Influence of a Single Amide Group on the Redox Function of *Pseudomonas aeruginosa* Cytochrome c_{551}

Yo-ta Takahashi,[1] Shin-ichi J. Takayama,[1] Shin-ichi Mikami,[1] Hajime Mita,[1]
Yoshihiro Sambongi,[2] and Yasuhiko Yamamoto*[1]
[1]*Department of Chemistry, University of Tsukuba, Tsukuba 305-8571*
[2]*Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima 739-8528*

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The influence of a single amide group located in the heme active site of *Pseudomonas aeruginosa* cytochrome c_{551} on its redox potential ($E^{\circ\prime}$) has been characterized through amino acid substitution of Asn64 with Gln or Ala. The $E^{\circ\prime}$ variation of ca. 60 mV among the proteins is attributed mainly to the alteration of the thermodynamic stability of the oxidized protein by the dipole and hydrogen-bonding ability of the amide group.

Proteins significantly modify the electrochemistry of hemes in cytochromes (cyts). The redox potentials ($E^{\circ\prime}$) of cyts vary by more than 700 mV, highlighting the paramount importance of $E^{\circ\prime}$ regulation by a surrounding protein for a wide variety of bioenergetic processes.[1] A prime difficulty in understanding the molecular mechanisms responsible for the $E^{\circ\prime}$ regulation in proteins is quantitative characterization of the physicochemical environment around the heme in a protein, which is created through a heterogeneous distribution of charges, dipoles, and non-uniform flexibility. Regarding such properties of proteins, amide groups have been shown to play various well-established roles in determining protein function such as the oxyanion hole of the serine proteases.[2] We characterize here the influence of a single amide group on the redox function of *Pseudomonas aeruginosa* cyt c_{551}[3] (PA), in which the heme Fe is coordinated to His and Met as axial ligands. According to the X-ray structure of PA,[3] the Asn64 side chain amide group is hydrogen bonded to both Ile48 CO and the axial Met61 S atom, and hence is anchored at the distance of ca. 0.53 nm from the heme Fe (see Supporting Information). In this study, Asn64 in PA was replaced by Gln (N64Q) and Ala (N64A), and then the effect of the distance between the amide group and the heme Fe on the $E^{\circ\prime}$ value has been determined from a comparative study among them.

We first analyzed the effects of the mutations on the heme active site of the protein by means of paramagnetic ^{1}H NMR (Figures 1a and 1b). The heme methyl proton shift pattern of the oxidized protein was greatly affected by the mutations (Figure 1a), demonstrating significant effects of the residue at position 64 on the heme electronic structure. In contrast, the spectral patterns of the cyanide adducts, in which the axial Met was replaced by cyanide ion, were remarkably similar to each other (Figure 1b). The heme electronic structure of the oxidized protein is affected by both axial His and Met coordination bonds, whereas that of the cyanide adducts depends only upon the His coordination.[4] Consequently, these NMR results indicated that the His coordination structure is conserved in these proteins and that the differences in the heme electronic structure among the oxidized proteins is largely due to the Met coordination.[5] The X-ray study indicated that the Fe–Met bond in PA is forced to be the *S* form due to the steric hindrance between Met61 and Asn64.[3] On the other hand, analysis of the heme

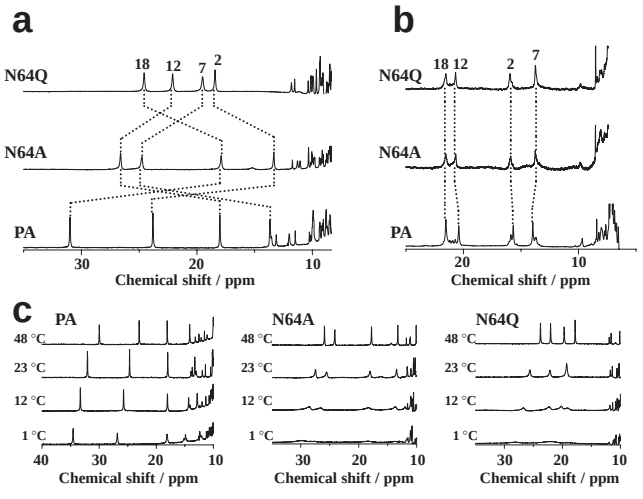

Figure 1. The downfield shifted portions of 600 MHz ^{1}H NMR spectra of the oxidized forms at 35 °C and pH 7.00 (a), and cyanide adducts at 35 °C and pH 9.80 (b) of PA, N64Q, and N64A. The assignments of heme methyl proton signals are indicated in the spectra. (c) The spectra of the oxidized proteins at pH 7.00 and the indicated temperatures.

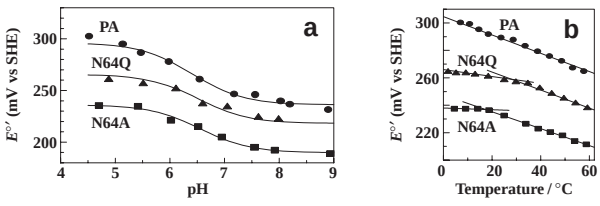

Figure 2. pH-Profile at 40 °C (a) and temperature dependence at pH 6.00 (b) of the redox potentials ($E^{\circ\prime}$) of PA (●), N64Q (▲), and N64A (■).

Table 1. Thermodynamic parameters of the redox reaction for the PA proteins at pH 6.00

	$E^{\circ\prime a}$ (mV vs SHE)	$\Delta H^{\circ\prime a}$/kJ mol^{-1}		$\Delta S^{\circ\prime a}$/J K^{-1} mol^{-1}	
PA	278	−47.0[b]		−64.5[b]	
		ΔH°(Low)	ΔH°(High)	ΔS°(Low)	ΔS°(High)
N64Q	253	−32.3	−44.1	−24.3	−63.5
N64A	221	−24.6	−40.1	−6.0	−59.4

[a]The experimental errors for redox potential ($\Delta E^{\circ\prime}$), enthalpy ($\Delta H^{\circ\prime}$), and entropy ($\Delta S^{\circ\prime}$) were ±5 mV, ±3 kJ mol^{-1}, and ±10 J K^{-1} mol^{-1}, respectively. [b]The value was more negative than that previously reported[8] possibly due to the effect of ionic strength on the $\Delta S^{\circ\prime}$ value.[14]

methyl proton paramagnetic shifts of oxidized N64Q and N64A suggested that their Fe–Met bonds are in the *R* form (see Supporting Information).[5] Hence, it could be assumed that Gln64 and Ala64 in the proteins do not exhibit steric hindrance against Met61 and are not hydrogen bonded to Met61.

We next measured the $E^{\circ\prime}$ values of the proteins at various pHs and temperatures (Figure 2).[6] At 40 °C, the $E^{\circ\prime}$ value of PA was higher by ca. 30 and ca. 60 mV than those of N64Q and N64A, respectively, throughout the pH range examined, although the pH-profiles of their $E^{\circ\prime}$ values reflected identical p*K* values attributable to the pK_a value of the heme 17-propionic acid side chain (Figure 2a).[7] In order to understand the differences in the $E^{\circ\prime}$ value among the proteins, the enthalpic ($\Delta H^{\circ\prime}$) and entropic ($\Delta S^{\circ\prime}$) contributions to the redox reaction were determined from the temperature dependence of their $E^{\circ\prime}$ values (Figure 2b). The plots of the $E^{\circ\prime}$ value of a protein against temperature generally give a straight line, when its structure is retained over the temperature range examined, as shown in the plots for PA.[8] In contrast, the plots for N64Q and N64A could be fitted by two straight lines with transition temperatures (*T*c) at ca. 25 and ca. 20 °C, respectively. Hence, two sets of values, $\Delta S^{\circ(\mathrm{Low})}$ and $\Delta H^{\circ(\mathrm{Low})}$, and $\Delta S^{\circ(\mathrm{High})}$ and $\Delta H^{\circ(\mathrm{High})}$, in the temperature ranges $< T$c and $> T$c, respectively, were determined for each of the proteins (Table 1). Thus, the temperature-dependence study of the $E^{\circ\prime}$ values of N64Q and N64A revealed the presence of two different protein structures possessing distinctly different thermodynamic properties to each other. The paramagnetically-shifted heme methyl proton signals of the oxidized forms of the proteins at lower temperatures exhibited anomalous line-broadening, whereas such line broadening was not observed for PA (Figure 1c). Similar line-broadening has been observed for HT and has been proposed to arise from an internal motion of the axial Met.[9] Hence, the dynamic nature of the Met coordination may be responsible for the appearance of the two different protein structures in the N64Q and N64A systems.

As shown in Table 1, the $\Delta S^{\circ\prime}$ value of PA was essentially identical, within experimental error, to the $\Delta S^{\circ(\mathrm{High})}$ values of N64Q and N64A. This finding indicated that the differences in the $E^{\circ\prime}$ value among the proteins are solely enthalpic in origin. The enthalpic regulation of the $E^{\circ\prime}$ values of the proteins could be explained in terms of the influence of an amide group on the thermodynamic stability of the ferricheme in the oxidized proteins. A model compound study demonstrated that the hydrogen bonding of an NH group to an S atom coordinated to the heme Fe elongates the Fe–S bond.[10] Hence, the stability of the ferricheme in the oxidized PA is expected to be lower than those in the oxidized N64Q and N64A. In addition, the Asn64 side chain NH in PA points toward the heme Fe^{3+} (see Supporting Information), and hence its dipole exerts electrostatic repulsion against the cationic ferricheme, which also contributes to lower the stability of the ferricheme in the protein. In contrast, the stability of the ferroheme in the reduced PA is not so significantly affected by the Asn64 NH dipole because of its electric neutrality. Thus, the Asn64 side chain of PA enhances the difference in the stability between the two redox forms, which in turn results in an increase in the $E^{\circ\prime}$ value of the protein. In the case of N64Q, due to the absence of Gln64–Met61 hydrogen bonding, the side chain amide group of Gln64 is likely to be located slightly further away from the heme Fe than that of Asn64 in PA. In fact, the Gln64[11] amide group in highly homologous *Hydrogenobacter thermophilus* cyt c_{552}[12] (HT) has been shown to be located at ca. 0.59 nm from the heme Fe.[13] Therefore, the stability of the ferricheme in N64Q is expected to be higher than that in PA. Furthermore, because of the lack of a side chain amide group for Ala64, the stability of the ferricheme in N64A could be even higher than that in N64Q. The observed ranking, N64A < N64Q < PA, in order of increasing $E^{\circ\prime}$ value, is consistent with our previous finding that a protein with higher stability in its oxidized form exhibits a lower $E^{\circ\prime}$ value.[7,8] Since a positive shift of >100 mV in the $E^{\circ\prime}$ value has been accounted for by the formation of a hydrogen bond between an NH group and an S atom coordinated to the heme Fe,[10] the $E^{\circ\prime}$ difference of ca. 30 mV between PA and N64Q suggested that the Asn64–Met61 hydrogen bonding in PA is rather weak.

In the present study, the influence of a single amide group near heme of PA on its redox function has been characterized. The results presented here provide novel insights into the functional regulation of the protein, which could be utilized for tuning the $E^{\circ\prime}$ value of the protein through protein engineering.

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