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Thermal stability of hydrophobic heme pocket variants of oxidized cytochrome *c*

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Abstract

Microcalorimetry has been used to measure the stabilities of mutational variants of yeast iso-1 cytochrome *c* in which F82 and L85 have been replaced by other hydrophobic amino acids. Specifically, F82 has been replaced by Y and L85 by A. The double mutant F82Y, L85A iso-1 has also been studied, and the mutational perturbations are compared to those for the two single mutants, F82Y iso-1 and L85A iso-1. Results are interpreted in terms of known crystallographic structures. The data show that (1) the destabilization of the mutant proteins is similar in magnitude to that which is theoretically predicted by the more obvious mutation-induced structural effects; (2) the free energy of destabilization of the double mutant, F82Y, L85A iso-1, is less than the sum of those of the two single mutants, almost certainly because, in the double mutant, the -OH group of Y82 is able to protrude into the cavity formed by the L85A substitution. The more favorable structural accommodation of the new -OH group in the double mutant leads to additional stability through (1) further decreases in the volumes of internal cavities and (2) formation of an extra protein–protein hydrogen bond.

Keywords: free energy; iso-1 cytochrome *c*; protein folding; yeast

The factors that determine the stability of the folded conformations of globular proteins are only partially understood. Some of the factors that have been identified and at least partially quantified include H-bonding (Shirley et al., 1992; Myers & Pace, 1996), hydrophobic interactions (Pace, 1992; Pace et al., 1996), and internal cavity formation (Karpusas et al., 1989; Eriksson et al., 1992; Xu et al., 1998). The latter two factors are especially important in defining the sequence and composition constraints for packing of hydrophobic cores in protein interiors (Ponder & Richards, 1987; Lim & Sauer, 1989). Additional effects, which have received somewhat less attention, are changes in side-chain entropy (Pickett & Sternberg, 1993; Doig & Sternberg, 1995) and changes in the entropy of bound water on folding (Dunitz, 1994; Williams et al., 1994; Zhang & Hermans, 1996).

Differential scanning microcalorimetry was used to measure the effects on protein thermostability of substitution of hydrophobic amino acid residues at two sites (positions 82 and 85) in the hydrophobic heme pocket region of yeast iso-1 cytochrome *c* (Fig. 1). Additionally, measurements of the stability of the protein with the double mutation (F82Y, L85A iso-1) were used to determine whether the effects of the two single mutations are additive or compensate for each other (Wells, 1990; Sondek & Shortle, 1992; Green & Shortle, 1993; Blaber et al., 1995). X-ray crystallographic analysis shows that the side chains of the amino acids at positions 82 and 85 are in contact (Lo et al., 1995a, 1995c), so the extent of any nonadditivity gives insight into the structural basis of cooperative interactions between protein components. The mutant proteins used in this study were F82Y, L85A iso-1; F82Y iso-1; and L85A iso-1. All of the mutant proteins are studied in a C102T pseudo wild-type iso-1 background in which Cys102, found in naturally occurring yeast strains, has been substituted with threonine (Cutler et al., 1987). Substitution or chemical blocking of Cys102 is necessary to prevent the formation of covalent disulfide-bonded dimers and allow folding studies of monomeric iso-1 cytochrome *c* (Zuniga & Nall, 1983).

Results

Reversibility of thermal transitions

For a reliable reversible thermodynamic description of protein stability, it is necessary that the experimentally measured unfolding transition be reversible. Repeated thermal scans of iso-1, L85A

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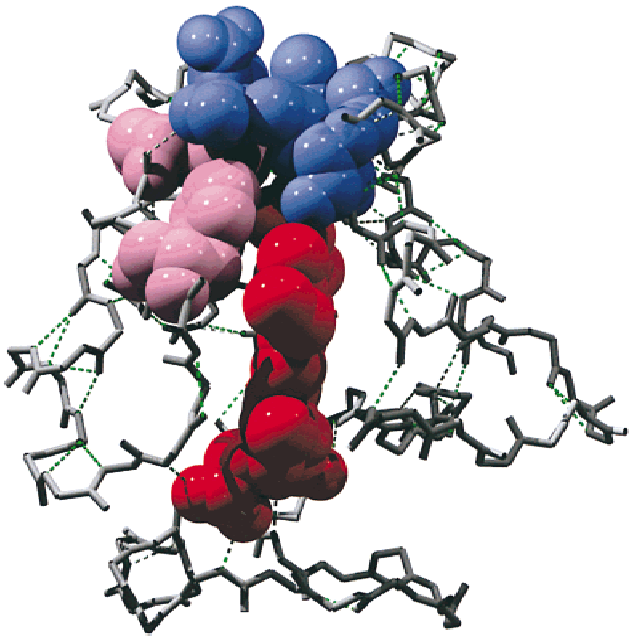

Fig. 1. Three-dimensional structure of yeast iso-1 cytochrome *c*. The positions of the mutational substitutions, F82 and L85, are shown in pink. Other residues, L9, R13, D90, and L94, which are perturbed by the mutations are shown in blue. Both sets of residues (pink and blue) pack against the heme (red). The backbone is shown in gray while H-bonds are indicated as green dashed lines. X-ray crystallography was used to determine the structures of the (C102T) pseudo wild-type iso-1 cytochrome *c* with the heme in the oxidized state (Berghuis & Brayer, 1992), the (C102) wild-type iso-1 cytochrome *c* with the heme in the reduced state (Louie & Brayer, 1990), and the mutant iso-1 cytochromes *c*, which are used in this study with their hemes in the reduced state (Lo, 1995; Lo et al., 1995a, 1995c).

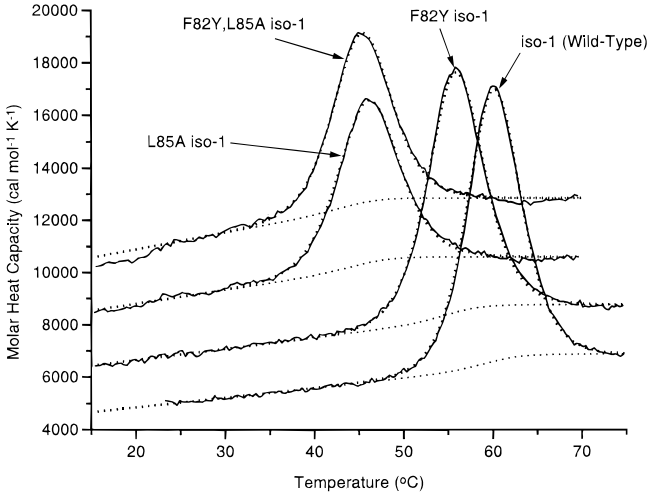

Fig. 2. DSC scans of iso-1 (wild-type), F82Y iso-1, L85A iso-1, and F82Y, L85A iso-1 cytochromes *c*, with the hemes in the oxidized state. Theoretical fits of the scans to a two-state unfolding model with a temperature dependent ΔC_P are shown as dotted lines. The merged extrapolations of the high- and low-temperature baselines across the transition regions are also shown as dotted lines. Protein concentrations were ~2 mg mL^{-1} (~160 μM). The buffer used was 0.1 M sodium phosphate, pH 6.0. The scan rate was 90 K h^{-1}. The data sets for the F82Y, the L85A, and the F82Y, L85A cytochromes *c* are displaced along the *y*-axis by 2,000, 4,000, and 6,000 cal mol^{-1} K^{-1}, respectively.

iso-1, F82Y iso-1, and F82Y, L85A iso-1 retain about 0.96 of the transitional enthalpy of the preceding scan showing that thermal unfolding is highly reversible. Highly reversible thermal unfolding transitions for the yeast cytochromes *c* near neutral pH contrast sharply with the irreversible thermal transitions exhibited by many other cytochromes *c* from higher eukaryotes (Potekhin & Pfeil, 1989).

Thermal unfolding of mutant and normal proteins

Differential scanning calorimetry (DSC) was performed to monitor the thermal unfolding transitions of the wild-type iso-1 cytochrome *c* and cytochromes *c* mutated in the heme pocket at positions 82 and 85. Calorimetric scans of the unfolding transitions of the pseudo wild-type iso-1, F82Y iso-1, L85A iso-1, and the double mutant, F82Y, L85A iso-1 cytochromes *c*, are shown in Figure 2. Also shown are the theoretical curves for a two-state transition in which $\Delta H^{vH}/\Delta H_m$ has been allowed to vary.

Calorimetric scans of the unfolding transitions of the iso-1 cytochromes *c* were used to obtain the calorimetric enthalpy ΔH_m, the van't Hoff enthalpy ΔH^{vH}, the melting temperature T_m, and the difference in the heat capacities of the folded and the unfolded conformations at the melting temperature ΔC_P (Table 1). The ratio of the van't Hoff enthalpy to the calorimetric enthalpy (β/MW) can be determined from these basic thermodynamic parameters. At pH 6.0 the values of T_m for the mutant cytochromes *c* are all lower than that of the wild-type cytochrome *c*. The nonadditivity of mutation-induced changes in stability is readily apparent on comparing T_m values: the T_m of the F82Y iso-1 single mutant is 4.2 °C lower than that of the wild-type protein, the T_m of the L85A single mutant is 13.7 °C lower than the wild-type protein, but the T_m of the F82Y, L85A iso-1 double mutant is only 0.7 °C lower than that of the L85A iso-1 single mutant.

Determination of ΔC_P

To compare ΔH and ΔS for proteins with different T_m's, it is necessary to measure ΔC_P so the ΔH and ΔS can be calculated for a common reference temperature. While ΔC_P can be determined, in principle, from fits of the data from a single calorimetry scan to a theoretical curve (i.e., Fig. 2), a more accurate value is obtained by measuring the temperature dependence of the enthalpy of unfolding over a wide temperature range. One way this is accomplished is by measuring ΔH_m as a function of pH where the T_m is strongly dependent on pH (Privalov, 1979). A value for ΔC_P can then be obtained from the slope of a plot of ΔH_m vs. T_m. For the wild-type iso-1 cytochrome *c*, the thermal unfolding transition was investigated between pH 3.0 and pH 5.0 using three different buffers. As the pH decreases from 5.0 to 3.0, the melting temperature and the calorimetric enthalpy decrease. Similar behavior is observed for iso-2 cytochrome *c* (Liggins et al., 1994) and for several other globular proteins (Privalov, 1979).

A graph of the calorimetric enthalpy of unfolding of the wild-type iso-1 cytochrome *c*, as a function of the melting temperature T_m, in three different buffers at a number of pH values gives ΔC_P = 1.66 kcal mol^{-1} K^{-1}. The correlation coefficients of the fit are r = 0.99 and r^2 = 0.98. The value of ΔC_P shows that ΔH has strong

Table 1. *Thermodynamic parameters of the unfolding transitions of heme pocket mutants of iso-1 cytochrome c*[a]

Protein	T_m (°C)	ΔH_m (kcal mol^{-1})	ΔH^{vH} (kcal mol^{-1})	$\Delta H^{vH}/\Delta H_m$	ΔS_m (cal mol^{-1} K^{-1})	ΔC_P (cal mol^{-1} K^{-1})
Iso-1	60.0	81 ± 4	110 ± 2	1.36 ± 0.04	243 ± 12	(260 ± 300)
F82Y, L85A iso-1	45.6	57 ± 3	92 ± 2	1.62 ± 0.04	178 ± 9	(310 ± 300)
F82Y iso-1	55.8	76 ± 4	103 ± 2	1.35 ± 0.04	232 ± 12	(580 ± 300)
L85A iso-1	46.3	57 ± 3	88 ± 2	1.55 ± 0.04	178 ± 9	(450 ± 300)

[a]Conditions are 0.1 M sodium phosphate, pH 6.0, 160 μM oxidized cytochrome *c*. T_m is the temperature at the midpoint of the unfolding transition (i.e., when $K = 1$). ΔH_m is the calorimetric enthalpy, ΔH^{vH} the van't Hoff enthalpy, and ΔS_m the entropy for unfolding. ΔC_P is the transitional change in the molar heat capacity obtained from fits to the calorimetry scans.

temperature dependence, but the excellent fit of the plot of ΔH_m vs. T to a straight line suggests that ΔC_P is largely temperature independent, at least over the temperature range for which ΔH_m has been measured (Liggins, 1997).

A more controversial means of measuring ΔC_P of unfolding is by plotting the ΔH_m vs. T_m for a series of iso-1 cytochromes *c* (wild-type and mutants) on the same graph and measuring the slope of the plot. The assumption being made here is that the introduction of a particular mutation affects the melting temperature T_m but not the enthalpy of unfolding at that temperature. This is a somewhat dubious assumption, particularly in view of the enthalpy/entropy compensation exhibited by composite cytochrome *c* mutant proteins (Liggins et al., 1994). The temperature range over which the enthalpies are measured can be expanded by measuring unfolding of the more thermostable reduced forms of iso-1 and iso-1 mutant proteins, and combining the data for both the oxidized and reduced forms of the proteins. This extends the temperature range for the plots of ΔH_m vs. T_m, but implicitly assumes that ΔH_m is independent of redox state, an equally dubious assumption. In view of the fact that there is, at present, *no* method whereby an indisputably accurate value of ΔC_P can be obtained, both assumptions are worth making for this series of single and double mutants of iso-1 cytochrome *c*. There is precedent for both assumptions. A linear dependence of ΔH_m on T_m has been reported for a series of proteins differing by point mutations (Hickey et al., 1991; Pielak et al., 1995), and redox state (Cohen & Pielak, 1995). Whatever errors these assumptions introduce are probably negligible since an analysis of different ΔH_m vs. T_m plots for determining ΔC_P indicates that the ΔC_P values obtained are generally the same within errors (Liggins, 1997). A graph of the calorimetric enthalpy of unfolding of mutant and the wild-type iso-1 cytochromes *c* with the heme in the oxidized and reduced states as a function of the melting temperature T_m gives a value of $\Delta C_P = 1.58$ kcal mol^{-1} K^{-1}. The correlation coefficients of the fit are $r = 0.99$ and $r^2 = 0.97$. This value of ΔC_P is the same as that determined by pH variation within the estimated 10% errors in ΔC_P and has the advantage of averaging any mutational or redox state effects on the value of ΔC_P.

Differences in stability of mutant and normal proteins

The enthalpies, entropies, and free energies of the unfolding transitions ($\Delta H(T_{ref})$, $\Delta S(T_{ref})$, and $\Delta G(T_{ref})$) for the wild-type and the seven mutant iso-1 cytochromes *c* have been evaluated at two reference temperatures (Table 2). These reference temperatures are: (1) 60.0 °C (the value of T_m for the wild-type); (2) 25 °C. The percentage errors in the thermodynamic quantities are four- to fivefold smaller when $T_{ref} = T_m$ (wild-type) than when T_{ref} is outside of the unfolding transition zone. The method of estimating ΔC_P from a series of mutant proteins implicitly assumes that mutational perturbations of $\Delta H(T_{ref})$ are negligible, an assumption born out by the data in Table 2.

Table 2. *Thermodynamic parameters of the unfolding transitions of heme pocket mutants of iso-1 cytochrome c*[a]

Protein/T_{ref}	$\Delta H(T_{ref})$ (kcal mol^{-1})	$\Delta S(T_{ref})$ (cal mol^{-1}K^{-1})	$\Delta G(T_{ref})$ (kcal mol^{-1})	$\Delta\Delta G(T_{ref})$ (cal mol^{-1} K^{-1})
$T_{ref} = 60$ °C				
Iso-1	81 ± 4	243 ± 12	0	0
F82Y, L85A iso-1	80 ± 3	249 ± 9	−3.1 ± 0.1	−3.1 ± 0.1
F82Y iso-1	83 ± 4	251 ± 12	−1.01 ± 0.05	−1.01 ± 0.05
L85A iso-1	79 ± 3	245 ± 9	−2.9 ± 0.1	−2.9 ± 0.1
$T_{ref} = 25$ °C				
Iso-1	26 ± 4	68 ± 12	5.5 ± 0.4	0
F82Y, L85A iso-1	24 ± 3	73 ± 9	2.6 ± 0.2	−2.9 ± 0.4
F82Y iso-1	27 ± 4	76 ± 12	4.8 ± 0.4	−0.7 ± 0.6
L85A iso-1	23 ± 3	69 ± 9	2.7 ± 0.2	−2.8 ± 0.4

[a]Enthalpies, entropies, free energies, and free energy changes (relative to wild-type iso-1) at $T_{ref} = 60$ °C (the T_m of the wild-type) and $T_{ref} = 25$ °C. These quantities are calculated using equations given by Becktel and Schellman (1987).

Estimation of errors in the thermodynamic parameters

To meaningfully interpret the thermodynamic data of protein unfolding that has been extrapolated to temperatures far removed from the melting temperatures of the proteins, it is essential to estimate the types and the magnitudes of the errors that are likely to occur when such extrapolations are made. The estimated errors in the values of $\Delta H(T_{ref})$, $\Delta S(T_{ref})$, and $\Delta G(T_{ref})$ arise from errors in the three input parameters: T_m, ΔH_m, and ΔC_P.

Differential scanning calorimetry can be used to make extremely accurate estimations of the melting temperature, which is the temperature at the peak of the unfolding curve (see Fig. 2). The errors in T_m, given in Table 1, were estimated to be $\pm 0.05\,^{\circ}\mathrm{C}$. The error in the enthalpy of unfolding at the melting temperature (ΔH_m) is somewhat larger. The calorimetric enthalpy of unfolding was used to calculate the values of $\Delta H(T_{ref})$, $\Delta S(T_{ref})$, and $\Delta G(T_{ref})$, which are shown in Table 2. The error in ΔH_m, given in Table 1, was estimated to be $\pm 5\%$ for the wild-type iso-1 cytochrome *c* and also for each mutant cytochrome *c*. This error estimate was determined from the scatter of values of ΔH_m, which were obtained from DSC scans of several samples of selected mutants.

Of all the thermodynamic parameters, the statistical errors in ΔC_P are the largest and generally fall in the range of 10–20% of the magnitude of ΔC_P. However, the uncertainty in how to measure ΔC_P can potentially lead to significant systematic errors in ΔC_P arising from the way it is evaluated (e.g., from fitting single scans, from pH dependence of ΔH_m, from point mutational perturbations of ΔH_m, from redox state dependence of ΔH_m). Other errors in ΔC_P are likely to be due to the assumptions made about ΔC_P: (1) ΔC_P is temperature independent; (2) the same value of ΔC_P can be used for the wild-type and also for all the mutant cytochromes *c* in the thermodynamic calculations. The calculations in Table 2 have been made using $\Delta C_P = 1.58$ kcal mol^{-1} K^{-1} since this value was determined from the largest data set, over the widest temperature range, and averages differences in ΔC_P resulting from temperature, redox state, and point mutations. We believe that this is the best choice for ΔC_P when comparing stabilities for a series of mutant proteins, in different redox states, over a wide temperature range.

It can be seen that the *absolute* values of the errors in $\Delta H(T)$, $\Delta S(T)$, and $\Delta G(T)$ are not large (Fig. 3). However, at the reference temperature of 25 °C, where the values of the $\Delta H(T)$ and $\Delta S(T)$ functions are near zero, the relative errors in $\Delta H(T)$ and $\Delta S(T)$ are large. This can be clearly seen by inspection of Table 2. On the other hand, both the absolute and the relative errors in the $\Delta G(T)$ function are not large. Therefore, it is reasonable to expect that any error in $\Delta G(T)$, which results from the assumptions made about ΔC_P, is similarly not large (Liggins, 1997). The errors in the $\Delta G(T_{ref})$ functions caused by the errors in ΔH_m are given in Table 2 for the wild-type, the F82Y single mutant, the L85A single mutant, and the F82Y, L85A double mutant cytochromes *c* at the reference temperatures of 60 and 25 °C.

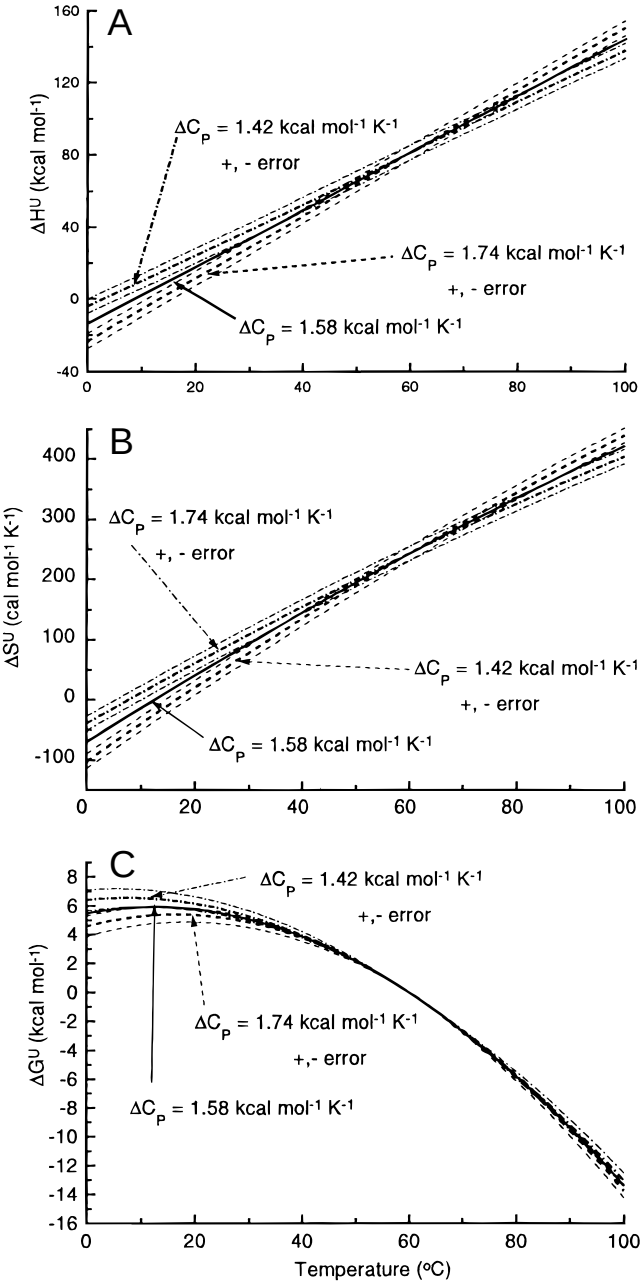

Fig. 3. The (**A**) enthalpy ΔH, (**B**) entropy ΔS, and (**C**) free energy of unfolding ΔG as a function of temperature T for wild-type iso-1 cytochrome *c*, with the heme in the oxidized state. The solution conditions were 0.1 M sodium phosphate buffer, pH 6.0. The functions $\Delta H(T)$, $\Delta S(T)$, and $\Delta G(T)$, calculated with $\Delta C_P = 1.58$ kcal mol^{-1} K^{-1} are denoted by the central solid lines. To show the effects of the estimated 10% uncertainty in ΔC_P, the thermodynamic functions are calculated for ΔC_P values of plus 10% (thick dashed line) and minus 10% (thick dash-dot line). Errors propagated from the error in ΔH_m (Table 1) are shown by the + error and the − error functions using the same line type (dashed and dash-dot) but narrower lines. For clarity, errors propagated from ΔH_m are not shown for the thermodynamic functions calculated with $\Delta C_P = 1.58$ kcal mol^{-1} K^{-1}.

Discussion

Features of the unfolding transitions

Three aspects of the measurements of thermostability are apparent. First, at the reference temperatures under standard solution conditions ($T_{ref} = 25\,^{\circ}\mathrm{C}$ or $T_{ref} = 60.0\,^{\circ}\mathrm{C}$), the mutant cytochromes *c* are less stable (i.e., they have smaller or more negative values of $\Delta G(T_{ref})$) than the wild-type cytochrome *c* (Table 2; Figs. 2, 4). Second, the double mutant F82Y, L85A iso-1 is less stable than the single mutant F82Y iso-1 and less stable or equal in stability to

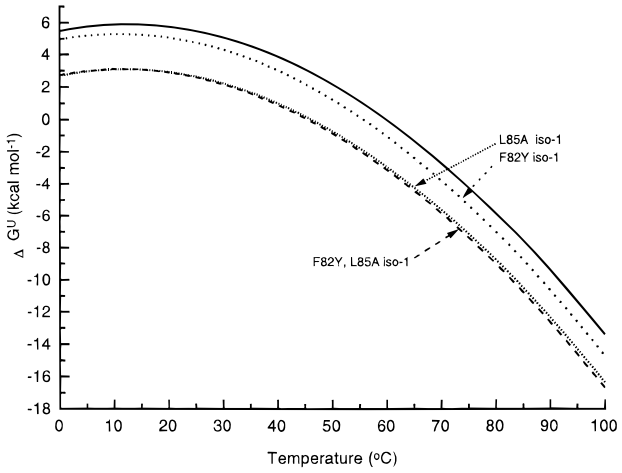

Fig. 4. Comparison of the free energies of unfolding ΔG as functions of temperature for two single substitution mutants, F82Y iso-1 and L85A iso-1; the corresponding double mutant, F82Y, L85A iso-1; and wild-type iso-1 cytochromes *c*. The hemes are in the oxidized state and the $\Delta G(T)$ function is evaluated using the calorimetric enthalpies of unfolding and a value of ΔC_P of 1.58 kcal mol^{-1} K^{-1}. The solution conditions were 0.1 M sodium phosphate buffer, pH 6.0. For all of the proteins, the errors are comparable to those given for wild-type iso-1 in Figure 3.

the single mutant L85A iso-1 cytochrome *c*. Third, in regards to their effects on stability the two mutations F82Y and L85A in the double mutant cytochrome *c* partially compensate for each other. The double mutant, F82Y, L85A iso-1 is only slightly less stable or is equal in stability to the single mutant L85A iso-1, and the sum of the mutation-induced decreases in stability of the two single mutant cytochromes *c* is greater than the mutation-induced decrease in stability of the double mutant cytochrome *c*: $\Delta\Delta G(T_{ref})$ (F82Y) + $\Delta\Delta G(T_{ref})$ (L85A) < $\Delta\Delta G(T_{ref})$ (F82Y, L85A); where $\Delta\Delta G(T_{ref})$ (mut) $\equiv \Delta G(T_{ref})$ (mut) − $\Delta G(T_{ref})$ (wt).

Measurement of ΔC_P

The difference between the heat capacities of the folded and the unfolded states of the protein (ΔC_P) can be measured in two ways: (1) by fitting the calorimetric data to thermodynamic expressions for the heat capacity in which ΔC_P ($\equiv \Delta C_P(T_m)$) appears as an adjustable parameter (Table 1, Column 7), or (2) by measuring the slope of the graph of the temperature dependence of the enthalpy of unfolding. For reasons that are not fully understood, the values of ΔC_P obtained in these two different ways are markedly different for many protein unfolding transitions (Connelly et al., 1991; Hu et al., 1992a).

The slopes of the pre-transition and the post-transition baselines of a typical calorimetric scan are different (Fig. 2), suggesting that ΔC_P is temperature dependent. Indeed, ΔC_P values obtained from curve fitting are often temperature dependent. This is somewhat puzzling because plots of the enthalpy of unfolding vs. T_m are usually linear, suggesting that ΔC_P *is* independent of temperature. In this work, plots of ΔH_m vs. T_m for cytochrome *c*, where T_m has been altered either by a change in the pH of the buffer, or by mutation and redox state, are both approximately linear. Within the estimated 10% error in ΔC_P, the values obtained by varying pH, $\Delta C_P = 1.66 \pm 0.17$ kcal mol^{-1} K^{-1}, or mutation and redox state, $\Delta C_P = 1.58 \pm 0.16$ kcal mol^{-1} K^{-1}, are the same. Nevertheless, we favor the value of $\Delta C_P = 1.58$ kcal mol^{-1} K^{-1} obtained from plotting ΔH_m vs. T_m when T_m is varied through mutational perturbation and redox state. The value of ΔC_P obtained in this manner is expected to average any actual differences that result from mutation or redox state, even if the differences are less than the error limits.

It should be noted that ΔC_P has previously been evaluated from the slope of a plot of ΔH_m vs. T_m, which was obtained by varying the pH value at which the oxidized wild-type (C102T) iso-1 cytochrome *c* is unfolded (Cohen & Pielak, 1994). A value of 1.37 kcal mol^{-1} K^{-1} was obtained for ΔC_P, which is 13% lower than our value. The same group obtains a value essentially identical to ours ($\Delta C_P = 1.60$ kcal mol^{-1} K^{-1}) when data from pH and mutational perturbation of T_m are combined in a single plot of ΔH_m vs. T_m (Pielak et al., 1995).

Compared to the values obtained by measuring the temperature dependence of T_m, the value of ΔC_P obtained from curve fitting is somewhat smaller and tends to fluctuate widely from scan to scan. A typical value is 0.53 kcal mol^{-1} K^{-1}, obtained from the scan of the wild-type (C102T) iso-1 cytochrome *c* shown in Figure 2. The reasons for this are not fully understood. The slope of the high-temperature baseline may be influenced by reactions not related to unfolding that occur preferentially at higher temperatures. One possibility is heme ligation by the amino terminus of the polypeptide chain (Hammack et al., 1998). Another might be peptide bond hydrolysis, deamidation of Asn or Gln, or other irreversible reactions. Also, small errors in fitting the high-temperature and the low-temperature baselines can result in large errors in ΔC_P. The fitting errors may well depend on the amount of baseline (i.e., temperature range) that it is reasonable to collect, and/or whether the baselines are linear, as is assumed. Thus, it is generally regarded as more reliable to measure ΔC_P from the temperature dependence of the enthalpy of unfolding (Privalov, 1979; Becktel & Schellman, 1987; Hu et al., 1992b).

Our assumption that ΔC_P is independent of temperature is likely to be an approximation in view of the fact that the ΔC_P for unfolding has been shown to depend on temperature for many proteins, including horse cytochrome *c* (Privalov & Makhatadze, 1990, 1992). Comparisons of the thermodynamic parameters at a common reference temperature near the transition temperature (e.g., 60 °C; Table 2) should be especially accurate, since the contributions of terms involving ΔC_P are very small near T_m. Extrapolations to temperatures far removed from the transition region (25 °C; Table 2, and the lower temperature region of Fig. 4) are, however, more suspect.

Comparison of calorimetric and van't Hoff enthalpies

A troubling aspect of the data presented in Table 1 is that the ratio of the van't Hoff enthalpy to the calorimetric enthalpy ($\Delta H^{vH}/\Delta H_m$) is greater than unity for all four cytochromes *c*. This behavior is similar to the behavior of the six cytochromes *c* studied previously (Liggins et al., 1994). For an ideal two-state transition, it is usually assumed the value of $\Delta H^{vH}/\Delta H_m$ should be equal to unity. It was hypothesized previously that the reason why the values of $\Delta H^{vH}/\Delta H_m$ are consistently greater than unity for the proteins studied here is because reversible intermolecular association occurs in the unfolded state. The observed dependence of T_m on the protein concentration (Liggins et al., 1994) was used to support this hypothesis. Reversible intermolecular association among

unfolded species provides a plausible explanation for the fact that $\Delta H^{vH}/\Delta H_m > 1$ for the cytochromes *c*, which were studied in this work. Recently, it has been suggested that, near neutral pH, the amino terminus of iso-1 can be a heme ligand in the unfolded protein (Hammack et al., 1998), a suggestion that provides a possible mechanism for oligomerization in the unfolded protein. If oligomers occur, the observation that ΔH_m for unfolding is independent of protein concentration (Liggins et al., 1994) indicates that the contribution of the oligomerization process to the calorimetric enthalpy of unfolding is negligible.

Recently, the interpretation of the value of $\Delta H^{vH}/\Delta H_m$ has been called into question by a theoretical analysis of the problem (Zhou et al., 1999). Examples were used to show that $\Delta H^{vH}/\Delta H_m = 1$ is not always a good indication of the absence of intermediates. It was also shown that a ratio of greater than one can occur for a simple monomolecular reaction, a result in conflict with the usual interpretation of $\Delta H^{vH}/\Delta H_m > 1$ as indicating molecular association. At the present time, the implications of a $\Delta H^{vH}/\Delta H_m \neq 1$ are not settled.

The stabilities of the proteins studied by scanning calorimetry have also been measured by Gdn·HCl-induced unfolding. The transitions have been analyzed using a two-state linear extrapolation model to obtain the unfolding free energy, $\Delta G_{H_2O}(T_{ref})$, at $T_{ref} = 20\,°C$ in the absence of Gdn·HCl (Raman, 1997). The measures of stability by Gdn·HCl unfolding and by calorimetry ($\Delta G_{H_2O}(T_{ref})$ and $\Delta G(T_{ref})$) are the same within errors. The agreement between the two ways of measuring stability adds support to our contention that it is ΔH_m and not ΔH^{vH} that yields the true unfolding enthalpy, and that the van't Hoff analysis yields erroneously high values (by up to 62%; Table 1) for the unfolding enthalpy. $\Delta H^{vH}/\Delta H_m$ values of greater than unity have been reported for several monomeric globular proteins (Kitamura & Sturtevant, 1989; Connelly et al., 1991; Xie et al., 1991; Plaza del Pino et al., 1992).

Differences in the structures of the mutant and the wild-type proteins

Inspection of the three-dimensional structures of the wild-type and the mutant cytochromes *c* provides possible explanations for the differences in their stabilities. The comparison of the structure of wild-type iso-1 cytochrome *c* (Louie & Brayer, 1990; Berghuis & Brayer, 1992) with those of the mutants (Louie & Brayer, 1989; Lo, 1995; Lo et al., 1995a, 1995c) reveals localized structural differences in the vicinity of the mutation sites. The effects of the mutations on the amino acid positional differences in the three-dimensional structure of the cytochrome *c* molecule for two single mutants (F82Y iso-1, L85A iso-1) and the corresponding double mutant (F82Y, L85A iso-1) are summarized in Figure 5. The structural changes lead to changes in the solvent accessible surface areas, solvent inaccessible cavities, side-chain entropies, hydrogen bonding, and bound water, all of which are known to affect thermodynamic stability.

Changes in solvent accessible surface areas of substituted residues

Table 3 gives the solvent accessible surface areas (ASAs) for the folded proteins of the residues at the mutation sites (positions 82 and 85). Estimates of the range in solvent ASAs are also given for the same residues in the unfolded state (Creamer et al., 1997). The change in ASA between the folded and unfolded states is a major factor governing solvation free energies of folding (Rose et al., 1985; Eisenberg & McLachlan, 1986). Although Table 3 lists only the changes in ASA for the mutated residues at positions 82 and 85, these two residues account for most of the ASA changes with comparatively minor changes elsewhere. Nevertheless, our estimation of the solvation free energy contributions to the stability differences ($\delta\delta G(sc_{tr})$ in Table 5) includes residues at positions 9, 13, 90, and 94, as well as residues 82 and 85. In principle, solvation free energies should be calculated including all the residues in the protein and any prosthetic groups. In practice, comparisons of closely related mutant proteins are more accurate when limited to those residues with significant structural changes. This is because a few poorly defined surface residues can have large effects on the accuracy of the calculations. A case in point are the Lys residues, parts of which are poorly defined in the electron density so that modeling the residue is somewhat arbitrary. In all cases the mutation-induced structural changes show no change or an increase in the ASA for the folded proteins. The ASAs for the unfolded proteins reflect the sizes of the residues, so that the smallest ASA is that of unfolded L85A iso-1 (Table 3).

Solvent inaccessible cavities

Wild-type iso-1 contains a sizable internal cavity in the heme pocket. All of the mutations discussed here have considerable effects on the shape and the size of this cavity. In the wild-type molecule, the cavity is bounded by one edge of the heme-porphyrin moiety and by the side chains of L32, I35, M64, and L98 (Louie & Brayer, 1990; Lo, 1995). The size of this cavity together with other cavities is given in Table 4. The size of the heme pocket cavity is independent of the oxidation-reduction state for wild-type but depends sensitively on the nature of the substituents at positions 82 and 85. In the extremes the cavity shrinks from 35^3 Å (wild-type) to 14 $Å^3$ (F82Y iso-1) and expands to as much as 40 $Å^3$ (L85A iso-1). For F82Y iso-1 the displacements caused by the shift and rotation of the Y82 side chain have decreased the size of the heme pocket cavity (Table 4). The volume decreases from 35 to 14 $Å^3$ and the ASA decreases from 6.1 to 0.8 $Å^2$. However, when all cavities are considered together, there is very little total change in cavity volumes. This results from the appearance of new cavities in the mutant proteins, which offset the loss in volume of the heme pocket cavity (Table 4; Columns 2, 4). For L85A iso-1 rearrangement of the side chains of L9, R13, and L94 in the vicinity of L85 (Fig. 5) causes the volume of the heme pocket cavity to be less than it might otherwise have been (Lo, 1995; Lo et al., 1995a). There is a modest increase in the total volume of all cavities from 53 $Å^3$ for wild-type to 66 $Å^3$ for L85A iso-1 (Table 4). For F82Y, L85A iso-1 the result of the two amino acid substitutions and a localized structural rearrangement is a heme pocket cavity with a total volume (15.0 A^3) that is considerably less than for wild-type iso-1 (35 A^3) or L85A iso-1 (40 A^3) (Table 4).

Theoretical bases for the stability differences

The structural factors, which can be expected to affect the mutation-induced changes in stability of this series of mutant cytochromes *c*, are: (1) the formation/abolition of hydrogen bonds (Myers & Pace, 1996); (2) the change in the surface area of hydrophobic groups that are buried upon folding (Fauchere & Pliska, 1983; Eriksson et al., 1992; Matthews, 1996); (3) the change in the volume/

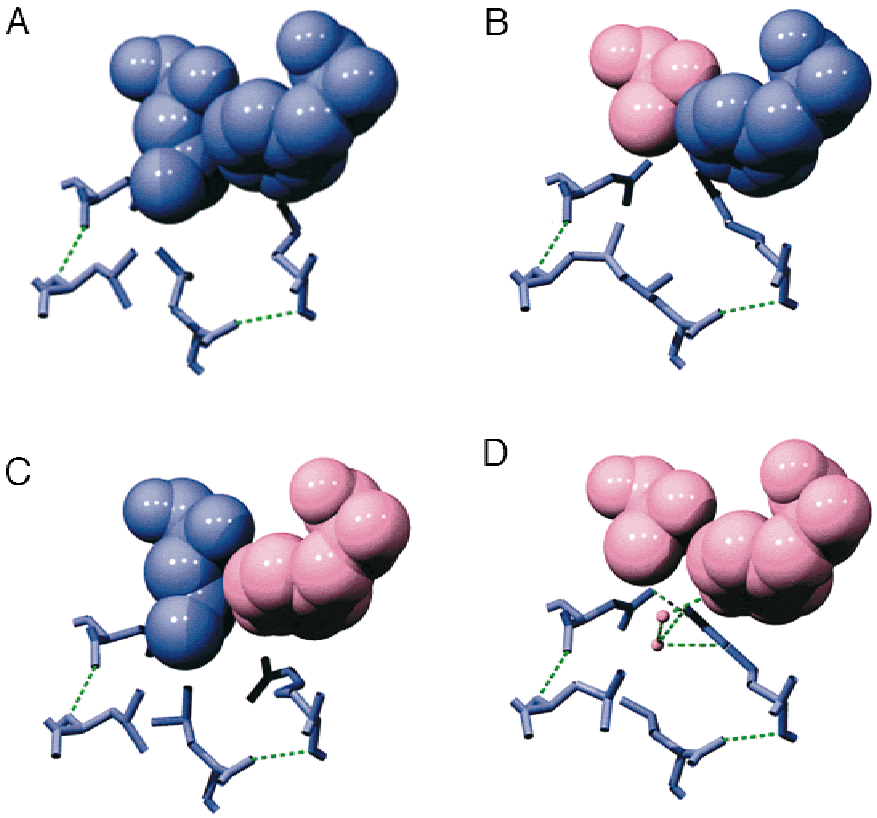

Fig. 5. Structural changes in iso-1 cytochrome *c* variants induced by mutations singly and together at two sites (positions 82 and 85). Structures of four proteins are compared: (**A**) iso-1 (wild-type), (**B**) L85A iso-1, (**C**) F82Y iso-1, and (**D**) the double mutant, F82Y, L85A iso-1. In **A** through **D** the normal (wild-type) residues are shown in blue, and mutational substitutions are shown in pink. **A:** The structure for iso-1 (starting in the lower left and proceeding clockwise) shows the residues L94, D90, L85, F82, R13, and L9. The mutated positions 85 and 82 are shown with space filling atoms while the remaining residues are stick models. H-bonds (green dashed lines) are shown between the amide N-H of L94 and the amide CO of the D90 and between the amide N-H of R13 and the amide CO of the L9. These two H-bonds are retained in the mutant proteins (**B**–**D**), as well as in cytochromes *c* from yeast iso-2, horse, tuna, and rice (Brayer & Murphy, 1996). **B:** There are three main differences in the structure of L85A iso-1 compared to the wild-type protein: (1) the side chain of L94 shifts into the new space near A85; (2) R13 moves toward A85 and is now in a position where the R13 side-chain NH2 nitrogen atom is only 3.61 Å from the D90 side-chain OD2 oxygen atom. Although the distance is greater than the usual H-bond distance, a large decrease in the thermal factor of the R13 side chain from 32.5 $Å^2$ for iso-1 to 16.7 $Å^2$ for L85A iso-1 indicates a new side-chain–side-chain interaction. (3) The L9 side chain adopts a new conformation as it shifts away from the mutation site in response to changes in conformation of L94 and R13. **C:** For F82Y iso-1 the Y82 side chain moves and rotates toward the surface of the protein to alleviate a steric conflict between the new hydroxyl group and the side chain of L85. The average side-chain thermal factor of the residue at position 82 increases from 16.9 $Å^2$ for the iso-1 (F82) to 25.2 $Å^2$ for F82Y iso-1 (Y82). The movement of Y82 allows the new hydroxyl group to H-bond with a surface water molecule. The L85 side chain remains in a conformation essentially the same as that of the wild-type (iso-1) protein. Both the R13 and L9 side chains take on new conformations. The new R13 conformation facilitates an increase in solvent exposure of Y82, and encourages displacement of the L9 side chain from the favored position of L9 in wild-type iso-1 (70% occupancy) to a minor alternative conformation (30% occupancy in wild-type) (Louie & Brayer, 1990). **D:** In the structure of F82Y, L85A iso-1, the Y82 side chain remains in the position of F82 in wild-type as the new (Y82) hydroxyl H-bonds to a new internal water molecule (WAT248). Another water (WAT224) H-bonds to the guanidinium group of R13 and to WAT248. As the R13 side chain moves closer to the D90 side chain, a new H-bond with an N to O distance of 3.3 Å is formed between the R13 NH2 and the D90 OD2. The thermal factor for R13 is 19.3 $Å^2$, which is much less than that of the wild-type protein (32.4 $Å^2$). In addition there are side-chain conformation changes for L9 and L94 (Lo et al., 1995a).

surface area of solvent inaccessible cavities (Eriksson et al., 1992; Matthews, 1996); (4) van der Waals interactions (Eriksson et al., 1992; Matthews, 1996); (5) the changes in the state of bound water molecules (Williams et al., 1994; Matthews, 1996; Zhang & Hermans, 1996); and (6) the change in the conformational entropy of amino acid side chains upon folding (Doig & Sternberg, 1995).

Approximate values for the changes in the free energy of unfolding that are expected for these mutant proteins have been calculated (Table 5). It must be understood that these values can only be, at best, approximations; it is not yet possible to predict the effects of these types of mutations on protein stability with exactitude (Myers & Pace, 1996). The theoretical and the experimental differences in the stability of yeast iso-1 cytochrome *c*, which are caused by the heme pocket mutations, are presented side by side in the final two columns of Table 5. The magnitudes of the calculated and observed stability changes are similar, suggesting that all major factors that relate mutational perturbations of structure to changes in thermodynamic stability have been identified. Inspection of the individual factors (listed in Table 5) suggests that the additional stability of F82Y, L85A iso-1 can be attributed to at least two

Table 3. *Solvent ASA of residues 82 and 85 in heme pocket mutants of iso-1 cytochrome c*[a]

	ASA (Å^2) of residue 82 and 85					
	Folded ASA			Unfolded ASA		
Protein	82	85	Total	82	85	Total
Iso-1(ox)	22.4	8.9	31.3	134–173	116–148	250–322
Iso-1	17.2	5.7	22.9	134–173	116–148	250–322
F82Y, L85A iso-1	34.0	1.0	35.0	149–186	66–100	215–285
F82Y iso-1	36.5	0.1	36.6	149–186	116–148	265–334
L85A iso-1	22.6	0.6	23.2	134–173	66–100	200–273

[a]The ASA in this table for folded proteins were obtained using the method of Connolly (1983, 1993) with a probe radius of 1.4 Å. The Protein Bank Data (PDB) coordinate files are from X-ray crystallography (Lo, 1995) of reduced proteins, except for iso-1(ox), which is the oxidized form (Berghuis & Brayer, 1992; Lo, 1995; Lo et al., 1995a). All waters and a sulfate anion were removed from the PDB files prior to calculation. Lower and upper bounds of unfolded protein ASAs are from Creamer et al. (1997).

Table 4. *Cavities in heme pocket mutants of iso-1 cytochrome c*[a]

Protein	Cavities (total #)	Volume heme pocket cavity (Å^3)	Total volume of cavities (Å^3)	ASA heme pocket cavity (Å^2)	Total ASA of cavities (Å^2)
Iso-1(ox)	6 (9)	34	138 (204)	5.0	16.3 (24.2)
Iso-1	2 (6)	35	53 (117)	6.1	7.9 (13.4)
F82Y, L85A iso-1	4 (8)	15	46 (204)	1.1	2.1 (29.2)
F82Y iso-1	4 (7)	14	50 (104)	0.8	2.7 (7.8)
L85A iso-1	3 (5)	40	66 (124)	8.4	9.7 (14.5)

[a]Cavities are measured with the crystallographic waters present and absent (in parentheses). When no numbers are in parentheses, the values are the same in the presence and absence of water. The "heme pocket cavity" is a prominent cavity bounded (in wild-type iso-1) by residues 32, 35, 64, 98, 102, and the heme. Volumes (Column 3) and ASAs (Column 5) are given for the heme pocket cavity. Cavity volumes and ASAs were measured using the method of Connolly (1983, 1993) with a probe radius of 1.2 Å. The PDB coordinate files are from X-ray crystallography (Lo, 1995) of reduced proteins, except for iso-1(ox), which is the oxidized form (Berghuis & Brayer, 1992; Lo, 1995; Lo et al., 1995a).

Table 5. *Summary of the theoretical free energies of stabilization/destabilization, relative to iso-1 imparted to the mutant iso-1 cytochromes c, at 25 °C, by the various structural parameters*[a]

Protein	$\delta\delta G(sc_{tr})$ (kcal mol^{-1})	$\delta\delta G(sc_S)$ (kcal mol^{-1})	$\delta\delta G$(cav) (kcal mol^{-1})	$\delta\delta G$(hb) (kcal mol^{-1})	$\delta\delta G$(bw) (kcal mol^{-1})	$\Delta\Delta G$(calc) (kcal mol^{-1})	$\Delta\Delta G$(exp) (kcal mol^{-1})
F82Y, L85A iso-1	−2.6	0.38	0.15	1.5	−4	−4.6	−2.9 ± 0.4
F82Y iso-1	−0.7	−0.40	0.07	0	0	−1.0	−0.7 ± 0.6
L85A iso-1	−1.8	0.77	−0.29	0	0	−1.3	−2.8 ± 0.4

[a]$\delta\delta G$ (Columns 2–6) represents the contribution to the free energy of unfolding for side-chain transfer, sc_{tr} (Eisenberg & McLachlan, 1986); side-chain entropy, sc_S (Pickett & Sternberg, 1993; Doig & Sternberg, 1995); cavity volumes, cav (Xu et al., 1998); changes in the number of hydrogen bonds (at 1.5 kcal mol^{-1}), hb (Myers & Pace, 1996); and changes in bound water (at 2 kcal mol^{-1}), bw (Dunitz, 1994; Williams et al., 1994; Zhang & Hermans, 1996). $\Delta\Delta G$ represents the calculated, calc (Column 7), and experimental, exp (Column 8), mutation-induced change in the free energy of unfolding.

factors: (1) supplementary decreases in the volumes of internal cavities and (2) formation of an extra protein–protein hydrogen bond. However, these two stabilizing factors may be partially offset by the destabilizing effect of additional bound waters.

Materials and methods

Strains of the yeast *Saccharomyces cerevisiae* expressing mutant pseudo wild-type (C102T) iso-1 were obtained from Dr. Michael

Smith of the University of British Columbia, Vancouver, Canada. Growth of yeast and purification of cytochrome *c* were performed as described previously (Nall & Landers, 1981; Nall, 1983). The purified stocks of cytochrome *c* were lyophilized and stored at −70 °C until needed. Methods for preparation of protein solutions, for scanning calorimetry, and for data analysis have been described in detail elsewhere (Liggins et al., 1994; Liggins, 1997).

X-ray crystallography and structural analysis

The X-ray crystallography analysis of the wild-type and mutant iso-1 proteins has been reported previously (Louie & Brayer, 1989, 1990; Lo, 1995; Lo et al., 1995a, 1995b, 1995c). The areas (ASAs) and volumes of the internal cavities were calculated from the structural data with a probe of radius 1.2 Å using the MSP programs (Connolly, 1983; Connelly et al., 1993). Solvent ASAs were calculated using a 1.4 Å probe radius. Changes in the solvation free energy of folding were calculated with a 1.4 Å probe by the method of Eisenberg and McLachlan (1986).

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