Thermodynamics of the Equilibrium Unfolding of Oxidized and Reduced *Saccharomyces cerevisiae* Iso-1-cytochromes *c*†

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ABSTRACT: We report thermodynamic data for the chemical denaturation of iso-1-cytochromes *c* from *Saccharomyces cerevisiae* having amino acid substitutions R38A, N52I, and F82S in all possible combinations. The guanidine hydrochloride denaturation of isolated proteins was monitored by fluorescence measurements. The redox potentials, $E^{\circ\prime}$, for both the folded and unfolded conformations have been measured. Free energy changes of chemical unfolding together with direct electrochemical measurement of the free energy changes of reduction for both the native and unfolded proteins yield a complete thermodynamic cycle, which includes four states of cytochrome *c*: oxidized folded, oxidized unfolded, reduced folded, and reduced unfolded. Completed cycles illustrate that the stability of cytochrome *c* to denaturing conditions is different for each amino acid substitution by an amount that depends on the heme oxidation state. Thus, the differential protein stability cannot be interpreted simply in terms of a hydrophobic effect, without also considering coupled Coulombic effects.

Cytochrome *c* is a well-characterized electron transfer protein (Moore & Pettigrew, 1990) with an iron porphyrin prosthetic group that readily toggles between the +3 and +2 oxidation states for function. The reduced and oxidized yeast iso-1-cytochrome *c* solution structures have been compared using ^{1}H NMR techniques (Gao et al., 1991), and high-resolution three-dimensional structural analyses of both oxidation states have also been completed (Berghuis & Brayer, 1992). Recently, our group addressed questions of the redox structure–function relationship (Hake et al., 1992) and the issue of thermodynamic stability of the oxidation states of horse heart cytochrome *c* with respect to chemical denaturation (Bixler et al., 1992). Hilgen-Willis et al. (1993) have confirmed this analysis with yeast iso-1-cytochrome *c*. In this study, we expand the implications of these studies.

Chemical denaturation combined with electrochemistry were used to study the effects of amino acid substitutions on protein stability. This approach allows dissection of the energetic effects of the amino acid substitutions in both the oxidized and the reduced states of the protein. The principle is analogous to the study by Lu et al. (1992), who dissected the effects of chemical modification on both the folded and unfolded conformations of the T4 lysozyme protein. Likewise, both the folded and unfolded conformations of cytochrome *c* have been studied in terms of substituting hydrophilic amino acids with hydrophobic amino acids (Bowler et al., 1993).

In addition to these and other interactions that affect the stability of the native and unfolded conformations of proteins such as cytochrome *c* [Muthukrishnan and Nall (1991) and references therein], we add that redox states must be clearly specified. The two oxidation states of cytochrome *c* impart energetic differences that alter the overall stability of cytochrome *c*. Depending on the amino acid substitutions, varying degrees of change in free energy of unfolding are observed for the reduced and oxidized states of cytochrome *c*.

In the present paper, we examine a suite of homologous cytochromes *c* with altered redox potentials. The changes in free energy of unfolding the oxidized and reduced substituted proteins relative to the normal protein, G_u^{III} and G_u^{II}, respectively, are calculated. The central question of this study is as follows: Do amino acid substitutions affect the stability to unfolding differentially in the oxidized and reduced states?

EXPERIMENTAL PROCEDURES

Protein Preparation. The altered cytochromes *c* were prepared by *in vitro* oligonucleotide-directed mutagenesis of plasmids pAB569 and pAB669 using the procedure outlined by Kunkel et al. (1987). Specifically, yeast shuttle plasmid pAB458 (Fetrow et al., 1989), which contains a *Bam*HI–*Hind*III fragment encompassing the *CYC1*$^+$ gene, was used to produce plasmids pAB569, which contains a C102A replacement, and pAB669, which contains N52I and C102A replacements (Hickey et al., 1991; Das et al., 1989). Scheme 1A,B illustrates how these plasmids were used in turn to produce the alterations described in this study. The oligonucleotides, OL** and OL‡, were synthesized with Applied Biosystems DNA synthesizer Model 380A and were used as mutagenic primers. The oligonucleotides are, respectively, 5′-AAGGTTGGTCCAAACTTGCATGGTAT-CTTTGGCGCCCACTCTGGTCAAGC-3′; and 5′-AA-GATGGCCAGCGGTGGG TTG-3′.

After the completion of *in vitro* mutagenesis, the desired change in the plasmid DNA was verified by plasmid DNA sequencing (dideoxy chain termination protocol using Sequenase kit version 2.0, United States Biochemical Corporation), and relevant primers were used to target the desired region of the gene. The plasmid containing the desired change (Scheme 1) was used to transform yeast strain B-6748 (*MAT***a** *cyc1-783::lacZ cyc7-::CYH2*$^+$ *ura3-52 his3-1 leu2-3 leu2-112 trp1-289 can1-100 cyh2*$^-$). In brief, the plasmid was

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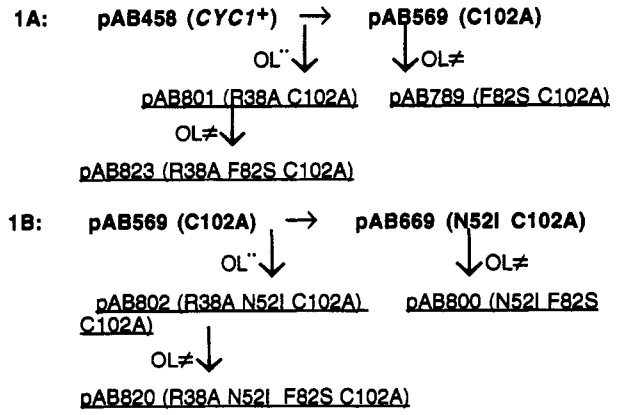
Scheme 1



integrated into the normal chromosomal position in yeast genomic DNA and subsequently excised, using the method described by Holzschu et al. (1987). The *cyc1-783::lacZ* segment in the yeast strain B-6748 was replaced by a functional *CYC1* allele. After Ura$^-$ strains were tested on diagnostic media, the desired Nfs$^+$ strains that had lost the *cyc1-783::lacZ* segment were chosen for DNA sequence analysis to verify the desired alteration. (Nfs$^+$ denotes growth on media with nonfermentable substrates.) DNA sequencing was particularly necessary to ensure that the C102A replacement was present because C102 is present in the *cyc1-783::lacZ* allele of the parent strain (Holzschu et al., 1987). The resultant yeast strains contained a single copy of the altered allele for the desired iso-1-cytochrome *c* protein.

Protein Isolation and Purification. The *Saccharomyces cerevisiae* strains having the desired alterations in iso-1-cytochrome *c* were grown in fermentors (New Brunswick Scientific, Model SF-116) at 30 °C following the procedure of Sherman et al. (1965). The extraction of crude cytochrome *c* used coarse Amberlite (Sherman et al., 1968), followed by FPLC purification of the crude extract. Some modifications are notable for the extraction of crude cytochrome *c*. The total Amberlite was added in three separate batches. First, one-half of the required amount of resin was allowed to equilibrate for an hour, followed by the addition of the other half in two aliquots, which were each allowed to equilibrate for 0.5 h. Other changes incorporated are described in Hickey et al. (1988).

FPLC purification of the crude protein, obtained after dialysis against $(NH_4)_2CO_3$ and centrifugation to remove precipitated proteins, was done using S-Sephacryl (Pharmacia) packed to approximately 15 cm (i.d. = 2.2 cm) and equilibrated with buffer A (100 mM potassium phosphate, pH 7.2). After the sample was loaded and unbound proteins were washed off with 200 mL buffer A, the iso-1-cytochrome *c* was eluted with a gradient using buffer B (100 mM potassium phosphate, and 0.8 M potassium chloride, pH 7.2). The gradient was set at 12–40% from 200 to 1500 mL, at a flow rate of 5.3 mL min^{-1}. The substituted proteins eluted at 20–30% buffer B. Fractions with a purity index ratio (410 nm/280 nm) of ≥4.0 were pooled, concentrated, and exchanged into 100 mM potassium phosphate (pH 7.0) using an Amicon ultrafiltration cell equipped with a YM-3 membrane filter (W. R. Grace Co., Beverly, MA). A single band (~12.5 kDa) was detectable on Coomassie blue stained SDS–polyacrylamide gels.

Chemical Denaturations. If necessary, the iso-1-cytochromes *c* were oxidized by the addition of a few grains of $K_3Fe(CN)_6$, which were then removed by eluting the sample on a small (1 mL) DE-52 column equilibrated in 100 mM potassium phosphate (pH 7.0) buffer. Samples for fluorescence measurements were prepared batchwise from buffer and a concentrated stock of 4.0 M guanidine hydrochloride (Gdn) made in the same buffer with the pH adjusted to 7.0. Iso-1-cytochrome *c* concentrations varied from 1 to 2 M.

Equilibrium unfolding of proteins by added Gdn was monitored at room temperature on a Perkin-Elmer MPF-2A fluorimeter set in the ratio mode. The excitation wavelength was set at 290 nm, the emission wavelength was set at 350 nm, and the slit width was 10 nm. Reversibility was verified by mixing of two samples of different concentrations, allowing equilibrium to be re-established (~10 min), and measuring the relative fluorescence emmission.

In order to report the concentration of Gdn required to half denature the protein, C_m, the data were analyzed as a two-state equilibrium transition between the native, N, and denatured, D, states: N ↔ D, as described by Hickey et al. (1988). The fraction of unfolded protein, $f_u = (X - X_N)/(X_D - X_N)$, is plotted vs Gdn concentration. X is the measured fluorescence value at a given Gdn concentration; X_N is the limiting fluorescence value of folded protein at 0 M Gdn; and X_D is the limiting value of unfolded protein, at the given Gdn concentration, obtained from a linear extrapolation of the data at high denaturation concentrations, as described by Pace (1986).

The data were fit to eq 1 (Santoro & Bolen, 1992), using the nonlinear least-squares fitting program from SigmaPlot Scientific Graphing Software (1992, Jandel Scientific, San Rafael, CA):

$$EM = \{(EM_N + m_N[Gdn]) + (EM_U + m_U[Gdn]) \exp[-(G^{III} + m_G[Gdn])/RT]\}\{1 + \exp[-(G^{III} + m_G[Gdn])/RT]\} \quad (1)$$

G_u^{III} is a measure of how much more stable the native conformation of the oxidized protein is than the unfolded denatured conformation, and m_G, the slope of the linear transition region of unfolding, is a measure of the cooperativity of the transition from the native to the denatured protein. EM is the measured fluoresence intensity; EM_N and EM_U are the intercepts of the pre- and post-unfolding baselines, respectively; m_N and m_U are the slopes of those baselines; and [Gdn] is the guanidine concentration.

Electrochemistry. Using established procedures (Burrows et al., 1991), cyclic voltammograms were obtained using a 4,4′-dithiodipyridine-modified 2 mm gold disk electrode with a platinum wire counter electrode. The potentials were measured relative to a saturated calomel electrode and are reported relative to SHE.

Cytochrome *c* concentrations ranged from 0.5 to 1.1 mM in 100 mM potassium phosphate, pH 6.0 and/or pH 7.0. After cyclic voltammograms of the folded protein were recorded, enough Gdn, ≥4.5 M, was added to completely unfold the cytochrome *c* and obtain the redox potential of the unfolded conformation. Wild-type iso-1-cytochrome *c* (Sigma) and the C102A variant were found to have equivalent $E°'$ values for both the folded and unfolded conformations. Thus, the C102A protein was used as the reference protein. Three proteins, with the following substitutions, C102A, N52I C102A, and R38A N52I F82S C102A, were studied at both pH 6 and pH 7 to determine the free energy associated with the reduction of the unfolded protein conformation. For both the wild type and these variants, there is a small constant (10 mV) shift in $E°$ between pH 6 (−188 mV) and pH 7 (−178

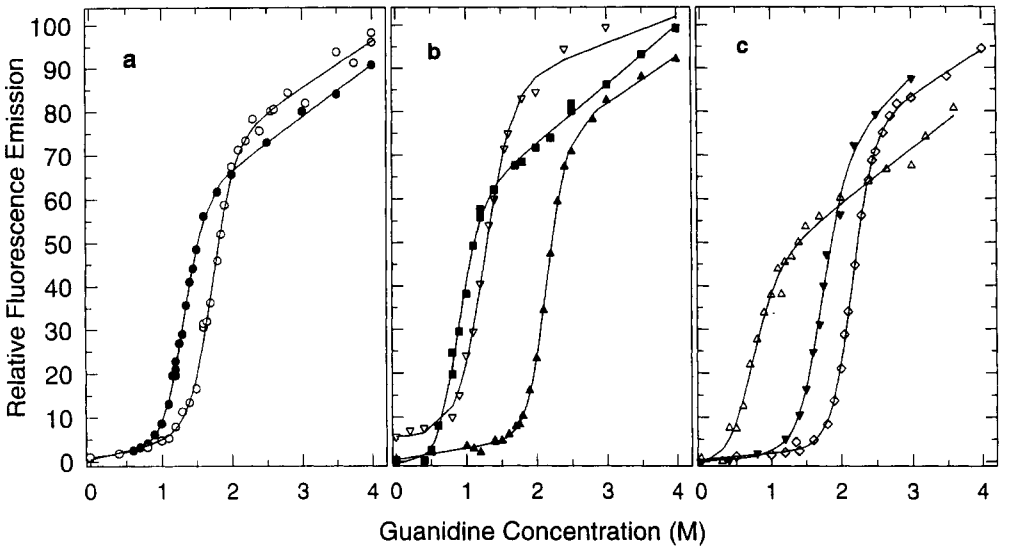


FIGURE 1: Guanidine hydrochloride induced unfolding of iso-1-cytochromes *c*: raw data fit to nonlinear least-squares analysis (—). (a) C102A-substituted protein (●) and R38A N52I F82S C102A substituted protein (○). (b) Proteins with two amino acid substitutions: F82S C102A (■); R38A C102A (▽); and N52I C102A (▲). (c) Proteins with three amino acid substitutions: R38A N52I C102A (◇); R38A F82S C102A (△); and N52I F82S C102A (▼).

Table 1: Thermodynamic Parameters for Guanidine Denaturation of Substituted Fe(III) Iso-1-cytochromes *c*[a]

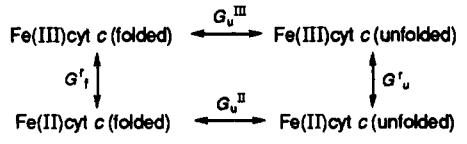
amino acid replacement in iso-1-cytochrome *c*	$E^{\circ\prime}$ (±2 mV) vs SHE	C_m ● 0.1 (M)	G_u^{III} ± 0.5 (kcal mol^{-1})	m_G ± 0.2	calculated G_u^{II} ± 0.5 (kcal mol^{-1})
C102A	285	1.3	5.0	−3.9	16.1
F82S C102A	247	0.9	3.5	−3.8	13.7
R38A C102A	239	1.4	3.8	−3.0	13.8
N52I C102A	231	2.1	9.0	−4.2	18.3
R38A N52I C102A	212	2.1	8.2	−3.8	17.6
R38A F82S C102A	203	0.8	2.3	−3.1	11.5
N52I F82S C102A	189	1.7	5.5	−3.2	14.4
R38A N52I F82S C102A	162	1.7	6.2	−3.6	14.4

[a] Values for $E^{\circ\prime}$ (25 °C in 0.1 M potassium phosphate, pH 7.0) and C_m (using a linear extrapolation method) were reported previously (Komar-Panicucci et al., 1992). Values for G_u^{III} and m_G were obtained from a nonlinear least-squares fit to the original data, as illustrated in Figure 1. Uncertainties in E°, C_m, and m_G are obtained from replicate determinations; (maximum) errors in ΔG_u determined by a propagation of error analysis.

mV). The $E^{\circ\prime}$ value for unfolded protein was determined at pH 7.0 for all other proteins.

Using the same procedure described here, the initial determination of the redox potentials for all of the proteins in a folded conformation is reported in Komar-Panicucci et al. (1992). The cytochrome *c* concentrations ranged from 0.2 to 0.5 mM in 0.1 M potassium phosphate (pH 7.0).

Data Analysis. Application of the following thermodynamic cycle permits the computation of the free energy of unfolding of the reduced state of cytochrome *c* (Bixler et al., 1992):



The value for G_u^{III} is obtained from Gdn denaturation of the oxidized form of cytochrome *c* (described earlier); G^r_f is the free energy associated with the electrochemical reduction of the folded conformation of cytochrome *c* obtained from electrochemical measurements; and G^r_u is a direct measure of the same for the unfolded conformation, which prior to the study of Bixler et al. (1992) had not been reported. The difference in free energy of unfolding between the oxidized and reduced proteins ($G_u = G_u^{III} - G_u^{II}$) was calculated, as previously described, from $G^r_f - G^r_u = -2.39nFE^{\circ\prime}$.

RESULTS

The process of unfolding using guanidine denaturation is known to reach equilibrium within 5 min (Bowler et al., 1993). Unfolding was shown to be reversible by a simple dilution procedure described in the Experimental Procedures section. Denaturation curves are shown in Figure 1, where the solid lines represent the nonlinear least-squares best fit of the raw data (Santoro & Bolen, 1992). The thermodynamic parameters G^{III} and m_G obtained from this analysis in the transition region are given in Table 1. Data for the C102A-substituted protein correspond well with those from a previous report (Hickey et al., 1991). The m_G values, which reflect the cooperativity of the unfolding transitions, are approximately the same for all of the substituted proteins.

Cyclic voltammograms for iso-1-cytochromes tested in both the folded and unfolded conformations at pH 6.0 are given in Figure 2, and corresponding data are given in Table 2. It appears that $E^{\circ\prime}$ for unfolded cytochrome *c* is independent of amino acid substitutions made in the heme environment. As shown in Figure 2, the reduction potential of the unfolded cytochromes *c* was the same for three variants: −188 mV vs SHE at pH 6.0. All chemical denaturations were done at pH 7.0. To correct for this change in conditions, we measured $E^{\circ\prime}$ for unfolded R38A N52I F82S C102A at pH 7.0, which was determined to be −178 mV vs SHE. Subsequent $E^{\circ\prime}$ values were also determined to be equal to −178 mV for all unfolded proteins at pH 7.0. Thus, we used the folded $E^{\circ\prime}$

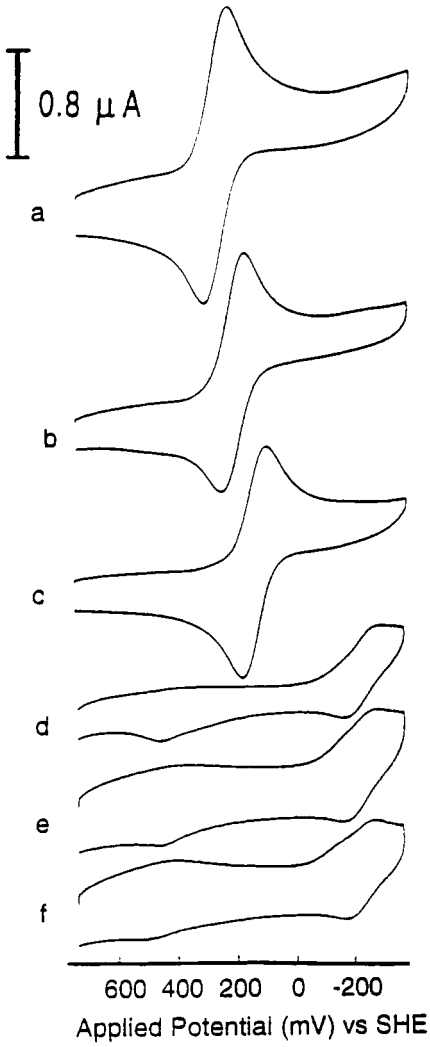


FIGURE 2: Cyclic voltammograms of substituted iso-1-cytochromes *c* (in 0.1 M potassium phosphate buffer, pH 6.0): (a) folded 0.9 mM C102A; (b) folded 0.9 mM N52I C102A; (c) folded 1.1 mM R38A N52I F82S C102A; (d) unfolded 0.6 mM C102A; (e) unfolded 0.7 mM N52I C102A; and (f) unfolded 0.8 mM R38A N52I F82S C102A.

Table 2: Stabilization Free Energy of the Reduced vs Oxidized States for Cytochromes *c*, G_u, and Calculated Changes in Free Energy of Altered Cytochromes *c* in the Oxidized and Reduced States Compared to the C102A-Substituted Protein, G_u^{III} and G_u^{II}

amino acid replacements	$G_u \pm 0.3$ (kcal mol^{-1})	$G_u^{III} \pm 0.7$ (kcal mol^{-1})	$G_u^{II} \pm 0.7$ (kcal mol^{-1})
C102A	−10.7		
F82S C102A	−9.8	−1.7	−2.7
R38A C102A	−9.6	−1.3	−2.4
N52I C102A	−9.4	+4.0	+2.7
R38A N52I C102A	−9.0	+3.1	+1.4
R38A F82S C102A	−8.8	−2.9	−4.8
N52I F82S C102A	−8.5	+0.5	−1.7
R38A N52I F82S C102A	−7.8	+1.2	−1.7

values reported in Table 1 to calculate the values for G_u^{II} that cannot be measured directly.

We present a complete set of free energy differences in Table 2. G_u is a measure of how much more stable the reduced conformation is to unfolding compared to the oxidized conformation, and G_u^{III} and G_u^{II} are, respectively, measures of the differential stabilities for the oxidized and reduced states of the substituted proteins compared to C102A cytochrome *c*. As can be predicted, Table 1 illustrates that when the redox potentials of the substituted proteins decrease, the values for G_u decrease. However, there is no obvious trend in the G_u^{III} or G_u^{II} values. For example, the N52I C102A-substituted protein is 4.0 kcal mol^{-1} more stable than the C102A-substituted protein in the oxidized state, but only 2.7 kcal mol^{-1} more stable in the reduced state. The R38A N52I F82S C102A-substituted protein is 1.2 kcal mol^{-1} more stable than the C102A-substituted protein in the oxidized state and 1.7 kcal mol^{-1} *less* stable in the reduced state.

The free energy changes of unfolding for all of the proteins in each oxidation state can be compared. Stabilization of the oxidized state, relative to the C102A-substituted protein, is observed to be as much as 4.0 kcal mol^{-1} (N52I C102A), and destabilization is as much as 2.8 kcal mol^{-1} (R38A F82S C102A). The reduced state was stabilized by as much as 2.7 kcal mol^{-1} (N52I C102A) and destabilized by as much as 4.7 kcal mol^{-1} (R38A F82S C102A).

DISCUSSION

The variants, with amino acid replacements in the heme environment of iso-1-cytochrome *c*, were constructed originally to assess the effects of redox alterations on the *in vitro* and *in vivo* functions of cytochrome *c*. The midpoint of guanidine-induced unfolding was determined in order to establish that the given amino acid replacements did not result in significant (global) unfolding. In fact, five out of seven of the proteins were at least as stable as, if not more stable than, the C102A protein to guanidine denaturation. Secondly, it was established, through temperature-dependent variations in the midpoint potentials of cyclic voltammograms, that the redox changes were not a consequence of entropic factors. These facts provided the grounds to address the effects of redox alterations on function (Komar-Panicucci et al., 1992, 1993). For this study, it is sufficient to state that all substituted cytochrome *c* proteins were functional *in vivo*, as indicated by growth on the nonfermentable carbon sources glycerol (YPG) and liquid lactate medium.

The function of cytochrome *c* is dependent on the oxidation state of the protein, and knowledge of the energetics of protein stability with respect to the oxidation state is central to our understanding of function. This study has shown that the contribution of individual amino acid replacements to the stability toward chemical denaturation varies between the oxidized and reduced proteins. The effects of amino acid replacements near the heme on the folding energetics of cytochrome *c* serve as a prototype for understanding how oxidation states affect protein stability.

Typically, there is an inclination to evaluate protein stability in terms of macroscopic parameters without regard for potentially significant changes in the oxidation state. For example, a remarkable amino acid replacement, N52I, was unveiled in our lab by classical genetic procedures (Das et al., 1989). When produced independently, it was found that the N52I substitution gives rise to a protein more stable to chemical denaturation (Hickey et al., 1991; Figure 1c) than the native C102A-substituted protein. Furthermore, the protein has an unprecedented increase in thermal stability (Das et al., 1989). When macroscopic parameters, evaluated in terms of guanidine denaturation and melting curves, were considered, results indicated that hydrophobic interactions may be the main factor for the enhanced stability for replacements at position 52. In this study, we have shown that the differential unfolding free energy induced by the N52I replacement is significantly larger for the oxidized protein than for the reduced protein (Table 2). The previous analysis of the stability of N52 replacements focused on the transfer free energy associated with the

replacement. The present results show that a more complex analysis is required. The order of unfolding free energies for the replacements studied is similar in the oxidized and reduced proteins, but not identical. For example, for the replacement R38A N52I F82S C102A, G^{III} is positive, indicating a net stability increase relative to the reference protein C102A, while G^{II} is negative, indicating a net stability decrease relative to the C102A protein. The transfer free energy per se should not depend on the oxidation state of the prosthetic group. Thus, if G_{transf} were the only important energy term, G^{III} and G^{II} would be equal.

Therefore, when comparing the effects of amino acid substitutions on protein stability to denaturing conditions, the reference protein oxidation state must be specified. Although the interactions that cause the destabilizations reported in this paper are not completely understood, the macroscopic cause and implications are clear. By changing the oxidation state of mutationally altered cytochromes *c*, the Coulombic interactions between the heme group and the polypeptide are significantly altered. This change in the electrostatics of the protein and the resultant changes in other residue–residue interactions destabilize the reduced state relative to the oxidized state of the protein. This is reflected in the G_u values. The cumulative effects of these (relative) destabilizations may be sufficient to overcome a normally favorable effect, such as burial of the hydrophobic group in the N52I replacement. Considering the complexity of protein folding (and unfolding), it is not surprising that a simple or predictable effect on the stability in each oxidation state may not be observed.

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