Probing weakly polar interactions in cytochrome *c*

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Abstract

Theoretical, statistical, and model studies suggest that proteins are stabilized by weakly polar attractions between sulfur atoms and properly oriented aromatic rings. The two sulfur-containing amino acids, methionine and cysteine, occur frequently among functional alleles in random mutant libraries of *Saccharomyces cerevisiae* iso-1-cytochrome *c* genes at positions that form a weakly polar aromatic–aromatic interaction in the wild-type protein. To determine if a weakly polar sulfur–aromatic interaction replaced the aromatic–aromatic interaction, the structure and stability of two variants were examined. Phenylalanine 10, which interacts with tyrosine 97, was replaced by methionine and cysteine. The cysteine was modified to form the methionine and cystine analog, *S*-methyl cysteine (Cys_{SMe}). Proton NMR studies indicate that changing Phe 10 to Met or Cys_{SMe} affects only local structure and that the structures of sulfur-containing variants are nearly identical. Analysis of chemical shifts and nuclear Overhauser effect data indicates that both sulfur-containing side chains are in position to form a weakly polar interaction with Tyr 97. The F10M and F10C_{SMe} variants are 2–3 kcal mol^{−1} less stable than iso-1-cytochrome *c* at 300 K. Comparison of the stabilities of the F10M and F10C_{SMe} variants allows evaluation of the potential weakly polar interaction between the additional sulfur atom of F10C_{SMe} and the aromatic moiety of Tyr 97. The F10C_{SMe};C102T variant is 0.7 ± 0.3 kcal mol^{−1} more stable than the F10M;C102T protein. The increased stability is explained by the difference in hydrophobicity of the sulfur-containing side chains. We conclude that any weakly polar interaction between the additional sulfur and the aromatic ring is too weak to detect or is masked by destabilizing contributions to the free energy of denaturation.

Keywords: cytochrome *c*; helix–helix packing; N-terminal helix; NMR; weakly polar interactions

The native state of a protein is stabilized by a large number of weak interactions. These include nonpolar interactions, weakly polar interactions, and hydrogen bonds. This study explores the role of weakly polar interactions in determining the structure and stability of *Saccharomyces cerevisiae* iso-1-cytochrome *c*. The choice of variants is based on random mutagenesis studies of the evolutionarily conserved interactions between the N- and C-terminal helices (Auld & Pielak, 1991; Fredericks & Pielak, 1993). These studies show that both sulfur-containing amino

acids, methionine and cysteine, are observed among the functional single missense mutations at positions 10 and 97. Here we report structural and thermodynamic studies of two variants,⁵ F10M;C102T and F10C;C102T.

Treatment of the F10C;C102T variant with methyl methanethiosulfonate yields the *S*-methyl cysteine (C_{SMe}, Cys_{SMe}) derivative F10C_{SMe};C102T:

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Abbreviations: COSY, correlated spectroscopy; Cys_{SMe} or C_{SMe}, *S*-methyl cysteine; GdnHCl, guanidine hydrochloride; NOE, nuclear Overhauser effect; NOESY, 2-dimensional NOE spectroscopy; δ , chemical shift; δ_{ps} , paramagnetic shift.

⁵ Variant proteins are described using the one-letter code (IUPAC, 1985). Multiple amino acid substitutions are separated by a semicolon (e.g., F10M;C102T). Throughout this paper, the numbering system for higher eukaryotic cytochromes *c* is used (Moore & Pettigrew, 1990). All variants also contain the C102T mutation. The C102T variant is used in place of the true wild-type protein because removal of the sole free-cysteine sulfhydryl renders the protein more amenable to *in vitro* studies (Betz & Pielak, 1992), without affecting its structure or function (Cutler et al., 1987; Gao et al., 1991a; Berghuis & Brayer, 1992).

The difference between *S*-methyl cysteine and methionine is that the γ -methylene of the latter is replaced by sulfur. The methylene-sulfur-methyl bond angle decreases only slightly, from 105.3° to 103.9°, upon replacing the methylene (Peterson et al., 1960; Kennard, 1973). *S*-methyl cysteine can also be considered a model for cystine in which the disulfide is present but the cross-link is absent. Provided that the F10M;C102T and the F10C_{SMe};C102T variants have similar structures and that the residue at position 10 maintains the close contact with Tyr 97 observed in the wild-type protein (Louie & Brayer, 1990), permutation of Phe 10 to methionine and *S*-methyl cysteine allows evaluation of a potential sulfur–aromatic interaction between Tyr 97 and the additional sulfur in Cys_{SMe}.

Examination of protein structures suggests that weakly polar interactions stabilize proteins by 1–2 kcal mol^{−1} at room temperature (Burley & Petsko, 1985, 1988; Reid et al., 1985). Interactions between two aromatic rings have been characterized theoretically (Jorgensen & Severance, 1990), in proteins (Singh & Thornton, 1985; Burley & Petsko, 1988; Serrano et al., 1991; Jasanoff et al., 1992; Anderson et al., 1993), in peptides (Armstrong et al., 1993), and in model systems (Cozzi et al., 1993). For two aromatic rings, a favorable interaction is thought to arise from the electrostatic attraction of the π -electrons and partially negatively charged carbon atoms of one ring with the partially positively charged protons of the other. An analogous weakly polar interaction has been proposed between aromatic protons and the electron lone pairs of sulfur in methionine, cysteine, and cystine (Morgan et al., 1978; Bodner et al., 1980; Reid et al., 1985; Burley & Petsko, 1988) and between aromatic residues and amino groups (Fong et al., 1993).

The geometry of aromatic side-chain interactions is defined using the coordinate system of Singh and Thornton (1985). The parameters that describe this geometry are the distance between the centers of the aromatic rings, the interplane angle, P , and the angle of elevation from the center of one ring to the center of the other, θ . In their examination of high-resolution crystal structures these authors found two preferred orientations referred to as “fully stacked” ($P \approx 70$ – 90° , $\theta \approx 62.5$ – 90°) and “moderately stacked” ($P \approx 45$ – 60° , $\theta \approx 45$ – 67.5°).

The packing of the N- and C-terminal helices is the most conserved structural element of cytochromes *c* (Mathews, 1985) and is thought to be present in an early folding intermediate of horse cytochrome *c* (Elöve et al., 1992). In iso-1-cytochrome *c* (Louie & Brayer, 1990; Berghuis & Brayer, 1992), the N- and C-terminal helices are approximately perpendicular and possess an evolutionarily invariant aromatic–aromatic pair, Phe 10 and Tyr 97, at the helix–helix interface. The side chain of Phe 10 is buried, but part of the Tyr 97 side chain, including its phenolic hydroxyl, is exposed to solvent. The parameters for this pair, $P \approx 45^\circ$ and $\theta \approx 53.5^\circ$, place these rings in the moderately stacked class. The distance between ring

centers, 5.9 Å, and the closest carbon–carbon distance between rings, 3.9 Å, are well within the accepted ranges defined by Singh and Thornton (1985). These criteria, together with the evolutionary conservation of the residues (Moore & Pettigrew, 1990), strongly suggest that this is a weakly polar aromatic–aromatic interaction where the π -cloud of Phe 10 interacts with the aromatic protons of Tyr 97 (Fig. 1; Kinemage 1).

We examined the effect of replacing Phe 10 with the sulfur-containing residues Met and Cys_{SMe} on the structure and stability of the variants. This allows us to test for weakly polar sulfur–aromatic interactions between (1) the δ -sulfur of Met 10 and the ring of Tyr 97, (2) the δ -sulfur of Cys_{SMe} 10 and the ring of Tyr 97, and (3) the γ -sulfur of Cys_{SMe} and the ring of Tyr 97.

Results

Degree of modification

Titration of the F10C;C102T variant with 5,5'-dithiobis-(2-nitrobenzoic) acid revealed 1.0 ± 0.1 mol of free sulfhydryl per mol of protein. After treatment with methyl methanethiosulfonate the variant exhibited less than 0.1 mol of free sulfhydryl per mol of protein. The amino acid sequence of the F10M;C102T variant was confirmed by Edman degradation of the first 16 residues (Auld, 1993).

Assignment of proton resonances

For the C102T variant, stereospecific assignments are available for 497 and 476 proton resonances in the reduced and oxidized forms, respectively (Gao et al., 1990, 1991a). For the F10M;C102T variant, 457 and 419 such assignments are available (Auld, 1993; provided on Diskette Appendix). Assignment of the reduced form of the F10C_{SMe};C102T variant is not possible because reduction of the iron coincides with reduction of the disulfide in the *S*-methyl cysteine side chain. Nevertheless, in the oxidized form, 340 proton resonances (including the N- and C-terminal helices) have been assigned (Auld, 1993; listing provided on the Diskette Appendix). With the exception of the first three residues of the N-terminal extension, Ser 2, and Gly 83, at least one assignment is available for each residue in at least one variant. From the total resonance assignments for both redox forms of the C102T and F10M;C102T variants, 382 assignments are common to all four. These 382 commonly assigned resonances were analyzed as described in the next section. Of these 382 resonances, 307 are assigned in the oxidized form of the F10C_{SMe};C102T variant. These 307 resonances were analyzed as described later.

Paramagnetic shift

NMR-derived structural information is normally limited to analysis of NOEs and coupling constants (Wüthrich,

Fig. 1. Superposition in stereo of the Cys_{SMC} C, CA, and CB atoms onto the corresponding atoms of Phe 10 from the crystal structure of the oxidized C102T variant (PDB2YCC.ENT; Berghuis & Brayer, 1992) using the dominant disulfide conformation (Thornton, 1981). In the orientation shown, the distances between the γ -sulfur and the closest δ - and ϵ -carbons of Tyr 97 are 2.7 Å and 3.2 Å. The corresponding distances for the δ -sulfur are 3.9 Å and 4.3 Å. The average distance from the ϵ -methyl protons and the closest δ -proton of Tyr 97 is 2.8 Å. The corresponding distance for the closest ϵ -proton of Tyr 97 is 3.3 Å.

1986). The presence of a low-spin iron in both redox forms of cytochrome *c*, however, allows the detection of subtle differences in structure by examination of the paramagnetic shift (Gao et al., 1991b). The paramagnetic shift, δ_{ps} , is defined as the chemical shift (δ) of a nucleus in the oxidized form minus that of the same nucleus in the reduced form (Williams et al., 1985; Feng et al., 1990; Gao et al., 1991a,b). For protons not directly attached to the heme or the iron ligands, δ_{ps} is dominated by pseudo-contact terms. These depend on the position of a proton relative to the unpaired electron present on the heme iron in the oxidized form. Therefore, comparison of δ_{ps} for a variant to δ_{ps} for the wild-type control, in this case the C102T variant, reveals the location of structural changes.

To facilitate comparison we define the term Δ_{para} as $\delta_{ps,F10M;C102T}$ minus $\delta_{ps,C102T}$. Significant values of Δ_{para} reveal regions that differ in structure. For 306 of the 382 available paramagnetic shifts, the change in δ_{ps} is within the error for the determination of the four chemical shifts required to calculate Δ_{para} (i.e., ± 0.04 ppm). In assessing the significance of the remaining values, each Δ_{para} was compared to $\delta_{ps,C102T}$ for that resonance. Of the 76 Δ_{para} values greater than ± 0.04 ppm, only 35 are greater than 30% of the magnitude of $\delta_{ps,C102T}$ for that resonance (Table 1). Comparisons between the δ_{ps} of the C102T and the F10C_{SMC};C102T variants cannot be made because reduction of the *S*-methyl cysteine side chain is concomitant with reduction of the heme iron.

Chemical shifts

Chemical-shift changes (relative to the C102T protein) in both redox forms of F10M;C102T range between 0.1 and 0.4 ppm and, in general, are localized to the interior residues of the N- and C-terminal helices. A comparison of

Table 1. Values of $\Delta_{para} > \pm 0.04$ ppm and $> \pm 0.3 \times \delta_{ps,C102T}$

Resonance	δ_{ps} (ppm)	Δ_{para} (ppm)
Lys -2 β^1	-0.11	0.15
Lys 4 NH	-0.17	-0.08
Ala 7 α	-0.06	-0.10
Ala 7 β	0.00	0.06
Lys 11 NH	0.07	-0.10
Lys 11 β^1	0.10	-0.05
Cys 14 NH	-0.16	-0.14
Leu 15 β^1	0.32	0.33
Leu 15 $\delta 2$	0.55	0.20
Pro 30 β^1	0.09	-0.05
Gly 34 NH	0.34	0.12
Gly 34 α^2	0.00	-0.07
Phe 36 β^1	0.00	-0.06
Phe 36 β^2	0.04	-0.14
Gly 37 NH	-0.29	-0.68
Arg 38 β^1	0.05	-0.05
Arg 38 ϵ	-0.09	0.07
His 39 NH	0.17	-0.22
His 39 β^1	0.28	-0.09
Gln 56 $\delta 2^1$	-0.14	0.06
Leu 58 α	0.09	-0.05
Glu 61 β^1	-0.25	0.30
Gln 63 $\delta 2^2$	0.06	0.05
Ser 65 β^1	0.10	0.07
Glu 66 β^1	0.16	0.10
Gln 70 β^2	0.45	-0.27
Met 80 α	-0.37	-0.15
Ala 81 NH	0.11	-0.06
Lys 86 NH	-0.13	-0.08
Lys 86 HA	-0.10	0.18
Gln 92 α	0.39	-0.65
Ile 95 $\gamma 2$	-0.25	-0.16
Leu 98 $\delta 2$	-0.18	-0.46
Glu 103 NH	0.10	-0.05

the chemical shifts in the oxidized forms of the F10M;C102T variant to those of the C102T variant is presented in Figure 2. Ninety-nine of the 307 commonly assigned resonances exhibit chemical-shift changes greater than ± 0.02 ppm. However, 71 of the 99, including the α proton of position 10, exhibit values of Δ_{para} that are less than ± 0.04 ppm. Because nearly 80% of the resonances that exhibit significant changes in chemical shift show insignificant changes in paramagnetic shift, many of the chemical-shift changes can be attributed to removal of ring-current effects (Johnson & Bovey, 1958; Perkins, 1982; Gao et al., 1991b) resulting from the replacement of Phe 10 with a nonaromatic residue. For the remaining resonances, there is correspondence between large values of Δ_{para} and large changes in chemical shift as seen by comparison of Table 1 and Figure 2.

Nearly identical chemical-shift changes, compared to the oxidized C102T variant, are observed for the oxidized F10C_{SMC};C102T variant. Comparison of the chemical shifts in the oxidized forms of the F10C_{SMC};C102T variant to those of the F10M;C102T variant are presented in Figure 3. Compared to Figure 2 there are fewer outliers, indicating that F10M;C102T and F10C_{SMC};C102T are nearly isostructural.

More quantitative information about the similarity of two proteins can be obtained from statistical analysis of chemical-shift changes. The average magnitude of the change in chemical shift for the 307 commonly assigned protons in the oxidized state of the proteins is 0.05 ppm for both the comparison of the F10M;C102T variant to the C102T variant and for comparison of the F10C_{SMC};C102T variant to the C102T variant. On the other hand, comparison of the sulfur-containing variants to each other yields an average magnitude of 0.03 ppm. The distribution of chemical-shift changes can be assessed by determination of the standard deviation of the mag-

Fig. 3. Proton chemical shift, δ , for the oxidized forms of the F10M;C102T variant versus the F10C_{SMC};C102T variant. A: All protons. B: Aliphatic protons. C: Amide protons.

nitude of chemical-shift changes. The standard deviation is ± 0.10 ppm when the F10M;C102T and F10C_{SMC};C102T variants are compared individually to the C102T variant. When the two sulfur-containing position 10 variants are compared to each other the standard deviation decreases to ± 0.05 ppm. The small standard deviation from comparison of the two sulfur-containing variants to the C102T protein indicates that all three proteins possess similar structures. The smaller standard deviation from comparison of the sulfur-containing variants to each other suggests that they are even more similar in structure.

NOEs

In two-dimensional NOE (NOESY) spectra, crosspeaks between the N- and C-terminal helices are observed for the C102T, F10M;C102T, and F10C_{SMC};C102T variants (Fig. 4). In addition to those shown in Figure 4, NOEs are observed between the β protons of the variant residues at position 10 and the Tyr 97 δ and ϵ protons. The NOEs are also observed between these β protons and two other resonances, those of the amide proton of position 10 and the δ methyl protons of Leu 94. Assignment of the ϵ -methyl proton resonances for Met 10 and Cys_{SMC} 10 is problematic because of the lack of scalar-coupling information. However, strong NOEs, absent from the spectra of the C102T variant, are observed between the benzoid protons of Tyr 97 and a resonance at 2.63 ppm in the oxidized form of the F10C_{SMC};C102T variant and 2.41 ppm in the oxidized form of the F10M;C102T variant. These resonances are assigned to the ϵ -methyl groups.

The flip rate of Tyr 97

In the C102T variant, the side chain of Tyr 97 is known to have a slow flip rate in the reduced state and probably pos-

Fig. 2. Proton chemical shift, δ , of the oxidized form of the C102T variant versus the F10M;C102T variant. A: All protons. B: Aliphatic protons. C: Amide protons.

Fig. 4. Portions of NOESY spectra showing crosspeaks between the aromatic protons of Tyr 97 and the methyl protons of both Ala 7 and Ala 101.

sesses an intermediate flip rate in the oxidized state (Eley et al., 1982; Alean et al., 1988; Nall & Zuniga, 1990). Several observations lead us to conclude that the side chain of Tyr 97 is in the fast-flipping regime ($>10^3 \text{ s}^{-1}$; Campbell et al., 1975; Wüthrich & Wagner, 1975) in the F10M;C102T and F10C_{SMC};C102T variants. First, only a single strong COSY crosspeak is observed between the δ and ϵ proton resonances of Tyr 97 in both oxidation states of the F10M;C102T variant and in the oxidized form of the F10C_{SMC};C102T variant. Second, strong NOEs are observed between both the δ and ϵ protons of Tyr 97 and its β protons in both oxidation states of the F10M;C102T variant and in the oxidized form of the F10C_{SMC};C102T variant.

Thermodynamic studies

Thermal and GdnHCl denaturation is reversible for the C102T, F10M;C102T, and F10C_{SMC};C102T variants (Betz & Pielak, 1992; Auld, 1993; Betz, 1993). A sample of the chemical denaturation data is shown in Figure 5. Values for the GdnHCl concentration at which half of the protein molecules are denatured, C_m , the dependence of ΔG on GdnHCl concentration, m_{GdnHCl} , and the free energy of denaturation at zero denaturant concentration, $\Delta G_{\text{d,H}_2\text{O}}$, are presented in Table 2. Examination of these data show that the sulfur-containing variants are 2–3 kcal mol⁻¹ less stable than the C102T variant, and the F10C_{SMC};C102T variant is 0.7 ± 0.3 kcal mol⁻¹ more stable than the F10M;C102T variant at 300 K. The error is the root mean square of the sum of the uncertainties in $\Delta G_{\text{d,H}_2\text{O}}$. Data from thermal denaturations (Table 3) rank the stability of the proteins in the same order

as GdnHCl denaturation data. Relative to the C102T variant, the temperature at which half the protein molecules are denatured, T_m , for the sulfur-containing variants decreases by more than 10 °C and the enthalpy of denaturation at T_m , ΔH_m , decreases by approximately 25 kcal mol⁻¹. Values of T_m and ΔH_m for the F10C_{SMC};C102T variant are significantly larger than those for the F10M;C102T variant.

Side-chain hydrophobicity

Two methods for assessing side-chain hydrophobicity were considered, transfer free energy and surface area. The experimentally determined free energies for transfer of the side-chain models from cyclohexane to water ($\Delta G_{\text{tr, chx} \rightarrow \text{H}_2\text{O}}$) are presented in Table 4. Surface area was considered because of its linear relationship to transfer free energy (Tuñón et al., 1992; Rose & Wolfenden, 1993) and the degree to which particular side chains are buried in proteins (Miller et al., 1987; Rose & Wolfenden, 1993). Although the relationship between molecular surface area and transfer free energy (Tuñón et al., 1992) is based on hydrocarbons, extending these observations to sulfur-containing side chains seemed justified because Lesser and Rose (1990) showed that carbon and sulfur are equally buried in proteins.

Molecular and solvent-accessible surface areas are presented in Table 4. Although the surface areas of the two sulfur-containing side chains are identical, S-methyl cysteine, which contains two polarizable sulfur atoms, is more hydrophobic by 0.7 ± 0.06 kcal mol⁻¹. Such a discrepancy would arise if the hydrophobicity of a disulfide

Table 2. Chemical denaturation data (pH 7.2, 300 K)

Protein	m_{GdnHCl} (kcal mol ⁻¹ M ⁻¹ ; ± 0.25)	C_m (M; ± 0.05)	$\Delta G_{\text{d,H}_2\text{O}}$ (kcal mol ⁻¹ ; ± 0.3)
C102T	4.2	1.5	6.3
F10M;C102T	4.2	0.8	3.4
F10C _{SMe} ;C102T	4.6	0.9	4.1

sulfur is more than that of an equivalent thioether sulfur. Comparing the value of $\Delta G_{\text{tr, chx} \rightarrow \text{H}_2\text{O}}$ for butane to those of the sulfur-containing model compounds (Table 4) shows that this is the case. In summary, for the side chains considered here, the correlation between surface area and transfer free energy does not hold because a disulfide sulfur is more hydrophobic than an equivalent thioether sul-

fur. Therefore, we restrict our analysis of hydrophobicity to transfer free energies.

Discussion

Comparing the structure of the C102T variant to the F10M;C102T variant

Several lines of reasoning lead to the conclusion that the global fold of cytochrome *c* is unchanged by the substitution of methionine for Phe 10. First, over 80% of the Δ_{para} values are within the error for determination of chemical shift. Second, values of Δ_{para} that are large with respect to $\delta_{\text{ps,C102T}}$ for the particular resonance are rare (Table 1). Third, NOEs are observed between the N- and C-terminal helices of each variant (Fig. 4). These observations suggest that the N- and C-terminal helices of the F10M;C102T variant pack in a manner similar to that observed in the wild-type protein, but there are subtle structural differences.

Insights into the structural differences are obtained by examining the data in Table 1 and Figure 4. The sign of Δ_{para} is predominately negative, indicating a general diminution in paramagnetic shift. This suggests that the polypeptide is less well packed around the heme in the F10M;C102T variant. Comparison of NOEs to calculated interproton distances (obtained by adding protons to the coordinates of the C102T variant [Berghuis & Brayer, 1992]) shows that the ϵ -methyl-group protons of Met 10 (and Cys_{SMe}) are closer to the ϵ protons of Tyr 97 than is expected from examination of the C102T variant. Specifically, the calculated distances between β protons of Phe 10

Fig. 5. A: Relative fluorescence versus GdnHCl concentration for the (1) C102T, (2) F10M;C102T, and (3) F10C_{SMe};C102T variants. The curves represent the nonlinear least-squares fits of the data to the two-state hypothesis assuming a linear relationship between ΔG and GdnHCl concentration (Schellman, 1978; Santoro & Bolen, 1988). B: A plot of ΔG versus GdnHCl concentration for the data in A.

Table 3. Thermal denaturation data

Protein, pH	ΔH_m (kcal mol ⁻¹ ; ± 4)	T_m (°C; ± 0.5)
C102T, pH 4.6	89.4	54.7
F10M;C102T, pH 4.6	63.0	37.6
F10C _{SMe} ;C102T, pH 4.6	63.6	38.7
C102T, pH 6.0	108.8	59.6
F10M;C102T, pH 6.0	75.7	47.5
F10C _{SMe} ;C102T, pH 6.0	81.4	48.3

Table 4. Surface areas and $\Delta G_{tr, chx \rightarrow H_2O}$ for side-chain models

Side chain	Model	Solvent-accessible surface area ^a (Å ²)	Molecular surface area ^a (Å ²)	$\Delta G_{tr, chx \rightarrow H_2O}$ ^b (kcal mol ⁻¹ ; ± 0.06)
Phe	Toluene	264	123	2.98
Ile	Butane	250	109	4.92
Met	Ethyl methyl sulfide	249	111	2.33
Cys _{SMc}	Methyl disulfide	249	111	2.99

^a Determined as described in text.^b Radzicka and Wolfenden (1988); Saunders et al. (1993).

and the benzoid protons of Tyr 97 are greater than 5 Å, yet NOEs are observed between the β protons of Met 10 (and Cys_{SMc}) and the benzoid protons of Tyr 97. This reorganization is in the direction expected for formation of a sulfur–aromatic interaction.

The observed reorganization is consistent with modeling studies (Fig. 1). That is, reorganization must occur to accommodate the side chain of Met 10 (and Cys_{SMc} 10) because simple substitutions show that the two sulfur-containing side chains cannot occupy the same space as the aromatic ring of Phe 10. In the position shown in Figure 1, one lone pair on the γ -sulfur is close (≈ 1.6 Å) to one of the lone pairs on the γ -sulfur of Cys 14. The other lone pair on the γ -sulfur of Cys_{SMc} 10 is in position to interact with the polar hydrogens of Tyr 97.

Further evidence for a weakening of the core comes from the observation that the flip rate of Tyr 97 increases upon substitution of Phe 10 with methionine (and S-methyl cysteine). Note that slow-flipping rings are not a requirement for weakly polar interactions (Serrano et al., 1991). Apparently, introduction of smaller residues at position 10 allows for faster flipping of the phenol moiety. This loosening is in accord with the decrease in stability (Tables 2, 3) observed upon changing Phe 10.

In eukaryotic cytochromes *c*, three helices contact each other to form a portion of the protein core. The N- and C-terminal helices contact each other at a 90° angle and the sixties helix packs against the C-terminal helix at a 60° angle (Kinemage 1). The N-terminal helix extends from Ser 2 to Cys 14, the sixties helix from Asp 60 to Thr 69, and the C-terminal helix from Lys 87 to the C-terminus, Glu 103 (Louie et al., 1988; Gao et al., 1990). From a comparison of eukaryotic cytochromes *c* to bacterial cytochrome *c*-551, Chothia and Lesk (1985) showed that substitutions at the helix–helix interfaces cause their reorientation. As predicted, residues at the interfaces exhibit significant changes in paramagnetic shift upon changing Phe 10 to methionine (Table 1). Chothia and Lesk also suggested that the reorientation of the helices between the prokaryotic and eukaryotic proteins is caused by replacement of residues that pack against the propionic acid side chains of the heme, residues 34–59 of eukaryotic cytochromes *c*. This region of the protein also exhibits signif-

icant changes in paramagnetic shift, lending support to this suggestion.

The side chain of Leu 15 is on the protein surface (Kinemage 1; Berghuis & Brayer, 1992). Its amide proton donates a hydrogen bond to the carbonyl of Phe 10 forming the C-cap of the N-terminal helix. The sign of Δ_{para} for the protons of Leu 15 suggests a movement of its side chain toward the heme upon changing Phe 10 to methionine. This local repacking is probably the result of introducing a smaller side chain at position 10 (Table 4; Fig. 1). Eriksson et al. (1992) have noted similar responses to cavity-creating mutations.

For certain protons, removing the aromatic ring of Phe 10 affects their chemical shift but not Δ_{para} . For instance, in the C102T variant, the side chain of Val 20 is approximately 4.0 Å from the aromatic ring of Phe 10. Removal of the aromatic ring yields large changes in chemical shift for all the side-chain proton resonances of Val 20, but none of these protons are captured by the screen used to construct Table 1, suggesting that only slight repacking has occurred.

In summary, changing Phe 10 to methionine does not upset the global fold of the protein, but small local changes do occur. These include a general weakening of the hydrophobic core brought about by adjustment of the N-terminal helix, the C-terminal helix, the sixties helix, and the region that contacts the heme propionates. Modeling (Fig. 1) suggests a reason for the repacking. Comparison of NOEs indicates that the interaction of the terminal helices remains intact and, specifically, the side chain of Met 10 interacts with the aromatic moiety of Tyr 97. All these data are in accord with the idea that the native fold determines side-chain packing, but different side-chain combinations are compatible with the fold (Behe et al., 1991; Lattman & Rose, 1993).

Comparing the structure of the F10M;C102T and F10C_{SMc};C102T variants

Although it is not possible to obtain paramagnetic shifts for the F10C_{SMc};C102T variant, comparisons of chemical shifts and NOEs between the oxidized forms of the three proteins suggest that the sulfur-containing vari-

ants have similar structures. First, comparison of Figures 2 and 3, along with the observation that large changes in chemical shifts are correlated with significant changes in paramagnetic shift, suggests strongly that the F10M;C102T and the F10C_{SMe};C102T variants possess nearly identical structures. Statistical analysis of chemical-shift data also shows that the F10M;C102T and F10C_{SMe};C102T variants are more similar to each other than they are to the C102T variant. Evidence that the N- and C-terminal helices are closely packed in these variants is obtained from examination of NOESY data (Fig. 4). Furthermore, the resonances of the Met 10 and Cys_{SMe} 10 ϵ -methyl protons are shifted downfield by 0.25 ± 0.03 ppm relative to the chemical shifts of model compounds (Bundi & Wüthrich, 1979; A.J. Saunders & G.J. Pielak, unpubl. obs.), suggesting that these groups are near the edge of an aromatic ring (Johnson & Bovey, 1958; Perkins, 1982; Wüthrich, 1986). Analysis of NOE and scalar coupling data shows that the aromatic ring belongs to Tyr 97. These observations and the fact that the sulfur-containing side chains are closer to the ring of Tyr 97 than is expected from examination of the crystal structure of the C102T variant are all consistent with the geometry of weakly polar interactions observed in proteins (Reid et al., 1985).

In summary, compared to the C102T protein, the F10M;C102T and F10C_{SMe};C102T variants exhibit changes in local structure and a change in the flip rate of Tyr 97. However, the near chemical-shift identity of the oxidized F10M;C102T and F10C_{SMe};C102T variants and the observation of similar NOEs for these two variants suggest that their structures are essentially identical. Further analysis of NOE data shows that the sulfur-containing side chains at position 10 are in close proximity to the aromatic side chain at position 97, analysis of chemical-shift data suggest they are well poised for a weakly polar interaction. Furthermore, modeling (Fig. 1) shows that both sulfurs of Cys_{SMe} 10 can interact with the side chain of Tyr 97.

Thermodynamic comparison of the C102T variant to the F10M;C102T and F10C_{SMe};C102T variants

Previous studies show that an aromatic–aromatic interaction is not required to produce a functional cytochrome *c* (Hampsey et al., 1986; Auld & Pielak, 1991; Fredericks & Pielak, 1993). Permutation of Phe 10 to Met and Cys_{SMe} should replace one weakly polar interaction with another. Examination of the thermodynamic data (Table 2) reveals that replacement of Phe 10 results in a 40–50% reduction in $\Delta G_{d,H_2O}$.

Clearly, it is not possible to simply replace this weakly polar aromatic–aromatic interaction with a weakly polar sulfur–aromatic interaction. In an attempt to explain the decreased stability, we considered differences in the hydrophobicity and steric interactions brought about by the

substitutions. For the discussion that follows it is important to note that the side chain of Phe 10 is buried and the side chains of Met 10 and Cys_{SMe} 10 appear to be buried within the helix–helix interface.

Assuming that hydrophobicity alone controls stability, the differences in $\Delta G_{tr, chx \rightarrow H_2O}$ suggest that the F10M;C102T variant should be 0.7 ± 0.06 kcal mol^{−1} less stable than the C102T variant, and that the F10C_{SMe};C102T variant should be as stable as the C102T variant. These differences in $\Delta G_{tr, chx \rightarrow H_2O}$, however, do not account for the decrease in $\Delta G_{d,H_2O}$. That is, the destabilization accompanying replacement of Phe 10 is not due solely to changes in hydrophobicity.

The fact that these simplifications underestimate the observed stability decreases suggests that other phenomena are also important. For instance, reorientations consistent with modeling and seen by NMR suggest that residues repack in response to the introduction of a smaller side chain at position 10 and this repacking may also alter the solvent exposure. Furthermore, data for the side-chain models do not contain information about all the entropy changes that accompany protein denaturation (Creighton, 1991).

Thermodynamic comparison of the F10M;C102T and F10C_{SMe};C102T variants

Analysis of chemical denaturation data (Table 2; Fig. 5) shows that the F10C_{SMe};C102T variant is 0.7 ± 0.3 kcal mol^{−1} more stable than the F10M;C102T variant at 300 K. Compared to the F10M;C102T variant, the F10C_{SMe};C102T variant also exhibits an increased T_m over a range of conditions (Table 3). Because the structures of the F10M;C102T and F10C_{SMe};C102T variants appear to be identical, comparison of the variants containing one or two sulfur atoms at position 10 allows evaluation of the potential weakly polar interaction involving the γ -sulfur of Cys_{SMe} and the ring of Tyr 97.

Analysis of the NMR data shows that the ϵ -methyl group of the residue at position 10, in both the F10M;C102T and F10C_{SMe};C102T variants, is in position to form a weakly polar interaction. Molecular modeling (Fig. 1) and NOESY data suggest that both sulfurs of *S*-methyl cysteine interact with the aromatic protons of Tyr 97. To investigate the extent to which the difference in stability can be attributed to a weakly polar sulfur–aromatic interaction rather than differences in hydrophobicity between the sulfur-containing side chains, we examined $\Delta G_{tr, chx \rightarrow H_2O}$. The difference in $\Delta G_{tr, chx \rightarrow H_2O}$ values (0.7 ± 0.06 kcal mol^{−1}; Table 4) completely accounts for the increased stability of the F10C_{SMe};C102T variant over the F10M;C102T variant.

Conclusions

A variety of studies have shown that naturally occurring weakly polar interactions stabilize proteins. Replacement

of Phe 10 with sulfur-containing residues causes substantial destabilization, yet produces biologically active cytochromes *c*. The destabilization can be partially explained by the difference in hydrophobicity between phenylalanine, methionine, and *S*-methyl cysteine. Factors that may account for the residual destabilization include: (1) removal of a favorable interaction between the rings of Phe 10 and of Tyr 97; (2) removal of favorable interactions between Phe 10 and its neighbors (e.g., cavity formation; Lee, 1993); (3) introduction of unfavorable interactions between the side chain of Met 10 or Cys_{SMc} 10 and the rest of the protein; and (4) changes in the entropy of denaturation that are not reflected in data for model compounds. Because of the local repacking of the interface between the N- and C-terminal helices, the data say nothing about the possibility of a weakly polar interaction between the position 10 δ -sulfur and the ring of Tyr 97. Modeling suggests a reason for the repacking, the sulfur-containing side chains at position 10 cannot occupy the same space as the benzoid ring of Phe 10. More detailed information about the structure and thermodynamic stability of the variants is required to dissect these factors.

Analysis of NMR data shows that the F10M;C102T and F10C_{SMc};C102T variants are nearly isostructural, that the sulfur-containing residues at position 10 are in close proximity to Tyr 97, and that these residues are well poised for a weakly polar interaction. Modeling indicates that both sulfur atoms of *S*-methyl cysteine can interact with the aromatic protons of Tyr 97. Therefore, replacement of Phe 10 with methionine or *S*-methyl cysteine allows evaluation of a notional sulfur–aromatic interaction between the γ -sulfur of Cys_{SMc} and the aromatic ring of Tyr 97.

The F10C_{SMc};C102T variant is 0.7 ± 0.3 kcal mol⁻¹ more stable than the F10M;C102T variant. Differences in hydrophobicity between Met and Cys_{SMc} completely account for the increased stability. A similar conclusion, that the interaction of two aromatic side chains in barnase is no stronger than is expected from their burial, was reported by Serrano et al. (1991). Our observation suggests that a weakly polar interaction between the γ -sulfur and Tyr 97 either is too weak to detect or is masked by compensating factors. A possible explanation for the weakness of this interaction comes from studies of model compounds. Comparison of $\Delta G_{tr, chx \rightarrow H_2O}$ values for models containing zero, one, and two sulfur atoms suggests that the additional sulfur of *S*-methyl cysteine is not as polar as the lone sulfur of methionine.

Materials and methods

Protein purification and modification

The F10M;C102T and F10C;C102T mutants were produced as described by Auld and Pielak (1991). Cyto-

chromes *c* were isolated based on the method of Mauk and Mauk (1989). The free-sulfhydryl present in the F10C;C102T protein was *S*-methylated using methyl methanethiosulfonate (Ramdas et al., 1986). The number of free sulfhydryls was determined by titration with 5,5'-dithiobis-(2-nitrobenzoic) acid (Habeeb, 1972). Ferricytochromes *c* were prepared as described by Betz and Pielak (1992).

NMR

The NMR experiments were performed at 2–4 mM protein in H₂O:D₂O (9:1), pH 7.2, and 300 K using a Bruker AMX 500 spectrometer with two-channel quadrature detection in t_2 and time-proportional phase incrementation (Marion & Wüthrich, 1983) in t_1 . Data sets consisted of 512 t_1 increments of 32–64 transients and 2K data points in t_2 . The spectral width was $\pm 4,032$ Hz in both dimensions. Data were processed on a Silicon Graphics Personal Iris using Felix 2.0 (Hare Software, Woodinville, Washington). Resolution enhancement was performed by a combination of trapezoidal and sinebell functions in t_1 and t_2 . The final digital resolution was 4.0 Hz point⁻¹. NOESY data were acquired with a mixing time of 133 ms. The error for determining chemical shifts was ± 0.01 ppm.

Denaturation

Chemical and thermal denaturations of ferricytochromes *c* were performed as described by Betz and Pielak (1992). Chemical denaturation data (fluorescence versus GdnHCl concentration) were fitted to a two-state model assuming a linear relationship between ΔG and GdnHCl concentration (Schellman, 1978). Thermal denaturation data (absorbance versus temperature) were fitted to a two-state model. Both procedures utilized a nonlinear least-squares approach assuming linear pre- and posttransition baselines (Shortle et al., 1988; Santoro & Bolen, 1988; Hughson & Baldwin, 1989). The GdnHCl denaturation studies of the C102T, F10M;C102T, and F10C_{SMc};C102T variants were performed in 50 mM sodium phosphate, pH 7.2, at 300 K. Characterization of the F10C;C102T variant did not prove possible because it denatures irreversibly, presumably because of its free sulfhydryl group. All other denaturation data regress to χ^2 values well within the 1% confidence limit (Havlicek & Crain, 1988). The values of ΔH_m , T_m , m_{GdnHCl} , C_m , and $\Delta G_{d,H_2O}$ were also calculated by hand as described by Pace et al. (1989). Manual fitting and nonlinear regression yielded identical results. Errors in m_{GdnHCl} , C_m , $\Delta G_{d,H_2O}$, T_m , and ΔH_m are estimated to be ± 0.25 kcal mol⁻¹ M⁻¹, ± 0.05 M, ± 0.3 kcal mol⁻¹, ± 0.1 °C, and ± 2 kcal mol⁻¹, respectively.

Surface area calculation and modeling

The molecular surface areas and solvent-accessible surface areas (Richards, 1977) for the side chains of phenylala-

nine, methionine, and S-methyl cysteine were calculated using the program GEPOL92 (Silla et al., 1991; Pascual-Ahuir et al., 1992) as described by Tuñón et al. (1992). The side chains were modeled by toluene and the extended forms of butane, ethyl methyl sulfide, and methyl disulfide. Bond angles and lengths are from Peterson et al. (1960) and Kennard (1973). Van der Waals radii are from Bondi (1964). Using the van der Waals radii from Lee and Richards (1971) changes our results by a maximum of 6% (data not shown). A probe radius of 1.4 Å was used. Modeling was performed using the IRIS workstation (see above) using InsightII (Biosym Technologies, San Diego, California).

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