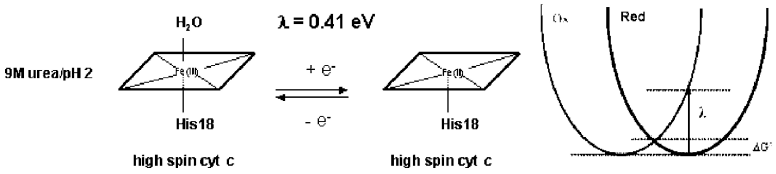
Article

Electrochemistry of Unfolded Cytochrome *c* in Neutral and Acidic Urea Solutions

Milan Fedurco, Jan Augustynski, Chiara Indiani, Giulietta Smulevich, Marin Antalk, Mikul Bn, Erik Sedlk, Mary C. Glascock, and John H. Dawson

J. Am. Chem. Soc., **2005**, 127 (20), 7638-7646• DOI: 10.1021/ja050321g • Publication Date (Web): 30 April 2005



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
ARTICLES
Published on Web 04/30/2005

Electrochemistry of Unfolded Cytochrome *c* in Neutral and Acidic Urea Solutions

Milan Fedurco,*,† Jan Augustynski,† Chiara Indiani,‡ Giulietta Smulevich,‡ Marián Antalík,§ Mikuláš Bánó,§ Erik Sedlák,|| Mary C. Glascock,⊥ and John H. Dawson⊥

Contribution from the Department of Chemistry, University of Geneva, CH-1211 Geneva, Switzerland, Department of Chemistry, University of Florence, Via della Lastruccia 3, 50019 Sesto Fiorentino (Fi), Italy, Department of Biophysics, Institute of Experimental Physics, Slovak Academy of Sciences, Watsonova 47, 043 53 Kosice, Slovakia, Department of Biochemistry, Faculty of Science, P.J. Safarik University, Moyzesova 11, 041 54 Kosice, Slovakia, and Department of Chemistry & Biochemistry, University of South Carolina, Columbia, South Carolina 29208

Received January 18, 2005; E-mail: milan_fedurco@freesurf.ch

Abstract: The present investigation reports the first experimental measurements of the reorganization energy of unfolded metalloprotein in urea solution. Horse heart cytochrome *c* (cyt *c*) has been found to undergo reversible one-electron transfer reactions at pH 2 in the presence of 9 M urea. In contrast, the protein is electrochemically inactive at pH 2 under low-ionic strength conditions in the absence of urea. Urea is shown to induce ligation changes at the heme iron and lead to practically complete loss of the α-helical content of the protein. Despite being unfolded, the electron-transfer (ET) kinetics of cyt *c* on a 2-mercaptoethanol-modified Ag(111) electrode remain unusually fast and diffusion controlled. Acid titration of ferric cyt *c* in 9 M urea down to pH 2 is accompanied by protonation of one of the axial ligands, water binding to the heme iron ($pK_a = 5.2$), and a sudden protein collapse (pH < 4). The formal redox potential of the urea-unfolded six-coordinate His18-Fe(III)-H_2O/five-coordinate His18-Fe(II) couple at pH 2 is estimated to be −0.083 V vs NHE, about 130 mV more positive than seen for bis-His-ligated urea-denatured cyt *c* at pH 7. The unusually fast ET kinetics are assigned to low reorganization energy of acid/urea-unfolded cyt *c* at pH 2 (0.41 ± 0.01 eV), which is actually lower than that of the native cyt *c* at pH 7 (0.6 ± 0.02 eV), but closer to that of native bis-His-ligated cyt b_5 (0.44 ± 0.02 eV). The roles of electronic coupling and heme-flattening on the rate of heterogeneous ET reactions are discussed.

Introduction

While several electrochemical studies[1−5] focused on the redox chemistry of cytochrome *c* (cyt *c*) in denaturant solutions and acids have appeared, the question of how protein unfolding affects heterogeneous electron-transfer kinetics at the metal/solution interface has yet to be systematically examined. Horse-heart cyt *c* (104 amino acids) is one of the most extensively studied metalloproteins in protein folding investigations.[6] The ferric and ferrous iron in the native form of protein are complexed by the porphyrin ring and two amino acid ligands, His18 and Met80, while the heme group is covalently attached via thioether bonds to the polypeptide backbone. This differs from bovine cyt b_5, where the relatively flat heme is held exclusively by two histidines, His39 and His63, while the heme propionates are oriented toward the solvent phase.[7,8] As evident from the split EPR *g*-values, the axial ligation in ferric cyt *c* completely removes degeneracy of d_{xy}, d_{xz}, d_{yz} orbitals in the Fe t_{2g} sub-shell.[9] The rhombic field of axial ligands and peptide backbone impose certain strain on the heme and force it to adopt twisted roof-like geometry,[10−12] differing from that seen in the reduced form of the protein.[13] Because ET kinetics depend on

† University of Geneva.
‡ University of Florence.
§ Slovak Academy of Sciences.
|| P.J. Safarik University.
⊥ University of South Carolina.

(1) Bixler, J.; Bakker, G.; McLendon, G. *J. Am. Chem. Soc.* **1992**, *114*, 6938−6939.
(2) Ferri, T.; Poscia, A.; Ascoli, F.; Santucci, R. *Biochim. Biophys. Acta* **1996**, *1298*, 102−108.
(3) Zhu, Y.; Dong, S. *Biochem. Bioenerg.* **1996**, *41*, 107−113.
(4) Pineda, T.; Sevilla, J. M.; Román, A. J.; Blázquez, M. *Biochim. Biophys. Acta* **1997**, *1343*, 227−234.
(5) Santucci, A.; Bongiovanni, C.; Mei, G.; Ferri, T.; Polizio, F.; Desideri, A. *Biochemistry* **2000**, *39*, 12632−12638.
(6) Bertini, I.; Rosato, A.; Turano, P. *J. Porphyrins Phthalocyanines* **2004**, *8*, 238−245.
(7) Durley, R. C. E.; Mathews, F. S. *Acta Crystallogr., Sect. D* **1996**, *52*, 65−76.
(8) (a) Muskett, F. W.; Kelly, G. P.; Whitford, D. *J. Mol. Biol.* **1996**, *258*, 172−189. (b) Muskett, F. W.; Whitford, D. The RCSB Protein Data Bank. NMR Structure of Bovine Cytochrome b_5 (PDB ID: 1HKO). Deposited: March 25, 2003. Online access at http://www.rcsb.org/pdb/.
(9) Gadsby, P. M. A.; Thomson, A. J. *J. Am. Chem. Soc.* **1990**, *112*, 5003−5011.
(10) Bushnell, G. W.; Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 585−595.
(11) Qi, P. X.; Beckman, R. A.; Wand, A. J. *Biochemistry* **1996**, *35*, 12275−12286.
(12) Banci, L.; Bertini, I.; Gray, H. B.; Luchinat, C.; Reddig, T.; Rosato, A.; Turano, P. *Biochemistry* **1997**, *36*, 9867−9877.
(13) Banci, L.; Bertini, I.; Huber, J. G.; Spyroulias, G. A.; Turano, P. *J. Biol. Inorg. Chem.* **1999**, *4*, 21−31.

10.1021/ja050321g CCC: $30.25

heme solvation as well as on the magnitude of related inner-shell Gibbs energy changes between the reactant and product states,[14,15] the relaxation of the heme triggered by the protein unfolding as well as the heme iron ligand exchange and spin-state changes are expected to influence the rate of ET reactions of cyt *c*. Because the redox transition of the native form of cyt *c* shows little reorganization of the internal ligand structure, the related heterogeneous ET kinetics remain unusually fast.[15] The question how the protein unfolding affects the magnitude of the reorganization energy of cyt *c* (III/II) couple will be addressed in the present study.

In our previous work,[16] we spectroscopically monitored the relaxation in the polypeptide strain during urea denaturation of cyt *c*. At pH 7 in 9 M urea, a heme flattening due to the formation of a bis-His-ligated heme was observed. In particular, a large intensity decrease and frequency changes of the out-of-plane and the thioether linkage modes in the low-frequency region of the resonance Raman spectrum of cyt *c* at pH 7.0 in 9 M urea were observed. At lower pH, the conversion of the bis-His-ligated cyt *c* to high spin forms was accompanied by a positive shift in the formal redox potential ($E^{\circ\prime}$) of ca. 130 mV. The sigmoidal shape of the $E^{\circ\prime}$ dependence on pH measured in 9 M urea was also reproduced by following the absorbance changes at 518−520 nm (CT1 band) and changes in the intensity of CD signal at 258 nm. The p$K_a \approx 5.2$ obtained from the titration measurements in acidic urea solutions correlates well with protonation of His33 in cyt *c* (p$K_a \approx 5.2$). Furthermore, magnetic circular dichroism (MCD) measurements provided the first direct experimental evidence for a six-coordinate His/H_2O-Fe(III) complex of urea-denatured cyt *c* below pH 5.2.[16] This is quite different from the five-coordinate aquo complex of denatured ferric cyt *c* reported in acidic guanidinium hydrochloride (GuHCl) solution.[17] In addition to the above-mentioned ligation changes, circular dichroism,[16,18,19] resonance Raman,[16,21−23] NMR,[24,25] fluorescence spectroscopy,[19,26−29] and small-angle X-ray scattering[30,31] evidence suggests that the acidic titration of cyt *c* in denaturating solutions is accompanied by marked conformational changes, significant increase in protein volume, and augmented charge on the protein surface. CD measurements[20] have shown that the ordered helical content in cyt *c* gradually disappears with increasing concentration of denaturant and the protein adopts a random-like structure at pH 2 in 9 M urea.

How do such dramatic changes in protein structure affect the electrochemical behavior of cyt *c*? Because electron transfer via tunneling in proteins is fastest via peptide chains and hydrogen-bonding contacts as opposed to direct through-space jumps,[32] the unfolding of cyt *c* should be accompanied by strong variation in the rate constant of the heterogeneous electron transfer. Intuitively, one would expect interfacial ET kinetics of cyt *c* in acid (pH 2) would be sluggish and electrode reactions would be highly irreversible. This should be especially true because the probability of water molecules entering the heme cavity is expected to be larger in acidic 9 M urea solutions where several amino acid residues undergo protonation, and one of the axial ligands (His33) in the ferric bis(His) complex is replaced by a water molecule. According to the modern theories of the heterogeneous electron transfer,[14] the entry of highly polar water molecules to the heme cavity should increase the barrier for the electron transfer by ca. 50%. Indeed, it was observed experimentally that acidification of the cyt *c* solution down to pH 2 (low ionic-strength conditions) leads to a complete loss of the electron exchange between the heme iron and 6-mercaptopurine-modified Au electrode.[4]

In the present work, horse heart cyt *c* has been found to undergo reversible one-electron transfer reactions at pH 2 in the presence of 9 M urea. Highly concentrated urea solutions are found to affect the structure of the gold/solution interface, suppress hydrogen evolution, and improve the electron-exchange between cyt *c* and the electrode. The main objective of the present work is to measure the heterogeneous constant of the electron transfer (k°) for the electroreduction of cyt *c* in acidic 9 M urea solutions. Conformational changes induced in the protein by urea have also been monitored by fluorescence spectroscopy and viscosimetry.

Experimental Methods

Horse heart cyt *c* (type VI, Sigma) for electrochemical experiments was used as received. Ferric cyt *c* used for spectroscopic experiments was fully oxidized by treatment with excess of potassium ferricyanide followed by gel filtration to remove the ferri/ferrocyanide.

All solutions were freshly prepared using analytical reagent grade chemicals and high purity water (18.2 MΩ cm resistivity, Millipore). Cyclic voltammetric measurements were performed in a two-compartment glass cell using the portable PC-based Eco-Tribo Polarographic Analyzer in a three-electrode configuration (Polaro-Sensors, Prague). All potentials were measured against a Ag/AgCl/Cl^- (3 M KCl) reference electrode (reported versus NHE). A platinum counter-electrode (large area Pt grid) was separated from a working electrode by a ceramic separator. A cross section of the Ag(111) low-index single-crystal rod oriented with 0.5° accuracy (Metal Crystals & Oxides, Ltd.), or a polycrystalline Au rod (Specpure 99.999%, Johnson Matthey), served as working electrodes (both, 3 mm in diameter). The electrode surface was prepared by polishing mechanically with fine emery papers and then with suspensions of 1.0 and 0.3 μm alumina powders. Alumina was removed from the electrode surface via gentle sonication in an ultrasonic bath and washing with copious amounts of distilled water. Before the measurements, the electrode surface was cleaned by cycling the potential into the hydrogen evolution region in 0.1 M $NaClO_4$ solution. Electrochemical measurements were performed in deaerated solutions, under an argon atmosphere, at 22 ± 1 °C.

(14) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265−322.
(15) Fedurco, M. *Coord. Chem. Rev.* **2000**, *209*, 263−331.
(16) Fedurco, M.; Augustynski, J.; Indiani, C.; Smulevich, G.; Antalík, M.; Bánó, M.; Sedlák, E.; Glascock, M. C.; Dawson, J. H. *BBA Proteins & Proteomics* **2004**, *1703*, 31−41.
(17) Yeh, S. R.; Han, S. W.; Rousseau, D. L. *Acc. Chem. Res.* **1998**, *31*, 727−736.
(18) Myer, Y. P. *Biochemistry* **1968**, *7*, 765−776.
(19) Hamada, D.; Kuroda, Y.; Kataoka, M.; Aimoto, S.; Yoshimura, T.; Goto, Y. *J. Mol. Biol.* **1996**, *256*, 172−186.
(20) Takahashi, S.; Yeh, S.-R.; Das, T. K.; Chan, C.-K.; Gottfried, D. S.; Rousseau, D. L. *Nat. Struct. Biol.* **1997**, *4*, 44−50.
(21) Yeh, S. R.; Takahashi, S.; Fan, B.; Rousseau, D. L. *Nat. Struct. Biol.* **1997**, *4*, 51−56.
(22) Yeh, S. R.; Rousseau, D. L. *Nat. Struct. Biol.* **1998**, *5*, 222−228.
(23) Yeh, S. R.; Rousseau, D. L. *J. Biol. Chem.* **1999**, *274*, 17853−17859.
(24) Russell, B. S.; Melenkivitz, R.; Bren, K. L. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8312−8317.
(25) Russell, B. S.; Bren, K. L. *J. Biol. Inorg. Chem.* **2002**, *7*, 909−916.
(26) Tsong, T. Y. *J. Biol. Chem.* **1974**, *249*, 1988−1990.
(27) Tsong, T. Y. *Biochemistry* **1975**, *14*, 1542−1547.
(28) Shastry, M. C.; Roder, H. *Nat. Struct. Biol.* **1998**, *5*, 385−392.
(29) Thomas, Y. G.; Goldbeck, R. A.; Kliger, R. S. *Biopolymers* **2000**, *57*, 29−36.
(30) Kataoka, M.; Hagihara, Y.; Mihara, K.; Goto, Y. *J. Mol. Biol.* **1993**, *229*, 591−596.
(31) Akiyama, S.; Takahashi, S.; Kimura, T.; Ishimori, K.; Morishima, I.; Nishikawa, Y.; Fujisawa, T. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1329−1334.
(32) Gray, H. B.; Winkler, J. R. *Q. Rev. Biophys.* **2003**, *36*, 341−372.

Cyclic voltammetry of cyt *c* in neutral urea solutions was conducted on a 2-mercaptoethanol-modified gold electrode in 0.01 M phosphate buffer containing 0.1 M $NaClO_4$. The solution pH of the 0.01 M phosphate buffer/0.1 M $NaClO_4$ solution was adjusted by adding dilute $HClO_4$. Thiol-modified working electrodes were prepared by dipping the Ag(111) or the Au electrode in 1 mM 2-mercaptoethanol solution in water for 2 min (prepared fresh before each modification of the electrode surface) followed by extensive rinsing under a stream of distilled water immediately before use. C_{11}-hydroxyalkanethiolate films on Ag(111) electrode were formed by dipping the electrode in 1.0 mM 11-hydroxy-1-undecanethiol solution in ethanol for 12 h followed by extensive rinsing of the chemically modified electrode in ethanol and 0.1 M $NaClO_4$ solution in water. Self-assembled mercaptoethanol monolayers block the adsorption of cyt *c* on gold and silver electrodes and avoid serious protein denaturation and spin state changes on the heme iron (as observed on bare silver surface using surface-enhanced Raman spectroscopy).[33]

The viscosity of urea/ferric cyt *c* solutions was measured by a contactless Couvette-type viscosimeter.[34] The device is able to measure viscosity at near constant shear rate, and its main advantage is lack of errors originating from surface phenomena, like surface tension or surface shear. The shear velocity is generated in the measured sample between two glass cylinders. The inner cylinder (rotor) and outer stationary cylinder are centered along the vertical axis. The rotor is electromagnetically equilibrated in the vertical direction positioned under the surface of the liquid. A small aluminum cylinder in the rotor is located in an outer magnetic field of rotating permanent magnets. The interaction between eddy currents and the rotating outer magnetic field causes the rotation of the rotor. The viscosity of the sample is calculated from the angular rotor velocity and the angular velocity of the permanent magnets. The above device measures the viscosity of a 1.6 mL sample volume to an accuracy of ~0.1%. Measurements of cyt *c* viscosity were carried out at a constant temperature of 25 °C and the shear rate of 65 s^{-1}. The reduced viscosity was calculated according to the equation $\eta_{red} = (\eta - \eta_0)/(c \cdot \eta_0)$, where η_0 is viscosity of the complex solvent at the same temperature and η is the solution viscosity measured for a given protein concentration, *c*. A relatively low protein concentration ($c = 3$ mg/mL) was used, so that the reduced viscosity was maintained very close to the intrinsic viscosity $[\eta] = \lim_{c \to 0} \eta_{red}$. The solution pH was adjusted by adding HCl directly to the measuring cell, and pH values were always measured before and after measurements. The solution pH was measured with a glass electrode (Radiometer America Electrodes) and the pH meter (EA 940 Expandable Ion Analyzer Orion Research, USA) or by a Sensorex glass electrode directly in the measuring cell.

Fluorescence spectra were obtained using a Shimadzu RF-5000 spectrofluorimeter. The excitation wavelength was 290 nm. The fluorescence of Trp-59 was followed at 340 nm.

Results and Discussion

Redox Chemistry of Cyt *c* in Neutral Urea Solutions. Figure 1 illustrates the inhibitive effect of urea on the electrochemical reduction of ferric cyt *c* at pH 7.0. As the concentration of urea increases, the diffusion-limited current for cyt *c* reduction strongly decreases. In 9 M urea, the faradaic peak current at ca. +0.22 V drops practically to zero. The experimental linear scan voltammograms of cyt *c* have been simulated[35] using: (a) $c_{ox} = 1.04$ mM, $D_{ox} = 1.2 \times 10^{-6}$ cm^2/s; (b) $c_{ox} = 0.91$ mM, 5.5×10^{-7} cm^2/s; (c) $c_{ox} = 0.74$ mM, 3.0×10^{-7} cm^2/s; (d) $c_{ox} = 0.37$ mM, 2.7×10^{-7} cm^2/s; (e) $c_{ox} = 0.09$ mM, 2.1×10^{-7} cm^2/s. It is evident from the electrochemical data shown in Figure 1 that the amount of His/Met-ligated protein in solution decreases from 100% to ca. 9% upon increase in the denaturant concentration from 0 to 9 M urea (see Table 1).

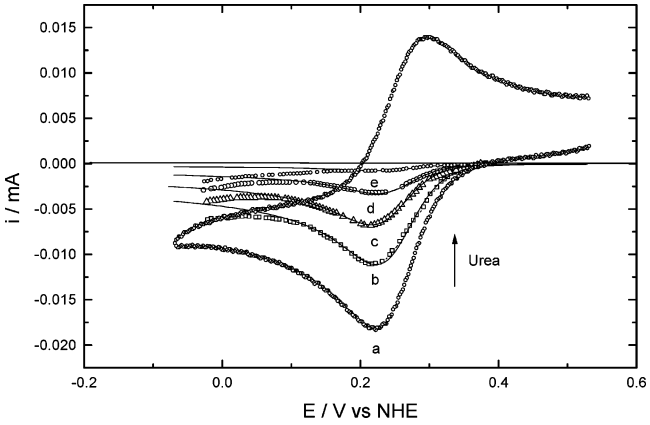


Figure 1. Cyclic voltammogram for 1.0 mM cyt *c* recorded in the absence of urea (a), and cathodic scans upon addition of (b) 5 M, (c) 6 M, (d) 7 M, (e) 9 M urea. Simulated linear scan voltammograms are shown as a solid line. Measured on a 2-mercaptoethanol-modified gold electrode in 0.01 M phosphate buffer/0.1 M $NaClO_4$ (pH 7.0). Scan rate: 100 mV/s.

The amount of His/Met-ligated cyt *c* remaining in solution at increasing urea concentration has also been determined by Russell and co-workers.[24] On the basis of 2D-NMR NOESY experiments, they reported very similar values of 85%, 67%, and 35% of His/Met-ligated form of cyt *c* in 5, 6, and 7 M urea, respectively. On the other hand, the absorbance change at 695 nm (porphyrin-to-iron $a_{2u}(\pi) \rightarrow d_z^2$ electronic transition) gives a much sharper drop in the concentration of the native protein above 6 M urea, 100%, 98%, 88%, 16%, and 3%.[16] The diffusion coefficient determined from the simulations of cyclic voltammograms is found to decrease much more rapidly than that determined from our viscosimetric measurements (Table 1, columns 2 and 3). Such differences might be due to changes in the size and shape of cyt *c* induced by the denaturation, which were neglected in the estimate of the diffusion coefficient from the viscosimetric data (Table 1, column 2). On the other hand, we conclude that some degree of protein unfolding is brought about by the high electric fields in the vicinity of the electrode surface (vide infra). The rapid decrease of the current at +0.22 V is due to three concomitant factors: (i) increase of the viscosity of the urea/water mixture with the increase of the denaturant concentration, (ii) variation in the effective radius of the protein and its charge during unfolding, and, most important, (iii) decrease in the effective concentration of His/Met ligated native cyt *c* (which undergoes electroreduction at +0.22 V).

Previous H/D-exchange NMR studies[40] have established that the conformation of cyt *c* in the denaturant solution is substantially perturbed even though the His/Met-ligation is still preserved in up to 5 M urea concentration. Nonetheless, from

(33) Oellerich, S.; Wackerbarath, H.; Hildebrandt, P. *J. Phys. Chem. B* **2002**, *106*, 6566−6580.
(34) Bánó, M.; Strhársky, I.; Hrmo, I. *Rev. Sci. Instrum.* **2003**, *74*, 4788−4795.
(35) Antigona CV simulation software package was kindly provided to us by L. Mottier (University of Bologna, Italy).
(36) Perl, D.; Jacob, M.; Bánó, M.; Stupák, M.; Antalík, M.; Schmid, F. X. *Biophys. Chem.* **2002**, *96*, 173−190.
(37) Hubbard, J. B.; Douglas, J. F. *Phys. Rev. E* **1993**, *47*, R2983−R2986.
(38) Zhou, H.-X. *Biophys. J.* **1995**, *69*, 2298−2303.
(39) Schejter, A.; Luntz, T. L.; Koshy, T. I.; Margoliash, E. *Biochemistry* **1992**, *31*, 8336−8343.
(40) Elöve, G. A.; Bhuyan, A. K.; Roder, H. *Biochemistry* **1994**, *33*, 6925−6935.

Table 1. Variations of the Viscosity of the Solution, Diffusion Coefficient, and Relative Amount of the His/Met-Ligated Ferric Cyt *c* as a Function of Urea Concentration

urea [M]	urea[a] η [mPa s]	cyt *c*[b] $10^{-7} \times D_{ox}$ [cm²/s]	cyt *c*[c] $10^{-7} \times D_{ox}$ [cm²/s]	%Met/His (electrochemistry)	%Met/His[d] (^{1}H NMR)	%Met/His[e] (695 nm)	%Met/His[f] (Trp fluorescence)
0	0.89	12	12	100	100	100	100
5	1.17	9.07	5.5	87	85	98	99
6	1.27	8.42	3	71	67	88	96
7	1.38	7.75	2.7	35	35	16	78
8	1.51	7.09				4	34
9	1.66	6.42	2.1	9		3	7

[a] Obtained by extrapolation from viscosimetric measurements (25 °C) reported in ref 36. [b] Diffusion coefficient calculated according to refs 37,38, supposing unchanged shape of cyt *c*. [c] From simulation of linear scan voltammograms. [d] Taken from ref 24. [e] Met80 absorbance recorded at 695 nm (taken from ref 16). [f] Trp59 fluorescence at 340 nm (taken from Figure 3, ref 39).

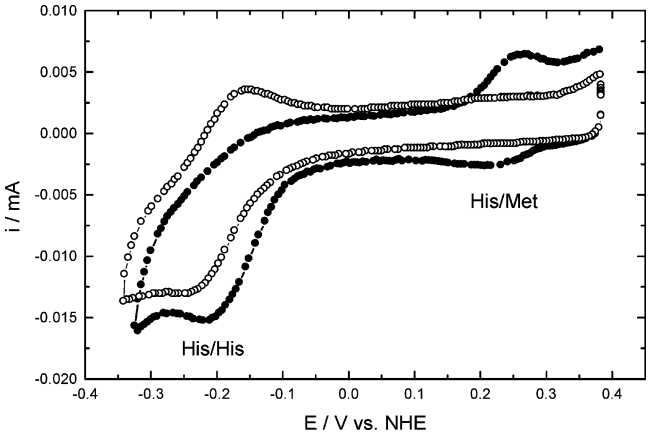


Figure 2. Effect of urea on the reversibility of His/His-Fe(III/II) couple of cyt *c*. Cyclic voltammograms for 1 mM cyt *c* recorded on 2-mercaptoethanol-modified Au electrode in 7 M urea (●), and in 9 M urea (○) containing 0.01 M phosphate buffer/0.1 M $NaClO_4$ (pH 7.0). Scan rate: 100 mV/s.

Figure 1, trace b, it is evident that the eventual conformational changes in the protein do not significantly affect the electron-transfer kinetics associated with His/Met-ligated cyt *c*. In fact, the cathodic peak with E_p at 0.22 V does not undergo displacement toward more negative potentials in 5 M urea and the electroreduction of cyt *c* remains fast and diffusion controlled. On the other hand, our simulations (solid lines in Figure 1, curves c−e) show that the abrupt decrease of the cathodic current at 0.22 V for urea concentrations $c_{urea} > 6$ M is not only due to increasing solution viscosity but is mainly due to ligand-exchange reactions coupled to conformational changes in the protein.

Cyclic voltammetry of cyt *c* on 2-mercaptoethanol-modified gold electrode in highly concentrated urea solutions ($c_{urea} > 6$ M), as employed in the present work, differs dramatically from highly irreversible behavior of cyt *c* observed previously on 4,4′-dithiopyridine-modified gold electrode.[3] Figure 2 illustrates typical cyclic voltammograms measured for cyt *c* in 7 M urea as compared to 9 M urea. It is observed that the addition of 7 M urea to the electrochemical cell causes significant lowering of the current at 0.22 V (see also Figure 1), while a new cathodic peak appears at ca. −0.2 V. The latter process is assigned to the electroreduction of bis(His)-Fe(III) to bis(His)-Fe(II) cyt *c*. The only anodic peak associated with the reoxidation of cyt *c* in 7 M urea is observed at ca. +0.27 V (absent in 9 M urea), which coincides with the reoxidation of the His/Met-Fe(II) to His/Met-Fe(III). This is quite surprising because in the liquid phase, dithionite-reduced ferrous cyt *c* is known to preserve its His/Met ligation in 9 M urea solution (pH 7).[16,41]

It is remarkable that the increase in urea concentration from 7 to 9 M results in improved reversibility of redox reactions (ca. −0.2 V) associated with the bis-His form of cyt *c* on Au electrode modified with 2-mercaptoethanol (Figure 2). A similar observation was described in earlier work by Bixler et al.,[1] who observed increased reversibility of the redox reactions of His/His-ligated cyt *c* on gold upon increasing the GuHCl concentration from 3.5 to 7 M (see Figure 1 in ref 1). No plausible explanation was given by the authors concerning the origin of this phenomenon. In the course of the present studies, we have realized that the lack of reversibility of cyt *c* in 7 M urea (and, possibly in 3.5 M GuHCl) might be due to parasitic electrode reactions rather to the metalloprotein itself. Thus, it appears that the main reason for the irreversibility of electrode reactions at potentials more negative than −0.1 V in 7 M urea is the cathodic reduction of water to molecular hydrogen and hydroxyl anion, which coincides with electrode reactions of the His/His-Fe(III/II) couple. Apparently, the increase in the bulk concentration of urea from 7 to 9 M leads to more efficient replacement of water molecules at the electrode/solution interface by urea and, therefore, favors electron-exchange reactions of cyt *c* as compared to discharge of water.

To prove the latter point, we have replaced the gold electrode by mercaptoethanol-modified Ag(111), a metal on which the hydrogen evolution due to water reduction at pH 7 commences at ca. 400 mV more negative potentials. Figure 3 shows that the $E^{\circ\prime}$ potential for the bis-His complex of cyt *c* that is present in 9 M urea is ca. 0.4 V more negative than that of the native form of the protein (identical to E° found on gold electrode). The cyclic voltammograms are much cleaner when not obscured by hydrogen evolution. As the result, the electrode reactions are reversible in the potential range studied and electron-transfer kinetics are very fast (diffusion controlled). The cathodic-to-anodic peak separation at −0.23 V vs NHE is found to be 60 mV, typical of a fast electrochemically reversible one-electron-transfer process. The fact that one is dealing with very rapid electron transfer is also apparent from the cyclic voltammogram recorded for cyt *c* in 9 M urea, with the peak-to-peak separation close to 59 mV even at the scan rate as high as 5 V/s (Figure 3, curve b). We have noticed that the rising cathodic part of the voltammogram recorded on silver electrode (indicated by an arrow in Figure 3, trace b) is very sensitive to traces of molecular oxygen. Therefore, prolonged purging of the electrolyte solution by inert gas was done before the degassed cyt *c* solution was

(41) Bhuyan, A. K.; Udgaonkar, J. B. *J. Mol. Biol.* **2001**, *312*, 1135−1160.

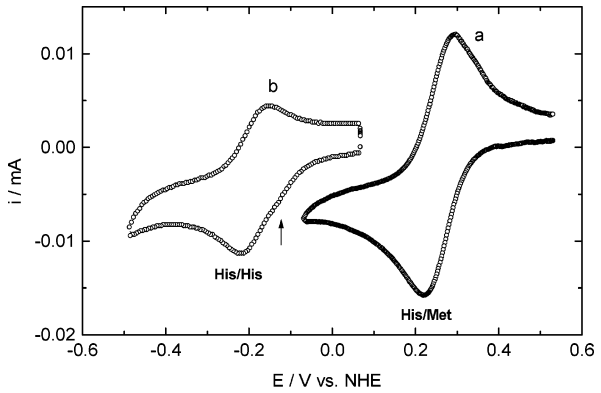


Figure 3. Comparison of cyclic voltammograms for 1.1 mM cyt *c* recorded on a 2-mercaptoethanol-modified gold electrode at 20 mV/s in the absence of urea (curve a), and on mercaptoethanol-modified Ag(111) electrode at 100 mV/s in the presence of 9 M urea (curve b). Measured in 0.01 M phosphate buffer/0.1 M $NaClO_4$ (pH 7.0).

introduced into the electrochemical cell. Cyt *c*/urea solution was then shortly purged by argon, and the inert gas was continuously blown through the headspace during the experiment. Despite such a procedure, traces of oxygen varied from experiment to experiment and were found very difficult to remove (due to the foaming of the protein solution).

Acidic Titration of Ferric Cyt *c* in Urea. Pioneering viscosity and fluorescence measurements by Tsong[26,27] at the beginning of the 1970's gave a qualitative basis of cyt *c* unfolding; however, no detailed knowledge existed at that time concerning ligation changes on the heme iron accompanying the process. Recent hydrogen-exchange NMR studies[42] shed some light on early stages of the acid-induced unfolding of the ferric cyt *c*. Accordingly, the omega loop (residues 40−57) in the protein, nested within the larger N-loop (residues 37−61), acts as a cooperative unfolding/refolding unit. Under high ionic strength conditions, gradual acidification of the cyt *c* solution down to pH 2 (no denaturant present) triggers reversible unfolding of the N-loop and results in a molten globule state with preserved His/Met-ligation. Fluorescent energy transfer (FET) measurements[43] revealed that at relatively low salt concentrations (<50 mM Na_2SO_4), both collapsed and extended polypeptides are present at equilibrium. As the salt content increases, the collapsed ensemble becomes even more compact, while the population of extended conformations decreases and practically disappears for the salt concentrations greater than 700 mM. This correlates with the fact that acid-unfolded cyt *c* is electrochemically inactive in 0.01 M HCl at pH 2 (low ionic strength conditions), while in the presence of >50 mM salt His/Met-ligated-protein shows quasi-reversible cyclic voltammetry and its formal redox potential is ca. 100 mV more cathodic to $E^{\circ\prime}$ of cyt *c* at pH 7.[4,5]

The situation during the acidic titration of ferric cyt *c* becomes radically different in the presence of a denaturating agent. This is due to variations in the extent of protein unfolding as well as ligation changes on the heme iron. Because urea is known to efficiently solubilize aromatic amino acid residues, these become gradually more and more exposed and protein loses its α-helical content. Figure 4 illustrates the pH dependence of Trp59

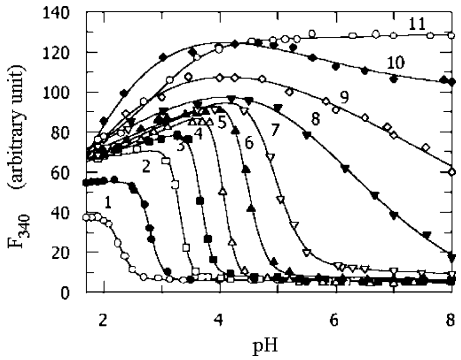


Figure 4. The pH dependence of the Trp59 fluorescence intensity, F_{340}, for 9.3 μM ferric cyt *c* in 10 mM phosphate taken as a function of urea. Curve 1, 0 M urea; 2, 1 M; 3, 2 M; 4, 3 M; 5, 4 M; 6, 5 M; 7, 6 M; 8, 7 M; 9, 8 M; 10, 9 M; 11, 10 M urea. Excitation wavelength: 290 nm.

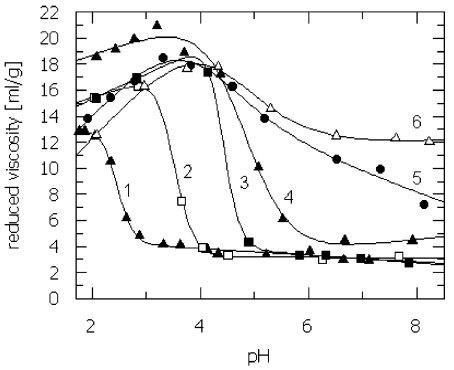


Figure 5. pH dependence of the reduced viscosity of 220 μM ferric cyt *c* in 10 mM phosphate at 25 °C at various urea concentrations. Curve 1, 0 M; 2, 2.9 M; 3, 5.1 M; 4, 5.9 M; 5, 7.8 M; 6, 8.7 M urea.

fluorescence monitored at different urea concentrations. In native cyt *c*, Trp59 is located in the vicinity of the heme, and, therefore, its fluorescence is strongly quenched by the chromophore. In the absence of urea, the cooperative conformational transition of ferric cyt *c* in low ionic media occurs with an apparent pK_a of 2.3 (Figure 4, curve 1). Upon increasing the urea concentration, the distance between Trp59 and the heme increases and the fluorescence signal of tryptophan strongly increases (Figure 4, curves 2−11). Because the Trp59 fluorescence in ferric cyt *c* is correlated to changes in the size of the protein associated with its unfolding, the fluorescence intensity increase is well correlated with the changes in the reduced viscosity of ferric cyt *c* (Figure 5), as a function of solution pH and urea concentration.

The reduced viscosity of native cyt *c* at pH 7 is found to be 2.9 mL/g. It varies very little with solution pH from 7 to 3 and increases to approximately 13 mL/g during the acidic transition. On the other hand, the reduced viscosity measured for ferric cyt *c* in 8.7 M urea (Figure 5, curve 6) rises steadily and linearly with the amount of high spin cyt *c*[16] between pH 8 and pH 4 (pH values corresponding to 0% and 93% His18-Fe(III)-H_2O, respectively). A further decrease of pH below 4 led to significant conformational changes in the protein as indicated by the sharp drop in reduced viscosity, consistent with a collapse of the polypeptide chains in the aquo complex of cyt *c*. This phenomenon clearly coincides with a sudden decrease in the Trp59 fluorescence below pH 4, as discussed above (Figure 4). The viscosity data suggest that a sharp (with respect to pH) acid-induced conformational change occurs in the protein below

(42) Krishna, M. M. G.; Lin, Y.; Rumbley, J. N.; Englander, S. W. *J. Mol. Biol.* **2003**, *331*, 29−36.
(43) Lyubovitsky, J. G.; Gray, H. B.; Winkler, J. R. *J. Am. Chem. Soc.* **2002**, *124*, 14840−14841.

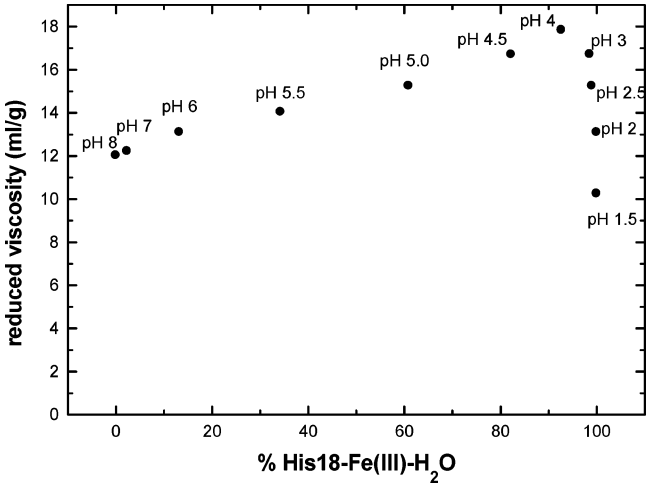


Figure 6. Reduced viscosity of ferric cyt *c* in 8.7 M urea from pH 1.5−8.0 plotted against the amount of high spin His-Fe(III)-H_2O complex detected at 620 nm during the acidic titration of 220 μM ferric cyt *c* in 10 mM phosphate at 25 °C.

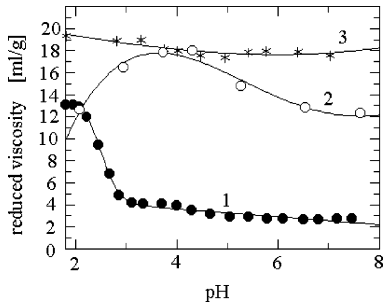


Figure 7. Viscosity dependence of ferric cyt *c* on pH in 10 mM phosphate at 25 °C. Curve 1, 0 M urea; 2, 8.7 M urea; 3, 6.0 M GuHCl. Concentration ferric cyt *c* 220 μM.

pH 4, where the majority of the cyt *c* species (<92%) already exist as high-spin His-Fe(III)-H_2O complex (Figure 6).

Despite the above-mentioned decrease in the reduced viscosity of cyt *c* when going from pH 4 to pH 2 (and sudden drop in Trp59 fluorescence), the electrochemical data did not suffer from any significant changes in the shape of cyclic voltammograms (except for ca. 10 mV shift in the $E^{\circ\prime}$ toward more anodic potentials).[16] Interestingly, no such collapse of the unfolded protein is noticed during the acidic titration of cyt *c* in 6 M GuHCl (Figure 7, compare curves 2 and 3). The observed differences likely reflect the fact that the protein exists as a five-coordinate aquo complex in GuHCl solutions,[17] while six-coordinate His18-Fe(III)-H_2O complex is formed in 9 M urea solutions.[16] Except for the above-mentioned differences in the heme ligation, there exist large differences in the ionic strength in the two denaturating solutions. Clearly, the heme solvation by polypeptide chains surrounding the heme is different in the two cases. It is currently not clear whether the polypeptide chain wraps around the heme or, eventually, adopts a different topology with the heme edge exposed to the aqueous phase.[44] Because the electron-transfer kinetics should depend on the degree of such heme exposure to water molecules as well as the heme-electrode distance, we decided to conduct related electrochemical experiments that would address the problem in detail.

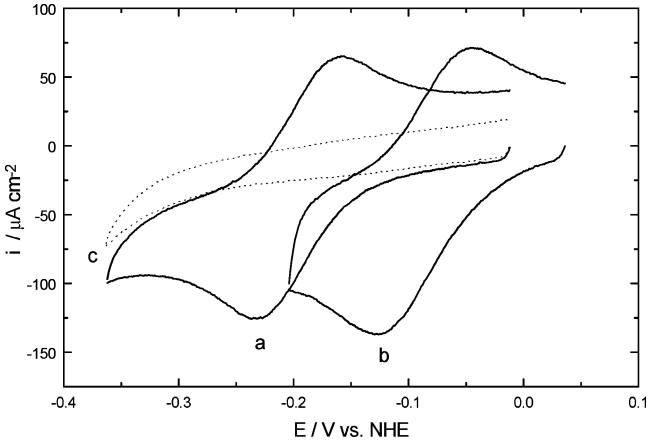


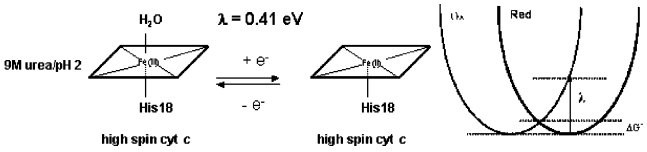
Figure 8. Cyclic voltammograms for 2 mM cyt *c* recorded on 2-mercaptoethanol-modified Ag(111) electrode in 0.01 M phosphate buffer containing: (a) 9 M urea, pH 7.0; (b) 9 M urea, pH 2.0. Dotted curve c was recorded in the absence of cyt *c*. Scan rate: 5 V/s.

It should be noted that no electrochemical measurements have been reported to date for cyt *c* in acidic urea solutions. This might be due to the fact that commonly used electrode-surface modifiers 4,4′-dithiodipyridine and 4,4′-bipyridine[45] undergo protonation below pH < 4 so that they cannot easily be used as electron-transfer promoters for cyt *c*. On the other hand, hydroxyl-terminated self-assembled monolayers of ω-hydroxyalkanethiols do not undergo acid/base reactions in acidic media, while still allowing for electron transfer across the metal/solution interface.[46] Electron energy loss spectroscopy (EELS) experiments by Gui et al.[48] have shown that dipping of Ag(111) in 2-mercaptoethanol at −0.1 V vs Ag/AgCl leads to the formation of close-packed self-assembled thiol monolayer with molecular packing density equal 0.70 nmol/cm^2 (very close to theoretical limiting packing density of 0.72 nmol/cm^2). Favorable hydrogen-bonding contacts between the hydroxyl groups of the chemisorbed mercaptoethanol and the highly hydrated cyt *c* molecules allow for very good electronic coupling between the metalloprotein and the electrode surface. It should be noted that Ag(111) is a superior electrode material as compared to gold due to the significantly larger overpotential for proton reduction, which would interfere otherwise with the redox reactions of cyt *c* in acidic urea solutions.

Figure 8 shows cyclic voltammograms for the bis-His complex (curve a) and the His/H_2O complex (curve b) of urea-denatured ferric cyt *c* recorded at 5 V/s. The formal redox potential for the His-Fe(III)-H_2O/His-Fe(II) couple is found to be ca. 130 mV more anodic as compared to $E^{\circ\prime}$ of the bis-His complex. Removal of perchlorate from 0.01 M HCl/9 M urea solution results in decreased reversibility of redox reactions due to low conductivity of the urea/HCl solution (ca. 100 mV peak-to-peak separation); however, it does not lead to the disappearance of the reduction/oxidation peaks. This is remarkable given that in the absence of urea, cyt *c* does not show any electrochemical activity at pH 2.0. Recovery of the electrochemical signal for cyt *c* and quasi-reversible voltammetric behavior[4,5]

(44) Smith, C. R.; Mateljevic, C.; Bowler, B. E. *Biochemistry* **2002**, *41*, 10173−10181.
(45) Hinnen, C.; Niki, K. *J. Electroanal. Chem.* **1989**, *264*, 157−165.
(46) Terrettaz, S.; Cheng, J.; Miller, C. J. *J. Am. Chem. Soc.* **1996**, *118*, 7857−7858.
(47) Cheng, J.; Terrettaz, S.; Blankman, J. I.; Miller, C. J.; Dangi, B.; Guiles, R. D. *Isr. J. Chem.* **1997**, *37*, 259−266.
(48) Gui, J. Y.; Stern, D. A.; Frank, D. G.; Lu, F.; Zapien, D. C.; Hubbard, A. T. *Langmuir* **1991**, *7*, 955−963.

Scheme 1. Electrochemical Electron Transfer Associated with Cyt *c* in the Acidic Urea Solution (pH 2)



is observed only upon increasing the ionic strength of the solution (i.e., in 100 mM $NaClO_4$), which induces formation of the A-form of cyt *c*, with the properties similar to those of the molten globule state (His18 and partially coordinated Met80 ligation).[49] The mixed spin nature of this state has been demonstrated by the features in the 620 and 695 nm bands and from Soret absorption spectra (392−405 nm).[4]

Kinetics of Cyt *c* Electroreduction in Urea. The experimentally measured heterogeneous electron-transfer rate constant ($k°$) is expected to vary dramatically with distance between the heme iron of cyt *c* and electrode surface. Depending on the urea concentration and pH, new electron-transfer pathways have to be established between the heme iron and protein surface. The quality of such newly established electron-tunneling pathways (i.e., their conductivity) should be reflected in the shape of the cyclic voltammogram as well as in the anodic-to-cathodic peak separation (ΔE_p). For example, assuming $k° \approx 1$ cm/s and distance-decay constant $\beta = 1.1$ Å^{-1},[50] an increase in the electron-transfer distance by 1 nm should be manifested by a ca. 6 orders of magnitude decrease in $k°$ and an increase in ΔE_p from ca. 0.06 to 1 V! Such high irreversibility of electrode reactions would render cyclic voltammetric measurements on Ag or Au practically impossible (violent hydrogen evolution at potentials exceeding −1.0 V).

As we have shown in the previous section, cyt *c* shows well-defined electrochemical behavior in 9 M urea down to pH 2. Because the heterogeneous rate constant, $k°$, cannot be extracted from the cyclic voltammogram recorded for cyt *c* on short chain 2-mercaptoethanol-modified electrodes at slow scan rates (diffusion-controlled ET reaction), the density of electronic states and the reorganization energy (λ) for cyt *c* may be obtained from deconvolution of a linear voltammetric scan on an electrode modified with long-chain ω-hydroxyalkanethiol.[46] The beauty of the latter method stems from the fact that the height of the activation barrier for the electron-transfer reaction $\Delta G^{\#}$ ($\sim\lambda/4$) may be determined from a single $i-E$ curve (voltammogram) with no need for measuring the whole series of rate constants as a function of temperature (Arrhenius plot). In the present work, we have exploited this approach to make an estimate of the reorganization energy for the His-Fe(III)-H_2O/His-Fe(II) couple of cyt *c* in 9 M urea at pH 2 (Scheme 1).

Relatively thick C_{11}-alkanethiolate monolayers are known to significantly slow the interfacial electron-transfer rate for cyt *c* by several orders of magnitude due to electron tunneling effects.[46] Electron transfer across the C_{11}-hydrocarbon spacer causes the peak potential (E_p) shift from −0.13 V to significantly more cathodic potentials (compare curves a and b in Figure 9A). The reorganization energy for the redox couple can be then obtained from the magnitude of such peak displacement. Figure

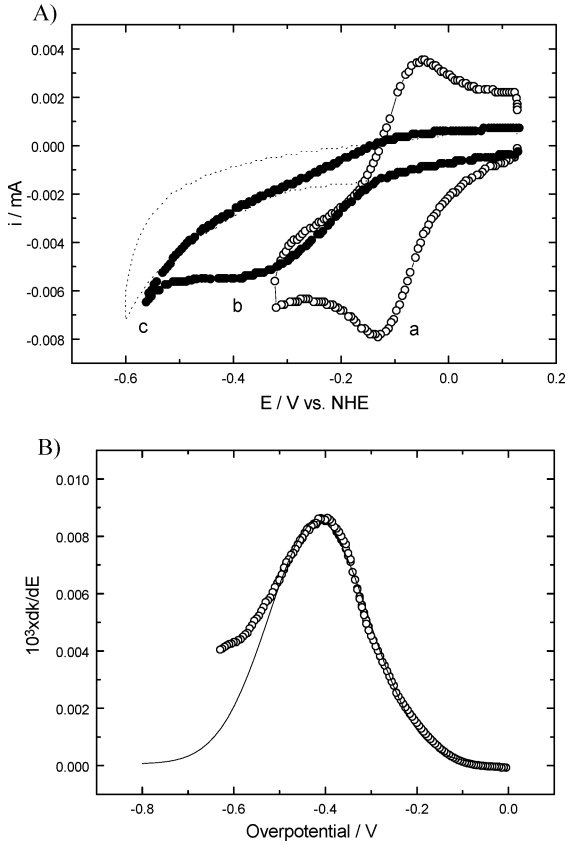


Figure 9. (A) Cyclic voltammograms for 1 mM cyt *c* recorded in 0.01 M phosphate buffer/0.1 M $NaClO_4$ containing 9 M urea (pH 2.0) on Ag(111) electrode modified by 2-mercaptoethanol (a) or 11-hydroxy-1-undecanethiol (b).[46] Scan (c) is the same as (b) but in the absence of protein. Scan rate 100 mV/s. (B) Reorganization energy for His/H_2O-Fe(III)/His-Fe(II) couple of cyt *c* obtained from the maximum of the derivative of the heterogeneous rate constant with respect to the overpotential based on the convolution analysis of curve b in Figure 9A.[46] Experimental data are shown as ○, and the Gaussian fit is represented by the solid line.

9B shows the variation of the rate constant for the reduction of unfolded cyt *c* in 9 M urea solution at pH 2.0. The rate constant of the heterogeneous electron transfer gradually increases with the increasing overpotential up to the value where the reaction driving force ($-\Delta G°$) becomes identical to the reorganization energy (λ). In the case of unfolded cyt *c*, such a condition is apparently reached at ca. −0.41 V, where the heterogeneous rate constant *k* reaches the maximum of 2×10^{-5} cm/s ($\alpha = 0.5$).

Because $-\Delta G° = \lambda$ at the maximum of the plot shown in Figure 9B, the exponential part of the Franck−Condon factor is effectively zero and the maximum rate constant, $k_{(max)}$, is proportional to the preexponential part of the rate expression

$$k_{(max)} = (4\pi^2/h)|H_{DA}{}^{eff}|^2/(4\pi\lambda k_B T)^{-1/2} \qquad (1)$$

where *h* is Planck's constant, k_B is the Boltzman constant, and $|H_{DA}{}^{eff}|^2$ is the maximum electronic overlap between the wave functions of propagating electrons at the Fermi level (donor states in the metal) and the acceptor states (i.e., highest occupied orbital of the heme iron). At energies exceeding the λ value for a metalloprotein, the rate constant decreases even though the reaction driving force is still increasing (Marcus inverted region).[14] The latter is in fact due to gradual decrease in the overlap between the wave functions of reactant and product,

(49) Goto, Y.; Calciano, L. J.; Fink, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 573−577.
(50) El Kasmi, A.; Wallace, J. M.; Bowden, E. F.; Binet, S. M.; Linderman, R. J. *J. Am. Chem. Soc.* **1998**, *120*, 225−226.

leading to lower probability of electron transfer and a strong decrease in the magnitude of the right-hand side of eq 1. Deviation of the current from the expected Gaussian shaped curve is most likely due to hydrogen evolution starting on silver in solution of pH 2 at potentials more negative than −0.5 V. It is evident from the location of the maximum on the Gaussian curve in Figure 9B that the reorganization energy for the urea-unfolded cyt *c* is 0.41 ± 0.01 eV (standard deviation of at least three independent measurements), about 0.17 eV lower than the previous estimate reported for the native cyt *c* at pH 7.0 (0.58 ± 0.03 eV).[46,47] This is consistent with our observation of a larger peak potential (E_p) displacement toward negative potentials for native cyt *c* (by ca. 200 mV) as compared to 9 M urea-unfolded protein at pH 2. Indeed, we find $\lambda = 0.6 \pm 0.02$ eV for the native form of cyt *c* at pH 7 on a 11-hydroxy-1-undecanethiol-modified Ag(111) electrode, the value being in excellent agreement with that obtained on gold electrode.[46]

The relatively low reorganization energy (ca. 0.6 eV) measured for the native cyt *c* is mainly the result of the hydrophobic protein interior in which the redox center (heme) is placed. The total reorganization energy, λ, for the native cyt *c* Fe(III/II) couple may be split into the outer-shell, λ_{os} (stabilization of the charge on the iron during the redox transition by polar amino acid residues and internal water molecules), and the inner-shell reorganization energy, λ_{is} (bond length and bond angle changes during the redox transition).

Muegge and co-workers[51] made an estimate of λ_{os} for the native cyt *c* in the order of 0.39−0.65 eV, where λ_{is} represents ca. 0.13 eV. It is hard to imagine how the combined effect of acid and urea would reshape the unfolded metalloprotein in such a way that the Fe(III/II) redox transition would actually take place in a "less polar" environment than that encountered in the interior of the native cyt *c*. On the other hand, the energy cost associated with λ_{is} might be lower in unfolded proteins because the resonance Raman spectra[16] suggest that the heme is more planar in His/His- and His/H_2O-cyt *c* complexes as compared to the distorted heme found in the native protein. However, the fact that electrode kinetics remain fast upon cyt *c* unfolding in urea cannot be explained exclusively due to lowering of λ_{is}, because its contribution to the total reorganization energy is expected to be relatively low. Our experimental evidence would tend to suggest that the acidic titration of cyt *c* below pH 4 causes protein collapse (Figures 4 and 5), likely to induce wrap-around geometry of polypeptide chains around the heme, with only limited heme solvation by bulk water. Reduction of Fe(III) then triggers removal of the water ligand from the heme iron; however, such high-to-low spin transition does not seem to lead to significant increase in λ_{is} energy. The magnitude of the k_{max} suggests that the electronic coupling of the electrode and the protein is excellent. Very good electronic coupling between the native (as well as unfolded) protein and the electrode is likely to be assured by favorable interactions of protonated amino acids (cyt *c* contains 22 lysine residues) with the highly hydrated OH-terminated alkanethiol monolayers. The heme edge of unfolded protein must be able to approach the C_{11}-thiolate-modified metal surface to distances below 1 nm. In the opposite case, electron-tunneling effects operating at larger separation distances would cause a rapid and exponential decrease in $k°$ with distance from the electrode surface.

Conclusions

As demonstrated in the present work, chemically modified gold and silver electrodes serve as a clean source of electrons and provide a unique possibility of measuring the reorganization energy of unfolded metalloproteins at electrified interfaces. Cyclic voltammetry allows one to monitor denaturant-induced ligation changes on the heme iron in cyt *c* and provides unfolded ferrous forms of the protein that are otherwise difficult to prepare with reducing agents. Electrochemical reduction of ferric cyt *c* also avoids interference due to the binding of dithionite decomposition products to the protein surface, which could result in protein stabilization (or destabilization) with respect to folding/unfolding reaction.

In the present work, acidic titration of the protein could be achieved down to pH 2 for the cyt *c*/urea system. Furthermore, highly concentrated urea solutions (9 M) were found to be beneficial to cyt *c* because the hydrogen evolution coming from the electroreduction of water was significantly suppressed on Au electrodes. Apparently, such parasitic reactions, and protonation of self-assembled thiol monolayers, prevented previous researchers from observing reversible redox chemistry for urea-unfolded His/His- and His/H_2O complexes of cyt *c*.

Viscosimetric measurements reported in the present work point out at major differences in the degree of unfolding of cyt *c* in 9 M urea as compared to 6 M GuHCl. A sharp acid-induced conformational change occurs in cyt *c* in 9 M urea below pH 4, where the protein already exists as a high spin His-Fe(III)-H_2O complex. This has been observed, although there is practically no significant difference regarding the potential range and reversibility of related electrode reactions. Thus, it is concluded that the extent of cyt *c* unfolding must be to some extent affected by the applied electric field at the electrode/solution interface. Such effects were noticed by Murgida and Hildebrandt[52] who studied potential-induced conformation and spin-state changes of cyt *c* on silver electrodes modified with negatively charged ω-carboxylalkanethiolate monolayers. However, the authors have failed to observe such changes using surface-enhanced Raman spectroscopy (SERS) on 2-mercaptoethanol-modified silver electrode as used in the present study. It should be emphasized that SERS is mostly sensitive to protein adsorbed directly on the electrode surface or, eventually, electrostatically bound to ionized monolayer, but is relatively insensitive to diffusing protein molecules in the vicinity of the electrode surface (as experienced in the present work).

High electric fields in the double layer region (up to 10^9 V/m) might affect the shape and dimensions of the unfolding protein. This could explain the faster decrease in the diffusion coefficient for cyt *c* with increasing urea concentration observed in the cyclic voltammetric experiment (Figure 1). This conclusion is also in line with the fact that under electrochemical conditions, we have achieved complete unfolding of ferrous cyt *c* in 9 M urea (cf., Figure 2), and obtained comparable reversibility of bis-His cyt *c* to that reported on Au electrode in 6 M GuHCl solution.[1] It should be noted that the above-mentioned "electrochemical" effects on the rate of protein unfolding might be of importance in studies utilizing short-chain 2-mercaptoethanol-

(51) Muegge, I.; Qi, P. X.; Wand, J.; Chu, Z. T.; Warshel, A. *J. Phys. Chem. B* **1997**, *101*, 825−836.

(52) Murgida, D. H.; Hildebrandt, P. *J. Phys. Chem. B* **2001**, *105*, 1578−1586.

modified electrodes. On the other hand, our kinetic measurements conducted on hydroxy-terminated C_{11} electrodes should not be affected by such effects. In the latter case, most of the electric field will drop within the C_{11} monolayer, so that reorganization energy estimates reported in the present work may be considered as pertinent to bulk unfolded cyt *c* species and free from any interaction with the source of free electrons.

Simple addition of urea to acidic cyt *c* solution results in metalloprotein with differing ligation on the heme iron (altered redox potential) and extremely fast interfacial ET kinetics. This phenomenon certainly merits further investigations and remains to be seen whether it also applies to myoglobin, cytochrome P450 family, and non-heme metalloproteins. If so, denaturant-unfolded proteins could find novel applications in the area of biosensing and in bulk catalysis.

Acknowledgment. This research was funded in part by grants from MIUR (COFIN 2001031798) (to G.S.), VEGA 3198 (to M.A.), and NIH GM 26730 (to J.H.D.).

JA050321G