As illustrated in Scheme 1, the formation of the His−N_{term} form is highly pH dependent. The bond between heme Fe and the N-terminal amino group (Fe−N_{term} bond) is broken as the N-terminal amino group is protonated under low pH conditions. Since cleavage of the Fe−N_{term} bond accompanies a change in the spin state of the heme Fe, that is, the ferric low spin (LS) His−N_{term} form is converted to a high spin (HS) ferriheme species,[20,25−27] this process can be readily and sensitively detected through observation of the Soret absorption change, and hence equilibrium constant K_3 in Scheme 1 is yielded by fitting of the data to the Henderson−Hasselbalch equation.[20,27,28] This equilibrium can be represented by a two-step process, that is, deprotonation of the N-terminal amino group and formation of the Fe−N_{term} bond, which can be assessed on the basis of equilibrium constants K_1 and K_2, respectively. Since K_1 can be estimated through the study of an appropriate model peptide, the K_2 value, which reflects the thermodynamic stability of the His−N_{term} form, can be obtained as $K_2 = K_3/K_1$, if $K_2 \gg 1$,[29] or on numerical fitting, if otherwise (see Materials and Methods). A change in the K_2 value leads to alteration of the thermodynamic stability of the His−N_{term} form, which in turn directly influences the energy of the denatured state of the protein. Obviously, the K_2 value is determined by the energy of the Fe−N_{term} bond and the conformational energy of an N-terminal polypeptide stretch composed of nine residues, that is, NEQLAKQKG. We have prepared HT mutants possessing a series of amino acid residues as the N-terminal residue, such as N1D, N1E, and N1G, to characterize the relationship between the thermodynamic stability of the His−N_{term} form and the properties of the N-terminal amino group of the polypeptide chain, and also to analyze the effect of a change in the thermodynamic stability of the His−N_{term} form on the overall protein stability.

In this study, we characterized the GdnHCl-induced unfolding of HT and its mutant proteins, that is, N1D, N1E, and N1G. We demonstrated that the thermodynamic stability of the His−N_{term} form increases with increasing basicity of the N-terminal amino group, and also that a protein with higher stability of the His−N_{term} form exhibits lower overall protein stability, as expected from the effect of the energy of the denatured state on the ΔG_{ND} value. Thus, the stability of the protein can be controlled by altering the basicity of the N-terminal amino group.

Materials and Methods

Protein Preparation. The wild-type HT and its mutants were produced using *Escherichia coli* and purified as reported previously.[30] The oxidized proteins were prepared by the addition of a 10-fold molar excess of potassium ferricyanide. GdnHCl was obtained as a powder from MP Biomedicals and used without purification. For the NMR measurements, GdnHCl-d_6 (Cambridge Isotopes) was used as received. We prepared 8.0 M GdnHCl solution, and its concentration was determined through the measurement of refractive index, as described previously.[31]

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Abbe NAR-1 refractometer (ATAGO CO., Ltd., Japan) was use for the measurement. GdnHCl solutions of desired concentrations were prepared by diluting 8.0 M stock solution. The pH of the sample was adjusted using 0.2 M KOH or 0.2 M HCl, and was monitored with a Horiba F-22 pH meter with a Horiba type 6069-10C electrode. The pH meter has been shown to underestimate the pH value of a bulk solution in the presence of a high salt concentration. A GdnHCl concentration ([GdnHCl])-dependent pH correction factor (ΔpH) was described as follows.[32]

$$\Delta\mathrm{pH} = -0.182 \times [\mathrm{GdnHCl}]^{1/2} + 0.161 \times [\mathrm{GdnHCl}] + 0.0055 \times [\mathrm{GdnHCl}]^2$$

Peptide Synthesis. The amino acid residues used for the synthesis were of the *L*-configuration, and purchased from Millipore Inc. The N^{α}-amino group was protected exclusively with a 9-fluorenylmethoxycarbonyl (Fmoc) group.[33,34] The side chain functional groups were protected with the following: a *t*-butyl group for Asp and Glu; a *t*-butyloxycarbonyl group for Lys; and a triphenylmetyl group for Asn and Gln. The peptide fragments were synthesized by means of solid phase methodology on a Perseptive Biosystems 9050 peptide synthesizer. To prepare peptide fragments, N^{α}-(9-fluorenylmethoxycarbonyl)-glycine *p*-alkoxylbenzyl alcohol polyethyleneglycol-modified polystyrene resin[35] (0.51 mmol Gly per gram resin (Watanabe Chemical Industries Inc.)) was used. After deprotection of the N-terminal Fmoc group with 20% piperidine in *N,N*-dimethylformamide, to obtain peptides with the desired sequences, each derived amino acid residue was coupled successively in the presence of *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate[36] on the resin.

The obtained peptide-resin was treated with a mixture consisting of 0.6 mL of *m*-cresol, 3.6 mL of thioanisol, and 25.8 mL of trifluoroacetic acid at room temperature for 2 h to cleave the peptide from the resin, as well as to deprotect the side chain functional groups. To the brown filtrate of the reaction mixture was added 70 mL of diethyl ether in an ice bath. The precipitate obtained on filtration was washed with diethyl ether several times and then dissolved in 0.5 M acetic acid. The obtained solution was lyophilized to give ~150 mg of product, which was used for NMR measurement without further purification because it exhibited higher purity than 95% on reversed-phase high performance liquid chromatography (RP-HPLC).

RP-HPLC was performed using a 5 μm particle size, 0.46 × 24 cm Lichrospher 100 RP-18 column (Merck) with a 0.46 × 1 cm RP-18 guard column (Merck) on a liquid chromatography system composed of a JUSCO-880 pump and a JUSCO-875 UV detector. Two isocratic solvent systems, mixtures of 0.1% trifluoroacetic and acetonitrile in different ratios (15% and 35% acetonitrile), were used to elute the peptides at a flow rate of 0.5 mL/min, and the effluent was monitored as to the UV absorption at 210 nm.

The molecular weights (*MW*) of the synthesized peptides were measured with a QStar Plusar i (Applied Biosystems). The experimental settings for the electron spray ionization (ESI) mass spectrometry were optimized automatically. The results were processed using the AnalystQS software with the instrument. Each sample was dissolved in distilled water to the concentration of ~10 μM, followed by direct injection into the ESI source with a syringe pump at a flow rate of 5 μL/min. Full mass spectra were acquired in the positive ionization mode over the *m*/*z* 100−1000 range. For example, in the case of P−N1G, two major ions were observed at *m*/*z* 320.2 and 479.8, which indicated $Z = 3$ and 2, respectively (see Supporting Information). The experimentally observed *MW*s were equal to the theoretically calculated values for the corresponding peptides.

^{1}H NMR Spectroscopy. NMR spectra were recorded on a Bruker Avance-600 FT NMR spectrometer operating at a ^{1}H frequency of 600 MHz. Samples for NMR measurements comprised ~1.0 mM protein in nominal ^2H_2O, together with 20 mM potassium phosphate buffer, p^{2}H 7.0. Typical spectra of oxidized cyts *c* required a 100 kHz spectral width, 32k data points, a ~10 μs 90° pulse, a ~1 s recycle time, and 2k scans, and the water signal was suppressed with a 100 ms presaturation pulse. Chemical shifts are given in parts per million (ppm) downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate with H_2O as an internal reference.

pH Titration of Denatured Proteins. Absorption spectra of 5 μM proteins were recorded on a Beckman DU 640 spectrophotometer. Cleavage of the Fe−N_{term} bond of the His−N_{term} form with decreasing pH was monitored at 25 °C as the pH dependence of the 399.5 nm absorbance ($A_{399.5}$) (see Supporting Information), and the results were fitted to the following equation to yield the pK_3 value,[20]

$$A_{399.5} = \{A_{399.5,\mathrm{LS}} + A_{399.5,\mathrm{HS}}[10^{n(\mathrm{p}K_3 - \mathrm{pH})}]\}/[1 + 10^{n(\mathrm{p}K_3 - \mathrm{pH})}]$$

where $A_{399.5,LS}$ and $A_{399.5,HS}$ are the 399.5 nm absorbance of the LS His−N_{term} form and the HS ferriheme species resulting from the loss of the Fe−N_{term} bond, respectively, and *n* is the number of protons involved in the process. But since the K_2 value of the proteins considered in the study are relatively small (see below), deprotonated high spin form emerges almost throughout the course of the pH titration for determining the pK_3 values of proteins. Consequently, the equilibrium constant determined by the fitting is not identical to K_3, but H^+ concentration ($[H^+]$) at an inflection point. We therefore labeled the equilibrium constant determined by the fitting as K_3'.

Determination of the K_2 Value. The K_2 values of the present proteins are not ≫1, and hence the K_2 values have been obtained on fitting. The equilibrium constant (K) for the formation of the His−N_{term} form at a given pH value is expressed as $K = K_2/(1 + ([H^+]/K_1))$, and hence plots of fraction of the His−N_{term} form, that is, $K/(1 + K)$, against pH can be fitted with the Henderson−Hasselbalch equation to yield the pK_3'' value as an inflection point. The pK_3'' values should be equal to the pK_3' values determined from the analysis of the pH dependence of the 399.5-nm absorbance (see above). Since the K_1 values for the present proteins were available through the studies of the model peptides, the K_2 value for each protein is determined as an optimal value which satisfies p$K_3'' =$ pK_3'.

GdnHCl-Induced Unfolding of Proteins. GdnHCl-induced unfolding of protein was carried out in 20 mM potassium phosphate buffer at 25 °C and was monitored as the absorption change at 686 nm, recorded using a Beckman DU 640 spectrophotometer (see Supporting Information). Assuming the linear dependence of energy (ΔG) on [GdnHCl], that is, $\Delta G = \Delta G_{ND} - m[\mathrm{GdnHCl}]$, where *m* is a constant, the results were fitted to a two-state model according to the following equation,[37]

$$\theta_{obs} = ((\theta_N + m_N[\mathrm{GdnHCl}]) + (\theta_D + m_D[\mathrm{GdnHCl}]) \times \exp\{-(\Delta G_{ND} - m[\mathrm{GdnHCl}])/RT\})/(1 + \exp\{-(\Delta G_{ND} - m[\mathrm{GdnHCl}])/RT\})$$

Where θ_{obs} is the observed absorbance, θ_N and m_N (θ_D and m_D) are the intercept and slope of a line drawn for the native (denatured) form with the assumption of a linear relationship between θ_{obs} and [GdnHCl], and R and T represent the gas

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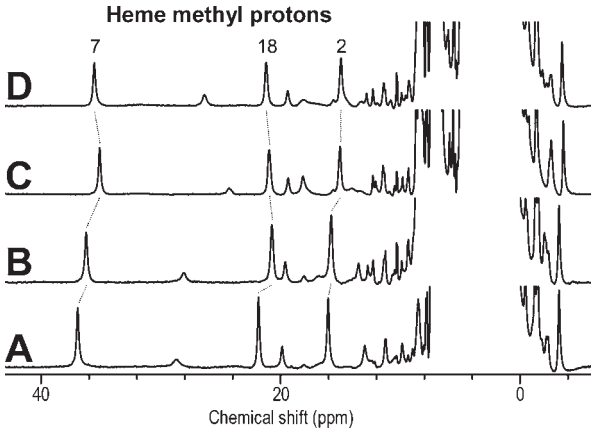

Figure 1. 600 MHz ^{1}H NMR spectra of the His−N_{term} forms of *H. thermophilus* cytochrome c_{552} (HT) (A), the N1D mutant (B), the N1E mutant (C), and the N1G mutant (D) at 25 °C and p^{2}H 7.00, in the presence of [GdnHCl-d_6] = 6.0 M. The assignments[25] of the heme methyl proton signals are indicated with the spectra, and the corresponding signals in the different spectra are connected by broken lines.

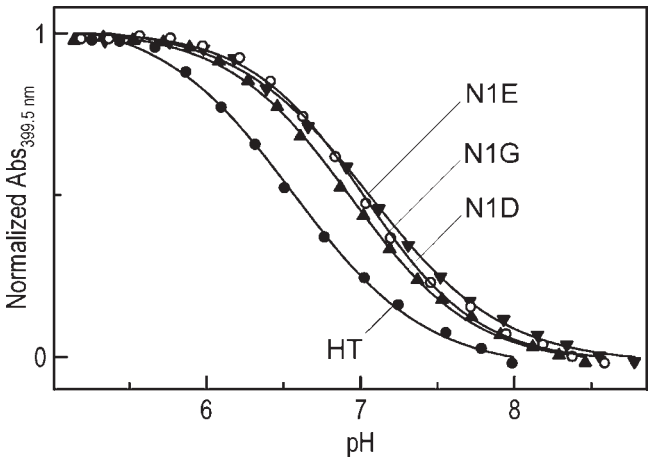

Figure 2. Plots of the normalized 399.5 nm-absorbance of oxidized HT (●), N1D (▲), N1E (▼), and N1G (○) against pH in the presence of [GdnHCl] = 6.0 M at 25 °C. The results for HT and N1E were taken from ref 20.

constant and absolute temperature, respectively. [GdnHCl] at the unfolding midpoint, C_m, was determined from the fitting.

Results

^{1}H NMR Spectra of Mutant Proteins. We first analyzed the effects of the amino acid substitutions on the heme active site structure of the native protein by ^{1}H NMR. In the ^{1}H NMR spectra of the oxidized cyts *c*, the resolved heme methyl and Fe-bound Met proton signals, which are highly sensitive to the heme active site structure,[38−42] were essentially unaltered by the amino acid replacements (see Supporting Information). These results indicated that, as in the cases of the mutant proteins previously studied, the heme active site structure is not affected by the replacement of the N-terminal amino acid residue.[20]

We next compared the heme electronic structures of the oxidized forms of the His−N_{term} forms of the mutant proteins with that of the wild-type HT through analysis of their ^{1}H NMR spectra (Figure 1). As has been demonstrated previously, the heme methyl proton signals of the His−N_{term} form of the protein, observed in 15−40 ppm, were slightly affected by the replacement of the N-terminal amino acid residue.[20] In addition, the signals emerging in the shift range characteristic of the HS ferriheme species,[25,26] that is, downfield of 50 ppm, were observed for the all denatured proteins at p^{2}H 7.0, and their intensities varied among the proteins (see Supporting Information). The interconversion between the His−N_{term} form and the HS ferriheme species in the denatured proteins takes place through cleavage/formation of the Fe−N_{term} bond.

Determination of the K_3' Values. The K_3' value can be determined through the analysis of pH dependence of the Soret absorption band (Figure 2 and see Supporting Information).[20,27] The pK_3' values obtained on fitting of the data to the Henderson−Hasselbalch equation are summarized in Table 1. The pK_3' values of the mutant proteins varied in the range of about 0.4 pH units between 6.66 ± 0.06 for the wild-type HT and 7.06 ± 0.03 for the N1E mutant protein. Thus, as has been reported previously,[20] the pK_3' value was affected by the N-terminal amino acid residue.

Estimation of the K_1 Values. We synthesized nonapeptides of which the amino acid sequences are identical to those of the N-terminal stretches of the proteins under consideration to estimate the pK_1 values of the mutant proteins. The pK_a values of the N-terminal amino groups of the peptides in [GdnHCl-d_6] = 6.0 M were determined from the pH-dependent profiles of the C_αH or C_βH proton NMR shifts of the N-terminal residues (see Supporting Information), as summarized in Table 1. The pK_a values for the peptides ranged from 7.50 ± 0.02[20] for the wild-type HT to 8.60 ± 0.04 for the N1G mutant. It is assumed that these pK_a values can be represented as the pK_1 values in Scheme 1.

Thermodynamic Stability of the His−N_{term} Form. With the assumption that the pK_a values of the N-terminal amino groups of the model peptides are equal to the pK_1 values of the corresponding N-terminal stretches of the proteins, the pK_2 values were obtained on fitting (see Materials and Methods), as shown in Table 1. The pattern of HT < N1D < N1E < N1G, in order of increasing the absolute value of the pK_2 value, indicated that the thermodynamic stability of the His−N_{term} form can be controlled by the N-terminal residue. Furthermore, the change in $A_{399.5}$ ($\Delta A_{399.5}$) upon the pH titration of the proteins in [GdnHCl] = 6.0 M, obtained relative to the absorbance of the HS ferriheme species adjusted to 1.0, is summarized in Table 1. The $\Delta A_{399.5}$ value for the wild-type HT was considerably smaller than those of the other proteins. In the case of the wild-type HT, substituting its pK_1 (7.50 ± 0.02) and pK_2 (−0.77 ± 0.06) values in $K = K_2/(1 + ([H^+]/K_1))$ yielded ~4.5 for the *K* value at pH 8.0, that is, the His−N_{term} form and HS ferriheme species coexist in a ratio of ~82:18 at equilibrium. Consequently,

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Table 1. Equilibrium Constants, $K_1 - K_3'$, of the Reactions Associated with the Formation of the His-N_{term} Forms of Denatured HT and Its Mutants in the Presence of [GdnHCl] = 6.0 M at 25 °C, the Change in the 399.5 nm Absorbance upon the pH Titration of the Proteins in [GdnHCl] = 6.0 M, and the Parameters for the GdnHCl-Induced Unfolding at 25 °C and pH 8.0 and 5.0

						pH 8.0			pH 5.0		
Cyt *c*	pK_3'[a,i]	pK_1[b,i]	pK_2[c]	ΔG_D[d] (kJ mol^{-1})	$\Delta Abs_{399.5}$[e]	C_m[f] (M)	m[g,i] (kJ mol^{-1} M^{-1})	ΔG_{ND}[h,i] (kJ mol^{-1})	C_m[f] (M)	m[g,i] (kJ mol^{-1} M^{-1})	ΔG_{ND}[h,i] (kJ mol^{-1})
HT	6.66 ± 0.06[j]	7.50 ± 0.02[j]	−0.77 ± 0.06[k]		0.28	4.5 ± 0.1	12 ± 1	55 ± 6	4.1 ± 0.1	13 ± 1	52 ± 5
N1D	6.93 ± 0.04	8.24 ± 0.03	−1.29 ± 0.04	1.2 ± 0.4	0.34	4.3 ± 0.1	12 ± 1	52 ± 5	4.0 ± 0.1	13 ± 1	51 ± 5
N1E	7.06 ± 0.03[j]	8.40 ± 0.03[j]	−1.32 ± 0.04[k]	0.7 ± 0.4	0.35	3.6 ± 0.1	13 ± 1	48 ± 5	3.9 ± 0.1	13 ± 1	49 ± 5
N1G	7.01 ± 0.02	8.60 ± 0.04	−1.58 ± 0.04	1.3 ± 0.4	0.33	4.0 ± 0.1	13 ± 1	51 ± 5	4.1 ± 0.1	13 ± 1	52 ± 5

[a] The K_3' value was obtained through the pH dependence of the absorption spectra. [b] The K_1 value was assumed to be equal to the pK_a value of the *N*-terminal amino group of the corresponding model peptide. [c] The K_2 value was obtained on fitting as described in Materials and Methods. The errors were calculated as $[(Error_{pK_1})^2 + (Error_{pK_3})^2]^{1/2}$. [d] The net stabilization of the His-N_{term} form of mutant at pH 8.0 relative to that of wild-type protein. The error for the individual mutant was calculated as $\{(2.3RT)^2[(Error_{pK_{2(HT)}})^2 + (Error_{pK_{2(mutant)}})^2]\}^{1/2}$. [e] The change in the 399.5 nm absorbance upon the pH titration of the proteins in [GdnHCl] = 6.0 M. The change was obtained relative to the absorbance of high spin ferriheme species adjusted to 1.0. [f] [GdnHCl] at the unfolding midpoint. The average values of three or four separate experiments are given with the standard deviations as the errors. [g] The rate of change of the energy of unfolding as a function of [GdnHCl], that is, $d\Delta G/d[GdnHCl]$. [h] The unfolding energy. [i] The average values of three or four separate experiments are given with the standard deviations as the errors. The fitting errors for determining the pK_3', pK_1, m, and ΔG_{ND} values were estimated to be < 0.3%, < 0.2%, < 5%, and < 5%, respectively. [j] Cited from ref 20. [k] The absolute values of the pK_2 values of HT and N1E mutant protein, obtained using the technique described in the present manuscript, are smaller by about 17% and 3% relative to those reported in ref 20, respectively.

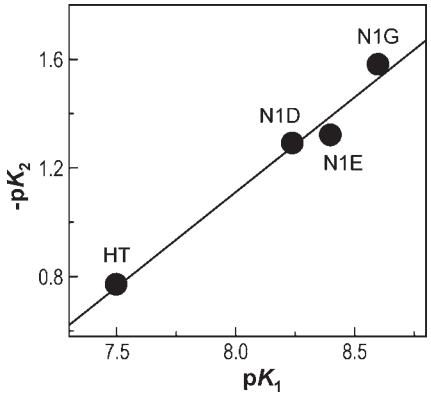

Figure 3. Plots of the pK_2 values against the pK_1 ones for HT and its mutants in the presence of [GdnHCl] = 6.0 M at 25 °C. The plots could be represented by a straight line expressed as $pK_2 = -0.70 \times pK_1 + 4.48$.

the small $\Delta A_{399.5}$ value for the wild-type HT is attributed to the presence of relatively high concentration of the HS ferriheme species at equilibrium.

The plots of the pK_2 values against the pK_1 ones could be represented by a straight line (Figure 3). These plots demonstrated that the thermodynamic stability of the His−N_{term} form increases with increasing basicity of the N-terminal amino group of the polypeptide chain, indicating that the strength of the Fe−N_{term} bond is a primary determinant for control of the thermodynamic stability of the His−N_{term} form.

Thermodynamic Stability of the His−N_{term} Form and Its Effect on the Overall Protein Stability. The His−N_{term} form can be considered as an ordered residual structure in the denatured protein.[20,25] The stability of the native protein is expected to be affected by that of the His−N_{term} form through its effect on the energy of the denatured state. In fact, as illustrated in Figure 4, the GdnHCl-induced unfolding curves of the mutant proteins at pH 8.0 deviated from that of the wild-type HT. The determined C_m, m, and ΔG_{ND} values of the proteins are listed in Table 1. The proteins at pH 8.0 are ranked as N1E < N1G < N1D < HT, in order of increasing C_m value as well as the ΔG_{ND} one. At pH 8.0, the net stabilization of the His−N_{term} form of the mutant proteins relative to that of the wild-type HT (ΔG_D) were estimated to be 0.7−1.3 kJ mol^{-1} (Table 1), and the ΔG_D value is likely to partly account for the difference in the ΔG_{ND} value between the wild-type and mutant proteins.

We then measured GdnHCl-induced unfolding curves of the wild-type HT and mutant proteins at pH 5.0 to characterize the effect of the protonation of the N-terminal amino group on the overall protein stability (Figure 4). Considering the pK_1 values of the N-terminal amino groups of these proteins, that is, $pK_1 = 7.50 \pm 0.02 - 8.60 \pm 0.02$, they are protonated at pH 5.0, and hence the protonation of the N-terminal amino group prevents the formation of the Fe−N_{term} bond, and hence the His−N_{term} form (Scheme 1 and Figure 2). As a result, the deviation of the GdnHCl-induced unfolding curves of the mutant proteins from that of the wild-type HT at pH 5.0 was considerably smaller than that at pH 8.0 (Figure 4). These results demonstrated that the formation of the His−N_{term} form in the denatured state influences the overall protein stability.

Discussion

Thermodynamic Stability of the His−N_{term} Form. The thermodynamic stability of the His−N_{term} form is reflected in the pK_2 value. Plots of the pK_2 values against the pK_1 ones (Figure 3) demonstrated that the thermodynamic stability of the His−N_{term} form is largely affected by the pK_1 (= pK_a) value of the N-terminal amino group of the polypeptide chain. The plots indicated that the thermodynamic stability of the His−N_{term} form increased by ∼4 kJ mol^{-1} with an increase in the pK_1 value of 1 unit. A similar relationship between the affinity of N-donor ligand to the heme Fe and its basicity has been reported for imidazoles, pyridines, and amines.[43−46] According to the empirical hard and soft acid and base principle, the binding of a hard amino group to

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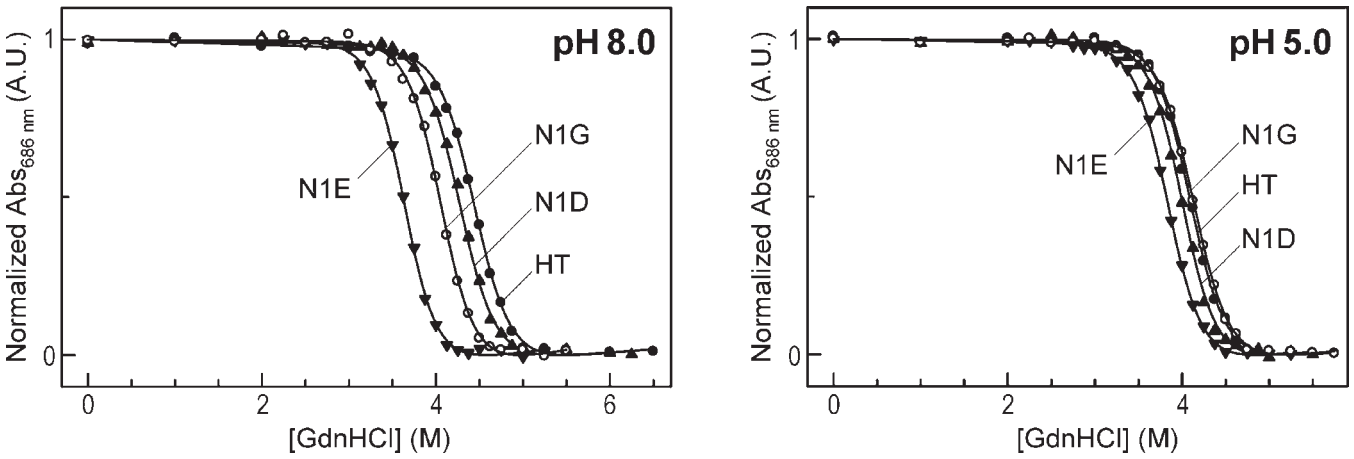

Figure 4. Plots of the normalized 686 nm-absorbance of oxidized HT (●), N1D (▲), N1E (▼), and N1G (○) against [GdnHCl] at 25 °C and pH 8.0 (left) and 5.0 (right).

hard Fe^{3+} becomes stronger with increasing basicity of the amino group. Thus, the present study demonstrated that this empirical principle is applicable to the formation of the His−N_{term} form in the denatured protein. This finding provides new insights into the control of the energetics of the denatured state of the protein.

Furthermore, the plots in Figure 3 can be represented by a straight line, which is expressed as $pK_2 = -0.70 \times pK_1 + 4.48$. Therefore, in the cases of the wild-type HT and its N-terminal amino acid residue mutant proteins, the pK_1 value of the N-terminal amino group should be > 6.4 for the stable formation of the His−N_{term} form. But, it is likely that the pK_1 criterion for the stable formation of the His−N_{term} form is affected by the structural properties of the N-terminal stretch such as the sequence and number of constituent amino acid residues.[10−12,20,47−49]

The formation of the His−N_{term} form affects the pathway and kinetics of the folding/unfolding of the protein. The pairing of two helices at opposite ends of the polypeptide chain is a highly conserved structural motif found in the cyt *c* family, and the association of the N- and C-terminal helices has been shown to accompany the first step of usual cyt *c* folding.[50−54] Further folding is expected to be prevented at this stage by the formation of the His−N_{term} form. In fact, it has been shown from the studies of yeast iso-1-cyt *c* mutant[27] and recombinant equine cyt *c*[28] not only that non-native ligation states of heme such as the His−N_{term} form impose kinetic traps that slow down refolding kinetics of the proteins, but also that chemical modification of N-terminal amino group to prevent the formation of the His−N_{term} form leads to considerable acceleration of the protein refolding. Furthermore, in the unfolding and refolding studies of HT and a homologous protein *Pseudomonas aeruginosa* cyt c_{551} (PA), it has been shown that these two proteins at pH 4.7 unfold and refold without kinetic traps,[22,55,56] and that the refolding of PA at pH 7.0 is considerably slower than that at pH 4.7.[55,56] Since, as in the case of HT, the His−N_{term} form has been shown to be formed in the denatured state of PA,[20] the slow refolding of PA at physiological pH would be due to the formation of the His−N_{term} form as a kinetic trap.

Effect of the Formation of the His−N_{term} Form on the Overall Protein Stability. Since the His−N_{term} form can be considered as an ordered residual structure in the denatured protein, its stability is expected to influence the energy of the denatured state of the protein, and hence the overall protein stability.[20,25] The studies on the GdnHCl-induced unfolding of the oxidized proteins revealed that the wild-type HT at pH 8.0 was destabilized on replacement of the N-terminal amino acid residue (Figure 4 and Table 1). Comparison of the pK_2 and C_m values among the wild-type HT, and N1D and N1G mutant proteins at pH 8.0 demonstrated that a protein with higher stability of the His−N_{term} form exhibits lower overall protein stability. The decrease in the stability of the wild-type HT caused by these mutations is likely to be due to the stabilization of the denatured state of the protein through strengthening of the Fe−N_{term} bond because of the increase in the basicity of the N-terminal amino group with the amino acid replacements. According to the X-ray structure of the wild-type HT,[22] the N-terminal Asn side chain does not undergo any particular intramolecular interaction that contributes to stabilization of the native conformation, and hence the energy of the native state is not likely to be so significantly affected by its replacement. On the other hand, the energy of the denatured state of the protein at pH 8.0 is affected by the thermodynamic stability of the His−N_{term} form, which can be controlled through the effect of the basicity of the N-terminal amino group on the strength of the Fe−N_{term} bond, as described above. This was confirmed by the results of the studies on the GdnHCl-induced

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unfolding of the proteins at acidic pH. At pH 5.0, the protonation of the N-terminal amino groups of the proteins prevents the formation of the His$-N_{term}$ form (Figure 2), and hence no ordered residual structure is expected to be formed in the denatured state. As a result, the energy of the denatured state of the protein, and hence the overall protein stability, should be nearly independent of the N-terminal amino acid residue. In fact, the C_m values obtained from the GdnHCl-induced unfolding curves of the mutant proteins at pH 5.0 were almost identical to the corresponding values of the wild-type HT (Figure 4 and Table 1), confirming that the overall protein stability is independent of the N-terminal amino acid residue, provided that the residue is not involved in a particular intramolecular interaction in either the native or the denatured state of the protein. Thus, the results obtained in the study on the GdnHCl-induced unfolding of the proteins at pH 8.0 demonstrated that the overall protein stability is controlled through the energy of the denatured state. In the present proteins, the energy of the denatured state is related to the thermodynamic stability of the His$-N_{term}$ form, which can be altered through the strength of the Fe$-N_{term}$ bond by changing the basicity of the N-terminal amino group of the polypeptide chain.

The effect of the formation of the His$-N_{term}$ form on the stability of the N1E mutant protein was somewhat different from the cases of the other mutant ones. Since the pK_2 values of the N1D and N1E mutants are similar to each other, these two proteins are expected to exhibit similar GdnHCl-induced unfolding curves. However, the stability of the N1E mutant protein at pH 8.0 was considerably lower than that of the N1D one, although the unfolding curves of the proteins at pH 5.0 were roughly similar to each other. The N-terminal Glu side chain carboxylate in the N1E mutant protein is capable of interacting with its amino group,[57] while such an intraresidue interaction does not occur in the N1D one. The anomalously lower stability of the N1E mutant protein compared with that of the N1D one would be in part attributed to the intraresidue interaction characteristic of the former protein, although detailed structural and thermodynamic characterization of the proteins is needed to elucidate structural factors responsible for the difference in the overall protein stability between them at pH 8.0.

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Conclusion

The characterization of the His$-N_{term}$ forms of the wild-type HT and its N-terminal amino acid residue mutant proteins revealed that the thermodynamic stability of the His$-N_{term}$ form was affected by the basicity of the N-terminal amino group of the polypeptide chain in such a manner that an increase in the pK_a value of the N-terminal amino group by 1 unit results in stabilization of the Fe$-N_{term}$ bond in the His$-N_{term}$ form by ~4 kJ mol^{-1}. This study also demonstrated that the overall protein stability can be manipulated through the energy of the denatured protein via a change in the thermodynamic stability of the His$-N_{term}$ form. Hence, the overall stability of the protein can be controlled through alteration of the basicity of the N-terminal amino group of the polypeptide chain.

Acknowledgment. We wish to thank Dr. Yoshihiro Sambongi (Hiroshima University) for the gift of the wild-type proteins. This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas (No. 21108505, "π-Space") from MEXT, the Yazaki Memorial Foundation for Science and Technology, and the NOVARTIS Foundation (Japan) for the Promotion of Science.

Supporting Information Available: The results of measurement of the molecular weights of the synthesized peptides, the absorption spectra, 565−795 nm, of oxidized HT at 25 °C and pH 8.0 and 5.0 in the presence of various [GdnHCl], the 600 MHz ^{1}H NMR spectra of oxidized HT, N1D, N1E, and N1G in 20 mM potassium phosphate butter, p^{2}H 7.00, at 25 °C, the 600 MHz ^{1}H NMR spectra of oxidized HT, N1D, N1E, and N1G in the presence of [GdnHCl-d_6] = 6.0 M at 25 °C and p^{2}H 7.00, the Soret absorption of oxidized HT, N1D, N1E, and N1G in the presence of [GdnHCl] = 6.0 M at 25 °C and the pH range of 5.2 to 8.6, the 600 MHz ^{1}H NMR spectra of the synthesized peptides in ^2H_2O at p^{2}H 3.0 and 25 °C, the ^{1}H NMR signal assignments for the synthesized peptides (P−N1D and P−N1G), the pH dependence of portions of the 600 MHz ^{1}H NMR spectra of the synthesized peptides (P−N1D and P−N1G) in the presence of [GdnHCl-d_6] = 6.0 M at 25 °C, the pH profiles of the *N*-terminal $C_\alpha H$ or $C_\beta H$ proton shifts of the synthesized peptides (P-HT, P−N1D, P−N1E, and P−N1G) in the presence of [GdnHCl-d_6] = 6.0 M at 25 °C, pH-profile of the normalized 399.5 nm-absorbance observed for HT protein of which N-terminal amino group was chemically modified to an α-keto acid to prevent the formation of Fe$-N_{term}$ bond, in the presence of [GdnHCl] = 6.0 M at 25 °C, and plots of the normalized 686 nm-absorbance of oxidized HT and N1G against [GdnHCl] at 25 °C and pH 8.0. This material is available free of charge via the Internet at http://pubs.acs.org.