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Proton Nuclear Magnetic Resonance as a Probe of Differences in Structure between the C102T and F82S,C102T Variants of Iso-1-cytochrome *c* from the Yeast *Saccharomyces cerevisiae*†

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ABSTRACT: Differences in chemical shifts and in nuclear Overhauser effects between the C102T and F82S,C102T variants of *Saccharomyces cerevisiae* iso-1-cytochrome *c* in both the reduced and oxidized forms are reported and analyzed. There is evidence for small conformational differences in both oxidation states of the double variant near position 82. Differences in structure are more evident in the oxidized forms of the variants. These differences extend to distant parts of the protein. It is concluded that the oxidized double variant has undergone a small rearrangement of several regions of the protein that are linked by a hydrogen-bond network. It is shown that the rearrangement involves hydrogen bonds associated with the two heme propionates and associated water molecules. The deductions from nuclear magnetic resonance data are compared with the differences in the crystal structures of the reduced forms of wild-type protein and the F82S variant [Louie, G. V., Pielak, G. J., Smith, M., & Brayer, G. D. (1988) *Biochemistry 27*, 7870-7876].

Studies of unnatural variants of cytochromes *c* have allowed existing ideas about the relationships between the sequence, structure, and function of this important class of electron transfer proteins to be tested and new ideas about the relationships to be proposed [for a review see Mauk (1991)]. Data from functional studies of variants can only be fully interpreted, however, with prior knowledge of the way changes in primary sequence affect tertiary structure and dynamics. One of the most well-studied variants of cytochrome *c* from both functional and structural standpoints is the F82S variant of *Saccharomyces cerevisiae* iso-1-cytochrome *c*. It has been found that this substitution reduces the reduction potential (Pielak et al., 1985; Rafferty et al., 1990) and stability of the protein (Pielak et al., 1987; Y. Gao, G. J. Pielak, J. Boyd, and R. J. P. Williams, manuscript in preparation) and alters the electron transfer properties [Liang et al., 1987, 1988; Rafferty

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et al., 1990; but see Concar et al. (1991)]. In this paper the structures of the C102T and the F82S,C102T variants are compared in both oxidation states by using proton nuclear magnetic resonance spectroscopy (NMR).[1]

Nearly complete proton assignments for the C102T variant have been reported in both oxidation states (Gao et al., 1990).[2] Comparison of the NMR spectra of oxidized with reduced states for horse (Feng et al., 1990a), tuna (Williams et al., 1985), and yeast iso-1-cytochromes *c* (Gao et al., 1991) has shown that there are many small changes from residue 40 to residue 85. This conclusion is in general agreement with the studies of Takano and Dickerson (1981a,b), who compared the crystal structures of the reduced and oxidized forms of tuna cytochrome *c*. The results from the crystallographic study of wild-type iso-1-cytochrome *c* (Louie et al., 1988a; Louie & Brayer, 1990) and the NMR studies of the C102T variant are in excellent agreement (Pielak et al., 1988; Gao et al., 1990, 1991). This gives a firm basis for the comparison of the C102T variant with the F82S,C102T variant.

For most proteins the determination of structure by NMR depends heavily on the nuclear Overhauser effect (NOE). However, cytochrome *c* possesses two attributes that allow chemical shift data to be used to an equal extent. First, the covalently bound aromatic heme moiety gives rise to large ring-current shifts. Second, the protein can exist in two different oxidation states. In the reduced form, the Fe(II) is diamagnetic (low spin, d^6), while in the paramagnetic oxidized form, the Fe(III) possesses one unpaired electron (i.e., low spin, d^5). Because the difference in structure between reduced and oxidized cytochrome *c* is small (Takano & Dickerson, 1981a,b; Williams et al., 1985; Feng et al., 1990a; Gao et al., 1991), the anisotropy of the paramagnetism allows the calculation of chemical shifts on the basis of the position of nuclei with respect to the unpaired electron. Differences between calculated and observed shifts can be interpreted in terms of changes in structure.

In this report NMR data are utilized to compare the structure of two variant forms of *S. cerevisiae* iso-1-cytochrome *c* that differ only at position 82. First, changes in the chemical shifts of heme and amino acid protons upon substitution of serine for phenylalanine at position 82 are analyzed in terms of ring-current, contact, and pseudocontact shifts. Second, changes in NOEs between the two variants are discussed in terms of structural changes. The conclusions are compared to those from the crystallographic study of the reduced form of the F82S variant (Louie et al., 1988b).

Materials and Methods

IUPAC–IUB nomenclature for amino acids (IUPAC–IUB Commission on Biochemical Nomenclature, 1970; IUPAC–IUB Joint Commission on Biochemical Nomenclature, 1985) and the heme (Commission on the Nomenclature of Biological Chemistry, 1960) is used throughout this article. The use of numeral superscripts to denote substituent groups of the heme was suggested by Bonnett (1978), and the use of letter superscripts to specify protons on the propionic acid side chains is based on Figure 2 of Chau et al. (1990).

NMR data were collected at pH 7 and 27 °C and interpreted exactly as described by Gao et al. (1990, 1991). The full set of assignments for the double variant is available as supplementary material. Differences in chemical shift are defined as the shift in the C102T variant minus that in the F28S,C102T variant. Paramagnetic shift is defined as the chemical shift in the oxidized protein minus that in the reduced protein and is referred to by the symbol δ_{obs}. Chemical shifts have an error or ±0.01 ppm. Therefore, the experimental error for differences between chemical shifts of identical protons in the two proteins is ±0.02 ppm and the error for differences in paramagnetic shifts is ±0.04 ppm.

Table I: Calculated g-Tensors for the C102T and F82S,C102T Variants

	g_{ax}	g_{eq}	α (deg)	β (deg)	γ (deg)
C102T	3.03	−2.32	347	−10	19
F82S,C102T	3.13	−1.88	332	−10	30

Pseudocontact shift arises because of charge–dipole interactions between the unpaired electron and the various protons. Equation 1 describes the pseudocontact shift for cytochrome *c* (Kurland & McGarvey, 1970):

$$\delta_{calc} = \frac{\beta^2 S(S+1)}{9kTr^3}[g_{ax}(3\cos^2\theta - 1) + 1.5g_{eq}\sin^2\theta\cos^2 2\phi] \quad (1)$$

where $g_{ax} = g_z^2 - {}^1/_2(g_x^2 + g_y^2)$ and $g_{eq} = g_x^2 - g_y^2$. g_x, g_y, and g_z are the principal components of the g-tensor. The *x*- and *y*-axes are in the heme plane while the *z*-axis is perpendicular to it. The angle θ is measured from the positive *z*-axis. The angle ϕ is measured clockwise in the *x*–*y* plane from the positive *x*-axis as viewed from the positive *z*-axis [see Williams et al. (1985)]. β is the Bohr magneton. The distance from the iron to the proton in question is *r*. Calculation of pseudocontact shifts for the C102T and the F82S,C102T variants were made independently by using the derived proton coordinates from the crystal structure of the reduced wild-type protein (Louie & Brayer, 1990), by the methods described by Gao et al. (1991). The calculated g-tensors for the two proteins are given in Table I. The change is very small.

A major problem in the use of NMR data, and this applies especially to analysis of chemical shifts, is the *quantitative* estimation of errors. This is not a problem of stating the accuracy, which is ±0.01 ppm. The problem lies in placing expectation limits on the calculated values of the pseudocontact shift, given that the susceptibility tensor in eq 1 comes from the experimental data. In other words, error in the calculated shift has to be expressed in terms of uncertainties in *r*, θ, and ϕ simultaneously. This problem has been faced by Feng et al. (1990a), who chose to illustrate the effects of error in δ_{calc} in terms of *r* for given values of θ and ϕ.

A different procedure is followed here. First, the square of the difference between δ_{obs} and δ_{calc} is examined because the minimization of the sum of the squared differences is used to drive the optimization of the g-tensor magnitude and orientation. For individual resonances (after optimization), the differences of the squares is given by $\Delta^2 = (\delta_{obs} - \delta_{calc})^2$. However, Δ^2 will yield a false impression when the absolute value of δ is small, no matter how such small differences arise (i.e., due to terms in either the denominator or numerator of eq 1). Therefore an additional screen for matching δ_{calc} and δ_{obs} was added:

$$\% = \frac{|\delta_{obs} - \delta_{calc}|}{\delta_{max}}(100)$$

where δ_{max} is the absolute value of the larger of the two shifts in the numerator. By combining these criteria, differences just

[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect.

[2] The C102T variant is used in place of the true wild-type protein because removal of the sole free cysteine nucleophile renders the variant more amenable to both structural and functional studies yet does not have a measurable effect on structure or function (Cutler et al., 1987; Pielak et al., 1988; Gao et al., 1990, 1991).

Table II: Heme Proton Chemical Shifts of the C102T and F82S,C102T Variants[a]

	reduced		oxidized	
proton(s)	C102T	F82S,C102T	C102T	F82S,C102T
2^1	3.49	3.60	7.80	9.42
3^1	5.23	6.01	−0.8	−0.14
3^2	1.45	1.93	−2.23	−1.43
5	9.33	9.99	nd	4.58
7^1	3.87	3.82	31.58	29.6
8^1	6.37	6.32	2.09	1.83
8^2	2.55	2.43	2.92	2.62
10	9.70	9.68	−0.24	0.03
12^1	3.55	3.53	10.82	11.27
13^{1a}	4.00	4.45	−1.45	−0.90
13^{1b}	4.22	4.22	nd	2.32
13^{2a}	2.68	2.67	2.72	3.12
13^{2b}	2.99	2.99	nd	nd
15	9.61	9.61	nd	7.37
17^{1a}	4.11	4.11	12.93	12.98
17^{1b}	3.60	3.62	14.96	14.62
17^{2a}	3.37	3.38	1.43	1.39
17^{2b}	2.70	2.68	−0.14	−0.12
18^1	2.30	2.30	34.58	32.39
20	9.13	9.22		1.82

[a] nd, assignment missing.

caused by large values of δ_{obs} or δ_{calc} are eliminated, while large effects on small values are retained. Only protons for which Δ^2 is greater than 0.02 and % is greater than 50 will be discussed further. There is only one explanation for cases where the values of Δ^2 and % are both large: the assumptions made in the comparison of δ_{calc} and δ_{obs} are wrong. As mentioned above, instances where Δ^2 is small but % is large could arise from cases where the absolute value of δ is small. On the other hand, large values of Δ^2 paired with small values of % could arise where values of δ_{obs} and δ_{calc} are large. Note that the screen used here is less stringent than the one we used previously (Gao et al., 1991), where differences of chemical shift greater than ±0.1 ppm were considered significant. However, in our previous paper all protons with a calculated pseudocontact shift of greater than 1.0 ppm were ignored. The introduction of % allows evaluation of protons possessing large pseudocontact shifts.

Discrepancies between the calculated pseudocontact shift and observed paramagnetic shift give information concerning regions where the solution structure of each of the two variants differs from the crystal structure of the reduced wild-type protein. To compare more directly the solution structures of the C102T and F82S,C102T variants, the parameter Δ_{F82S} was examined, where Δ_{F82S} is defined as

$$\Delta_{F82S} = (\delta_{calc} - \delta_{obs})_{C102T} - (\delta_{calc} - \delta_{obs})_{F82S,C102T}$$

If there is no discrepancy between the calculated and observed data for the two variants, or if the discrepancy is the same for the two proteins, Δ_{F82S} is zero. To evaluate significance, both Δ^2 and % were calculated for each value of Δ_{F82S} with the same limits described above.

Results

Differences in the Chemical Shift of Heme Protons. A list of chemical shifts of heme protons for both variant proteins in the reduced and oxidized states is given in Table II. These assignments are in excellent agreement with the homologous assignments for the horse protein [Feng et al. (1990b) and references therein] and for the acetylated derivative of iso-1-cytochrome c (Busse et al., 1990).

The major determinants of secondary shift for heme protons in the reduced protein are ring-current and other diamagnetic as well as electrostatic fields (e.g., hydrogen bonds; Perkins, 1982) brought about by nearby amino acid residues. Changes in the ring-current field experienced by heme protons in the double variant may arise from reorientation or replacement of aromatic amino acid residues with respect to the heme. Differences in chemical shifts of heme protons in the reduced proteins greater than 0.1 ppm are observed for protons 2^1, 3^1, 3^2, 5, 8^2, and 13^{1a} (Table II). The first five resonances are all near the side chain of Phe-82. The downfield shifts of 2^1, 3^1, 3^2, and 5 and the upfield shift of 8^2 in the F82S variant are readily explained by the replacement of the aromatic moiety at position 82 by serine with the concomitant loss of ring-current shift. It is concluded that these changes in chemical shift are direct effects of the substitution. On the other hand, the 13^{1a} proton is well removed from the region of substitution and must represent a remote effect, presumably a small conformational change. As discussed below, this small change occurs in the region of the protein extending from Ser-82 to the heme propionates and is transmitted through the network of hydrogen bonds extending from residues 78–80 to the heme propionates.

In the oxidized protein, proton chemical shift is controlled not only by ring-current effects but also by interactions of the unpaired electron with protons. These interactions give rise to contact and pseudocontact shifts. The latter are discussed under Materials and Methods. Contact shift is restricted to protons that are bound to the heme (i.e., heme and ligand protons), and the theory has been described by Wüthrich (1970).

Protons 3^1, 3^2, 8^2, and 10 exhibit similar changes in chemical shift of greater than 0.1 ppm in the oxidized and in the reduced protein. This approximately equal change in shift observed for both the reduced and oxidized forms of the double variant occurs near the site of the substitution at position 82 and is again suggestive of an alteration in the ring-current shift. Protons of the oxidized heme with large contact shifts in both variants are likely to be sensitive to small changes in structure. Therefore changes noted at 2^1, 7^1, 12^1, 17^{1b}, and 18^1 are taken to indicate that there is a very small change in the **g**-tensor of the iron. This conclusion is confirmed by the data in Table I. It is not possible to interpret these changes in contact shift in detail.

Comparison of Chemical Shifts of Amino Acid Protons within the Reduced Variants. For the vast majority of protons, differences in chemical shifts between the reduced states of the two variants are less than ±0.1 ppm. Residues possessing protons whose chemical shifts differ by ±0.1 ppm or greater are located on the structure of iso-1-cytochrome c (Louie & Brayer, 1990) in Figure 1. (A full listing of differences in chemical shift greater than ±0.1 ppm for both the reduced and oxidized proteins is available as supplementary material.) Changes in chemical shift between homologous protons of the two variants may be brought about directly, through a change in diamagnetism resulting from the substitution of phenylalanine by serine at position 82, and indirectly, by rearrangements brought about by reorganization of other diamagnetic fields. The observed changes are associated with the top left- and right-hand sides of Figure 1 and extend down the left side, leaving residues 19–50 unchanged.

Comparison of Chemical Shift of Amino Acid Protons within the Oxidized Variants. Many of the residues exhibiting differences of greater than or equal to ±0.1 ppm in chemical shift between the oxidized proteins (Figure 2) are also those observed for the reduced proteins (Figure 1). However, there are a greater number of protons in the oxidized proteins that exhibit significant differences. This could be caused by more

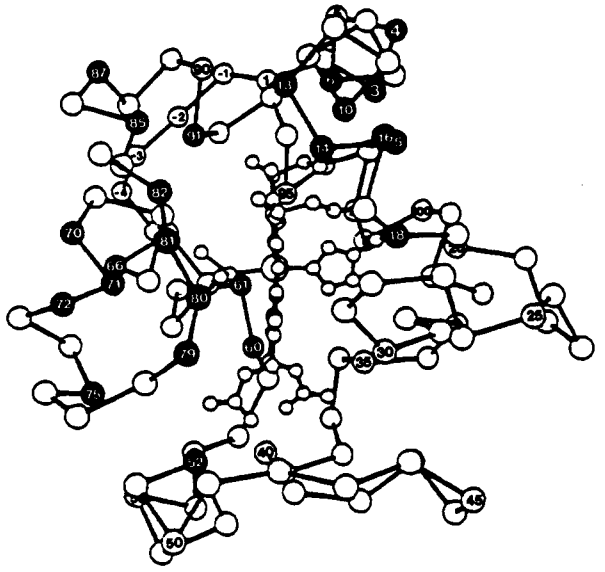

FIGURE 1: Residues with chemical shift differences of greater than or equal to ±0.1 ppm for the reduced variants (filled circles) superimposed on the backbone of reduced iso-1-cytochrome *c* [adapted from Louie and Brayer (1990)].

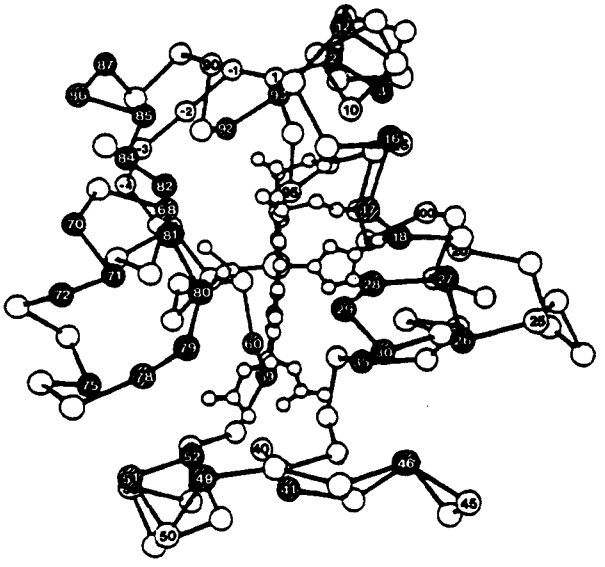

FIGURE 2: Residues with chemical shift differences of greater than or equal to ±0.1 ppm for the oxidized variants superimposed on the backbone of reduced iso-1-cytochrome *c* [adapted from Louie and Brayer (1990)]. Differences unique to the oxidized protein are striped.

severe structural changes in this oxidation state. However, chemical shift in the oxidized proteins is controlled by an additional factor, the influence of the unpaired electron.

Calculated Pseudocontact Shift and Observed Paramagnetic Shift. We have previously published the analysis of the paramagnetic shifts for the C102T protein, using both experimental and calculated data to assess changes (Gao et al., 1991). Calculation of paramagnetic shift was done independently by using the experimental paramagnetic shifts from the two variants and calculated pseudocontact shifts derived from the coordinates of the reduced wild-type protein kindly provided by Gary D. Brayer (Louie & Brayer, 1990). Differences between the calculated and observed values captured by the screen (described under Materials and Methods) are given in Table III. As can be seen by comparison of the first and second columns in Table III, protons from residues 2, 15, 16, 29, 35, 36, 39, 41–43, 51–53, 55–57, 59, 60, 63, 65, 68, 70, 78, and 79 exhibit significant values of Δ^2 and % in *both* proteins. The data set for the C102T variant agrees with that for the F82S,C102T variants in that the same regions of the protein are affected. The observation of redox-state changes for the residues listed above is in excellent agreement with previous studies of the C102T variant as well as tuna and horse cytochromes *c* (Gao et al., 1991; Williams et al., 1985; Feng et al., 1990a).

Comparison of Calculated Pseudocontact Shift and Observed Paramagnetic Shift for the Two Variants. Changes in ring-current fields should affect equally chemical shifts in both the reduced and oxidized forms of the proteins because only small conformational differences occur between the two oxidation states of either variant. That is, changes in ring-current shifts are, to a first approximation, not present in paramagnetic shift data. Therefore, analysis of changes in paramagnetic shift between the two proteins will be especially informative. Furthermore, only small conformational changes are observed between the reduced forms of the variants and these small changes are mostly near the site of the substitution. This simplifies the analysis of the two oxidized proteins via paramagnetic shift differences because the reduced proteins can be used as equally valid and equivalent diamagnetic blanks. (In effect there is a separate diamagnetic blank for each variant.) The reason for using the crystal structure of the reduced wild-type protein as the blank for both proteins is that the conformational changes observed by crystallography when the two reduced variants are compared (Louie et al., 1988a), are not all found in solution from examination of chemical shift data (vide infra).

Values of Δ_{F82S} captured by the screen (right-hand column of Table III) correlate with the chemical shift data for the reduced and oxidized variants (Figures 1 and 2). Significant values of Δ_{F82S} are identified for protons from residues within 5 Å of the protons of Phe-82, including residues 13, 68, 71, 72, 84, and 85. Significant values of Δ_{F82S} are also observed for protons from residues 18, 28–30, 35, 43, 49, 65, 67, 70, 74, 75, 78, 79, and 86, in spite of the fact these protons are all more than 5 Å from the site of the mutation. Protons belonging to His-18 and residues 28–30 exhibit large (>±1 ppm) pseudocontact shifts and are therefore very sensitive to the electronic environment of the heme. These protons are possibly captured by the screen because of the small change in **g**-tensor (Table I).

Differences in NOEs between the Two Variants. There are very few differences in NOEs between the proteins. Differences in NOEs between the reduced forms of the variants are listed in the upper panel of Table IV and are indicative of changes only in the immediate vicinity of residue 82. To illustrate the difficulties in using the NOE data to uncover changes, the NOEs involving the amide protons of residues Gly-29, Leu-68, Lys-79, and Gly-41 in the oxidized form of the two variants are presented in the lower panel of Table IV. These residues were chosen because the amide protons of the first three exhibit some of the largest values of Δ_{F82S}. Gly-41 was chosen because, like Lys-79, its amide proton is directly involved in hydrogen bonds to the heme propionates (Table V). NOEs involving the amide of Gly-41 are unchanged except those to the side-chain amide protons of Asn-52. In the C102T protein NOEs to both these Asn-52 protons are very strong, but in the F82S,C102T protein these NOEs are absent. This absence is not caused by a structural change because the side-chain amide protons of Asn-52 themselves are not seen in the oxidized form of the double variant. This may reflect a faster exchange rate for these protons in the F82S,C102T double variant. This finding reflects a difficulty

Table III: Protons with Values of Δ^2 Greater Than or Equal to 0.02 and Values of % Greater Than or Equal to 50

residue		C102T		F82S,-C102T		Δ_{F82S}		residue		C102T		F82S,-C102T		Δ_{F82S}	
		Δ^2	%	Δ^2	%	Δ^2	%			Δ^2	%	Δ^2	%	Δ^2	%
Ser-2	α			0.06	69			Asn-56	NH	0.05	86	0.03	91		
	β^1	0.05	66	0.09	74				$\delta 2^2$	0.03	120	0.53	93		
	β^2	0.05	61	0.05	62			Val-57	NH	0.03	156	0.03	132		
Phe-10	ϵ^1	0.54	65	nd[a]					α	0.12	87	0.07	86		
	δ^2	0.28	102	nd					β	0.07	80	0.06	84		
Arg-13	α					0.04	99	Leu-58	α			0.02	185		
Cys-14	NH	0.10	67					Trp-59	$\zeta 2$	0.05	53	0.07	71		
Leu-15	β^1			0.12	59				$\epsilon 3$	0.03	69				
	β^2	0.09	67	0.37	58			Asp-60	NH	0.37	134	0.26	142		
Gln-16	β^1	0.07	56	0.05	59			Glu-61	β^2	0.06	114				
	β^2			0.04	61			Asn-63	β^1	0.05	180	0.03	187		
	γ^1	0.05	66						β^2	0.03	145				
His-18	NH					0.09	74		$\delta 2^1$	0.03	81	0.03	86		
	α					0.05	93	Ser-65	β^1			1.82	123		
Val-28	NH	0.18	86						β^2	0.09	148	4.25	109	0.03	288
	α					0.02	106	Glu-66	β^1	0.07	91				
	β					0.16	96		β^2	0.04	63				
	γ^1					0.05	95	Tyr-67	β^2					0.04	108
Gly-29	NH	0.03	71	0.14	71	0.09	62	Leu-68	NH					0.06	141
Pro-30	α	0.05	55						α	0.11	66				
	β^1	0.02	61			0.08	86		γ			0.03	64		
	β^2					0.14	59	Asn-70	β^1	0.03	51				
Leu-32	α			0.49	88				β^2			0.10	62	0.10	99
Ile-35	NH	0.04	135						$\delta 2^1$	0.11	52	ND			
	$\gamma 1^2$			0.72	75			Pro-71	α					0.03	103
	δ					0.26	70		γ^1					0.09	103
Phe-36	α	0.03	56	0.04	57				δ^2	0.29	55				
	β^2			0.04	63			Lys-72	NH					0.07	152
Arg-38	α	0.13	87						β					0.03	103
	β^2	0.02	64					Tyr-74	α	0.22	50				
	ϵ	0.14	94	ND					β^2					0.02	102
His-39	NH	0.08	123	0.07	161			Ile-75	NH					0.04	97
	α	0.13	87	0.08	74			Thr-78	α					0.02	73
	β^1			0.05	163				β					0.05	51
Ser-40	α	0.09	64						$\gamma 1$	0.95	87	0.46	60	0.06	64
Gly-41	NH	1.11	146	0.77	191			Lys-79	NH	0.37	87	0.02	171		
Gln-42	β^1	0.03	67						α	0.11	79	0.02	59	0.04	71
	β^2	0.03	70					Ala-81	NH	0.03	62			0.05	103
	$\epsilon 2^2$	0.03	70	0.02	62				β	0.06	125				
Ala-43	NH	0.16	67	0.10	52			Pos-82	NH	0.04	87				
	β					0.02	93		α	0.31	72				
Thr-49	γ^1			0.05	78	0.15	83	Gly-84	α^1			0.03	57		
Ala-51	NH	0.09	102	0.04	105				α^2			0.08	59	0.09	101
Asn-52	α	0.07	55	0.14	67			Leu-85	NH			0.08	54	0.05	79
	β^2	0.05	85	0.03	58				α			0.29	159		
Ile-53	α	0.14	149	0.17	164			Lys-86	α					0.02	99
	$\gamma 1^1$			0.02	52			Lys-87	β^2			0.24	76		
Lys-55	NH	0.02	194	0.02	176			Glu-103	β^1			0.10	98		
	β^1	0.04	83	0.04	91				β^2			0.13	110		

[a] ND, assignment missing.

in the use of NOE comparisons where changes in relaxation rather than distance could be involved. For Leu-68, the strength of the NOEs to protons from Ser-65, Tyr-67, and Thr-69 are much weaker in the double variant. Also for the amide of Lys-79, NOEs to protons on Thr-49, Thr-78, and Met-80 are much weaker. These diminutions in NOEs may be caused by changes in hydrogen bonding and/or changes in the relaxation of these protons.

Discussion

Reduced Proteins. Many of the significant differences in chemical shifts between homologous protons in the two reduced proteins are in the immediate vicinity of position 82 (Figure 1). Most changes can be attributed to alteration of the ring-current field brought about by the removal of the Phe-82 without invoking major structural reorganization. Although NOE data (Table IV) are indicative of a small altered conformation in the vicinity of Met-80 (Table VI), there are substantially more changes in chemical shift (Figure 1) related to some structure change that NOE measurements do not reveal. Clearly, in this case at least, chemical shifts are more sensitive than NOEs in locating structural changes in solution. However, chemical shifts, in general, are more difficult to interpret.

A comparison of structural changes determined from the difference between the crystal structure of the reduced wild-type protein and that of the crystal structure of reduced F82S variant (Louie et al., 1988a,b) with the changes in chemical shift between the reduced C102T and F82S,C102T variants (i.e., the differences in solution structures) is presented in Table VI. This comparison shows that all crystallographic changes are not reflected in the chemical shift data and that all changes in the shift data are not reflected in changes in crystal coordinates.

Specifically, Louie et al. (1988b) observed reorganization in the immediate vicinity of position 82, in agreement with the NMR data presented here, but they also observed changes in structure near the propionic acids of the heme. These

Table IV: Differences in NOEs between the C102T and F82S,C102T Variants[a]

resonance	cross peak to	cross-peak intensity C102T	cross-peak intensity F82S,C102T
	Reduced		
Met-80 amide	Lys-79 amide	–	w
	Ala-81 amide	vw	–
	Hem-10	w	–
Hem-2^1	Cys-14 α	–	vw
	Leu-32 δ^1, δ^2	m, vw	s, m
	Leu-94 δ^1, δ^2	–, vs	vs, s
	Leu-98 δ^2	m	s
	Hem-3^1	–	w
Hem-3^2	Cys-14 α	w	m
	Leu-68 δ^2	m	s
	Met-80 γ^1, γ^2	–, –	w, w
	Met-80 ϵ	m	vs
	Phe-82 β^1, β^2	–, –	s, m[b]
	Leu-85 δ^1, δ^2	–, w	m, m
	Hem-20	–	w
Hem-12^1	Hem-13^{2b}	vw	–
	Oxidized		
Gly-29 amide	His-18 α	w	–
	Val-28 β	s	–
Gly-41 amide	Asn-52 $\delta 1/\delta 2$	s	–
	His-39 α	w	–
Leu-68 amide	Ser-65 α	m	w
	Tyr-67 β^1	s	w
Leu-68 amide	Thr-69 amide	s	m
	Leu-85 δ^1	w	–
Lys-79 amide	Thr-49 γ^2	s	w
	Thr-78 α, β, γ^1	s, w, s	m, –, –
	Met-80 amide	s	–
	Met-80 α	w	–

[a] –, no NOE; vw, very weak; w, weak; m, medium; s, strong; vs, very strong. [b] With Ser-82 instead of Phe-82.

Table V: Hydrogen-Bond Networks Involving the Heme Propionates in the Reduced Wild-Type Protein[a]

inner		outer	
17^{3a}	17^{3b}	13^{3a}	13^{3b}
Tyr-48 η	Gly-41 amide	Thr-49 $\gamma 1$	Thr-49 amide
Wat-121	Asn-52 $\delta 2$	Thr-78 $\gamma 1$	
Wat-168	Trp-59 $\epsilon 2$	Lys-79 amide	

Wat-121	Wat-168	Wat-166
Hem-17^{3a}	Arg-38 η	Asn-52 $\delta 2$
Arg-38 ϵ		Tyr-67 η
His-39 carbonyl		Thr-78 $\gamma 1$
Gln-42 amide		

[a] Adapted from Louie and Brayer (1990).

changes included movement of Phe-36, Trp-59, and Met-64. Analysis of the NMR data (chemical shifts and NOEs) suggests minimal conformational changes in this region of the reduced protein. NOEs to the side-chain protons of Phe-36, Asn-52, Trp-59, and Met-64 are unchanged between the two proteins in both oxidation states (data not shown), and the chemical shifts of assigned resonances in this region change by less than ±0.1 ppm, with the exception of the chemical shift of one of the propionic acid side chains (Table II). This is not what would be expected from the movements shown by the crystal structure determination. Conversely, several shift changes of considerable magnitude between the two proteins do not relate to changes observed between the two crystal structures. In all, there are 26 residues whose protons exhibit changes in chemical shift upon substitution of serine for phenylalanine at position 82. Significant differences in the chemical shift of amide protons are observed for 12 of these residues, suggesting that many of the differences between the two variants lie in small alterations in hydrogen bonding. In particular, the regions involved lie on the Met-80 side of the heme as shown in Figure 1. An example is the amide proton of Lys-72 that is hydrogen-bonded to an internal water molecule (conserved among rice, tuna, and yeast iso-1-cytochromes *c*) that is itself hydrogen-bonded to the carbonyl of Phe-82 and side-chain amide of Asn-70 (Louie & Brayer, 1990). All the distant hydrogen-bond changes appear to connect with the substitution at position 82 via the heme, its propionates, and Met-80. These findings indicate that the crystal and solution structure differ somewhat.

Oxidized Proteins. Comparison of Figure 2 with Figure 1 shows that more differences in chemical shift are present in the oxidized than in the reduced proteins. These additional changes could reflect additional structural changes, or they may be mainly caused by the greater chemical shift dispersion caused by the paramagnetic nature of the oxidized protein. It is possible to compare the crystal structure of cytochrome *c* to the solution structure by using crystallographic coordinates along with the **g**-tensor values to calculate the pseudocontact shift and then comparing this calculated value to the experimental paramagnetic shift. Discrepancies between calculated and observed shifts for each protein give an indication of where each protein differs from the crystal structure of the reduced wild-type protein. The two different reduced proteins are used as diamagnetic blanks for the calculations of pseudocontact shift. Significant values of Δ_{F82S} therefore reflect a difference between the oxidized forms of the C102T and the F82S,C102T variants in solution. Discussion is limited to the largest differences as defined by the screening procedure outlined under Materials and Methods. Note that there is no way to compare the crystal structures because they are not available for the oxidized proteins.

Table VI: Comparison of Crystallographic Data for the Reduced F82S Variant with NMR Data for the Reduced F82S,C102T Variant

residue	crystallography: change in hydrogen bonding on changing Phe-82 to Ser-82	crystallography: movement	NMR change in chemical shift (ppm)
Arg-13	hydrogen bond formed between guanidinium and Gly-84		α, 0.11
Phe-36		0.5 Å	no change
Asn-52	loss of hydrogen bond to buried water	0.5 Å; water moves 1.4 Å toward Fe	α, 0.18; δ's unassigned in F82S,C102T
Trp-59 $\epsilon 1$	loss of hydrogen bond to heme 17^{3a} oxygen	≈15° rotation about X1; distance between $\epsilon 1$ and heme 17^3 oxygen 0.7 Å	no change
Trp-59 β's, $\zeta 2$, $\zeta 3$		>1 Å	no change
Met-64		sulfur 0.6 Å; ϵ 0.8 Å	β^1, 0.09
Met-80		ϵ, 0.7 Å and becomes accessible to solvent	β^1, 0.1; γ^1, 0.07; ϵ, –0.07
Gly-83		≈0.6 Å	unassigned
Gly-84	see Arg-13		
Leu-85		δ^1, 0.7 Å; δ^2, 1.2 Å; becomes accessible to solvent	α, 0.74; δ^1, 0.19; δ^2, 0.16
Hem-3^2		displaced into heme pocket by 0.8 Å	–0.48

The values of Δ_{F82S} given in Table III map onto the molecule in much the same way as the chemical shift data anomalies for the oxidized proteins (Figure 2). It is concluded first that no major changes occur within the N- and C-terminal helices of the oxidized F82S,C102T variant because significant values of Δ_{F82S} are not observed for any residues belonging to this region with the exception of Ser-2. Second, many of the same residues under the heme–Met-80 side of the protein are affected, as is the case for the reduced F82S,C102T variant, although the effects are both more numerous and often larger. Third, an additional region around residues 28–35 exhibits shift changes. Of the 22 residues in Table III that show significant values of Δ_{F82S}, 10 possess amide or exchangeable protons with significant values of Δ_{F82S}, again demonstrating alteration in hydrogen bonding between the variants. Fourth, exchangeable protons from residues 43, 49, 78, and 79 identified in the Δ_{F82S} screen share a common feature—they are all involved in the hydrogen-bonding network related to the heme propionates (Takano & Dickerson, 1981a,b; Louie et al., 1988a; Louie & Brayer, 1990; Table V).

The extra region of the oxidized protein involved in the structural modification is around residues 28–30. It may be that this region is sensitive due to a greater flexibility in the oxidized state. The region is connected to the heme through close contact at Val-28 and hydrogen bonding from Pro-30 to His-18. Changes within the hydrogen-bonding network of this region are also observed when the oxidized and reduced states of wild-type cytochromes *c* are compared (Takano & Dickerson, 1981a,b; Williams et al., 1985; Feng et al., 1990; Gao et al., 1991). Therefore, the substitution of serine for phenylalanine at position 82 increases the conformational differences between the oxidized and reduced proteins, and this exaggeration occurs mainly in the oxidized form of the F82S,C102T variant. Consideration of the facts that the Met-80 side chain is less constrained, especially in the oxidized state, and that there are remote conformational changes involving the heme propionates leads to the suggestion that the transmission of the conformational changes involves the hydrogen-bonding network linking Thr-78 and Lys-79 to the remote parts of the protein (Table V).

Conclusions

The substitution of serine for Phe-82 appears to be largely a simple replacement of an aliphatic for an aromatic side chain in the reduced form of the F82S,C102T variant with some structural reorganization in the region of the substitution shown in Figure 1. In the oxidized protein there is a more noticeable change in this region (Figure 2) extending from position 82 via the hydrogen-bond network that differentiates the reduced and oxidized proteins. This agrees with the changes of stability of this region of the protein to temperature, substitution, and pH (Pielak et al., 1987; Y. Gao, G. J. Pielak, and R. J. P. Williams, manuscript in preparation).

Analysis of the F82S variant has generated interesting points concerning methods of study and fundamental knowledge concerning structural changes that can occur upon amino acid substitution. The crystallographic approach and the NMR shift probe are in fair but not full agreement. The shift probe approach is more sensitive to changes in hydrogen bonding. The alternative NMR approach using NOEs is obviously not sensitive enough or could even be misleading due to changes in relaxation properties.

Concerning knowledge of the effects of amino acid substitution on the reactions of proteins, the following points are relevant where the electron is considered the substrate for cytochrome *c*: (1) In the substrate-bound (reduced) form, the effects of substitution are small but noticeable even at considerable distances from the binding site, here the iron. (2) In the substrate-free (oxidized) form, the effects of the substitution are larger and at considerable distances from the iron. (3) Both the binding constant for substrate, which here is the redox potential, and the kinetics of electron transfer have been examined for the F82S and F82G substitutions (Pielak et al., 1985; Rafferty et al., 1990; Concar et al., 1991) where a 40-mV shift and only small changes in rate constants are observed. It is obviously not possible to interpret such changes with confidence because there are differential changes in both the oxidation states of the protein.

It will be interesting to see the effects of amino acid substitutions on substrate-free and -bound forms of other proteins when studied in the depth achieved here with a combination of crystallography and NMR. Is it the case that networks of hydrogen bonds are very sensitive to amino acid substitutions in many proteins?

Supplementary Material Available

Three tables, giving the proton assignments for the reduced and oxidized F82S,C102T variant and the differences in chemical shift greater than ±0.1 ppm between the F82S, C102T and C102T proteins in both the reduced and oxidized states (14 pages). Ordering information is given on any current masthead page.

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