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Using entropies of reaction to predict changes in protein stability: tyrosine-67-phenylalanine variants of rat cytochrome *c* and yeast Iso-1 cytochromes *c*

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Abstract

Using the voltammetric method of square-wave voltammetry, a direct electrochemical examination was made of the wild type and Tyr67Phe mutant of both rat cytochrome *c* and yeast iso-1-cytochrome *c*. In addition to determining the equilibrium reduction potential ($E^{0\prime}$) for each cytochrome, the entropy of reaction, $\Delta S^{0\prime}_{\mathrm{Rxn}}$($\Delta S^{0\prime}_{\mathrm{Rxn}} = S^{0\prime}_{\mathrm{Red}} - S^{0\prime}_{\mathrm{Ox}}$), for the reduction process was determined via the non-isothermal method. Having determined $\Delta S^{0\prime}_{\mathrm{Rxn}}$ and $E^{0\prime}$, $\Delta H^{0\prime}$ was calculated. For rat cytochrome *c*, it was found that $\Delta S^{0\prime}_{\mathrm{Rxn}} = -43$ J mol^{-1} K^{-1} for the wild type and -53 J mol^{-1} K^{-1} for the Tyr67Phe variant, with the $\Delta H^{0\prime}$ for both the wild type and variant nearly identical, indicating that the changes in reduction potential and probably stability are due to changes in $\Delta S^{0\prime}_{\mathrm{Rxn}}$. In contrast the measured $\Delta S^{0\prime}_{\mathrm{Rxn}}$ for yeast iso-1-cytochrome *c* demonstrated significant changes in both entropic and enthalpic contributions in going from wild type to mutant cytochrome *c*. The entropy of reaction provides information regarding the relative degree of solvation, and very likely the degree of compactness, of the oxidized state versus the reduced state of the redox protein. A thermodynamic scheme and stability derivation are presented that show how the entropies of reaction of wild type versus variant cytochromes contribute to and predict changes in stability in going from oxidized to reduced protein. For yeast iso-1-cytochrome *c*, the thermodynamically predicted change in stability was very close to the experimentally observed value, based on previous differential scanning calorimetric stability measurements. While such data is not available for rat cytochrome *c*, consideration of the enormously increased local stability of the rat oxidized cytochrome *c* variant predicts that the reduced rat variant will be even more stable than the already stabilized oxidized variant.

Keywords: Entropy of reaction; Rat cytochrome c; Iso-1-cytochrome *c*; Variant; Mutant; Yeast; Voltammetry; Square-wave voltammetry; Direct electrochemistry; Electrochemistry; Heme proteins; Redox; Stability; Thermodynamics

Abbreviations: Cyt *c*, cytochrome *c*; E_s, the step potential; E_{sw}, the square-wave amplitude or pulse potential; $f = \tau^{-1}$ frequency, where τ is the time required for one complete cycle; SHE, standard hydrogen electrode; VAR, variant; WT, wild type.

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1. Introduction

Cytochrome *c* is a small (MW 12 400) heme-containing protein that carries out its physiological function in the intermembrane space of mitochondria [1,2]. It has an equilibrium reduction of +260 mV versus the standard hydrogen electrode (SHE). Its most important function is providing electrons to cytochrome *c* oxidase, which in turn reduces oxygen to water in the terminal respiratory chain. It is also the substrate for a number of physiological reductants (cytochrome *c* reductase, cytochrome b_5, sulfite oxidase and cytochrome b_2) and oxidants (cytochrome *c* oxidase and cytochrome *c* peroxidase). Cyt *c*, probably the most widely studied protein, has been examined from many perspectives [1,2]. Cyt *c* continues to be an important redox protein, not only for the study of protein structure–function relationships, but also for a continued understanding and elucidation of biological electron-transfer mechanisms. Cyt *c* possesses electrochemical and spectroscopic properties that are ideal for exploring the details of redox protein thermodynamic behavior and associated electron-transfer reactions.

In explaining variation in the redox potentials among different types of cytochromes, including cytochromes c, investigators have proposed several factors: (a) the polarity of the heme environment [3]; (b) the accessibility of the heme to the solvent [4]; (c) the strength of the axial ligand field [5]; (d) local [6] and global [7] electrostatic interactions [8,9]; and (e) the influence of heme propionate solvation [10].

When further considering site specific mutations of cyt *c*, several effects often result. The reduction potential is often shifted, usually to a more negative value and rarely to a more positive value. The protein can become more or less stable in regard to its global stability as measured by denaturation by urea or guanidine hydrochloride. Studying changes in stability in going from wild type to mutant and oxidized to reduced state, is a fundamental way to evaluate the cytochrome's functional activity, and understand the sequence determinants of stability. Its alkaline pK_a (the inflection point pH where the 695 nm band, ascribed to the methionyl–80 sulfur–Fe bond, is lost) is shifted, usually to a lower pH. Its UV-visible absorption spectra in the oxidized and reduced state can also be altered. A less obvious effect of single or double site specific mutations is the observed change in entropy of reaction, $\Delta S^{0\prime}_{\mathrm{Rxn}}$. The $\Delta S^{0\prime}_{\mathrm{Rxm}}$ is difference in the standard entropy of the reduced state and oxidized state of the cyt *c*: $\Delta S^{0\prime}_{\mathrm{Rxn}} = S^{0\prime}_{\mathrm{Red}} - S^{0\prime}_{\mathrm{Ox}}\Delta S^{0\prime}_{\mathrm{Rxm}}$ provides information regarding the degree of solvation of the prosthetic group upon reduction and possibly the degree of compactness of each oxidation state. $\Delta S^{0\prime}_{\mathrm{Rxn}}$ explicitly provides the entropic contribution to the observed equilibrium reduction potential, $E^{0\prime}$, of the variant cyt *c*.

One cyt *c* variant that has been explored extensively is the Tyrosine67Phenylalanine (Tyr67Phe) variant. Interestingly, this variant has been prepared and evaluated for yeast iso-1-cyt *c* [11,12], cyt *c* [13] and horse cyt *c* [14]. In the case of the Tyr67Phe yeast iso-1 cyt *c* variant (referred to and abbreviated as iso-1 or iso-1-cyt *c*), the X-ray crystallographic structures were determined for both the reduced and oxidized forms. While no structures are available for Tyr67Phe rat cyt *c*, NMR spectroscopic experiments by Luntz et al. [13] were done to explore the effects upon the hemochrome structure of the protein after losing the –OH of tyrosine 67. In this paper we report an electrochemical entropic study of both the wild type and Tyr67Phe variant of both rat (mammalian) rat cyt *c* and yeast iso-1-cyt *c*. A free energy thermodynamic scheme and general equation are also presented to show how the entropies of reaction of the wild type and variant cytochromes both contribute to and predict changes in stability in going from oxidized to reduced protein.

2. Experimental methods

2.1. Electrochemical methods: square-wave voltammetry

Square-wave voltammetry (SWV) is a sensitive electrochemical method which provides a high signal-to-noise response [15]. Osteryoung and co-

workers have been largely responsible for developing square-wave voltammetry and making it accessible for analytical research [16,17]. Most recently, SWV has been applied to the study of iron-sulfur proteins and enzymes [18–20]. Square-wave voltammetry is significantly more sensitive than cyclic voltammetry [16]. In square-wave voltammetry (Fig. 1) a potential is applied over time in the form of a square-wave superimposed on a staircase. The applied potential is progressively stepped in fixed increments, E_s (the step potential), and pulsed positively and negatively at each step with potential E_{sw}, the square-wave amplitude. This is all done at a particular frequency $f = \tau^{-1}$, where τ is the time for one full cycle. The resultant current is measured at the end of each negative (reducing, cathodic) and positive (oxidizing, anodic) pulse (○). Overall, the voltammetric scan is either in the positive (oxidizing) or negative (reducing) direction. For each applied potential step, the difference in current is calculated between the positive and negative currents generated from the applied potential pulses. Experimentally, the positive and negative currents are collected separately and then the differences are taken for all pulses to provide the observed 'net' square-wave voltammogram. Typical SW voltammograms are shown in Fig. 2. The potential at which the voltammetric peak is observed

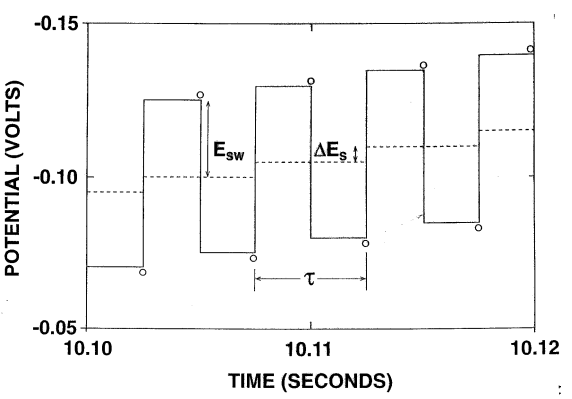


Fig. 1. Square wave voltammetric input wave form. E_{sw} is the square wave amplitude from half peak-to-peak, E_s the staircase step potential, t the time for one complete cycle, and the square wave frequency f is the reciprocal of t. The current is measured at the end of each potential pulse, which is indicated by the small circle.

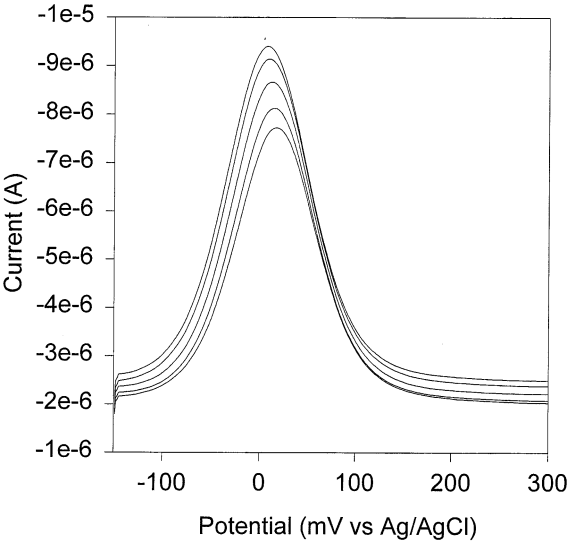


Fig. 2. Typical square wave voltammograms of 1.0 mM rat Tyr67Phe cytochrome c in 50 mM Tris/100 mM NaCl buffer, pH 7.0 over the temperature range 4–25°C. For each voltammogram, positive scan from $E_i = -150$ mV vs. Ag/AgCl to $E_f = +300$ mV vs. Ag/AgCl, $E_{sw} = 25$ mV, $f = 15$ Hz and $E_s = 1$ mV.

defines the equilibrium reduction potential, $E^{0\prime}$. The width at half height is a measure of reversibility. The redox reaction is reversible when $W_{1/2} = 90/n$ mV, with n being the number of electrons transferred per equivalent. The height of the square-wave voltammetric peak is proportional to relative concentration of that particular species. All events take place within the thin 'reaction layer' at the electrode surface. Electrochemical experiments shown in this work were demonstrated to be diffusion controlled, since the peak current (i_p) versus $\tau^{-1/2}$ was linear (data not shown).

2.2. Instrumentation

All electrochemical experiments were done with a BAS 100 B/W electrochemical analyzer (Bioanalytical Systems). All potentials are reported versus the standard hydrogen electrode, SHE. In Fig. 2 where potentials are shown with respect to the experimentally used Ag/AgCl/1 M KCl reference electrode ($E^{0\prime} = 230$ mV versus SHE), the SHE potentials are obtained by adding 230 mV. The

buffer used throughout this work, unless otherwise stated, was 50 mM Tris/100 mM NaCl, pH 7.0. Tris–HCl, Tris–Base, and NaCl were obtained as ultra-pure reagents (J.T. Baker). Only triple distilled water (Purification Systems) with a conductivity of 18 Mohms cm^{-1} was used. Final protein concentrations were typically about 0.5–1 mM. Microdialysis (typically of 100 μl of sample) was used to change buffers. A disk of 3 mm diameter gold electrode (99.999%, Johnson Matthey) encased in a casting resin sheath served as the working electrode. The electrode was polished with 0.3 micron alumina slurries (Buehler, Lake Bluff, IL) and rinsed with distilled water to remove polishing material and to further clean the electrode surface. After the gold electrode surface was polished, it was modified by placing it in a 5 mM solution of bis(4-pyridyl) disulfide (BPD) (Aldrich) promoter for 10 min. The electrode was then washed with distilled water and used immediately. This procedure apparently bonds the sulfur atoms of the BPD promoter to the electrode surface and follows the work of Taniguchi et al. [21]. The BPD modified gold electrode has also been used by Burrow et al. [22] to study similar cytochromes *c*. A promoter facilitates electron transfer between the cytochrome and the electrode surface but is not electrochemically active itself.

A specially designed anaerobic semimicro-cell was used for the electrochemical experiments [18,19] and permits samples of about 25–30 μl volume to be examined. The small volume of sample is effectively transfixed between the working, reference and auxiliary electrodes. Oxygen was removed by passing wet 99.99% pure argon over the sample and through the cell for 20 min prior to the experiment. The reference electrode used was an Ag/AgCl/1 M KCl electrode. The counter electrode was 18 gauge platinum wire. All redox potentials are corrected to the SHE unless otherwise noted.

2.3. Entropic and enthalpic contributions to the reduction potential of the cytochromes c by non-isothermal electrochemical method

An additional semimicro-cell, similar to that described above, was fabricated with a water jacket surrounding the main body. Its temperature was varied and controlled with an RM6 Lauda/Brinkman thermostated water bath. The reference electrode, which was also water jacketed, was maintained at a constant 25°C with a second thermostated water bath. This permitted rapid and precise control of the temperature of the cytochrome sample for non-isothermal electrochemical experiments. The advantage of non-isothermal electrochemical measurements of the reduction potential, $E^{0\prime}$, as a function of temperature is that the slope of $E^{0\prime}$ versus T is directly proportional to the standard entropy change, $\Delta S^{0\prime}_{\mathrm{Rxn}}$ [10]. In the non-isothermal experiment, only the sample changes temperature, while the temperature of the reference electrode is kept constant. Only the bottom 1.5 cm of the reference electrode assembly, with a porous vycor glass tip, was in the temperature controlled cell and made contact with the cytochrome solution, which was brought to equilibrium at each new temperature at which the $E^{0\prime}$ was determined by square-wave voltammetry. The thermal junction potentials were evaluated experimentally and observed redox potentials were determined to be within ± 0.3 mV of their true values over the temperature range from 2 to 40°C. Since mutant cytochromes *c* are sometimes less stable than their wild types, the experimental temperature excursion was confined to the range of 3–30°C. For each wild type cytochrome, ten completely separate temperature excursions were done and each with a different sample. The same was done for the two Tyr67Phe mutants (rat and iso-1) but with five completely separate temperature excursions and samples. In Table 1, the average of the measured values are shown with $\pm$ SD.

To distinguish folding/unfolding events from electron transfer events, an 'et' subscript was used to denote all redox/electron transfer events. In the non-isothermal experiment, the electron-transfer reaction evaluated is the reduction of cyt *c*, with one electron transferred ($n = 1$).

$$\mathrm{Cyt}\ c_{\mathrm{Ox}} + \mathrm{e}^- \rightleftarrows \mathrm{Cyt}\ c_{\mathrm{Red}} \quad (1)$$

$$\text{where: } \Delta G^{0\prime}_{\mathrm{et}} = -nFE^{0\prime} = \Delta H^{0\prime}_{\mathrm{et}} - T\Delta S^{0\prime}_{\mathrm{et}} \quad (2)$$

$\Delta G^{0\prime}_{\mathrm{et}}$ is the free energy for the reduction process, $\Delta H^{0\prime}_{\mathrm{et}}$ and $\Delta S^{0}_{\mathrm{et}}$ are the enthalpic and entropic contributions to $\Delta G^{0\prime}_{\mathrm{et}}$, n is the number of elec-

trons transferred per equivalent, and F is the Faraday's constant in coulombs. The change in $E^{0\prime}$ as a function of temperature under appropriate conditions [23] with the non-isothermal method, leads to:

$$d\Delta G^{0\prime}_{et}/dT = -nF(dE^{0\prime}/dT) = \Delta S^{0\prime}_{Rxm} \quad (3)$$

where $\Delta S^{0\prime}_{Rxn}$ is the entropy of reaction. Furthermore, $\Delta S^{0\prime}_{Rxn}$ is the difference between the standard entropies of the reduced and oxidized forms of the protein:

$$\Delta S^{0\prime}_{Rxn} = S^{0\prime}_{Red,et} - S^{0\prime}_{Ox,et} \quad (4)$$

$\Delta S^{0\prime}$, the standard entropy change at 25°C, was calculated from $\Delta S^{0\prime}_{Rxm}$ according to Taniguchi et al. [24]:

$$\Delta S^{0\prime}_{et} = \Delta S^{0\prime}_{Rxn} - 66.5 \text{ J mol}^{-1} \text{ K} \quad (5)$$

Since $\Delta G^{0\prime}_{et} = -nFE^{0\prime}$, $\Delta G^{0\prime}_{et}$ can be calculated from the reduction potential of the cytochrome; $\Delta H^{0\prime}_{et}$ is then obtained from $\Delta S^{0\prime}_{et}$ and $\Delta G^{0\prime}_{et}$. For an excellent discussion of entropic contribution to the reduction potentials of heme proteins see Bertrand et al. [10] and Taniguchi et al. [24]. The very negative entropy change in going from the oxidized to reduced state has been ascribed to increased solvation of the reduced form of the protein [10,25].

2.4. Preparation of the Tyr67Phe rat variant

Both the wild type and Tyr67Phe variants of rat cyt *c* and yeast iso-1-cyt *c* were prepared as previously described [13]. For the rat variant, the terminal amino group was not acetylated but lysine 72 is trimethylated as a result of being expressed in yeast. In the original work on the Tyr67Phe mutant, this variant is referred to as the RnC-II fraction, since a RnC-I fraction which has both terminal acetylation and trimethylation of the lysine 72 [13] was also observed.

3. Experimental results

3.1. Square-wave voltammetry of the wild type and Tyr67Phe rat cytochromes c and yeast iso-1-cytochromes c

The square-wave voltammetry of the wild type rat cytochrome with an observed $E^{0\prime} = +261$ mV (data not shown); this value is within 2 mV of the $E^{0\prime} = +259$ mV reported earlier [13]. In Fig. 2 is shown a typical data set of the square-wave voltammetry of rat Tyr67Phe variant at different temperatures. Even over a wide potential range, no other voltammetric peaks were observed for the Tyr67Phe variant, thus, indicating that no redox dependent ligand exchanges occurred. The potentials in Fig. 2 are shown with respect to the Ag/AgCl/1 M KCl reference electrode and are converted to potentials with respect to the standard hydrogen electrode (SHE) by adding +230

Table 1
Reduction potentials, entropies of reaction, entropic and enthalpic contributions to $E^{0\prime}$ of wild type and Tyr67Phe variant of both rat cytochrome *c* and iso-1-cytochrome *c*[a]

Cyt *c*	$E^{0\prime}$ (mV)	$\Delta S^{0\prime}_{Rxn}$ J mol$^{-1}\cdot$K^{-1}, (cal mol$^{-1}\cdot$K^{-1})	T$\Delta S^{0\prime}$ kJ mol^{-1}, (kcal mol^{-1}), [mV]	$\Delta H^{0\prime}$ kJ mol^{-1}, (kcal mol^{-1}), [mV]
WT rat	+261 ± 2	−43 ± 1 (−10.2)	−32.6 (−7.9) [+331][a]	−58.3 (−13.9) [+592]
Tyr67Phe rat	+236 ± 2	−53 ± 3 (−12.7)	−35.6 (−8.8) [+361]	−58.8 (−14.1) [+597]
WT iso-1	+280 ± 2	−57 ± 4 (−13.6)	−36.8 (−8.8)	−64.4 (−15.3)
Tyr67Phe iso-1	+220 ± 1 (−6.7)	−28 ± 2 (−6.7)	−28.2 (−6.7)	−49.9 (−11.7)

[a] The T$\Delta S^{0\prime}$ and $\Delta H^{0\prime}$ contributions are also noted in units of mV and their difference provides the observed reduction potential, $E^{0\prime}$: $\Delta G^{0\prime} = -nFE^{0\prime} = \Delta H^{0\prime} - T\Delta S^{0\prime}\cdot T = 298$ K.

mV to the observed potentials. All discussion and analysis of the potentials are with respect to the SHE reference.

3.2. Non-isothermal determination of the entropy of reaction, $\Delta S^{0\prime}_{Rxn}$

Using the cell/electrode assembly described above, square-wave voltammetric non-isothermal determination of the entropy of reaction, $\Delta S^{0\prime}_{\mathrm{Rxn}}$, was first made for purified horse heart cyt *c*. The entropy of reaction obtained for horse heart cyt *c*, -48 J mol^{-1} K^{-1} (data not shown), was in excellent agreement with values obtained previously at similar ionic strengths and obtained both electrochemically and calorimetrically [10,24,26]. The entropies of reaction and associated thermodynamic values were also determined for the wild type and Tyr67Phe variant of both rat (Fig. 2 and Table 1) cytochrome and yeast iso-1-cyt *c* (data not shown, but results summarized in Table 1). For all cytochromes the net reaction evaluated was: $\mathrm{Met_{80}}$–$\mathrm{Fe^{3+}}$–$\mathrm{His_{18}} + \mathrm{e^-} \rightarrow \mathrm{Met_{80}}$–$\mathrm{Fe^{2+}}$–$\mathrm{His_{18}}$ and the $E^{0\prime}$ of each (see Table 1 for the observed $E^{0\prime}$) was followed as a function of temperature. The $\Delta S^{0\prime}_{\mathrm{Rxn}}$ of rat Tyr67Phe variant was found to be -53 J mol^{-1} K^{-1} compared to -43 J mol^{-1} K^{-1} for the wild type (Fig. 3). The results for Tyr67Phe variant and wild type yeast iso-1-cytochromes *c* were substantially different, since their values were found to be inverted relative to rat: $\Delta S^{0\prime}_{\mathrm{Rxn}} = -28$ J mol$^{-1}\cdot$K^{-1} and -57 J mol$^{-1}\cdot$K^{-1}, respectively (Table 1). Somewhat different values for $\Delta S^{0\prime}_{\mathrm{Rxn}}$ of wild type and Tyr67Phe variant iso-1-cytochromes *c* were obtained in earlier work [11,24], since the non-isothermal experiments were done at either a different pH or ionic strength than that used in this work.

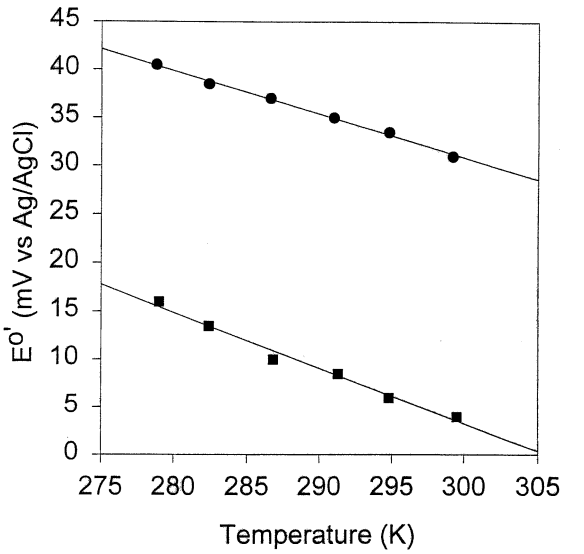


Fig. 3. Non-isothermal determination of ΔS_{n} for rat cytochromes *c* based on single temperature excursions for wild type and variant rat cytochrome *c*. 1.0 mM protein in 50 mM Tris/100 mM NaCl buffer, pH 7.0. $E^{0\prime}$ was determined every 4°C after thermal equilibrium was obtained over the ramp of 5–25°C. $E^{0\prime}$ was plotted versus temperature and the ΔS_{n} was determined from the slope of the linear regression line. The circles are data from rat wild type cyt *c* and the squares are data from rat Tyr67Phe variant. For these two typical data sets: $\Delta S^{0\prime}_{\mathrm{Rxn,WT}} = -43.3$ J mol$^{-1}\cdot$K and $\Delta S^{0\prime}_{\mathrm{Rxn,Tyr67Phe}} = -55.1$ J mol$^{-1}\cdot$K, respectively.

4. Discussion

4.1. Square-wave voltammetry of the wild type and Tyr67Phe variant of rat cytochrome c

In the square-wave voltammetry the rat wild type cyt *c* and its Tyr67Phe variant, the observed reduction potentials were: $E^{0\prime} \simeq +258$ mV and $E^{0\prime} \simeq +231$ mV versus SHE, respectively (Fig. 2 and Table 1). As is true for nearly all variants of cyt *c*, the reduction potential of the variant is lower than that for the wild type. For example in a large series of Phe 82 mutants of yeast iso-1-cyt *c*, their $E^{0\prime}$'s were lowered 10–42 mV [27]. In the case of the Tyr67Phe, it is helpful to review some of the previously measured physical properties of the wild type and variant cytochrome before considering a thermodynamic scheme to interpret the experimental results.

4.2. Local and global stability

There are two types of stability for cyt *c*: local and global. In turn there are three measures of local stability which involve the left side of the molecule and the 695 nm band due to the me-

thionyl sulfur–Fe^{3+} bond, shown in Fig. 4. The 695 nm absorbance (charge transfer) band has been assigned to the Met–80–S–Fe^{3+} bond [28]. When temperature is increased, the Fe–S bond is eventually disrupted. This two-state process usually takes place at temperatures that are lower than those required for the thermal global denaturation of the protein [29] and is endothermic. The temperature at which half of the 695 nm band intensity is lost is termed the $T_{1/2}$. The higher the observed $T_{1/2}$, the greater the local stability of this bond and, hence, the greater the stability of the left side of the molecule, shown in Fig. 4. A second important measure of local stability is the alkaline pK_a. As pH is increased, the Met–80–S–Fe^{3+} bond is eventually disrupted and results in the displacement of the Met 80 side chain from iron coordination [30], probably displaced by a lysine ligand [31]. The pK_a is the point

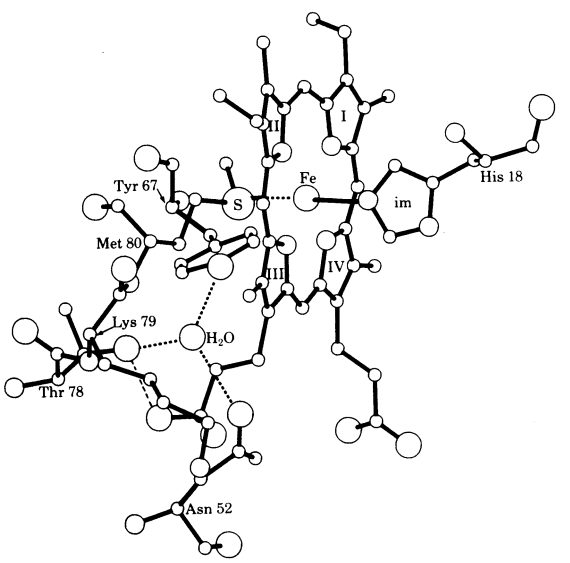


Fig. 4. Diagram of the structure of tuna cytochrome *c* [43,44] in the region of Tyr67Phe. Fe indicates the heme iron atom, Im shows the imidazole side chain of histidine 18 and S is the sulfur atom of methionine 80. Histidine 18 and methionine 80 are the axial ligands of the heme, from the 'right' and 'left' sides of the protein, respectfully. The amino acid residues are indicated in three letter code placed near the α-carbon atoms. H_2O marks Water 166 which is hydrogen bonded to tyrosine 67, asparagine 52 and threonine 78. This figure reprinted with permission from Biochemistry 32 (1992) 8336–8343, Copyright 1992, American Chemical Society.

of inflection of the sigmoidal curve. The higher the alkaline pK_a, the greater the local stability. There is a more complicated acidic pK_a, in which one follows the loss of the 695 nm band as the pH is lowered. The lower the acidic pK_a, the greater the protein's local stability.

The most widely used measure of global stability for cyt *c* is its denaturation by either urea or guanidine hydrochloride. Denaturation is followed through the increase of fluorescence emission of tryptophane-59 [32] as the protein is unfolded. When cyt *c* is folded, the Trp-59 is close enough to the heme iron to have its fluorescence emission completely quenched. As the protein unfolds, Trp-59 moves further and further away from the heme iron, with increasing fluorescence emission. These titrations are analyzed as a two-state system (folded and unfolded) and from the titration curves, the free energy of unfolding, $\Delta G_{D,aq}$, in the pure buffer can be estimated. While nearly all such titrations have been done for the ferric cyt *c*, a few studies have been done for the ferrous (reduced) forms of horse heart cyt *c* [33,34]. For ferric horse heart cyt *c* with urea as denaturant, $\Delta G_{D,aq,Ox} = 9.5$ kcal mol^{-1}, but for the ferrous protein, $\Delta G_{D,aq,Red} =$ 18.4 kcal mol^{-1}. The theoretical basis for this quantitative method of global stability is the heteropolymer theory [35–37]. The titrations with denaturant are analyzed by a non-linear least squares fit of the data to the equation:

$$-RT \ln [(F - F_o)/(F_\infty - F)]$$

$$= \Delta G_{D,aq} - m \cdot [\text{urea}]$$

This expression [38] results from interpreting the effect of urea as a displacement of the two-state equilibrium between the native (folded) and unfolded forms of the protein [39]. F_o, F, and F_∞ are the fluorescence in pure buffer at finite concentrations of urea, and at saturation of the effect, respectively. $\Delta G_{D,aq}$ is the free energy of unfolding in pure buffer. The slope, m, of the straight line obtained in this type of titration is a parameter proportional to the difference in the exposure to the solvent of hydrophobic residues in the native versus the unfolded states of the protein.

Table 2
Different measures of local [13] stability ($pK_{a,acid}$, $pK_{a,alkaline}$, $T_{1/2}$, and $\Delta G^{*}_{Ox/695nm}$) and global [45] stability ($\Delta G_{D,ag}$, m) of wild type and Tyr67Phe rat cytochrome *c*

Cyt *c*	$E^{0\prime}$ (mV)	$pK_{a,acid}$ (pH)	$pK_{a,alkaline}$ (pH)	$T_{1/2}$	$\Delta G_{Ox/695nm}$ (kcal mol^{-1})	$\Delta G_{D,aq}$ (kcal mol^{-1})	m (kcal mol^{-2})
WT	+259	2.8	9.5	60	1.2	9.15	1.21
Tyr67Phe	+224	2.1	10.7	90	2.7	7.19	0.86

Another important approach for evaluating global stability is thermal denaturation as seen by differential scanning calorimetry (DSC) [12]. It is considered to be the least ambiguous method [40], since DSC measures the thermodynamic properties of the unfolding transition directly. One of the key requirements for using DSC, however, is that the unfolding process be reversible and this is often the case for wild type and mutant cytochromes *c*.

4.3. Earlier results with Tyr67Phe variant

In Table 2 are shown some of the earlier results obtained for the physical properties of both the wild type and Tyr67Phe rat cyt c [11,13]. In going from wild type to the Tyr67Phe variant, the surprising results are: (1) the local stability (methionyl sulfur–Fe^{3+} bond) is greatly enhanced with the $T_{1/2}$ rising from 60 to 90°C; and (2) the global stability actually decreases. For the wild type protein, the lone water molecule, shown in Fig. 4, is hydrogen bonded to three key residues: Tyr 67, Thr 78, and Asn 52. X-ray crystallographic work on oxidized mammalian cytochrome [11] shows that this water (formally called water 166) is present and appears to be present in the oxidized state for all eukaryotic cytochromes *c* [41]. For wild type yeast iso-1-cyt *c* it is present in both redox states. This hydrogen bond network is thought [11] to fine tune the redox potential of the wild type protein. In the rat Tyr67Phe variant, Luntz et al. [13] proposed, from NMR spectroscopic experiments, that the water is missing in the oxidized form of the protein. In contrast, Berghuis et al. [11] observed that in the reduced state of the yeast iso-1-cyt *c* Tyr67Phe variant, there are two water molecules in this region that form an even more complex hydrogen bond structure, in which the additional water takes over the function of the tyrosyl–OH group. While Luntz et al. [13] concluded that the water molecule in this region is absent in the oxidized form of rat cyt *c*, Berghuis et al. [11] were also not able to observe with X-ray crystallography either of these two waters in the oxidized Tyr67Phe iso-1-cyt *c*. Presumably these waters, if present, are mobile and in motion, making it impossible to structurally resolve them [11]. Further work is needed to determine whether or not there are any hydrogen bonded waters in this region for either rat or yeast Tyr67Phe cytochromes in the oxidized state.

4.4. The thermodynamics of how the entropy of reaction of cytochrome c contributes to the observed change in stability in going from oxidized to reduced protein

While it is an empirical fact that: (1) wild type cytochromes *c* are more stable in the reduced state compared to the oxidized state [34,42]; and (2) that the entropy of reaction for this process is invariably negative [10,24]. It is less obvious how changes in entropy ($\Delta S^{0\prime}_{et}$) and enthalpy ($\Delta H^{0\prime}_{et}$) derived from $\Delta S^{0\prime}_{Rxn}$ play a role in experimentally observed changes in protein stability in going from the oxidized to reduced state, or wild type to variant, or both. In Scheme 1, all the reversible reactions of interest are shown, but with the arrows pointing so as to form a thermodynamic cycle. The stability of any protein is a function of free energy and is a measure of the amount of work needed to go from the folded (F) to the unfolded (U) state of the protein: $\Delta G^{0\prime}_{F\rightarrow U}$. In Scheme 1, the full cycle for cyt *c* shown: Cyt $c_{F,Ox} \rightarrow$ Cyt $c_{F,Red} \rightarrow$ Cyt $c_{U,Red} \rightarrow$ Cyt $c_{U,Ox} \rightarrow$ Cyt

$c_{F,Ox}$; thus, there are two stability measurements ($\Delta G^{0\prime}_{F\rightarrow U,Red}$ and $\Delta G^{0\prime}_{F\rightarrow U,Ox}$) and two redox processes (Scheme 1). Using sign to note the direction of the reaction and traversing the entire path so that $\Delta G^{0\prime}_{Total}=0$, these free energies are summarized in equation as:

$$\Delta G^{0\prime}_{et,F}+\Delta G^{0\prime}_{F\rightarrow U,Red}-\Delta G^{0\prime}_{et,U}-\Delta G^{0\prime}_{F\rightarrow U,Ox}=0 \quad (6)$$

But $\Delta G^{0\prime}_{F\rightarrow U,Red}$ and $\Delta G^{0\prime}_{F\rightarrow U,Red}$ are really just the free energies of stability, so Eq. (6) can be written as:

$$\Delta G^{0\prime}_{et,F}+\Delta G^{0\prime}_{Stab,Red}-\Delta G^{0\prime}_{et,U}-\Delta G^{0\prime}_{Stab,Ox}=0 \quad (7)$$

Reorganizing to obtain the change in free energy in going from the oxidized to the reduced state:

$$\Delta\Delta G^{0\prime}_{Stab}=\Delta G^{0\prime}_{Stab,Red}-\Delta G^{0\prime}_{Stab,Ox}=\Delta G^{0\prime}_{et,U}-\Delta G^{0\prime}_{et,F} \quad (8)$$

To determine the change in free energy in going from wild type to variant cyt *c*, two forms of Eq. (8) can be written:

$$\Delta\Delta G^{0\prime}_{Stab,VAR}=\Delta G^{0\prime}_{et,U,VAR}-\Delta G^{0\prime}_{et,F,VAR} \quad (9)$$

$$\Delta\Delta G^{0\prime}_{Stab,WT}=\Delta G^{0\prime}_{et,U,WT}-\Delta G^{0\prime}_{et,F,WT} \quad (10)$$

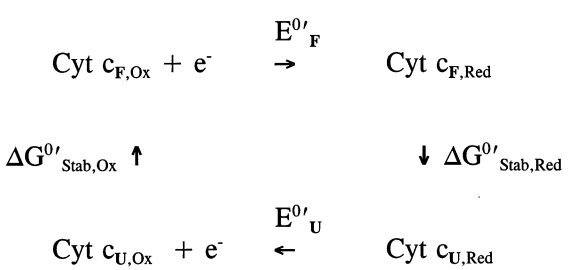
At this point, it is assumed that for the unfolded protein the mutation of the variant has minimal effect on its redox behavior and will behave the same as wild type protein; or simply: $\Delta G^{0\prime}_{et,U,WT}\simeq\Delta G^{0\prime}_{et,U,VAR}$. With this assumption and subtracting Eq. (10) from Eq. (9), the difference in stability (folded to unfolded) in going from the oxidized to reduced and wild type to variant is:

$$\Delta\Delta\Delta G^{0\prime}_{Stab}=\Delta G^{0\prime}_{et,F,WT}-\Delta G^{0\prime}_{et,F,VAR} \quad (11)$$

When Eq. (11) is expanded to show the enthalpic and entropic contributions from electron transfer process for each $\Delta G^{0\prime}_{et}$, we obtain:

$$\Delta\Delta\Delta G^{0\prime}_{Stab}=\Delta H^{0\prime}_{et,F,WT}-T\Delta S^{0\prime}_{et,F,WT}-\Delta H^{0\prime}_{et,F,VAR}+T\Delta S^{0\prime}_{et,F,VAR} \quad (12)$$

With this simple derivation, it is clear that the overall change in stability (folded to unfolded) in going from wild type to variant and from oxidized to reduced cyt *c*, is simply the differences in the entropic and enthalpic contributions to the redox potential of the wild type and variant cyt *c*.



Scheme 1. Relationship between reduction potentials, free energies of unfolding (stability), or both oxidized and reduced, and folded (F) and unfolded (U) cytochromes *c*.

4.5. Stability changes for wild type and Tyr67Phe yeast iso-1-cytochrome c

The case of wild type and variant yeast iso-1-cyt *c* provides a test of this derivation (Eq. (12)) and of Scheme 1, since: (1) the electrochemical data show clearly that there are changes in both the enthalpic and entropic contributions to the electron transfer free energy; and (2) thermal stability measurements are available for both oxidized and reduced states of both the wild type and Tyr67Phe iso-1-cyt *c* [12]. Experimentally in our work it was found that for wild type: $\Delta S^{0\prime}_{Rxn}=-57$ J mol$^{-1}\cdot$K^{-1} and $\Delta H^{0\prime}=-64.4$ kJ mol^{-1}. For the Tyr67Phe variant, however, $\Delta S^{0\prime}_{Rxn}=-28$ J mol$^{-1}\cdot$K^{-1} and $\Delta H^{0\prime}=-49.9$ kJ mol^{-1}. When these results and the associated entropies and enthalpies, as summarized in Table 3, were used in the full expression for $\Delta\Delta\Delta G^{0\prime}_{Stab}$ (Eq. (12)), the calculated value for $\Delta\Delta\Delta G^{0\prime}_{Stab}$ was -1.4 kcal mol^{-1}. This value of $\Delta\Delta\Delta G^{0\prime}_{Stab}=-1.4$ kcal mol^{-1} is remarkably close to $\Delta\Delta\Delta G^{0\prime}_{Stab,Ox\rightarrow Red}=-1.3$ kcal mol^{-1} determined experimentally by Lett et al. [12] via differential scanning calorimetry. Eq. (12) correctly predicts that the overall (global) stability of the variant is lower than the wild type protein, even though there still is a slight increase in the local stability of the oxidized variant versus the oxidized wild type as indicated by an increase in the $T_{1/2}(T_m)$ of the variant. Interestingly, these results also support the original approximation that the redox properties of the unfolded wild type and variant cytochromes are the same.

Table 3
Thermodynamic parameters of unfolding for wild type and Tyr67Phe yeast iso-1-cytochrome for both redox states as measured by DSC and referenced to oxidized wild type iso-1-cytochrome c: free energies of denaturation, ΔG_D [12][a]

Parameter	WT iso-1	Tyr67Phe iso-1
$E^{0\prime}$ (mV)	+290	+234
Oxidized		
T_m (°C)	53.6	58.3
ΔG_D (kcal mol^{-1})	0.0	1.5
Reduced		
T_m (°C)	80.3	81.1
ΔG_D (kcal mol^{-1})	8.6	8.8
Oxidized vs. (to) reduced $\Delta G_{D,Ox\rightarrow Red}$ (kcal mol^{-1})	8.6	7.3
$\Delta\Delta G^{0\prime}_{Stab} = 7.3-8.6 = -1.3$ kcal mol^{-1}		(wild type more stable than variant by 1.3 kcal mol^{-1} when going from Ox → Red)

[a] Data from Table 2 of reference [12].

4.6. Stability changes for wild type and Tyr67Phe rat cytochromes c

Experimentally, for the wild type and variant rat cyt *c*, it was observed that within experimental error $\Delta H^{0\prime}_{et,F,VAR}$ was nearly identical to $\Delta H^{0\prime}_{et,F,WT}$, i.e. $\Delta H^{0\prime}_{et,F,VAR} \simeq \Delta H^{0\prime}_{et,F,WT}$. Thus, for rat cyt *c* the changes in stability in going from the wild type to the variant cytochrome are controlled primarily by changes in entropy. In making a comparison to the iso-1-cytochromes noted above, thermal denaturation studies have not been done for the rat cytochromes *c*. Nonetheless, the large difference in stability between the variant and wild type cytochromes *c* as viewed from the vantage point of the thermal denaturation of the Met–80–S–Fe^{3+} bond seen at 695 nm ($T_{1/2}$: wild type, 60°C and variant 90°C) does provide us with some predications regarding the difference in stability of the reduced states of the wild type versus the variant. Using the experimentally determined $T\Delta S^{0\prime}_{et}$ and $\Delta H^{0\prime}_{et}$ values in Table 1, $\Delta\Delta\Delta G^{0\prime}_{Stab} = -0.60$ kcal mol^{-1}. In this calculation, we predict that the overall (global) thermal stabilities of the wild type cyt *c* is, again, more stable by 0.6 kcal mol^{-1} than the variant, in spite of the fact that the local stability of the oxidized variant is much greater than the wild type.

The thermal titration of the 695 nm spectroscopic band by Luntz et al. [13] (Table 2) can also be used as an approximation of thermally induced unfolding (partial). Luntz et al. [13] evaluated the $T_{1/2}$ thermal titration curves to provide free energies of stabilities ($\Delta G_{Ox/695nm}$ and see Table 2) for the wild type and variant in the oxidized state only. They determined that this change in stability was: $\Delta\Delta G^{0\prime}_{Stab,Ox} = +1.5$ kcal mol^{-1}, i.e. the oxidized variant is much more stable than the oxidized wild type. Since $\Delta\Delta\Delta G^{0\prime}_{Stab,Ox\rightarrow Red}$ (-0.6 kcal mol^{-1}) = $\Delta\Delta G^{0\prime}_{Stab,Ox}$ ($+1.5$ kcal mol^{-1}) − $\Delta\Delta G^{0\prime}_{Stab,Red}$, we can calculate $\Delta\Delta G^{0\prime}_{Stab,Red}$. The calculated $\Delta\Delta G^{0\prime}_{Stab,Red} = +2.1$ kcal mol^{-1}. This calculation provides the surprising prediction that if we could measure the stability of the reduced protein via methionyl–80 sulfur–Fe^{2+} bond breaking (or possibly even through DSC), then we expect to find that the Tyr67Phe variant is even more stable than the wild type in the reduced state than it is in the oxidized state! These results are quite different than iso-1-cytochromes where the reduced variant and wild type differed by only +0.2 kcal (Table 3). In order to more rigorously test the usefulness and validity of the electrochemically determined $\Delta S^{0\prime}_{Rxn}$ and their associated $\Delta S^{0\prime}$ and $\Delta H^{0\prime}$ terms, differential scanning calorimetric studies are in progress for both oxidation states of both wild type and Tyr67Phe variant of rat cyt *c*.

In conclusion, we have shown that electrochemically determined entropies of reaction can indeed inform us regarding the changes in protein stability as the cyt *c* is taken from the oxidized to the reduced state, or from wild type to variant, or

both. The related questions: (1) what is the relationship is between the stabilities determined thermally versus those done with chemical denaturant; and (2) what are the thermodynamic relationships, if any, between local and global stabilities, are both being investigated by evaluating other cyt *c* and iso-1-cyt *c* variants with both DSC and the nonisothermal determination of their entropies of reaction.

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