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How does reorganization energy change upon protein unfolding? Monitoring the structural perturbations in the heme cavity of cytochrome *c*

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Abstract

In several classes of proteins the redox center provides an additional intrinsic biophysical probe that could be used to study the protein structure and function. In present report reorganization energy (λ, as a parameter describing electron transfer properties) was used to study the protein structural changes around the heme prosthetic group in cytochrome *c* (cyt *c*). We attempted to monitor the value of this parameter upon the unfolding process of cyt *c* by urea, during which it was increased sigmoidally from about 0.52 to 0.82 eV for native and unfold protein, respectively. Results indicate that by structural changes in the heme site, λ provides a complementary tool for following the unfolding process. Assuming a reversible two-state model for cyt *c* unfolding, ΔG_{H_2O}, C_m and *m* values were determined to be 8.32±0.7 kcal mol^{-1}, 1.53± 0.19 $kcalmol^{-1}M^{-1}$ and 5.03 M, respectively.

Keywords: Reorganization energy; Heme proteins; Electron transferring; Cytochrome *c*; Protein unfolding

1. Introduction

Electron transfer is one of the most fundamental processes in chemistry and biology, and accordingly it has attracted significant attention from researchers in diverse disciplines in recent years. In biological systems, there are several classes of proteins that contain a redox center and all probably optimized via evolution for a specific purpose [1]. The redox center provide these proteins with an additional intrinsic biophysical probe which is absent in other proteins and worth to be considered as an alternative tool in studying the protein structure and function.

Experimental studies of electron transfer process of protein yield a unique number so called the electron transfer rate constant, k_{ET}. To delineate various factors that give rise to k_{ET}, appropriate theoretical models have been formulated. Theoretical aspects of nonadiabatic electron transfer process are well established within the classical, semiclassical, and quantum mechanical framework [2–12]. In all these theories the first step is partitioning of k_{ET} into two factors: the electronic factor (H_{DA}) and the nuclear factor (FC) as shown in Eq. (1):

$$k_{ET} = \left(\frac{2\pi}{}\right) H_{DA}^2 FC \quad (1)$$

The first factor, H_{DA}, contains information about the total (direct and indirect) interaction of the donor-acceptor electronic wave functions. The factor FC is related to the fluctuations and relaxation of the nuclear polarization of the medium. In classical treatment of electron transfer, a key parameter in the FC factor is the reorganization energy (λ) defined as the free energy change associated with the relaxation of the entire set of nuclear coordinates surrounding the redox sites as the charge distribution changes from the reactant to the product state [12]. Based on this definition, one can expect λ to be sensitive to the structural changes occur in the microenvironment of the redox sites.

In this report we used λ (as a parameter describing electron transfer properties) to study the conformational changes in protein structure around the heme prosthetic group in cytochrome *c* (cyt *c*). To examine the sensitivity of λ to the structural changes in the heme site of cyt *c*, we attempt to monitor the

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value of this parameter upon the unfolding process of the protein. We used horse heart cyt *c* as a protein model since it is one of the most extensively studied metalloprotein in protein folding and electrochemical investigations [13–22]. Cyt *c* is a monomeric metalloprotein composed of 104 amino acids. It is an electron carrier in the respiratory chain [23,24] and an activator in the programmed cell death [25–27]. Although it is not categorized as an enzyme, bearing a heme in its structure, it shows significant peroxidase activity. The iron in the native form of protein is complexed by the porphyrin ring and two amino acid ligands, His 18 and Met 80. The heme group is covalently attached via a thioether bond to the polypeptide backbone. Upon unfolding, the highly polar water molecules penetrate into the heme cavity, and so heme meets a surrounding medium with higher dielectric constant. Since the electron transfer kinetics and accordingly the reorganization energy depend on the heme solvation, the unfolding process is expected to be detectable through λ measurement.

2. Experimental

2.1. Chemicals

Horse heart cyt *c* was purchased from Merck. 2-mercaptoethanol ($HOCH_2CH_2SH$), from Merck, and 11-mercapto 1-undecanol ($HO–(CH_2)_{11}SH$) from Sigma-Aldrich were respectively abbreviated as C_2 and C_{11} and used as received. All other chemicals were analytical grade reagents.

2.2. Protein determination

Cyt *c* solutions were made in 0.01 M phosphate buffer containing 0.1 M $NaClO_4$ at neutral pH. The concentration of the oxidized cyt *c* was determined experimentally using the molar absorption coefficient of 106,000 $M^{-1}cm^{-1}$ at 409 nm [20].

2.3. Electrochemical measurements

All cyclic voltammograms were recorded using a Potentiostat/Galvanostat (model 263-A, EG&G, USA) and a single-compartment electrochemical cell equipped with a platinum rod auxiliary electrode, an Ag/AgCl reference electrode (containing 1 M KCl) and a polycrystalline gold disk working electrode with a disk diameter of 2 mm. Prior to each electrochemical measurements, the following pretreatments were preformed sequentially: First, the gold electrode was mechanically polished with fine emery papers and then with suspensions of 10 and 0.3 μm alumina powders, respectively, to a mirror finish observed. Alumina was removed from the electrode surface via gentle sonication in an ultrasonic bath and subsequent washing with copious amounts of distilled water. The electrode was further cleaned with exposure to 60 °C sulfochromic acid as described previously [28,29]. All experimental solutions were de-aerated by highly pure nitrogen for 10 min, and a nitrogen atmosphere was kept over the solutions during measurements.

Modified gold electrodes were prepared by dipping the electrode in a 20 mM freshly prepared mercaptoethanol solution in water for 10 min, followed by extensive rinsing under a stream of distilled water immediately before using. The modified electrodes with long chain modifier, were formed by dipping the electrode in a 20 mM C_{11} solution in ethanol for 12 h followed by extensive rinsing in ethanol and then in 0.1 M $NaClO_4$ solution in water. The self-assembled monolayer block the adsorption of cyt *c* on the gold surface and avoid serious protein denaturation and spin state changes on the heme iron [30].

2.4. Determination of λ

Kinetic parameters were determined using a procedure mainly based on the method reported by Miller [28,29]. We used self-assembled ω-hydroxyalkanethiol monolayer to control the electroreactivity of cyt *c* at the Au electrode surface. Cyclic voltammograms of cyt *c* solutions were recorded using C_{11} modifier-coated gold electrode. The driving force dependence of the electron transfer rate was then used to determine λ of the species in solution. Diffusion coefficient and formal potential for each cyt *c* solution was obtained using a C_2-modified gold electrode. To extract the potential dependence of the intrinsic heterogeneous electron transfer rate, the effect of diffusion on the voltammetric peaks was corrected through convolution technique [31–33]. The derivative of the heterogeneous electron transfer rate constant versus potential is proportional to the density of electronic states distribution (DOS) [34]. Theoretically, DOS is a Gaussian function of potential [28,29]. The peak of the DOS plot gives a direct measure of λ of the redox molecule [28,29]. This procedure was repeated for a series of 1 mM cyt *c* solutions containing different urea concentrations.

3. Results and discussion

Study of cyt *c* direct electrochemistry is hampered due to the poor electroactivity of redox proteins at electrode surfaces. It is generally believed that the electroinactivity of cyt *c* solutions occurs due to the strong adsorption of the protein onto the

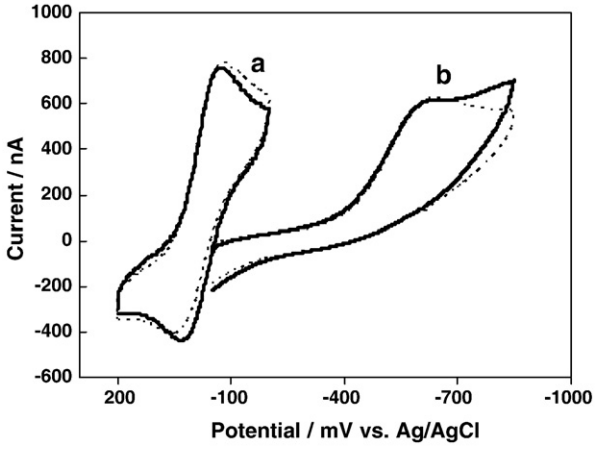

Fig. 1. Cyclic voltammograms of 1.014 mM native [solid curves] and refolded [dotted curves] cyt *c* solutions on modified gold electrode in 0.01 M phosphate buffer containing 0.1 M $NaClO_4$ [pH 7, scan rate 100 mVs^{-1}]. a and b represent the voltammograms obtained using C_2 and C_{11} modified Au electrodes, respectively.

electrode surface. However, the protein adsorption passivates the electrode. To overcome this problem, one successfully used strategy is the use of ω-hydroxyalkanethiol monolayer at the Au electrode surface. Fig. 1 shows the cyclic voltammograms of cyt *c* using two different ω-hydroxyalkanethiol (C_2 and C_{11}) coated gold electrodes. As seen in Fig. 1 the cyclic voltammograms obtained for the native and refolded cyt *c* are similar (solid and dotted curves, respectively) indicating that the cyt *c* unfolding is a reversible process as reported earlier [8]. As seen in Fig. 2, Au electrodes coated with C_2 give a quasi-reversible voltammetric response for cyt *c* solution. At the scan rate of 20 mV/s the $E_{1/2}$ (calculated from the midpoint between the cathodic and anodic waves) was found to be 81 mV (vs. Ag/AgCl). The cathodic and anodic peaks were separated by a ΔEp of 74 mV. Fig. 2 (inset) shows the plot of cathodic and anodic peak current (i_{pc}) against square root of the scan rate ($v^{1/2}$). As seen both peak currents change linearly with $v^{1/2}$ in the v region of 20–600 mV/s indicating that the electrochemical reaction of cyt *c* at these modified electrodes is a diffusion-controlled redox process.

The use of ω-hydroxyalkanethiol-coated Au electrodes also offers another useful advantage which allows one to control the absolute heterogeneous electron transfer rate of the cyt *c* solution. By increasing the number of methylene groups within the thiol used to form the monolayer, the electron transfer reaction from the electrode to the redox center is forced to proceed at longer distance and consequently slowing overall rate. That's why the voltammograms obtained using the Au electrodes coated with C_{11} are completely irreversible and their cathodic peaks show substantial shifts to negative potentials (Fig. 1). In the case of our study this effect is desirable since heterogeneous electron transfer reactions are too fast to be measured at bare electrode and should be slowed down to be measured over a wide range of electrode potentials. By choosing a sufficiently thick thiol monolayer, one can slow the absolute heterogeneous electron transfer rate to an extent at which diffusion limitations are minimized. The driving force

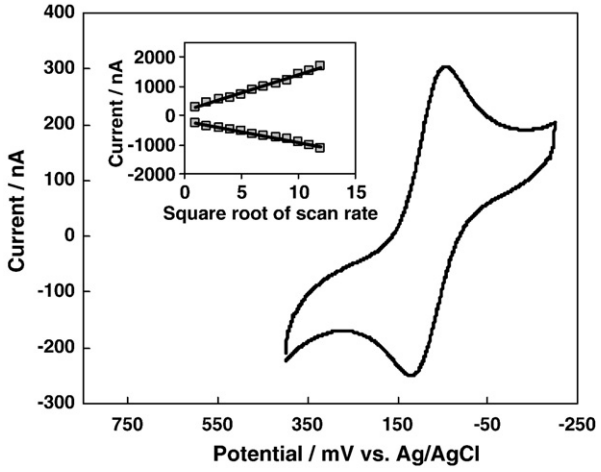

Fig. 2. Quasi-reversible voltammetric responses of cyt *c* solution using C_2 modified Au electrode. The experiments were carried out in the 0.01 M phosphate buffer containing 0.1 M $NaClO_4$ [pH 7] at the scan rate of 20 mV/s. Inset: scan rate dependence of the cathodic and anodic peak currents of cyt *c* solution for the same electrode.

dependence of the electron transfer rate can then be used to determine λ of the species in solution.

According to Marcus theory [5], the activation energy of heterogeneous electron transfer is given by Eq. (2):

$$\Delta G^* = \frac{\lambda}{4}\left[1 + \frac{nF(E - E^0)}{\lambda}\right]^2 \tag{2}$$

where, n is the number of transferred electrons, $E - E^0$ is the overpotential, and λ is the reorganization energy. λ represents the energy necessary to transform the nuclear configuration in the reactant and the solvent to those of the product state [35]. λ is usually separated into inner-shell (λ_{is}) and outer-shell (λ_{os}) components as shown in Eq. (3):

$$\lambda = \lambda_{is} + \lambda_{os} \tag{3}$$

The inner-shell component (Eq. (4)) depends on the degree of electron delocalization in the redox centre i.e. on the effective reactant and product radii, and the outer-shell component (Eq. (5)) depends on the polarizability of the medium surrounding the redox center:

$$\lambda_{is} = \sum_j \frac{f_j^r f_j^p}{f_j^r + f_j^p}\left(\Delta q_j\right)^2 \tag{4}$$

$$\lambda_{os} = (\Delta e)^2\left[\frac{1}{2a} - \frac{1}{2r}\right]\left[\frac{1}{\varepsilon_{op}} - \frac{1}{\varepsilon_s}\right] \tag{5}$$

where f_j^r and f_j^p are respectively the *j*th normal mode force constants in the reactants and products, Δq_j is the change in equilibrium value of the *j*th normal coordinate, Δe is the charge transferred from reactant to the electrode, a is the radius of the reactant, r is the distance from the centre of the reactant to the electrode surface, ε_s and ε_{op} are the static and optical dielectric constants of the solvent, respectively [4].

This model (Eq. (3)) has been previously used to measure the λ of native cyt *c* solutions [36]. To examine this theoretical model by the experimental measurement of λ upon protein unfolding, we have to consider the structural details of cyt *c* and the changes which occur during this process. The heme group in cyt *c* locates interior of the protein in a hydrophobic pocket [37], which effectively shields the heme from the solvent and results in the rigidity of the iron surroundings (protein residues and solvent molecules). Therefore, the λ of electron transfer reaction of cyt c is low i.e. 0.58 eV [36]. Although there are six long-lived water molecules in the heme cavity of cyt *c*, they are effectively separated from the heme iron by several residues located in the hydrophobic heme pocket of the protein, including aromatic residues of tyrosine, tryptophan, and phenylalanine [38,39]. Upon unfolding, the structural integrity of the heme pocket is lost and the polar solvent surrounds the heme moiety. According to the Eq. (5) such changes in the surrounding increases the value of λ. This equation predicts that the more polarizable environment, the larger will be the λ. Thus, λ is expected to be a homogeneous function of the exposed surface area of the heme.

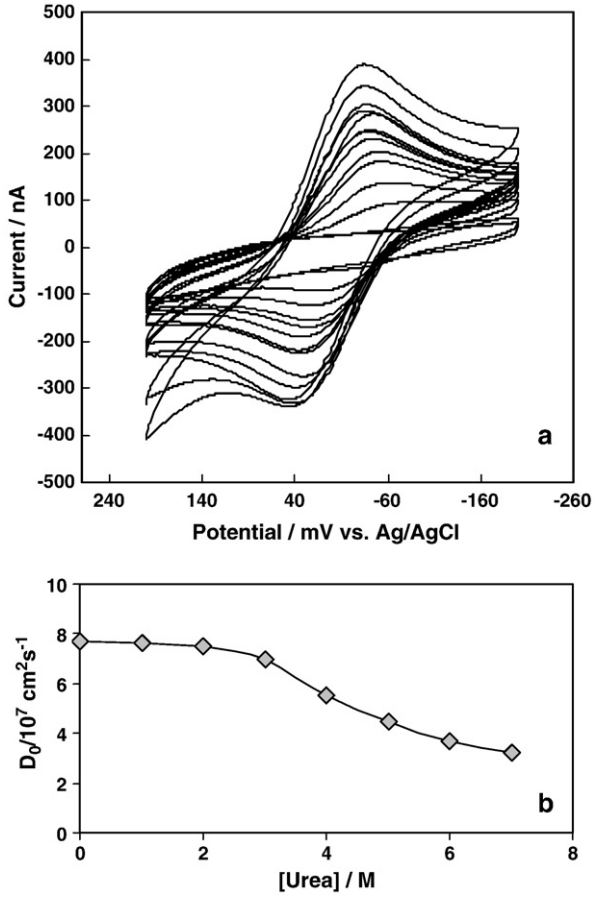

Fig. 3. a) Cyclic voltammograms of 1.014 mM cyt *c* recorded in the presence of urea. The currents were measured on a C_2 modified gold electrode in 0.01 M phosphate buffer containing 0.1 M $NaClO_4$ (pH 7) with a scan rate of 100 mVs^{-1}. The voltammograms from outside to inside were obtained in the presence of urea concentrations of 0, 1, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, and 9 M, respectively. b) Diffusion coefficient of cyt *c* as a function of urea concentration. These values were calculated from voltammograms of Fig. 3a.

It has been shown that urea with 9 M concentration unfolds the cyt *c* almost completely and produces a structure resembling random coil [40]. Native conformation of cyt *c* is at low-spin state His18-Met80 ligation. In the presence of 9 M urea, cyt *c* retains its spin state, although its Met80 ligand is replaced with another strong field ligand, His33 [21]. This indicates that upon unfolding, the overall structure of the heme does not change. In agreement, it has shown that λ_{os} for cyt *c* is ca. 45 times larger than the corresponding λ_{is} [41]. Thus, we can conclude that in cyt *c*, the heme surrounding plays the main role in the magnitude of λ quantity and accordingly one can assume that almost the entire change in λ arises from the outer-shell component (Eqs. (3–5)). Assuming a two-state model for cyt *c* unfolding [42], at any concentration of urea, each cyt *c* molecule is either folded or unfolded, but when urea concentration changes from zero to 9 M, the ratio of the folded-state molecular population to the population of the unfolded state varies from one to zero. So, the observed λ of a cyt *c* solution can be evaluated by:

$$\lambda = \lambda_N f_N + \lambda_U f_U \quad (6)$$

where, λ_N and λ_U are the reorganization energy of the folded and unfolded states, and f_N and f_U are partial fraction of these states, respectively.

To measure the quantity of λ through the unfolding process of cyt *c*, we combined the Miller method with the above assumptions. The use of two-state model for the analysis is allowed providing the reversibility of the unfolding process. As seen in Fig. 2 the cyclic voltammograms obtained for the native and refolded cyt *c* are similar (solid and dotted curves, respectively) indicating that the cyt *c* unfolding is a reversible process as reported earlier [18]. The use of short ω-hydroxyalkanethiol-coated Au electrode simplifies the electrochemical characterization of the cyt *c*. The resulting reversible to quasi-reversible voltammetric waves allows one to obtain both the cyt *c* redox potential and diffusion constant. Fig. 3a shows the cyclic voltammograms of cyt *c* under the effect of various concentrations of urea using C_2-modified Au electrode. Upon raising the urea concentration, both the cathodic and anodic peak currents (i_{pc} and i_{pa}) decrease. From these voltammograms we obtained the redox potential and diffusion constant of cyt *c* at each concentration of urea. The resulting values are summarized in Fig. 3b. The diffusion coefficient of cyt *c* in the absence of urea was calculated to be 7.69×10^7 cm^2/s. This value agrees well

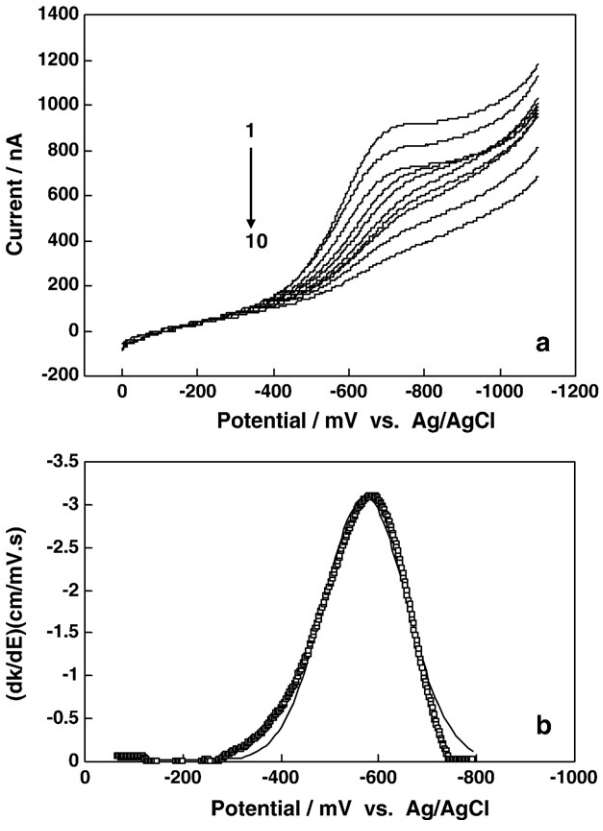

Fig. 4. a) Cathodic waves of the cyclic voltammograms of cyt *c* solution in the presence of urea on C_{11} modified gold electrode. The urea concentrations from 1 to 10 were 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 M, respectively. Other experimental conditions were the same as Fig. 3. b) Density of electronic states diagram for native cyt *c* calculated as the derivative of the heterogeneous electron-transfer rate constant with respect to the formal potential. The solid curve is the best fit Gaussian distribution to the experimental data.

with the published reports [16,22]. The diffusion coefficient of cyt *c* as a function of urea concentration as depicted in Fig. 3b were used for correcting diffusion limitation in convolution method.

To investigate the redox kinetics of cyt *c* upon unfolding, a gold electrode coated with C_{11} was applied. Fig. 4a represents the voltammetric curves of cyt *c* under the influence of different concentrations of urea. These voltammograms were first corrected for the effect of diffusion through the convolution technique and then normalized to both the unit concentration and electrode area to convert the currents to heterogeneous rate constants, exactly according to Miller method. The derivative of the heterogeneous electron transfer rate constant as a function of the overpotential gives a measure of the density of electronic states function for the solution redox molecule. Fig. 4b shows a representative derivative plot for the native cyt *c*. The Marcus theory of electron transfer predicts that the density of electronic states distribution should be Gaussian in shape. The solid curve through the data points corresponds to the best fit Gaussian curve of the data. As seen in Fig. 4b, the density of electronic states distribution for the oxidized cyt *c* closely follows the expectations of the Marcus theory. The peak of the density of electronic states distribution occurs at the reorganization voltage and so, the maximum value of this plot gives a direct measure of the λ of the redox molecule. Analysis of the shown plot in Fig. 4b gives the value of 0.52 eV for native cyt *c* which is close to the value previously reported by Miller group [37].

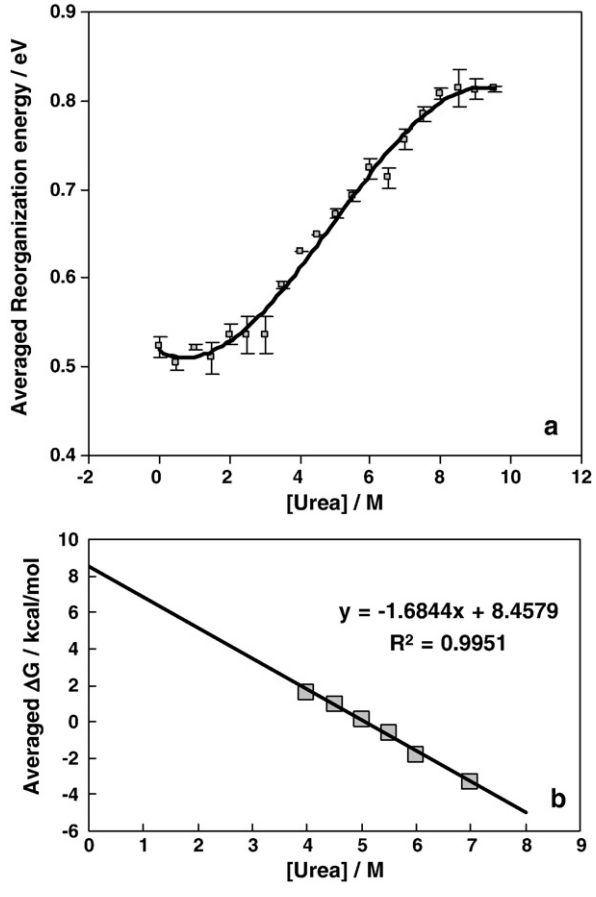

Fig. 5. a) Reorganization energy of cyt *c* solution as a function of urea concentration. Measurements were carried out in the same condition as Fig. 3 and the values of λ were calculated according to the method explained in the text. Each point is the average of three independent measurements. b) The variation of unfolding free energy for cyt *c* vs. different concentrations of urea. Extrapolation to zero concentration of urea gives $\Delta G[H_2O]$ value which is a good estimate for the conformational stability of the protein.

Table 1
Comparison between electrochemical reorganization energy and Soret absorption as probes for studying cyt *c* unfolding process

Probe	$\Delta G^{a}_{H_2O}$ [kcal mol^{-1}]	m^{a} [kcalmol^{-1}M^{-1}]	C^{a}_{m} [M]	Reference
Soret absorption	8.02±0.43	1.28±0.07	6.26	[19]
Soret absorption	7.6	1.116	6.9	[42]
λ^{a}	8.32±0.7[b]	1.53±0.19[b]	5.03[b]	Current work

[a]For definitions see text.
[b]The values are extracted from Fig. 5 [For details see text].

The λ values for cyt *c* as a function of urea concentration are summarized in Fig. 5a. The resulting urea denaturation curve was analyzed assuming a two-state mechanism. From the data in the transition region, the free energy of unfolding, ΔG, was calculated and found to vary linearly with urea concentration (Fig. 5b). The ΔG-[Urea] plot was analyzed using the linear extrapolation method (Eq. (7)):

$$\Delta G = \Delta G_{H_2O} - m[\text{Urea}] \quad (7)$$

where, m is a measure of the dependence of ΔG on urea concentration. $\Delta G_{(H_2O)}$ is the value of ΔG at zero concentration of urea and estimates the conformational stability of the protein. Table 1 lists the urea mid-transition concentration [C_m], $\Delta G_{(H_2O)}$, and m values obtained in this study and compares them with those obtained by spectroscopy. Comparison shows that the value measured electrochemically for protein stability agrees well with the previously reported values, but the m value is greater than that obtained by spectroscopy, and to some extent, C_m is lower [42,43]. More precise analysis suggests that the observed difference in C_m originates from the expansion of the transition range in electrochemistry (3–8 M urea) with respect to that of spectroscopy (5–8 M urea).This means that in the range of 3–5 M urea, where the spectroscopic probes show no transition, electrochemistry indicates the beginning steps of protein unfolding process. A plausible explanation for the electrochemically observed unfolding below 5 M urea is the higher sensitivity of λ to the polarizability of heme environment. Entrance of one or more solvent molecules into heme cavity, or proximity of interior water molecules to the iron ion, or even increasing freedom degree of amino acid residues near the heme group are all reflected in the value of λ. In agreement, it is reported that the major denaturation transition in the unfolding profile of cyt *c* is preceded with another small transition centered below 4–5 M urea [42]. During the small preceding transition, the heme crevice is loosened on both sides of the heme plane so as to permit the penetration of solvent molecules and small anions. This effect increases the polarizability of heme environment and thus the value of λ_{os}. Concurrent to this phenomenon, there is a definite destabilization of the

polypeptide conformation as well as of the heme coordination sphere which in turn could affect the λ_{os} value. Taken together, the urea-induced cyt *c* unfolding process includes a first minor "structural click" followed by a more extensive unfolding transition. The early small conformational perturbation is not easily detected by some of the spectroscopic methods [44,45] whereas it can be manifested by the value of λ in electrochemical measurements. Hence, it seems that voltammetry is sensitive enough to monitor the unfolding process of heme proteins and could be considered as an alternative probes for studying the structural changes at heme environment.

4. Conclusion

When a heme protein unfolds, the highly polar water molecules penetrate into the heme cavity, and so heme meets a surrounding medium with higher dielectric constant. Marcus theory predicts that in such a situation the λ value increases. Our study provides experimental evidences demonstrating how λ changes upon protein unfolding. Raising the concentration of urea as a suitable denaturant for cyt *c*, the λ value increased sigmoidally producing a denaturation curve similar to those obtained by spectroscopic methods. Evaluation of the results indicates that as a sensitive probe to the structural changes occurs in the heme site of cyt *c*, λ provides an alternative tool for following the unfolding process. In summary, the experimental findings presented here may provide new insights into how electron transfer properties of redox proteins could be used as an intrinsic biophysical feature to study the protein structure and function.

Acknowledgment

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