The Role of a Conserved Water Molecule in the Redox-dependent Thermal Stability of Iso-1-cytochrome c*

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Eukaryotic cytochromes c contain a buried water molecule (Wat166) next to the heme that is associated through a network of hydrogen bonds to three invariant residues: tyrosine 67, asparagine 52, and threonine 78. Single-site mutations to two of these residues (Y67F, N52I, N52A) and the double-site mutation (Y67F/N52I) were introduced into *Saccharomyces cerevisiae* iso-1-cytochrome c to disrupt the hydrogen bonding network associated with Wat166. The N52I and Y67F/N52I mutations lead to a loss of Wat166 while N52A and Y67F modifications lead to the addition of a new water molecule (Wat166) at an adjacent site (Berghuis, A. M., Guillemette, J. G., McLendon, G., Sherman, F., Smith, M., and Brayer, G. D. (1994) *J. Mol. Biol.* 236, 786–799; Berghuis, A. M., Guillemette, J. G., Smith, M., and Brayer, G. D. (1994) *J. Mol. Biol.* 235, 1326–1341; Rafferty, S. P., Guillemette, J. G., Berghuis, A. M., Smith, M., Brayer, G. D. G., and Mauk, A. G. (1996) *Biochemistry*, 35, 10784–10792). We used differential scanning calorimetry (DSC) to determine the change in heat capacity ($\Delta C_p$), and the temperature dependent enthalpy ($\Delta H_i$) for the thermal denaturation of both the oxidized and reduced forms of the iso-1 cytochrome c variants. The relative stabilities were expressed as the difference in the free energy of denaturation ($\Delta G_p$) between the wild type and mutant proteins in both redox states. The disruption of the hydrogen bonding network results in increased stability for all of the mutant proteins in both redox states with the exception of the reduced Y67F variant which has approximately the same stability as the reduced wild type protein. For the oxidized proteins, $\Delta G_p$ values of 1.3, 4.1, 1.5, and 5.8 kcal/mol were determined for N52A, N52I, Y67F, and Y67F/N52I, respectively. The oxidized proteins were 8.2–11.5 kcal/mol less stable than the reduced proteins due to a redox-dependent increase in the entropy of unfolding.

Water in and around proteins is recognized as being important to protein structure, function and stability (4–6). Surface and bound water molecules have been identified in protein structures using crystallographic methods and NMR spectroscopy. Eukaryotic cytochromes c, the paradigms of electron transfer proteins, are ideally suited for investigating the structural and functional purposes of water-protein interactions (7). Crystallographic studies performed on the oxidized and reduced states of both tuna and yeast iso-1-cytochrome c proteins have indicated that a conserved and internally bound water molecule (Wat166), along with the surrounding hydrogen bond network are central to the structural transition of cytochrome c between oxidation states (8, 9). The water molecule is adjacent to the heme and the hydrogen bonding network which is composed of conserved residues Asn$^2$, Tyr$^2$, and Thr$^2$, which are also hydrogen bonded in ferrocytochrome c to the Met$^2$ sulfur which is one of the two heme iron ligands. The oxidation-reduction or redox potential that determines the direction of electron flow between electron transfer proteins is dependent upon the heme ligands and the surrounding peptide (10). In addition, the functional properties of cytochrome c are dependent on the oxidation state of the protein, and knowledge of the energetics of protein stability with respect to oxidation state is central to our understanding of function (11). For example, the addition of an electron to ferricytochrome c results in modified functional properties including a significant increase in stability (12).

Several studies using classical genetic procedures or site directed mutagenesis have shown that the hydrogen bond network and Wat166 modulate redox potential and the stability of the protein (13–20). The high resolution three dimensional structures of the reduced and oxidized states of yeast iso-1-cytochromes c carrying mutations at position 52 and/or 67 have been recently reported (1–3). When compared to the wild type protein structure, these mutants show significant changes in their hydrogen bonding networks adjacent to the heme as well as either the displacement of the conserved internally bound Wat166 or the addition of a second internally bound water molecule.

Recent investigations have shown that depending on the amino acid substitutions, varying degrees of change in the free energy of unfolding are observed for the two redox states of cytochrome c (11, 21). To further understand the function of the internally bound Wat166 and the associated hydrogen bonding network, we performed thermal denaturation studies on the two redox forms of a suite of homologous mutants that disrupt this internal network. The amino acid substitutions affected the stability of unfolding differently in the oxidized and reduced states of the proteins. Our results indicate that both redox forms of the protein should be investigated to better assess the effect of a mutation.

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1 The abbreviations used are: Wat166, invariant water 166 in the structure of yeast iso-1 cytochrome c; DSC, differential scanning calorimetry; $\Delta C_p$, change in molar heat capacity between native and unfolded states; $T_m$, midpoint for the transition; $\Delta H_{m,i}$ change in apparent (van’t Hoff) enthalpy at $T_m$; $\Delta G_p$, change in Gibb’s free energy of denaturation; $\Delta S_p$, change in entropy of denaturation; $\Delta S_{m,i}$, change in apparent (van’t Hoff) entropy at $T_m$; $\Delta H_{m,i}$, change in enthalpy of denaturation.
Site-directed Mutagenesis, Protein Expression, and Purification—Site-directed mutagenesis was used to generate the mutant forms of yeast iso-1-cytochrome c as previously reported (22, 23). The mutations were carried out on an earlier modified version of the naturally occurring yeast iso-1-cytochrome c gene in which the codon for cysteine at position 102 was replaced with that of threonine. The resulting product does not have problems with dimerization and autoreduction during purification (24). For the purposes of this study, the C102T variant will be referred to as the wild-type protein. Iso-1-cytochrome c was expressed in the Saccharomyces cerevisiae strain GM3C-2 and purified as described previously (24, 25). The primary structures and purity of the mutants were verified using electrospray ion mass spectroscopy on a Fisons Instruments VG Quattro II triple quadrupole mass spectrometer using the Mass Lynx program and by scanning absorption spectroscopy. Purified proteins were stored at −80 °C until needed.

Preparation of Cytochrome c Solutions—All DSC experiments were performed in 50 mM sodium acetate buffer, pH 4.7. In the preparation of fully oxidized samples, 50 equivalents of potassium ferricyanide was added to approximately 6 mg of protein in the dialysis tubing. Proteins were dialyzed against sodium acetate buffer using several buffer changes to remove the oxidizing agent and the sample was subsequently brought to a protein concentration of between 50 and 150 μM. Over this limited range of protein concentrations, the thermodynamic parameters were observed to be the same when performed on different samples of wild-type cytochrome c in both oxidation states. Similar results have been reported by Liggins et al. (26).

For the preparation of the fully reduced proteins, the samples were dialyzed as above and 50 equivalents (7.88 mM final concentration) of dithiothreitol was added to both the protein sample and the running buffer immediately before performing the DSC experiment. Protein concentrations were determined using a Varian model 2200 spectrophotometer prior to scanning using quartz cuvettes with 1-cm optical paths. The absorbance at 410 nm was measured and the protein concentration calculated, using a molar extinction coefficient of ε410 = 106.1 × 10^3 M−1 cm−1 (27) for both the oxidized and reduced protein samples.

Microcalorimetry—DSC was performed using a MicroCal DS-2 differential scanning calorimeter. Protein samples were filtered through a 0.22-μm filter and then both the protein samples and running buffer were thoroughly degassed prior to filling the calorimeter cells. The scans were performed under a nitrogen gas environment at a scan rate of 60 °C h−1. Each oxidized cytochrome c sample was subjected to series of scans from 15 °C to 75 or 85 °C while the reduced samples were scanned from 15 to 105 °C. Generally, six scans were performed on each protein sample to determine the degree of reversibility. Each of the protein variants gave reproducible results showing reversible denaturation.

Profiles of specific heat (C_p) were analyzed using the Origin program (v. 2.90) supplied by MicroCal, Inc. The van’t Hoff or apparent transitional enthalpy DHvH at Tm (one half completion of the transition) was determined for all the repetitive scans performed on each protein sample by curve fitting. The repeated DSC scans for each sample showed estimated errors for Tm and ΔHvH of less than 0.3 °C and 3% of the average, respectively. The first scan was used for determination of the thermodynamic parameters as the protein concentration was accurately known. After scanning to 75 or 105 °C, the degree of reversibility was less than 100%, which was evident from the small decrease of the calorimetric enthalpy of each subsequent scan, so an accurate concentration of native protein for the subsequent scans of the sample was not attainable. When corrected for the losses from irreversible unfolding of protein, the thermodynamic parameters obtained from repeated scans were in agreement with those obtained from the first scan of the same sample.

Isothermal Thermodynamics Functions—Thermodynamic parameters were determined at the Tm, the temperature at which half the protein molecules are completely denatured. The value of ΔCp,D, the change in heat capacity between the native and unfolded states, was determined by linear least-squares fitting of the Tm and the ΔHvH values to the equation,

\[ \Delta H_vH = \Delta C_p, T_m \cdot T_m + b \]  

(Eq. 1)

The change in entropy at Tm was calculated using the equation,

\[ \Delta S_vH = \frac{\Delta H_vH}{T_m} \]  

(Eq. 2)

The experiment was performed in 50 mM sodium acetate at pH 4.7 and a scan rate of 60 °C h−1. The thermal scans are for wild type oxidized (--), wild type reduced (---), N52I/Y67F oxidized (--), and N52I/Y67F reduced (---) cytochrome c.

The enthalpies of denaturation for the cytochrome c variants were determined at a given reference temperature T using the equation,

\[ \Delta H_D = \Delta H_vH + \Delta C_p(T - T_m) \]  

(Eq. 3)

The entropy of denaturation at the same reference temperature was calculated using the equation,

\[ \Delta S_D = \Delta S_vH + \Delta C_p \ln \left( \frac{T}{T_m} \right) \]  

(Eq. 4)

The free energy of denaturation at temperature T (ΔG_D) was determined from the DSC data using the integrated form of the Gibbs-Helmholtz equation with a temperature-independent ΔCp (28):

\[ \Delta G_D = \Delta H_D \left( 1 - \frac{T}{T_m} \right) - \Delta C_p \left( T_m - T \right) \ln \left( \frac{T}{T_m} \right) \]  

(Eq. 5)

RESULTS

Thermal denaturation studies were performed under both oxidizing and reducing conditions. The DSC profiles of the specific heat (C_p(T)), not corrected for intrinsic base line curvature, are shown in Fig. 1 for the reduced and oxidized forms of the wild type and N52I/Y67F iso-1-cytochromes c, respectively. Generally, the thermodynamic parameters (Tm, ΔHvH) obtained from repeated scans of a given sample were in good agreement with those obtained from the first scan. The reversibility of unfolding for the oxidized proteins was about 95% of the first scan. In contrast, the reversibility of the reduced proteins was about 75% of the first scan. A small proportion of the protein became oxidized during the thermal scans of the reduced proteins. This was detected in the DSC scan of ferrocytochrome c as a minor transition at the Tm of the oxidized protein. Similar results have been reported for the thermal denaturation of yeast iso-2-ferrocytochrome c (21).

The thermodynamic parameters of the unfolding transition for each of the samples are shown in Table I. These were calculated for the reduced and oxidized forms of the wild-type cytochrome c and the N52I/Y67F mutants.
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DSC experiments performed in 50 mM sodium acetate at pH 4.7. The thermodynamic parameters for the two redox forms of the wild type protein is the average of separate experiments. The data for the mutant proteins were obtained from a single experiment so the errors are not listed explicitly.

Table I

| Oxidized | Wild type\(^a\) | N52A\(^a\) | N52I\(^a\) | Y67F\(^a\) | Y67F/N52I\(^a\) |
|----------|-----------------|-------------|-------------|-------------|-----------------|
| \(T_m\) (°C) | 53.6 ± 0.3 | 57.6 | 66.1 | 58.3 | 70.9 |
| \(\Delta H_m\) (kcal mol\(^{-1}\)) | 48 ± 1 | 55 | 69 | 56 | 81 |
| \(\Delta S_m\) (cal mol\(^{-1}\) K\(^{-1}\)) | 100 ± 1 | 110 | 123 | 111 | 133 |
| \(\Delta S_m/\Delta H_m\) | 307 ± 4 | 333 | 363 | 335 | 387 |

Reduced

| \(T_m\) (°C) | 80.3 ± 0.3 | 82.6 | 87.2 | 81.1 | 91.2 |
| \(\Delta H_m\) (kcal mol\(^{-1}\)) | 89 ± 7 | 95 | 100 | 99 | 119 |
| \(\Delta S_m\) (cal mol\(^{-1}\) K\(^{-1}\)) | 138 ± 2 | 144 | 149 | 140 | 161 |
| \(\Delta S_m/\Delta H_m\) | 391 ± 4 | 405 | 413 | 395 | 442 |
| \(\Delta H_m/\Delta S_m\) | 1.6 ± 0.3 | 1.5 | 1.5 | 1.4 | 1.4 |

\(\Delta G_0 = \left( \frac{\Delta H_m}{T_m} \right) | T_{m,WT} - T_{m,WT} | \) (Eq. 6)

The DSC scans on different samples of the wild type protein in both oxidation states gave consistent values for the \(T_m\) and apparent change in enthalpy \(\Delta H_m\). Four experiments were performed on the reduced form of the protein and two experiments were performed on the oxidized protein as summarized in Table I. For comparative purposes between the mutant and wild type forms of cytochrome \(c\), the apparent enthalpies were used to calculate the entropies and Gibbs free energies of unfolding (see Tables I and II).

A comparison of the thermodynamic quantities of different proteins necessitates that they be compared at the same reference temperature (29). The calculation of these thermodynamic parameters for proteins with different values for \(T_m\) requires knowledge of the temperature dependence of \(\Delta H\) and \(\Delta S\), which can be determined from \(\Delta C_p\), the change in heat capacity between the native and unfolded states of the protein. Since \(\Delta C_p\) values calculated from a single DSC scan are inaccurate due to the difficulty in obtaining the proper baseline, the change in heat capacity was determined by taking the slope from a plot of \(\Delta H_m\) versus \(T_m\). The change in heat capacity for the two redox forms of the proteins were determined separately since the \(T_m\) obtained for the different samples cover a wide range of 38 °C. The \(\Delta C_p\) for the ferric proteins was calculated from the slope of a \(\Delta H_m\) versus \(T_m\) plot to be 1.8 ± 0.1 kcal mol\(^{-1}\) K\(^{-1}\) while that of the reduced proteins was 1.9 ± 0.2 kcal mol\(^{-1}\) K\(^{-1}\). The two slopes had correlations of 0.99 and 0.98, respectively. Values from 1.4 kcal mol\(^{-1}\) K\(^{-1}\) (12) to 2.0 kcal mol\(^{-1}\) K\(^{-1}\) (30) have been reported for the \(\Delta C_p\) of yeast iso-1-cytochrome \(c\). Our value for \(\Delta C_p\) was used to calculate the thermodynamic parameters for the denaturation of the cytochrome \(c\) variants at the reference temperature of 53.6 °C which is the \(T_m\) of the oxidized wild type protein (Table II). This reference temperature was used for comparative purposes because it gives the free energy changes of unfolding relative to the wild type oxidized protein and reduces the uncertainty associated with using the estimated value of \(\Delta C_p\) in Equation 5.

The value of \(\Delta C_p\) has been reported to change for mutant proteins (28, 31). Due to the uncertainty associated with determining the value of \(\Delta C_p\), each of the mutant proteins, the change in free energy of denaturation was calculated assuming that \(\Delta H\) and \(\Delta S\) are independent of temperature which requires only the apparent enthalpy of the wild type protein and the \(T_m\) of the wild type and mutant proteins. With these parameters the change in the \(\Delta G_0\) values can be approximated using the equation derived by (32).

\(\Delta G_0 = \left( \frac{\Delta H_m}{T_m} \right) | T_{m,WT} - T_{m,WT} | \) (Eq. 6)

For the purposes of the present investigation, \(\Delta H_m\) and \(T_m\), WT are the apparent enthalpy and \(T_m\) for the oxidized wild type protein. \(T_m\), WT is the \(T_m\) for the reduced wild type protein or for either redox form of the mutant proteins. The result of this calculation gives the change in the free energy of denaturation relative to the wild type oxidized protein. It had been previously reported that when compared to values obtained by more rigorous methods this simple equation gave a good approximation of the relative change in free energy of denaturation for several mutant iso-1-cytochromes \(c\) (31). The values we obtained using this equation are quite similar to the ones determined using Equation 5 especially for the oxidized forms of the different variants. The free energy values (\(\Delta G_{290°F}\)) obtained using Equation 6 show greater divergence for the reduced form of the protein which is likely because of the greater difference in the \(T_m\) (up to 38 °C) when compared to the wild type ferricytochrome \(c\). Overall, these results indicate that the values obtained using the integrated form of the Gibbs-Helmholtz equation (Equation 5) give a good approximation of the thermodynamic parameters for the proteins used in our investigation.

A comparison of the free energies of denaturation for the different oxidized proteins indicate that the mutations had a stabilizing effect. All four variants involved replacement of the native residues with more hydrophobic amino acids based on their experimentally measured transfer free energies from octanol to water (33). The mutant proteins were from about 1 kcal mol\(^{-1}\) (N52A and Y67F) to over 5 kcal mol\(^{-1}\) (N52I/Y67F) more stable than the wild type protein (Table II). The order of increasing stability for the oxidized proteins based on the free

\(^a\) Cytochromes all have C102T modification.

\(^b\) Errors in \(T_m\) are estimated to be ± 0.2 °C.
energies of unfolding was wild type < N52A ~ Y67F ≈ N52I < N52I/Y67F. The free energies of denaturation for the reduced mutant proteins were from about 0.4 kcal mol⁻¹ (Y67F) to around 4 kcal mol⁻¹ more stable than the wild type protein.

The order of increasing stability for the ferrous form of the mutant proteins were from about 0.4 kcal mol⁻¹ (Y67F) to 1.3 kcal mol⁻¹ for each additional –CH₂– group introduced by the mutation of a residue that does not contribute to the dipole that stabilizes the negatively charged propionate and the loss of the oxidation state dependent conformational change about the pyrrole A propionate. These results are consistent with N52 being part of the two hydrogen bonding networks centered around Wat166 and the pyrrole A propionate.

DSC experiments performed in 50 mM sodium acetate at pH 4.7, T_ref, the reference temperature equal to 53.6 °C, the T_m of the wild type oxidized protein. Values for redox potentials are at pH = 6.0, 25 °C, μ = 0.1 versus standard hydrogen electrode.

**Table II**

**Thermodynamic parameters of unfolding at reference temperature**

| Parameter          | Wild-type⁺ | N52A⁺ | N52I⁺ | Y67F⁺ | Y67F/N52I⁺ |
|--------------------|------------|-------|-------|-------|------------|
| E_m (mV)           | 290        | 257²  | 232²  | 234²  | 234²       |
| Oxidized T_m (°C)  | 80.3 ± 0.3 | 82.6  | 87.2  | 81.1  | 91.2       |
| ∆H_D (kcal mol⁻¹)  | 53.6 ± 0.3 | 57.6  | 66.1  | 58.3  | 70.9       |
| ∆S_D (cal mol⁻¹ K⁻¹) | 100 ± 1   | 103   | 100   | 103   | 101        |
| ∆G_D (kcal mol⁻¹)  | 0.0        | 1.3   | 4.1   | 1.5   | 5.8        |
| ∆G_D_e (kcal mol⁻¹) | 0.0        | 1.2   | 3.8   | 1.4   | 5.3        |
| Reduced T_m (°C)   | 246 ± 3    | 248   | 233   | 246   | 241        |
| ∆H_D (kcal mol⁻¹)  | 8.6 ± 2    | 8.5   | 10.9  | 8.8   | 12.9       |
| ∆S_D (cal mol⁻¹ K⁻¹) | 246       | 10.3  | 8.4   | 11.5  |            |
| ∆G_D (kcal mol⁻¹)  | 8.2        | 7.7   | 6.5   | 7.0   | 6.2        |
| ∆G_D_e (kcal mol⁻¹) | 8.2        |       |       |       |            |

⁺ Cytochromes all have C102T modification.
² Value for E reported previously in Rafferty et al. (3).
³ Value for E reported previously in Guillemette et al. (23).
⁴ Value for E reported previously in Berghuis et al. (1).
⁵ Value calculated using Equation 6.

**DISCUSSION**

Protein stability is dependent upon several factors including the number of intramolecular hydrogen bonds, the hydrophobicity of residues buried within the protein, segmental mobility and the environment of internally bound water molecules. Our investigation was undertaken to further understand how the relative stability of the two oxidation states of cytochrome c are affected by some of these factors. We were especially interested in the function of a conserved internally bound water molecule and its associated hydrogen bonding network. When compared to the wild type cytochrome c, none of mutations affect the overall peptide fold of the protein (1–3). The conformational differences observed are localized at the site of the mutation and in the region about the pyrrole A propionate. The structure about the pyrrole A propionate has previously been reported to be sensitive to the oxidation state of the protein (9).

The order of increasing stability for the two redox forms of a protein (10). The N52I, Y67F, and N52I/Y67F mutant proteins all have about the same redox potential but show different thermodynamic parameters (Table II). The oxidized forms of the N52I and N52I/Y67F are significantly more stable than their wild type counterparts while the Y67F oxidized mutant shows only a marginal increase in stability. These variants have the same redox potential because of differences in the relative stability of the reduced forms of the proteins. The first two mutations cause a large increase in stability while the latter variant has about the same stability as the wild type protein. A similar comparison of thermodynamic parameters for each redox pair can be used to justify why the Y67F mutant has a 15 mV lower redox potential than N52I despite the fact that the oxidized forms of both proteins are of approximately equal stability.

The free energies of denaturation ∆G_D were found to be larger for the reduced relative to the oxidized forms of all the proteins (Table II). When compared at the reference temperature, the oxidation dependent differences appear mainly due to a smaller change in unfolding entropy for the reduced forms of the proteins.

The order of increasing stability for the reduced mutant proteins were from about 0.4 kcal mol⁻¹ (Y67F) to around 4 kcal mol⁻¹ more stable than the wild type protein. The order of increasing stability for the two redox forms of a protein (10). The N52I, Y67F, and N52I/Y67F mutant proteins all have about the same redox potential but show different thermodynamic parameters (Table II). The oxidized forms of the N52I and N52I/Y67F are significantly more stable than their wild type counterparts while the Y67F oxidized mutant shows only a marginal increase in stability. These variants have the same redox potential because of differences in the relative stability of the reduced forms of the proteins. The first two mutations cause a large increase in stability while the latter variant has about the same stability as the wild type protein. A similar comparison of thermodynamic parameters for each redox pair can be used to justify why the Y67F mutant has a 15 mV lower redox potential than N52I despite the fact that the oxidized forms of both proteins are of approximately equal stability.

The free energies of denaturation ∆G_D were found to be larger for the reduced relative to the oxidized forms of all the proteins (Table II). When compared at the reference temperature, the oxidation dependent differences appear mainly due to a smaller change in unfolding entropy for the reduced forms of the proteins.
Depending on how firmly the water molecule is bound to the protein, the decreased entropy has a free energy cost of between 0 and 2 kcal mol\(^{-1}\) (37). While the displacement of Wat166 is entropically favorable, the decreased segmental mobility should lead to an increased entropy of unfolding. Even when taking all of these factors into account, it is not possible to fully explain the increased stability due to the N52I mutation. A similar conclusion has been drawn from the investigation of the N52I mutant of yeast iso-2-cytochrome \(c\) (21). In contrast to yeast cytochromes \(c\), the replacement of the invariant N52 residue by isoleucine in rat cytochrome \(c\) destabilizes the folded state relative to the unfolded state of the protein by 2.8 kcal/mol (38). Due to the complexity of the association between protein structure and stability, a better understanding of the effect of this and other mutations will require detailed investigation using cytochromes \(c\) from a variety of sources.

When the N52I replacement is combined with the Y67F mutation, the double mutant shows an even greater stability. One reason for the increased stability is likely due to the introduction of a more hydrophobic residue at position 67 in addition to the displacement of Wat166 which is the result of the N52I mutation. It is surprising that the results for the oxidized double mutant are additive while those of the reduced protein appear to be almost synergistic. The Y67F replacement results in the burial of a more hydrophobic residue within the protein which is energetically favorable. In the oxidized protein, this factor will be countered by the unfavorable effect of increasing the hydrophobicity in the environment of the positively charged heme.

Both Y67F and N52A mutations result in the addition of an internally bound water molecule. The effect on the stability of both proteins was found to be redox dependent. When comparing the ferrous proteins, only the N52A mutant showed a small increase in the stability despite the entropic cost of incorporating an additional water molecule. The relative increase in stability of this mutant results from the increased hydrophobicity of the mutant residue and the stabilization of the charged propionate by Wat300. In the oxidized state, both mutants show about the same increase in their relative stabilities when compared to the wild type protein. This was unexpected since the redox potential of the N52A mutant is 15 mV higher than that of the Y67F variant. Furthermore, when the stability of the heme crevice was measured by monitoring the pH at which
the Met^{50}SD-heme iron ligand of the oxidized protein is broken, the heme crevice stability of N52A is slightly less than the wild type protein while the Y67F mutant shows a significant increase in stability (1). Based upon the latter observation, it could be suggested that the N52A oxidized protein is less stable than its Y67F counterpart. The increase in the free energy of unfolding for the N52A protein is likely due to the combination of the removal of the asparagine residue that destabilizes the positively charged iron (34) as well as the factors proposed for the reduced protein.

When compared at the same reference temperature, all of the variants show an increase in the entropy of unfolding upon oxidation (Table II). In the oxidized state of the wild type protein, three segments of the polypeptide chain show increased mobility (9). Our results for the entropy of unfolding do not account for the oxidation state dependent changes in oxidation (Table II). In the oxidized state of the wild type protein, three segments of the polypeptide chain show increased mobility (9). The authors proposed that the stability differences between the two redox forms of wild type iso-1-cytochrome c result from differences in the transitional entropies (12). The authors proposed that the mobility of the polypeptide in this region of the protein do not account for the oxidation state dependent increase in the entropy of unfolding. The change in oxidation state affects the coulombic interactions between the heme group, the polypeptide and the solvent (39). The higher positive charge carried by the oxidized heme alters the solvation of the protein which may result in the larger $\Delta S_D$. It has been reported that the stability differences between the two redox forms of wild type iso-1-cytochrome c result from differences in the transitional entropies (12). The authors proposed that the folded oxidized wild type protein may bind in excess of 6 additional surface water molecules relative to the reduced form of the protein. The entropic cost of the additional bound water molecules could result in the relatively larger $\Delta S_D$ observed for each of the oxidized proteins used in the present investigation.

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