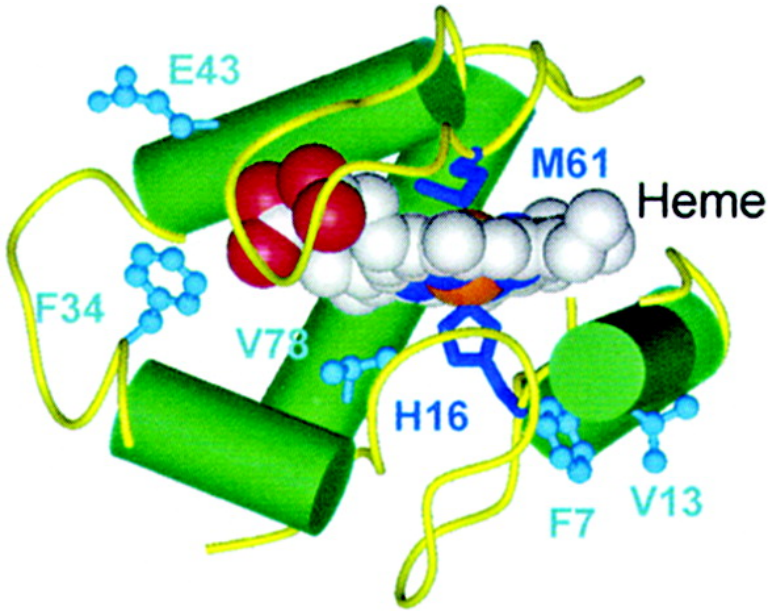
Communication

Relationship between Redox Function and Protein Stability of Cytochromes *c*

Norifumi Terui, Naoki Tachiiri, Hitomi Matsuo, Jun Hasegawa, Susumu Uchiyama, Yuji Kobayashi, Yasuo Igarashi, Yoshihiro Sambongi, and Yasuhiko Yamamoto

J. Am. Chem. Soc., **2003**, 125 (45), 13650-13651• DOI: 10.1021/ja035682f • Publication Date (Web): 21 October 2003



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 5 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
COMMUNICATIONS

Published on Web 10/21/2003

Relationship between Redox Function and Protein Stability of Cytochromes *c*

Norifumi Terui,[†] Naoki Tachiiri,[†] Hitomi Matsuo,[†] Jun Hasegawa,[‡] Susumu Uchiyama,[§] Yuji Kobayashi,[||] Yasuo Igarashi,[⊥] Yoshihiro Sambongi,*[,▽] and Yasuhiko Yamamoto*[,†]

Department of Chemistry, University of Tsukuba, Tsukuba 305-8571, Japan, Daiichi Pharmaceutical Co., Ltd., Edogawa-ku, Tokyo 134-8630, Japan, Graduate School of Engineering, Osaka University, Suita 565-0871, Japan, Graduate School of Pharmaceutical Science, Osaka University, Suita 565-0871, Japan, Department of Biotechnology, University of Tokyo, Tokyo 113-8657, Japan, and Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima 739-8528, Japan

Received April 17, 2003; E-mail: yamamoto@chem.tsukuba.ac.jp; sambongi@hiroshima-u.ac.jp

Understanding the molecular mechanisms responsible for regulation of the redox potentials ($E^{\circ\prime}$) of proteins is a problem of immense fundamental and practical importance. Monoheme Class I cytochromes *c* (cyts *c*), in which heme Fe is coordinated to His N and Met S atoms as axial ligands at the redox center, are some of the best characterized redox active proteins.[1] Homologous Class I cyts *c*, thermophilic *Hydrogenobacter thermophilus* cytochrome c_{552}[2] (HT) and mesophilic *Pseudomonas aeruginosa* cytochrome c_{551}[3] (PA), exhibit a unique thermodynamic property, i.e. despite their structural similarity together with their 56% sequence identity, the oxidized form of HT is significantly more stable than that of PA, as reflected by the large difference in denaturation temperature (T_m) (Table 1).[4a,b] Site-directed mutants of PA, for which amino acid substitutions were selected with reference to the corresponding residues in HT, exhibited thermostabilities between those of PA and HT[4b−e] (Table 1). Among the cyts *c* examined, overall protein stability correlated well with structural features of hydrophobic protein interior.

NMR and optical characterization of the heme active sites in the oxidized (paramagnetic) forms of PA, its mutants, and HT have revealed that the overall protein stability correlates well with the stability of the Fe−S coordination bond.[4f] Here, we illustrate a series of studies involving temperature-dependent cyclic voltammetry (CV) and ^{1}H NMR spectra of the cyts *c*. On the basis of a scrutiny of relationships among the overall protein stability, Fe−S bond stability, and redox properties, we propose a molecular mechanism underlying redox regulation in the cyts *c*.

To determine the relationship between the redox properties and overall protein stability,[5] we first carried out electrochemical measurements at 25 °C using CV.[6] The resulting $E^{\circ\prime}$ values were in the range of +0.2 − +0.3 V, i.e. similar to those reported for other Class I cyts *c*[7a−c] (Table 1). The $E^{\circ\prime}$ values for the PA mutants, F34Y single (sm) and F7A/V13M/F34Y /E43Y/V78I quintuple (qm), were lower than that of the wild-type, corresponding to an increase in overall protein stability. These results indicate that, upon mutation, the oxidized forms are stabilized more than the reduced. Thus, the $E^{\circ\prime}$ values of cyts *c* are determined by the overall stability of the oxidized forms, which are significantly affected by the mutation(s), as manifested in their T_m values (Table 1).

On electrochemical measurement at 5−85 °C, we observed CV curves below 70 and 80 °C for PA and sm, respectively. In contrast, those for qm and HT were detected up to 85 °C. Plots of $E^{\circ\prime}$ against temperature ($E^{\circ\prime}-T$ *plots)* for PA proteins (wild-type and the two mutants) gave straight lines at lower temperatures and exhibited downward curvature with increasing temperature, while those for HT exhibited a straight line over the temperature range examined (Figure 1). The temperature at which the plots started to show curvature correlated with the overall protein stability, as measured the denaturation temperature (Table 1). The curvature observed for the PA proteins indicates that the oxidized form becomes thermodynamically more stable than the reduced as the thermal denaturation proceeds. This is because the oxidized heme becomes more stable with removal of the hydrophobic heme environment on denaturation, as demonstrated by model studies.[7c,8]

From the $E^{\circ\prime}-T$ *plots*, we estimated the enthalpic ($\Delta H^{\circ\prime}$) and entropic ($\Delta S^{\circ\prime}$) contributions to the $E^{\circ\prime}$ value (Table 1).[7a−c] For the PA proteins, the $\Delta H^{\circ\prime}$ values increased consistently with increasing stability of the protein, whereas the $\Delta S^{\circ\prime}$ values remained essentially unchanged. These results suggest that the effect of the mutations in the PA protein on the $E^{\circ\prime}$ value is mainly an enthalpic

Table 1. Denaturation Temperatures and Thermodynamic Parameters of the Redox Reaction for the Cyts *c* Examined

cyt *c*	T_m[a] (°C)	$T_{m(Fe-S)}$[b] (°C)	$E^{\circ\prime}$[c] (V vs SHE)	$\Delta H^{\circ\prime}$[c] (kJ mol^{-1})	$\Delta S^{\circ\prime}$[c] (J K^{-1} mol^{-1})
PA	50.4	78[d]	0.291[e]	−42	−47
sm	66.4	89	0.254	−38	−48
qm	83.9	>100[d]	0.205	−34	−48
HT	91.8	>100[d]	0.215[e]	−31	−35

[a] The denaturation temperature of the oxidized protein in the presence of 1.5 M guanidine hydrochloride.[4b−e] [b] The dissociation temperature of the Fe−S coordination bond.[4f] [c] The experimental errors for the redox potential ($E^{\circ\prime}$), reduction enthalpy ($\Delta H^{\circ\prime}$), and reduction entropy ($\Delta S^{\circ\prime}$) were ±0.005 V, ±3 kJ mol^{-1}, and ±5 JK^{-1}mol^{-1}, respectively. The values were obtained from the plots presented in Figure 1. [d] Obtained from ref 4f. [e] The values were slightly different from those reported[11a,b] due to possible differences in experimental conditions.

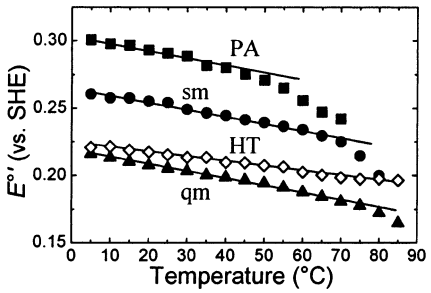


Figure 1. Temperature dependence of the redox potentials ($E^{\circ\prime}$) of PA (■), sm (●), qm (▲), and HT (◇). $E^{\circ\prime}$ values decrease with increasing temperature. The plots of $E^{\circ\prime}$ vs *T* are satisfactorily represented as straight lines, although those for the PA proteins at higher temperatures exhibit a downward curvature.

[†] Department of Chemistry, University of Tsukuba.
[‡] Daiichi Pharmaceutical Co., Ltd.
[§] Graduate School of Engineering, Osaka University.
[||] Graduate School of Pharmaceutical Science, Osaka University.
[⊥] Department of Biotechnology, University of Tokyo.
[▽] Graduate School of Biosphere Science, Hiroshima University.

10.1021/ja035682f CCC: $25.00

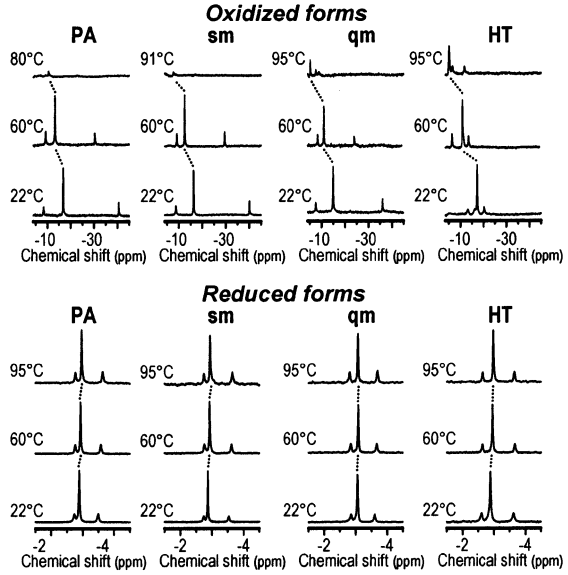


Figure 2. ^{1}H NMR signals of the resolved Fe-bound Met side-chain protons of PA, sm, qm, and HT at pH 6.00 and the indicated temperatures. The signals of the reduced and oxidized forms are shown at the bottom and top, respectively. The corresponding Fe-bound Met $C_\epsilon H_3$ proton signals[12] are connected by dotted lines.

contribution. In addition, the least negative $\Delta H^{\circ\prime}$ value observed for HT was consistent with its highest stability among the oxidized forms of the proteins (Table 1). Furthermore, the least negative $\Delta S^{\circ\prime}$ value of HT (Table 1) indicates that enthalpy−entropy compensation occurs in the redox thermodynamics as found in the other electron-transfer proteins such as cupredoxins[9] and rubredoxins.[10]

Next, we directly compared the stability of the heme active site where the electron-transfer reaction occurs in cyts *c*. On ^{1}H NMR spectroscopy, signals due to Fe-coordinated Met side-chain protons are often resolved in the spectra of both the oxidized and reduced forms of cyts *c*. The temperature-dependent appearance of these signals facilitates direct comparison of the stability of the heme active site. Such Met signals were observed up to 95 °C in the spectra of the reduced forms of all the cyts *c* examined (Figure 2), indicating their high structural stability around the heme active site under the conditions used for the electrochemical analysis.

In contrast to the reduced form, Met signals were barely observed in the NMR spectrum of the oxidized form of PA at 80 °C (Figure 2), suggesting that its heme active-site structure was no longer retained above this temperature. Met signals could be observed up to 91 °C for sm (Figure 2), demonstrating that the denaturation temperature for the heme active site in sm increased by >10 °C due to the mutation, F34 to Y. The stability of the heme active site in the oxidized form of PA was further enhanced by quintuple mutations, Met signals being exhibited up to 95 °C (Figure 2), although the signals were not as sharp as observed for the reduced form at the same temperature. These NMR results led to two conclusions: (1) In individual proteins, the heme active site of the reduced form is more stable than that of the oxidized one. (2) The mutations that stabilize the overall protein structure of the PA protein are also responsible for the stability of the heme active site in the oxidized form. Furthermore, as previously demonstrated,[4f] the stability of the heme active site in the oxidized forms of the proteins, as determined by NMR, positively correlates with the stability of the Fe−S bond, which was inferred from the dissociation temperature ($T_{m(Fe-S)}$) obtained through variable temperature measurement of the 695-nm absorption band (Table 1).

These findings, together with those described in our previous study,[4f] demonstrate that the overall protein stability, Fe−S bond stability, and redox property of cyt *c* are mutually related to each other. The protein conformational change induced by the mutations, which increases the overall stability of the oxidized form, happens to determine the Fe−S bond stability.[4f] Since the overall protein stability of the reduced form is less affected by the mutations than that of the oxidized one, the $E^{\circ\prime}$ values of the mutants decrease with respect to that of the wild-type PA in an enthalpic manner. Generally, oxidized heme is less favorable in the hydrophobic environment of the heme protein pocket compared with reduced heme. In the mutants, the oxidized heme is stabilized to some extent through the stronger Fe−S bond, leading to the regulation of the redox properties in the proteins. This is how the protein structure influences functionality of the cyts *c*.

Acknowledgment. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (A), "Dynamic Control of Strongly Correlated Softmaterials" (413/14045203), from MEXT.

Supporting Information Available: Additional figures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Moore, G. R.; Pettigrew, G. W. *Cytochromes c: Evolutionary, Structural, and Physicochemical Aspects*; Springer-Verlag: Berlin, 1990. (b) *Cytochrome c*: *A Multidisciplinary Approach*; Scott, R. A., Mauk, A. G., Eds.; University Science Books: Sausalito, CA, 1996.
(2) Sanbongi, Y.; Ishii, M.; Igarashi, Y.; Kodama, T. *J. Bacteriol.* **1989**, *171*, 65−69.
(3) Matsuura, Y.; Takano, T.; Dickerson, R. E. *J. Mol. Biol.* **1982**, *156*, 389−409.
(4) (a) Hasegawa, J.; Yoshida, T.; Yamazaki, T.; Sambongi, Y.; Yu, Y.; Igarashi, Y.; Kodama, T.; Yamazaki, K.; Kyogoku, Y.; Kobayashi, Y. *Biochemistry* **1998**, *37*, 9641−9649. (b) Hasegawa, J.; Shimahara, H.; Mizutani, M.; Uchiyama, S.; Arai, H.; Ishii, M.; Kobayashi, Y.; Ferguson, S. J.; Sambongi, Y.; Igarashi, Y. *J. Biol. Chem.* **1999**, *274*, 37533−37537. (c) Hasegawa, J.; Uchiyama, S.; Tanimoto, Y.; Mizutani, M.; Kobayashi, Y.; Sambongi, Y.; Igarashi, Y. *J. Biol. Chem.* **2000**, *275*, 37824−37828. (d) Uchiyama, S.; Hasegawa, J.; Tanimoto, Y.; Moriguchi, H.; Mizutani, M.; Igarashi, Y.; Sambongi, Y.; Kobayashi, Y. *Protein Eng.* **2002**, *15*, 455−461. (e) Sambongi, Y.; Uchiyama, S.; Kobayashi, Y.; Igarashi, Y.; Hasegawa, J. *Eur. J. Biochem.* **2002**, *269*, 3355−3361. (f) Yamamoto, Y.; Terui, N.; Tachiiri, N.; Minakawa, K.; Matsuo, H.; Kameda, T.; Hasegawa, J.; Sambongi, Y.; Uchiyama, S.; Kobayashi, Y.; Igarashi, Y. *J. Am. Chem. Soc.* **2002**, *124*, 11574−11575.
(5) HT was isolated from *H. thermophilus* cells,[4a] and PA and the mutants were produced using *Escherichia coli* and purified as previously reported.[4b,c]
(6) The cyclic voltammograms of the proteins were obtained as described previously (Taniguchi, I.; Iseki, M.; Eto, T.; Toyosawa, H.; Yamaguchi, H.; Yasukouchi, K. *Bioelectrochem. Bioenerg.* **1984**, *13*, 373−383). The protein concentration was about 0.5 mM in 20 mM phosphate buffer, pH 6.0, and 0.1 M $NaClO_4$. All potentials were referenced to the standard hydrogen electrode (SHE).
(7) (a) Battistuzzi, G.; Borsari, M.; Sola, M.; Francia, F. *Biochemistry* **1997**, *36*, 16247−16258. (b) Battistuzzi, G.; Borsari, M.; Sola, M. *Eur. J. Inorg. Chem.* **2001**, 2989−3004. (c) Battistuzzi, G.; Borsari, M.; Ranieri, A.; Sola, M. *J. Am. Chem. Soc.* **2002** *124*, 26−27.
(8) Tezcan, F. A.; Winkler, J. R.; Gray, H. B. *J. Am. Chem. Soc.* **1998**, *120*, 13383−13388.
(9) Battistuzzi, G.; Bellei, M.; Borsari, M.; Canters, G. W.; de Waal, E.; Jeuken, L. J. C.; Ranieri, A.; Sola, M. *Biochemistry* **2003**, *42*, 9214−9220.
(10) Zheng, H.; Kellog, S. J.; Erickson, A. E.; Dubauskie, N. A.; Smith, E. T. *J. Biol. Inorg. Chem.* **2003**, *8*, 12−18.
(11) (a) Ueyama, S.; Isoda, S.; Hatanaka, H.; Shibano, Y. *J. Electroanal. Chem.* **1996**, *401*, 227−230. (b) Cuttruzzolá, F.; Arese, M.; Ranghino, G.; van Pouderroyen, G.; Canters, G.; Brunori, M. *J. Inorg. Biochem.* **2002**, *88*, 353−361.
(12) Wüthrich, K. *NMR in Biological Research: Peptides and Proteins*; North-Holland Publishing Company: Amsterdam, 1976.

JA035682F