Articles

Importance of a Conserved Hydrogen-Bonding Network in Cytochromes *c* to Their Redox Potentials and Stabilities†

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ABSTRACT: To understand the determinants of redox potential and protein stability in *c*-type cytochromes, we have characterized two mutations to a highly conserved tyrosine group, tyrosine-75, of *Rhodobacter capsulatus* cytochrome c_2. Mutant Y75F was designed to test the importance of the tyrosine hydroxyl group to the typically high redox potentials of the cytochromes c_2 while maintaining a hydrophobic core. Mutant Y75C was designed to test the importance of a large hydrophobic group to redox potential by replacing an aromatic group with a small nonpolar group. Both mutants exhibit spectral and redox properties indicating that their heme environments have been perturbed. The kinetics of reduction by lumiflavin semiquinone and photooxidation by *Rhodobacter sphaeroides* photosynthetic reaction centers have been used to demonstrate that both mutants are structurally analogous to the wild-type protein at the active site of electron transfer. Different degrees of relative stability of the mutants toward a denaturant have been observed with the order being Y75C < wt < Y75F in the oxidized state and Y75C < Y75F < wt in the reduced state. These results are discussed in light of the recent structure determination of the *R. capsulatus* wild-type ferrocytochrome c_2 to suggest that *R. capsulatus* tyrosine-75, or its equivalent in other species, is part of a conserved hydrogen-bonding network which plays an important role in maintaining high redox potentials and protein stability of cytochromes *c* in general.

In biological electron-transfer reactions, differences between the redox potentials of reacting proteins provide the driving force or free energy for the reaction. Among electron-transfer proteins, the cytochromes *c* are a group of ubiquitous, well-studied electron-transfer proteins found in prokaryotic and eukaryotic organisms alike (Meyer & Kamen, 1982). The determinants of heme protein redox potentials are especially interesting due to their large diversity of over 800 mV or 17 kcal/mol (Churg & Warshel, 1986). Among the factors which appear to be responsible for the observed diversity in redox potentials of cytochromes *c* are the nature of the ligating groups to the heme (Xavier et al., 1981) and the microenvironment of the heme (Kassner, 1972). In the case of the class I *c*-type cytochromes which possess the same heme ligands, the heme environment as provided by the protein moiety is responsible for over 470 mV of the observed variation (Cusanovich et al., 1988).

The cytochromes c_2 are a subclass of the class I *c*-type cytochromes which exhibit a high degree of structural homology to eukaryotic cytochromes *c* although their sequence homology is typically less than 40% (Salemme et al., 1973a; Ambler et al., 1979). The cytochromes c_2 generally function as soluble electron carriers between membrane-bound redox centers under photosynthetic (Dutton & Jackson, 1972) and respiratory growth conditions (Beccarini-Melandri et al., 1978). The high-resolution structures of cytochromes c_2 from *Rhodospirillum rubrum* and *Paracoccus denitrificans* have been determined (Salemme et al., 1973a; Bhatia, 1980; Timkovich & Dickerson, 1976), and the structures from *Rhodobacter capsulatus* (Holden et al., 1987), *Rhodobacter sphaeroides* (Allen, 1988), and *Rhodopseudomonas virdis* (Miki et al., 1986) are currently under study by X-ray crystallography. In addition, the structures of cytochromes *c* from horse, tuna, rice, and yeast have previously been determined (Dickerson et al., 1971; Takano et al., 1973; Oichi et al., 1983; Louie et al., 1988). Taken together, these structures provide detailed molecular information on the cytochromes *c* in general which make them attractive candidates for structural and functional studies.

Interestingly, the cytochromes c_2 generally exhibit higher and more variable redox potentials than the analogous eukaryotic cytochromes *c* (Pettigrew et al., 1978). For example, the cytochromes c_2 from *P. denitrificans*, *R. capsulatus*, and *Rhodopila globiformis* display redox potentials of +250, +370, and +470 mV, respectively (Kamen & Vernon, 1955; Pettigrew et al., 1978; Meyer et al., 1983). On the other hand, the cytochromes *c* from yeast, tuna, and horse possess redox potentials of +260 mV (Pettigrew et al., 1975a). Initial comparisons of the cytochrome structures from *R. rubrum*, horse, and tuna suggested that the differences in redox potentials are the result of a more hydrophobic heme environment in *R. rubrum* cytochrome c_2, due to the absence of a specific bound water molecule found in the heme environment of the horse and tuna cytochromes *c* (Salemme et al., 1973b). According to this model, the oxidized state of cytochrome c_2 is more destabilized with respect to cytochrome *c*, and hence the redox potential is raised. In *R. rubrum* cytochrome c_2, the oxidized state has been proposed to be partially stabilized by an electrostatic interaction between an evolutionarily conserved

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tyrosine hydroxyl group and the sulfur atom of the heme ligating methionine (Salemme et al., 1973b). Interestingly, the only known naturally occurring substitution of the tyrosine is a phenylalanine substitution found in *Euglena* mitochondrial cytochrome *c* which displays a somewhat low redox potential of 244 mV (Pettigrew et al., 1975b).

To understand the role of this highly conserved tyrosine (tyrosine-75 in *R. capsulatus* cytochrome c_2), we have employed a genetic system to generate site-directed mutants of *R. capsulatus* cytochrome c_2 (Caffrey, 1991). In this system, mutant cytochrome genes harbored on a plasmid with broad-host specificity (Ditta et al., 1982) are introduced into a *R. capsulatus* strain which lacks a functional chromosomal copy of the gene for cytochrome c_2 (Daldal et al., 1986). Mutant Y75F,[1] which removes the hydroxyl group of tyrosine-75 without removing the hydrophobic core, was chosen to test the importance of the proposed hydroxyl–sulfur electrostatic interaction in stabilizing the oxidized state of *R. capsulatus* cytochrome c_2. In addition, the importance of a large hydrophobic core was tested by generation of mutant Y75C which replaces the aromatic group with a small nonpolar group. In what follows, the effects of these mutations on the *R. capsulatus* cytochrome c_2 heme environment, redox potential, structural integrity at the active site, and relative protein stability to denaturant are described. These results, in light of the recent structural information on *R. capsulatus* ferrocytochrome c_2, demonstrate that the high redox potentials of cytochromes c_2 are not due to the absence of a water molecule found in eukaryotic cytochromes *c* and that the determinants of redox potential are much more complex. Further, it appears that *R. capsulatus* tyrosine-75 participates in a evolutionarily conserved hydrogen-bonding network which is important to the stability of the reduced cytochrome state.

Materials and Methods

Protein Preparations. Site-directed mutagenesis and genetic manipulations to produce mutant cytochromes Y75C and Y75F in *R. capsulatus* strains are described elsewhere (Caffrey, 1991). *R. capsulatus* strains were grown under photosynthetic conditions in 1-L bottles under saturating light, or under respiratory growth conditions in a 16-L fermentor (Virtis Model 43-100). All cultures were grown on RCVB medium (Weaver et al., 1975). For respiratory growth, the 16-L fermentor culture was supplemented with 150 mg of thiamine hydrochloride, 2.3 mg of biotin, and approximately 60 mL/h of an acidic nutrient solution that contained malic acid (1.5 M), ammonium hydroxide (0.35 M), potassium phosphate (0.33 M), magnesium sulfate (30 mM), calcium chloride (17 mM), iron sulfate (1.5 mM), 2.5% trace metal solution (Weaver et al., 1975) to maintain a constant pH during growth and silicone defoamer (Mazer Chemicals) as needed. When required, cultures were also supplemented with 2.5 μg/mL tetracycline. For photosynthetically grown cultures, wet cell yields were approximately 5 g/L; for aerobically grown cells, wet cell yields were approximately 40 g/L. Cell extracts were prepared by a French press as in Bartsch (1971) or by sonication (5 × 1 min on ice, Heat Systems–Ultrasonics, Inc., cell disruptor Model W-220F with a Heat Systems–Ultrasonics, Inc., probe Model H-1). Wild-type and mutant cytochromes were purified as in Bartsch (1971) except that some extracts were first chromatographed on Bio-Rex 70 50–100-mesh resin (Bio-Rad). In preparations using this column, extracts were first acidified to pH 4.8 in 5 mM acetic acid before application to the column which was preequilibrated in 2 mM acetic acid, pH 4.8. The column was then washed with double-distilled H_2O, and samples were eluted with 1 M K_2HPO_4. Final protein purity indexes ($A_{276\text{-ox}}/A_{416\text{-red}}$) were 0.18 for wild type and mutants. Heme type and extinction coefficients at 550 nm were determined by the alkaline pyridine ferrohemochrome method (Bartsch, 1971). These analyses demonstrated that both mutants contained *c*-type heme as expected, with difference extinction coefficients in the reduced α region that were identical with those for wild-type cytochrome c_2 (19 mM^{-1} cm^{-1}). Molecular weights and isoelectric points of the cytochromes were determined by using the Pharmacia Phastsystem. In these analyses, the wild-type and mutant cytochromes possessed isoelectric points (7.1) and molecular weights (13.5K) that were indistinguishable from each other. *R. sphaeroides* photosynthetic reaction centers were prepared from strain R26 chromatophores as in Feher and Okamura (1978).

Spectroscopy, Redox Potential, and Kinetic Measurements. Cytochromes were oxidized by a crystal of potassium ferricyanide and reduced by a few crystals of sodium dithionite. Oxidants or reductants were subsequently removed by repeated ultrafiltration (Amicon YM5 membrane) or anion exchange (Bio-Rad AG-1-X8) followed by ultrafiltration. All visible and ultraviolet spectra (250–600 nm) were taken on a Cary 15 spectrophotometer at a bandwidth of 0.5 nm with a 1-cm path length. For visible and ultraviolet wavelength spectra, protein concentrations were between 5 and 10 μM. Circular dichroism spectra in the near-ultraviolet (240–400 nm) were taken on an Aviv circular dichroism spectropolarimeter Model 60 DS with protein concentrations at 30 μM in 20 mM Tris-HCl (pH 7.5), 40 mM NaCl. Near-infrared spectra (650–750 nm) were taken on an HP 8452A diode array spectrophotometer with protein concentrations at 60 μM in 50 mM PO_4 (pH 6.0). Midpoint redox potentials were determined by using potassium ferricyanide/ferrocyanide mixtures with spectrophotometric determination of the amount of cytochrome reduction (Pettigrew et al., 1975b). Redox titrations were carried out in 50 mM PO_4 (pH 7.0) at room temperature using a value of 414 mV as the redox potential of potassium ferro-ferricyanide (O'Reilly, 1973). Estimates of the uncertainties are on the order of ±4 mV based on replicate analyses. The kinetics of reduction of oxidized cytochromes by lumiflavin semiquinone were analyzed as described in Meyer et al. (1983). Photooxidation of cytochromes by *R. sphaeroides* photosynthetic reaction centers were in 10 mM Tris-HCl (pH 8.0), 0.025% LDAO, 70 mM NaCl, 1 mM sodium ascorbate, and 100 μM UQ_0 at room temperature.

Protein Denaturation. Guanidine hydrochloride denaturation of the cytochromes was examined by the change in the circular dichroism signal at 220 nm on an Aviv circular dichroism spectropolarimeter Model 60 DS. Denaturation analyses were performed on oxidized and reduced samples at a protein concentration of 2.0 μM in 20 mM Tris-HCl (pH 7.5), 40 mM NaCl at 20 °C which were preequilibrated 20 min in denaturant. Estimates of ΔG_u^* (free energy of unfolding in the absence of denaturant), *m* (cooperativity of unfolding), and C_m (midpoint concentration of denaturant required to unfold half of the protein) were obtained as previously described (Knapp & Pace, 1974; Schellman, 1978). In this analysis, a two-state equilibrium is assumed with K_u (equilibrium constant of unfolding) being defined as $(X_{obs} - X_i)/(X_f - X_{obs})$ where X_{obs}, X_i, and X_f are the observed, initial,

[1] Abbreviations: wt, *R. capsulatus* wild-type cytochrome c_2; Y75C or Y75F, tyrosine-75 replaced by cysteine or phenylalanine, respectively; UQ_0, 2,3-dimethoxy-5-methylbenzoquinone; Gdn-HCl, guanidine hydrochloride.

Table I: Spectral Properties of the *R. capsulatus* Wild-Type and Mutant Cytochromes c_2

property	cytochrome wt	Y75C	Y75F
visible maxima (nm)			
Fe^{2+}	417/522/550	417/523/551	417/523/552
Fe^{3+}	412/522	411/522	412/522
CD transitions (nm)			
Fe^{2+} heme L	262	262	262
Fe^{2+} heme N	329	334	329
Fe^{2+} W-L_a	292	292	292
Fe^{2+} Y=O+800L_b	280	280	280
Fe^{3+} W-L_a	301	301	301
Fe^{3+} Y-L_b	284	284	284
E_{696} (mM^{-1} cm^{-1})	1.1 ± 0.1	1.0 ● 0.1	1.0 ● 0.1

and final circular dichroism signals at 220 nm, respectively. The parameters ΔG_u^* and m are estimated from linear regressions of ΔG_u (the free energy of unfolding at a given concentration of Gdn-HCl) versus [Gdn-HCl] using the equations $\Delta G_u = -RT \ln K_u$ and $\Delta G_u = \Delta G_u^* - m$[Gdn-HCl] where R is the gas constant and T is the temperature in degrees kelvin. By definition, at C_m, $\Delta G_u = 0$ and thus $C_m = \Delta G_u^*/m$. From replicate analyses, the uncertainties of m and C_m were estimated to be ±0.19 kcal·L/mol^2 and ±0.06 mol/L, respectively. The uncertainities in ΔG_u^* are then ±0.30 kcal/mol for the ferricytochrome determinations and ±0.86 kcal/mol for the ferrocytochrome determinations. The larger errors in ferrocytochrome ΔG_u^* are due to extrapolation over a larger Gdn-HCl concentration range (e.g., 4.5 versus 1.6 M). The parameter $\Delta\Delta G_u'$ is estimated from the difference between the ΔG_u values of the wild-type and mutant cytochromes at a Gdn-HCl concentration midway between their C_m values. At these points, the errors in $\Delta\Delta G_u'$ are estimated to be ±0.03 kcal/mol for the ferricytochromes and ±0.43 kcal/mol for the ferrocytochromes.

Results

Spectroscopy. To ascertain changes in the electronic environment of the heme, the visible wavelength maxima of the mutants were examined and compared to the *R. capsulatus* wild-type cytochrome c_2 (Table I). In the oxidized state, Y75C exhibited a slight blue shift of 1 nm in the Soret peak in contrast to Y75F which displayed no spectral changes (>0.5 nm) from the wild-type cytochrome c_2. On the other hand, both ferrocytochrome mutants displayed red-shifted α and β peaks (1–2 nm). These spectral changes imply that the heme environments of Y75C and Y75F have been perturbed with respect to the wild-type cytochrome c_2.

The heme L and M transitions in near-ultraviolet circular dichroism spectra (250–400 nm) of cytochromes *c* have been shown previously to be more sensitive than absorption spectroscopy to perturbations of the environment of the heme group and aromatic groups (Myer & Pande, 1978; Watkins, 1986). In this region, the *R. capsulatus* wild-type ferrocytochrome c_2 has a maximum due to the heme L transition at 262 nm, a minimum due to the heme N transition at 329 nm, an inflection due to the tyrosine 0+800 L_b transitions at 280 nm, and an inflection due to the tryptophan L_a transition at 292 nm (Figure 1 and Table I). In Figure 1a, the spectra of Y75F are compared to the wild-type cytochrome. The heme and aromatic transitions of Y75F occur at similar wavelengths to the wild-type cytochrome, indicating that their environments have not been perturbed. Note, however, that the intensity of the 280-nm transition is reduced in Y75F which is consistent with the removal of one of the five tyrosines present in wild-type cytochrome c_2. In the case of Y75C, the heme L transition occurs at the same wavelength as the wild-type and Y75F cytochromes c_2, but the heme N transition is red-shifted 5 nm, indicating that the heme environment has been perturbed (Figure 1b and Table I). The wavelengths of the Y75C aromatic transitions are not measurably altered, suggesting that their environments have not been perturbed but the intensity of the 280-nm transition is reduced. In contrast to the ferrocytochrome results, no differences in wavelength maxima were observed between the wild-type, Y75F, and Y75C ferricytochrome near-ultraviolet circular dichroism spectra (Table I), but the intensities of the mutant 280-nm transitions were decreased as expected.

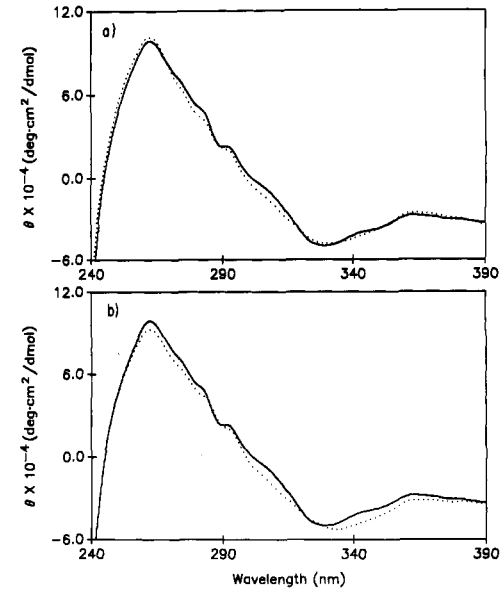

FIGURE 1: Near-ultraviolet circular dichroism spectra of the (a) wt (solid line) and Y75F (dotted line) ferrocytochromes and the (b) wt (solid line) and Y75C (dotted line) ferrocytochromes. Buffer conditions were 20 mM Tris-HCl (pH 7.5), 40 mM NaCl at 25 °C.

The presence of an absorption maximum near 695 nm is thought to reflect bonding between the methionine sulfur atom and the heme iron atom (Schechter & Saludjian, 1967). To ascertain whether ligation of the methionine to the heme iron group has been altered (*R. capsulatus* methionine-96), we have compared the near-infrared spectra of the mutant and wild-type cytochromes. Both mutants exhibit absorption maxima at 696 nm with extinction coefficients that cannot be distinguished from wild-type cytochrome c_2 (Table I). These findings suggest that the methionine-96–heme iron bond is intact in Y75C and Y75F and that the presence of a tyrosine or aromatic group at this position does not contribute to the position or extinction coefficient of this band.

Redox Potentials. Since the redox potentials of heme proteins are influenced by the solvent exposure and local protein environment of the heme (Kassner, 1972; Churg & Warshel, 1986), comparison of the redox potentials of wild type and mutants is a sensitive assay to detect changes in the heme environment. In Figure 2, the Nernst plots for the redox titrations of Y75F, Y75C, and the wild-type cytochromes at pH 7.0 are shown, and their measured midpoint redox potentials are compared in Table II. Titration of the near-infrared absorbance (i.e., the alkaline transition) yielded pK values of 8.9, 10.7, and 8.9 for the wild-type, Y75F, and Y75C ferricytochromes c_2 (data not shown), and thus the redox potential determinations are not expected to be pH-sensitive at pH 7.0 (Pettigrew et al., 1978). First note that the measured redox potential of the purified *R. capsulatus* wild-type cytochrome c_2 is +367 mV which is identical with the pre-

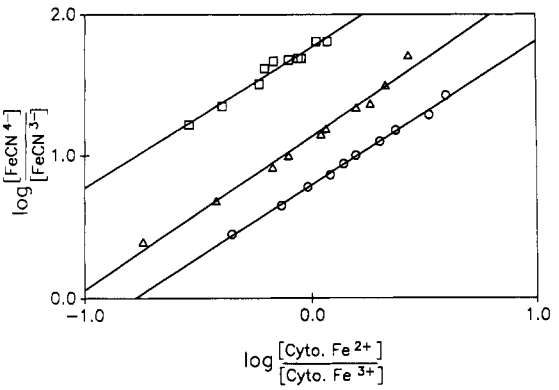

FIGURE 2: Oxidation–reduction titrations of wt (○), Y75C (△), and Y75F (□) in 50 mM PO_4 (pH 7.0) at room temperature.

Table II: Redox Properties of the *R. capsulatus* Wild-Type and Mutant Cytochromes c_2

cytochrome	E_0' (mV)	LF• k_2 (M^{-1} s^{-1})[a]	RC k_2 (M^{-1} s^{-1})[b]
wt	367	$(8.3 \pm 0.7) \times 10^7$	$(2.4 \pm 0.3) \times 10^8$
Y75C	348	$(6.4 \pm 0.4) \times 10^7$	$(2.2 \pm 0.2) \times 10^8$
Y75F	308	$(5.2 \times 0.3) \times 10^7$	$(2.2 \times 0.4) \times 10^8$

[a] Second-order rate constant for cytochrome reduction by lumiflavin semiquinone (LF•). [b] Second-order rate constant for cytochrome photooxidation by *R. sphaeroides* photosynthetic reaction centers (RC).

viously reported value of +368 mV (Pettigrew et al., 1975b). Interestingly, both mutants exhibit decreased redox potentials with the order being Y75F < Y75C < wt. The redox potential of Y75F has been decreased by a larger amount than Y75C (59 versus 19 mV). The observation that both mutants display decreased midpoint potentials suggests either that the oxidized states of the mutants have been stabilized with respect to the wild-type cytochrome or that the reduced states of the mutants have been destabilized with respect to the wild-type cytochrome (Churg & Warshel, 1986). Furthermore, the varying degrees of changes and the chemical nature of the mutations indicate that the redox-dependent stabilities have been affected to different degrees and presumably in different manners.

Cytochrome Reduction by Lumiflavin Semiquinone. The rate constants for reduction of *c*-type cytochromes by flavin semiquinones have been previously used to distinguish between classes of *c*-type cytochromes as well as to detect significant steric or electrostatic effects with a class (Tollin et al., 1986). To test whether the active sites of electron transfer of the two mutants are structurally analogous to the wild-type cytochrome c_2, the second-order rate constants of their reaction with lumiflavin semiquinone were examined (Table II). Both mutants displayed reduced reaction rate constants with the order being Y75F < Y75C < wt. In Figure 3, the second-order rate constants of *R. capsulatus* wild-type cytochrome c_2, mutants Y75C and Y75F, and a number of other class I *c*-type cytochromes are plotted as a function of the difference in redox potential between the lumiflavin semiquinone and a particular cytochrome. In addition, a theoretical curve is included which has previously been demonstrated to establish a semiempirical relationship between second-order reaction rate constants and redox potential differences within the class I *c*-type cytochromes (Tollin et al., 1986). The decrease in the mutant rate constants is in qualitative agreement with their decrease in redox potential; however, their deviation from the theoretical curve (two to four standard deviations) may indicate perturbation of other factors in the electron-transfer reaction (e.g., perturbation of the reorganizational energy).

Photooxidation by Photosynthetic Reaction Centers. Under photosynthetic growth conditions, the physiological role of

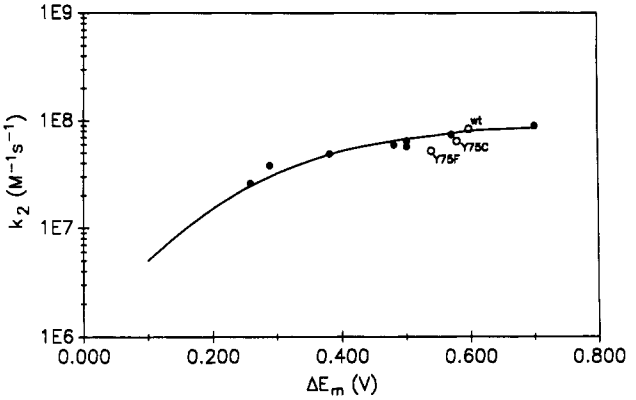

FIGURE 3: Second-order reaction rate constants of wt, Y75F, and Y75C reduction by lumiflavin semiquinone as a function of the difference between the reactant redox potentials. Included are rate constants for a number of other class I *c*-type cytochromes and a semiempirical relationship between rate constants and redox potentials. Experimental conditions were as in Meyer et al. (1983).

Table III: Denaturation Properties of the *R. capsulatus* Wild-Type and Mutant Cytochrome c_2

cytochrome	C_m (M)	m (kcal·L/mol²)	ΔG_u* (kcal/mol)	$\Delta\Delta G_u'$ (kcal/mol)
oxidized				
wt	1.57	2.37	3.71	
Y75C	1.44	2.57	3.71	−0.30
Y75F	1.71	2.59	4.42	+0.35
reduced				
wt	4.51	1.65	7.45	
Y75C	2.24	1.96	4.40	−4.10
Y75F	3.32	1.43	4.75	−1.83

cytochromes c_2 is to transfer electrons between the reduced cytochrome bc_1 complex and photoactivated reaction center. To test for structural perturbations in the mutant active sites of electron transfer (i.e., exposed heme edge), the second-order rate constants of the photooxidation of Y75C, Y75F, and wild-type cytochromes c_2 by *R. sphaeroides* photosynthetic reaction centers were determined (Table II). First note that the wild-type value of 2.4×10^8 M^{-1} s^{-1} is similar to the value reported for *R. sphaeroides* cytochrome c_2 of 1.5×10^8 M^{-1} s^{-1} determined under similar experimental conditions (Moser & Dutton, 1988). From the data listed in Table II, it is clear that the phenylalanine and cysteine substitutions have little effect on the second-order reaction rate constant. Thus, these mutations have not induced structural changes to a degree that measurably affects the reaction between cytochrome c_2 and a physiological partner. Furthermore, the rate constants for the mutants were not sensitive to their lower redox potentials, which is in contrast to the lumiflavin semiquinone kinetic experiments that were presented above. Moreover, these results are in contrast to a previous study of *c*-type cytochrome reactions with the protein flavodoxin (Tollin et al., 1986). In this previous study, the rate constants of electron transfer from the semiquinone of *Clostridium pasteurianum* flavodoxin to a number of *c*-type cytochromes (including *R. capsulatus* cytochrome c_2) indicated that redox potential effects were greater in the flavodoxin–cytochrome reactions (i.e., protein–protein reactions) than observed for the flavin–cytochrome reactions (Tollin et al., 1986). In the case of Y75C and Y75F, the effects of redox potential appear to be smaller in the reaction center–cytochrome reaction than the lumiflavin–cytochrome reaction.

Guanidine Hydrochloride Denaturation. Protein denaturation studies are useful in studying the effects of site-directed mutations on protein stability [for a recent review, see Alber,

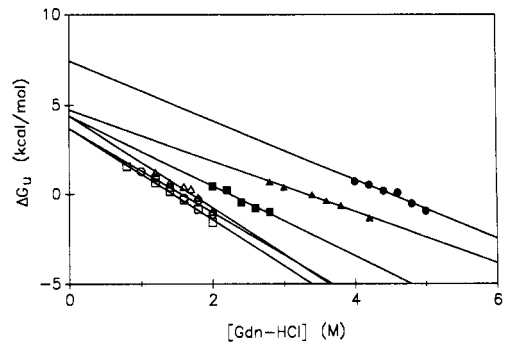

FIGURE 4: Derived ΔG_u as a function of [Gdn-HCl] to determine the parameters C_m, ΔG_u*, and m. Symbols correspond to wt (○), Y75F (△), and Y75C (□) ferricytochromes c_2 and wt (●), Y75F (▲), and Y75C (■) ferrocytochromes c_2. Buffer conditions were 20 mM Tris-HCl (pH 7.5), 40 mM NaCl, and 0–6.0 M Gdn-HCl at 25 °C.

(1989)]. In the present study, we have examined the relative stabilities of Y75C, Y75F, and wild-type cytochromes c_2 by monitoring the circular dichroism signal at 220 nm, which primarily reflects α-helical content, with increasing concentrations of guanidine hydrochloride. The use of this signal and denaturant has previously been shown to be useful in studies of yeast wild-type and mutant iso-1 cytochromes c (Hickey et al., 1988). In Figure 4, plots of ΔG_u as a function of [Gdn-HCl] for Y75C, Y75F, and wild-type cytochromes in both redox states are shown. In Table III, C_m, ΔG_u*, and m of wild-type and mutant cytochromes derived from linear regressions of the data in Figure 4 are listed. In addition, the parameter $\Delta\Delta G_u'$, which is the difference in the ΔG_u values of the mutant and wild-type cytochromes at a Gdn-HCl concentration midway between their C_m values (see Materials and Methods), is included in Table III. If the m value is unchanged in a mutant, then this parameter has the advantage of minimizing errors in estimating the difference in stabilities between wild-type and site-directed mutants because it is estimated in the [Gdn-HCl] region in which K_u is directly determined and not extrapolated over a large region as is ΔG_u*. Since the m values of the wild type and mutants do not deviate more than two standard deviations (Table III), the $\Delta\Delta G_u'$ values for the mutants are used to compare their stabilities relative to the wild-type cytochrome in the same redox state.

First note that the wild-type ferrocytochrome is significantly more stable to denaturant than the ferricytochrome with the difference estimated to be 3.74 kcal/mol using the ΔG_u* values. This result is in agreement with observations on eukaryotic cytochromes c which have indicated that reduced cytochromes c are more stable and rigid than oxidized cytochromes c [see Takano and Dickerson (1981) and references cited therein]. In the case of Y75F, the ferricytochrome is 0.35 kcal/mol more stable but the ferrocytochrome is 1.83 kcal/mol less stable with respect to the wild-type cytochrome. Therefore, substitution of tyrosine-75 by phenylalanine affects stability in a redox-dependent nature. In the case of Y75C, the ferricytochrome is 0.30 kcal/mol less stable and the ferrocytochrome is 4.10 kcal/mol less stable with respect to the wild-type cytochrome. Thus, substitution of tyrosine-75 by cysteine has destabilized both redox states although the reduced state is most affected.

Discussion

The spectroscopic data on mutants Y75F and Y75C indicate that removal of the aromatic group or oxygen atom perturbs the electronic environment of the cytochrome c_2 heme group but does not significantly alter ligation of the methionine to the heme. These observations are consistent with the data for the equivalent Y67F mutations of rat and horse cytochromes c which have demonstrated that both mutants exhibit spectral changes at visible wavelengths but an unperturbed absorbance in the near-infrared (Luntz et al., 1989; Wallace et al., 1989). However, the significant decrease in the redox potentials of Y75F and Y75C indicates that their heme environments have been perturbed. In the case of Y75F, this result is in agreement with the redox potentials of other cytochromes possessing the equivalent substitution. For example, in *Euglena* mitochondrial cytochrome c, the redox potential is decreased by 16 mV (Pettigrew, 1973). Moreover, the redox potentials of the Y67F mutations of rat and horse cytochromes c are decreased by 35 mV (Luntz et al., 1989; Wallace et al., 1989). Consequently, a general observation is that substitution of this tyrosine by other residues perturbs the heme environment and results in a decrease in redox potential. Note that this effect is the opposite of that proposed from the *R. rubrum* cytochrome c_2 structure (Salemme et al., 1973b). If an electrostatic interaction between the conserved tyrosine (e.g., *R. capsulatus* tyrosine-75) and the methionine sulfur stabilized the oxidized state, removal of the hydroxyl (e.g., substitution of phenylalanine for tyrosine) would be expected to destabilize the oxidized state and thus raise the redox potential. The fact that this is not the case in the *R. capsulatus* cytochrome c_2 mutants as well as the rat and horse cytochrome c mutants implies than an electrostatic interaction between the tyrosine hydroxyl and the methionine sulfur does not occur.

The lumiflavin semiquinone and *R. sphaeroides* photosynthetic reaction center kinetic studies of Y75F and Y75C indicate that both mutants are structurally analogous to the wild-type protein at the active site of electron transfer and that *R. capsulatus* tyrosine-75 does not play a critical role in cytochrome c_2 electron-transfer reactions. These observations are consistent with our previous results (Caffrey, 1991) which demonstrated that mutants Y75F and Y75C were functional in vivo, i.e., were competent to accept electrons from reduced cytochrome bc_1 complexes and donate electrons to photoactivated reaction centers. Similarly, the equivalent rat cytochrome c mutant (Y67F) has been shown to react in vitro with cytochrome c oxidase (Luntz et al., 1989), and the equivalent horse cytochrome c mutant (Y67F) has been shown to react in vitro with cytochrome c reductase (Wallace et al., 1989). Recent two-dimensional NMR and X-ray crystallography studies of wild-type cytochrome c_2 and Y75F have indicated that there are no large structural differences between the two proteins (to be reported elsewhere). Together these results suggest that *R. capsulatus* tyrosine-75, or its equivalent in other species, is not critical to the structure or function of cytochromes c in general and that substitution of the tyrosine with phenylalanine or cysteine does not introduce significant structural perturbations which substantially alter the kinetics of electron transfer.

The most striking result of the present study is the redox-dependent instability of the tyrosine mutation α helices. Note that the relative destabilization of the reduced state of both mutants is qualitatively consistent with their decrease in the redox potential; i.e., stabilization of the oxidized state and destabilization of the reduced state will result in a decrease in the redox potential. The components of protein stability which are perturbed by mutations to interior groups include hydrophobic effects, van der Waals forces, backbone conformation, hydrogen bonds, and local polarity (Sandberg & Terwilliger, 1989). Hydrophobic effects can be estimated from model studies of the free energy differences in transferring different amino acid side chains from a hydrophobic medium

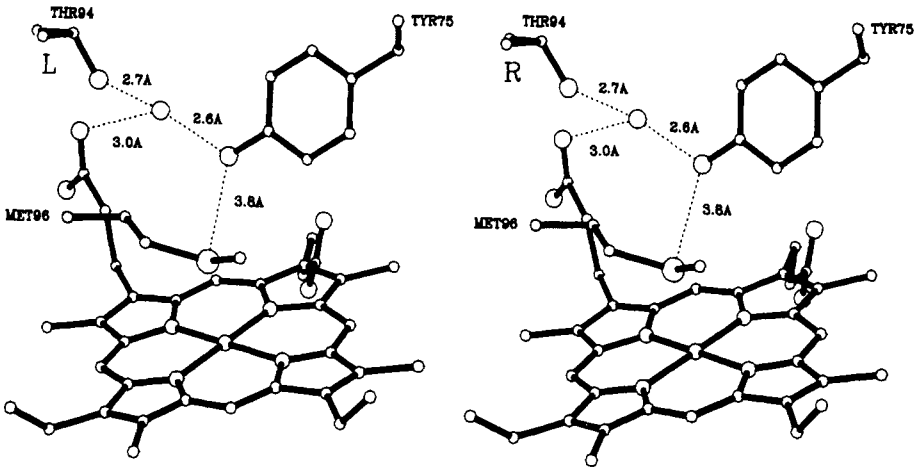

FIGURE 5: Stereo drawing of the *R. capsulatus* wild-type ferrocytochrome c_2 hydrogen-bonding network. Depicted are the heme, tyrosine-75, threonine-94, methionine-96, and water-41. For reference, the distance between the hydroxyl of tyrosine-75 and the sulfur of methionine-96 has been included.

such as octanol to a hydrophilic medium such as water (Fauchere & Pliska, 1983). According to these criteria, substitution of phenylalanine or cysteine for tyrosine-75 would result in 1.14 and 0.79 kcal/mol of stability due to the more hydrophobic nature of phenylalanine and cysteine (Fauchere & Pliska, 1983). Thus, the nonhydrophobic forces can be estimated by subtracting the expected energy due to hydrophobic effects from the observed stability. In the case of Y75F, the nonhydrophobic forces are estimated to be −0.79 and −2.97 kcal/mol in the ferri and ferro states, respectively (0.35 minus 1.14 and −1.83 minus 1.14). In the case of Y75C, the nonhydrophobic forces are estimated to be −1.09 and −4.89 kcal/mol in the ferri and ferro states, respectively (−0.30 minus 0.79 and −4.10 minus 0.79). The observation that the magnitude of the packing effects for both mutations is dependent upon their redox state implies that changes in local polarity are the primary component of the instability of the reduced state.

To interpret the larger degree of mutant destabilization in the reduced state, it is useful to examine the structure of the *R. capsulatus* ferrocytochrome c_2 heme environment (Holden et al., unpublished results). In Figure 5, the hydrogen-bonding network between a bound water molecule (water-41) and *R. capsulatus* residues 75 and 94 and the front propionate is shown. The presence of this water molecule calls into question the supposition that cytochrome c_2 redox potentials are generally higher than those of the eukaryotic cytochromes *c* due to the absence of this water molecule in *R. rubrum* cytochrome c_2 (Salemme et al., 1973b). Consequently, the reasons for the generally higher redox potentials of the cytochromes c_2 must be more complex than the presence or absence of this water molecule in the heme environment. From the X-ray crystallographic structure of the wild-type *R. capsulatus* ferrocytochrome c_2, the distances between the water-41 oxygen and the oxygens of the proposed hydrogen-bonding groups are 2.6–3.0 Å (Holden et al., unpublished results). Also, note that in *R. capsulatus* cytochrome c_2 the tyrosine-75 oxygen to methionine-96 sulfur distance is 3.8 Å in contrast to the 3.0-Å distance observed in tuna cytochrome *c* and *R. rubrum* cytochrome c_2 (Takano & Dickerson, 1981; Salemme et al., 1973b). Consequently, an electrostatic interaction between these groups, which stabilizes the oxidized state (i.e., decreases the redox potential), is inhibited by the larger distance in *R. capsulatus*.

Comparison of the heme environment of *R. capsulatus* cytochrome c_2 to those of tuna cytochrome *c* and *R. rubrum* cytochrome c_2 suggests similarities in their hydrogen-bonding networks. For example, in *R. capsulatus* cytochrome c_2, the hydrogen-bonding network is very similar to that of tuna cytochrome *c* in which an equivalent bound water is hydrogen-bonded to asparagine-52, tyrosine-67, and threonine-78 (Takano & Dickerson, 1981), the equivalents of *R. capsulatus* isoleucine-57, tyrosine-75, and threonine-94. Moreover, hydrogen-bonding networks which are identical with the tuna cytochrome *c* network are also observed in the cytochromes *c* from rice, yeast, and horse (Oichi et al., 1983; Louie & Brayer, 1990; Bushnell et al., 1990). Interestingly, in *R. rubrum* cytochrome c_2, the hydroxyl of tyrosine-52 (the equivalent of *R. capsulatus* isoleucine-57 and tuna asparagine-52) replaces the observed water molecule in *R. capsulatus* and tuna cytochromes *c*. As a consequence, the *R. rubrum* hydrogen-bonding network consists of tyrosine-52, tyrosine-70, serine-89, and methionine-96 (Bhatia, 1981). Therefore, it appears that evolution has conserved similar but nonexact hydrogen-bonding schemes in this region of the cytochrome which sometimes involve an internal water molecule. In Y75F and Y75C, one of the hydrogen-bonding partners is absent, and thus the hydrogen-bonding network does not form or is perturbed. As a consequence, the ferro states of Y75F and Y75C are destabilized. This proposition is supported by the absence of water-41 (HOH41) in the Y75F ferrocytochrome crystal structure (Holden et al., unpublished results). In light of the present study, formation of the conserved hydrogen-bonding network may have the net effect of stabilizing the cytochrome by bringing together distant regions of the protein backbone and the heme moiety (e.g., positions 75 and 94 of *R. capsulatus* cytochrome c_2). Further, the results of this study support the hypothesis that mutations affecting the internal water position will also perturb the cytochrome *c* redox potential (Louie & Brayer, 1989).

The observation that the Y75F and Y75C ferri states are not destabilized to similar degrees as their ferro states implies that the hydrogen-bonding network presented in Figure 5 does not exist in the wild-type oxidized state or that another side chain replaces tyrosine-75 as a hydrogen-bonding partner. Structural characterization of the wild-type and mutant ferricytochromes *c* is necessary to further elucidate this point. Nonetheless, substitution of *R. capsulatus* tyrosine-75 by phenylalanine appears to destabilize the reduced state by disruption of an internal hydrogen-bonding network involving an internal water molecule. Note that the idea of the phenylalanine mutation disrupting binding of the water molecule

was previously presented for the equivalent rat cytochrome *c* Y67F mutation (Luntz et al., 1989). However, this study only considered the effects of this mutation on the oxidized state and concluded that the reasons for conservation of this tyrosine were not apparent. In light of the present results, conservation of the tyrosine is important in stabilization of the ferro, not the ferri, state. During the course of evolution, tyrosine has been selected for as opposed to other hydrophobic groups such as phenylalanine, tryptophan, leucine, and isoleucine due to its hydrogen-bonding capabilities. Furthermore, by stabilizing the ferrocytochrome state, the conserved tyrosine has the net effect of maintaining high redox potentials in cytochromes *c*. Future mutations to substitute tyrosine-75 with other hydrophobic and potential hydrogen-bonding groups can test the ability of such groups to maintain a high redox potential or increase it without disrupting the cytochrome structure or stability. The structural characteristics of wild-type and mutant Y75F cytochromes c_2 are being further characterized by X-ray crystallography and two-dimensional NMR techniques to understand the mechanism for the destabilization of the reduced state by phenylalanine. It is anticipated that high-resolution structures of these proteins and other future mutations at this and other positions in the heme environment of *R. capsulatus* cytochrome c_2, and the complementary mutagenic studies of eukaryotic cytochromes *c*, will give insight into the determinants of the large diversity of redox potentials in biological molecules and the reasons for the high degree of evolutionary conservation of groups in the heme environment. Finally, it will be interesting to determine if internal water molecules which are part by hydrogen-bonding networks are important to the stabilities of other proteins.

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Registry No. Heme, 14875-96-8; lumiflavin semiquinone, 34533-61-4; cytochrome *c*, 9007-43-6; cytochrome c_2, 9035-43-2; tyrosine, 60-18-4.

References

Alber, T. (1989) *Annu. Rev. Biochem. 58*, 765–798.
Allen, J. (1988) *J. Mol. Biol. 204*, 495–496.
Ambler, R., Daniel, M., Hermoso, J., Meyer, T., Bartsch, R., & Kamen, M. (1979) *Nature (London) 278*, 659–660.
Baccarini-Melandri, A., Jones, O., & Hauska, G. (1978) *FEBS Lett. 86*, 151–154.
Bartsch, R. (1971) *Methods Enzymol. 23*, 344–363.
Bhatia, G. (1981) Ph.D. Thesis, University of California, San Diego.
Bushnell, G., Louie, G., & Brayer, G. (1990) *J. Mol. Biol. 214*, 585–595.
Caffrey, M. (1991) Ph.D. Thesis, University of Arizona, Tucson.
Churg, A., & Warshel, A. (1986) *Biochemistry 25*, 1675–1681.
Cusanovich, M., Meyer, T., & Tollin, G. (1988) *Adv. Inorg. Biochem. 7*, 37–92.
Daldal, F., Cheng, S., Applebaum, J., Davidson, E., & Prince, R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2012–2016.
Dickerson, R., Takano, T., Eisenberg, D., Kallai, O., Samson, L., Copper, A., & Margoliash, E. (1971) *J. Biol. Chem. 246*, 1511–1535.
Ditta, G., Stanfield, S., Corbin, D., & Helinsky, D. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7347–7351.
Dutton, P. L., & Jackson, J. B. (1972) *Eur. J. Biochem. 30*, 495–510.
Fauchere, J. L., & Pliska, V. (1983) *Eur. J. Med. Chem. 18*, 369–375.
Feher, G., & Okamura, M. (1978) in *The Photosynthetic Bacteria* (Clayton, R., & Sistrom, W., Eds.) pp 349–386, Plenum Press, New York.
Hickey, D., McLendon, G., & Sherman, F. (1988) *J. Biol. Chem. 263*, 18292–18305.
Holden, H., Meyer, T., Cusanovich, M., Daldal, F., & Rayment, I. (1987) *J. Mol. Biol. 195*, 229–231.
Kamen, M., & Vernon, L. (1955) *Biochim. Biophys. Acta 17*, 10–22.
Kassner, R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2263–2267.
Knapp, J., & Pace, C. (1974) *Biochemistry 13*, 1289–1294.
Louie, G., & Brayer, G. (1989) *J. Mol. Biol. 209*, 313–322.
Louie, G., & Brayer, G. (1990) *J. Mol. Biol. 214*, 527–555.
Louie, G., Hutcheon, W., & Brayer, G. (1988) *J. Mol. Biol. 199*, 295–314.
Luntz, T., Schejter, A., Garber, E., & Margoliash, E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3524–3528.
Meyer, T., & Kamen, M. (1982) *Adv. Protein Chem. 35*, 105–212.
Meyer, T., Przysiecki, C., Watkins, J., Bhattacharyya, A., Simondsen, R., Cusanovich, M., & Tollin, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6740–6744.
Miki, K., Saeda, M., Masaki, K., & Kasai, N. (1986) *J. Mol. Biol. 191*, 578–580.
Moser, C., & Dutton, P. (1988) *Biochemistry 27*, 2450–2461.
Myer, Y., & Pande, A. (1978) *The Porphyrins*, Vol. III, pp 271–322, Academic Press, New York.
Oichi, H., Hata, Y., Tanaka, N., Kakudo, M., Sakurai, T., Aihara, S., & Morita, Y. (1983) *J. Mol. Biol. 166*, 407–418.
O'Reilly, J. (1973) *Biochim. Biophys. Acta 292*, 509–515.
Pettigrew, G. (1973) *Nature (London) 241*, 531–533.
Pettigrew, G., Aviram, I., & Schejter, A. (1975a) *Biochem. J. 149*, 155–167.
Pettigrew, G., Meyer, T., Bartsch, R., & Kamen, M. (1975b) *Biochim. Biophys. Acta 430*, 197–208.
Pettigrew, G., Bartsch, R., Meyer, T., & Kamen, M. (1978) *Biochim. Biophys. Acta 503*, 509–523.
Salemme, F., Freer, S., Xuong, N., Alder, R., & Kraut, J. (1973a) *J. Biol. Chem. 248*, 3910–3921.
Salemme, F., Kraut, J., & Kamen, M. (1973b) *J. Biol. Chem. 248*, 7701–7716.
Sandberg, W., & Terwilliger, T. (1989) *Science 245*, 54–57.
Schechter, E., & Saludjian, P. (1967) *Biopolymers 5*, 788–790.
Schellman, J. (1978) *Biopolymers 17*, 1305–1322.
Takano, T., & Dickerson, R. (1981) *J. Mol. Biol. 153*, 95–115.
Takano, T., Kallai, O., Swanson, R., & Dickerson, R. (1973) *J. Biol. Chem. 248*, 5234–5255.
Timkovich, R., & Dickerson, R. (1976) *J. Biol. Chem. 251*, 4033–4046.
Tollin, G., Meyer, T., & Cusanovich, M. (1986) *Biochim. Biophys. Acta 853*, 29–41.
Wallace, C., Mascagni, P., Chait, B., Collaen, J., Paterson, Y., Proudfoot, A., & Kent, S. (1989) *J. Biol. Chem. 264*, 15199–15209.
Watkins, A. (1986) Ph.D. Thesis, University of Arizona, Tucson.
Weaver, P. F., Wall, J., & Gest, H. (1975) *Arch. Microbiol. 105*, 207–216.
Xavier, A., Moura, J., & Moura, I. (1981) *Struct. Bonding (Berlin) 43*, 187–213.