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Structural significance of an internal water molecule studied by site-directed mutagenesis of tyrosine-67 in rat cytochrome *c*

(protein stability/cytochrome *c* structure–function relationships)

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ABSTRACT **The tyrosine-67 to phenylalanine mutated rat cytochrome *c* is similar to the unmutated protein in its spectral, reduction potential, and enzymic electron-transfer properties. However, the loss of the 695-nm band, characteristic of the ferric form of the normal low-spin physiologically active configuration, occurs 1.2 pH units higher on the alkaline side and 0.7 pH unit lower on the acid side. Similarly, the heme iron-methionine-80 sulfur bond is more stable to temperature, with the midpoint of the transition being 30°C higher, corresponding to an increase in ΔH of 5 kcal/mol (1 cal = 4.184 J), partially mitigated by an increase of 11 entropy units in ΔS. Urea has only slightly different effects on the two proteins. These phenomena are best explained by considering that the loss of one of the three hydrogen-bonding side chains, tyrosine-67, asparagine-52, and threonine-78, which hold an internal water molecule on the "left, lower front" side of the protein [Takano, T. & Dickerson, R. E. (1981) *J. Mol. Biol.* 153, 95–115], is sufficient to prevent its inclusion in the mutant protein, leading to a more stable structure, and, as indicated by preliminary proton NMR two-dimensional phase-sensitive nuclear Overhauser effect spectroscopy analyses, a reorganization of this area. This hypothesis predicts that elimination of the hydrogen-bonding ability of residue 52 or 78 would also result in cytochromes *c* having similar properties. It is not obvious why the space-filling structure involving the internalized water molecule that leads to a destabilization energy of about 3 kcal/mol should be subject to extreme evolutionary conservation, when a more stable and apparently fully functional structure is readily available.**

Some of the invariant and highly conserved amino acid residues of mitochondrial cytochromes *c* are of special interest because of their close relation to the prosthetic group. Thus, tyrosine-67, located on the left side of the heme, is next to one of the two axial heme iron ligand residues, methionine-80 (1, 2); the other is histidine-18 (see Fig. 1).

Tyrosine-67 is interesting for the following reasons. (*i*) Its phenolic hydroxyl may be hydrogen-bonded to the methionine-80 sulfur (1, 2), which could contribute to maintaining the sulfur atom in its heme iron liganding position. (*ii*) A crystallographically identified buried water molecule that appears to be hydrogen-bonded to the three highly conserved residues tyrosine-67, asparagine-52, and threonine-78 may be involved in regulating some of the chemical properties of the heme protein (1, 2). (*iii*) Nitration (3, 4) or iodination (5) of tyrosine-67 drastically alters various heme-linked properties, suggesting that this residue plays an important role. The only known mitochondrial cytochrome *c* in which residue 67 is not a tyrosine, but a phenylalanine, is the cytochrome *c*-558 of *Euglena gracilis* (6). This cytochrome *c* has several unique heme-linked properties (7), but because of numerous other substitutions it is impossible to decide which are responsible for the observed differences.

The present report examines the effects of replacing tyrosine-67 with phenylalanine in rat cytochrome *c* on the stability of the methionine-80 sulfur-heme iron bond and on the thermal and urea-induced denaturation of the protein. It concludes that a major role of tyrosine-67 is to provide one of the three hydrogen bonds holding an internal molecule of water, the other two coming from asparagine-52 and threonine-78 (1, 2), and that this relatively unstable space-filling structure plays a significant role in the properties of the cytochrome *c* hemochrome.

MATERIALS AND METHODS

Expression of Rat Cytochrome *c* in Yeast and Large Scale Preparation. The RC9 processed pseudogene of rat cytochrome *c*, which translates into the normal rat cytochrome *c* amino acid sequence (8), was kindly provided by R. Scarpulla (Northwestern University). It was used along with a 0.33-kilobase DNA fragment containing the yeast *CYC1* promoter, isolated from YCpCYC1(2.4) (9), which was kindly provided by R. Zitomer (State University of New York, Albany), and an *Escherichia coli*–yeast multicopy shuttle vector, YEp13 (10), to assemble an expression vector, YEp(-50)RC9. The mutant RC9 coding sequence was obtained by oligomer-directed mutagenesis by using the method of Zoller and Smith (11); its DNA sequence was determined (12) and it was used to construct the expression vector YEp(-50)RC9(Y67F). When transformed into the yeast strain GM-3C-2 [*trp1-1, leu2-2, leu2-112, his4-519, cyc1-1, cyp3-1, (cyc7⁻), gal⁻*] (13), which expresses neither iso-1 nor iso-2 cytochrome *c*, both vectors confer the ability to grow readily on glycerol and lactate.

The media and fermentation procedure described by Stewart *et al.* (14) were adapted to 200-liter cultures. The cytochrome *c* was extracted and purified as described for yeast cytochrome *c* (15), with final purification on an HPLC cation-exchange column (SynChropak CM300, SynChrome, Lafayette, IN) (16).

Analytical Methods. Optical and circular dichroic spectra were recorded with Hitachi 557 and Jasco 600 (Jasco, Easton, MD) instruments, respectively. Proton NMR, one-dimensional longitudinal relaxation measurements, and two-dimensional phase-sensitive nuclear Overhauser effect spectroscopy (2D-NOESY) analyses (100-ms mixing time) were carried out with a Varian model XL-400 spectrometer. Oxidation–reduction potentials were measured by the method of mixtures (17). Sequential Edman degradation was carried out on a model 477A protein sequencer with a model 120A phenylthiohydantoin analyzer (Applied Biosystems). The kinetics of the reaction with cytochrome oxidase were

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Abbreviations: NOESY, phase-sensitive nuclear Overhauser effect spectroscopy; 2D, two dimensional.

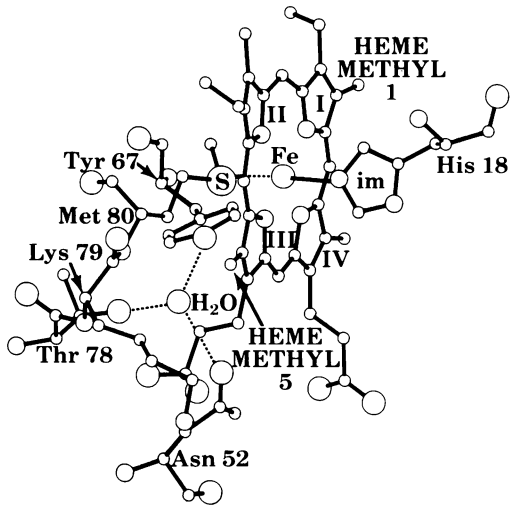

FIG. 1. Diagram of portions of the structure of cytochrome *c* relevant to effects of the tyrosine-67 to phenylalanine substitution, taken from the structure of the outer molecule of tuna ferricytochrome *c* determined by Takano and Dickerson (1, 2). The molecule is viewed from the front with the heme plane tilted to the left, so that the imidazole axial ligand of histidine-18 has moved closer to the viewer than the sulfur axial ligand of methionine-80 on the other side of the heme plane. Fe, heme iron atom; I, II, III, and IV, the corresponding pyrrole rings, respectively; S, the sulfur atom of methionine-80; im, the imidazole ring of histidine-18; H_2O, the internal water molecule hydrogen-bonded (dashed lines) to the side chains of residues 52, 67, and 78. Circles, other than those already listed, represent from largest to smallest, oxygen, nitrogen, and carbon atoms, respectively. The amino acid residues are indicated in three-letter code placed near their α-carbon atoms.

determined polarographically at pH 7.9 (25 mM Tris acetate) (18). Computer graphics were obtained by the HYDRA (19), or FRODO program, utilizing the tuna ferricytochrome *c* coordinates of the outer molecule (1, 2), on an Evans and Sutherland (Salt Lake City) model PS 390 graphics display system.

RESULTS

General Properties of Recombinant Wild-Type and Mutant Cytochromes *c*. Both wild-type and mutant preparations obtained from yeast cultures by recombinant procedures were separated by HPLC cation-exchange chromatography into two fractions. The first fraction off the column, comprising about 25% of the total, did not react in the Edman degradation. It had a chymotryptic N-terminal decapeptide that chromatographed, by reverse-phase HPLC on a C_{18} column (Waters μBondapak) under an acetonitrile gradient in 100 mM potassium phosphate (pH 7.2) (20), precisely like the corresponding peptide of natural rat cytochrome *c*. The second fraction showed the sequence of the natural rat protein for 10 cycles of Edman degradation. Thus the first fractions, RNc-I and RNc(Y67F)-I, were N-terminally acetylated, whereas the second fractions, RNc-II and RNc(Y67F)-II, were unblocked. The mutant proteins had the amino acid composition of the wild-type proteins, except for the change resulting from the residue substitution. In addition, both fractions of the wild-type and mutant proteins displayed a sharp resonance at 3.4 ppm representative of nine protons in the NMR spectrum. This unique resonance, not observed with any naturally occurring mammalian cytochrome *c*, is identical to that observed with fungal proteins and has been assigned to the N^{ϵ}-trimethyl group of lysine-72 (21). Thus, in contrast to the small degree of trimethylation observed with *in vitro* systems (22), yeast cells are capable of complete trimethylation of lysine-72 in a mammalian cytochrome *c*. All the experiments described below were carried out with the unblocked proteins.

The RNc(Y67F)-II mutant had spectroscopic features very similar to those of RNc-II, including identical ratios of the absorbance of the α, β, and Soret bands. Notable differences were as follows: (*i*) In the Soret region, the isosbestic point between the spectra of the ferrous and ferric forms (23) was shifted by 2 nm to the red, to 412 nm. (*ii*) The optical absorption maximum of the ferric protein in the far red, commonly described as the 695-nm band (24), was also shifted by 2 nm to the red, to 697 nm.

The reduction potential of the mutant protein was lower than that of the wild-type by 35 mV (Table 1). This did not appear to be detrimental either to the function of the protein in sustaining the growth of yeast or to the kinetics of its reaction with cytochrome *c* oxidase. The K_m values of the high-affinity phase (18) were the same for the wild-type and mutant proteins, while the V_{max} for the mutant was 25% higher (Table 1), indicating that the complex between RNc(Y67F)-II and cytochrome *c* oxidase reacted to that extent faster with the reductant *N,N,N',N'*-tetramethylphenylene diamine (25).

Effect of the Mutation on the Heme-Linked Ionizations. The native structure of the ferricytochrome *c* crevice is altered by heme-linked ionizations both at acid and alkaline pH values (26), resulting in cleavage of the methionine sulfur-heme iron bond and in disappearance of the 695-nm band that is associated with ligation of a low-spin ferric heme iron by bivalent sulfur (27, 28). Titrations of the effects of pH on the absorbance of the 695-nm band showed (Table 1) that the pK_a value of the alkaline ionization was higher in RNc(Y67F)-II than in RNc-II by 1.2 pH units, whereas that of the acid ionization was lower by 0.7 pH unit.

Thermally Induced Change of the 695-nm Band. Loss of the 695-nm band upon heating (24) results from removing methionine-80 from iron ligation and presumably replacing it by a group, X, of as yet unknown nature:

$$\text{Met-S-Fe}^{3+}\text{-imidazole-His} \rightleftarrows \text{X-Fe}^{3+}\text{-imidazole-His} \quad [1]$$

Thermal titrations of the 695-nm band of RNc-II and RNc(Y67F)-II were performed. To analyze the data, the 695-nm absorbance at low temperatures (20°C or lower) was assigned to the Fe-S state. The end-points at high temperature could not be reached because of precipitation, even when the latter was delayed by the presence of 0.5 M sodium phosphate (6), so that the absorbance of the Fe-X state was taken as that of the cyanide complex that also lacks the 695-nm band (24). The results were analyzed by plotting ΔG as a function of T, where $\Delta G = -RT \ln K$ and K is the equilibrium constant for Eq. 1. The entropy change at each T was estimated from $(d\Delta G/dT)_p = -\Delta S$; ΔH was obtained from $\Delta G = \Delta H - T\Delta S$. For both the wild-type and the mutant proteins, plots of ΔG against T showed two distinct portions: an initial linear segment (linear least squares regression coefficient $R = -0.996$ to -0.998), followed by a nonlinear segment that was observed up to the temperatures at which the proteins precipitated (Fig. 2). For all the temperatures in

Table 1. General properties of recombinant wild-type and mutant rat cytochromes *c*

Cytochrome *c*	Acid pK_a^{695}, pH	Alkaline pK_a^{695}, pH	E_0, mV	K_m, nM	V_{max}
RNc-II	2.8	9.5	+259	10.5	43
RNc(Y67F)-II	2.1	10.7	+224	10.5	54

K_m and V_{max} are for the high-affinity phase of the polarographic assay for the reaction with cytochrome oxidase. V_{max} is expressed as electrons per monomeric oxidase per s.

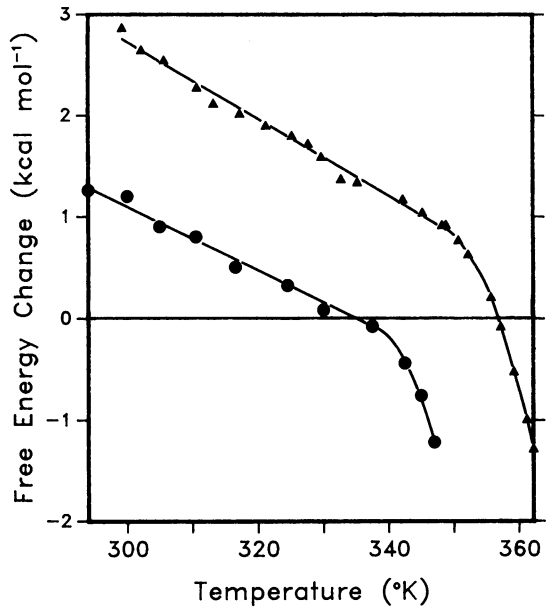

FIG. 2. Plot of the free-energy changes for the equilibrium between the Fe-S and Fe-X states of RNc-II (circles) and RNc-(Y67F)-II (triangles), as a function of the absolute temperature. The measurements of the absorbance of the 695-nm band were carried out in 0.1 M sodium phosphate (pH 7.0).

the linear region, ΔH remained constant, implying that the observed change was not due to general unfolding of the molecule (29), and it was assumed to represent the equilibrium given in Eq. 1. This is further supported by the fact that the linearity of the plots was interrupted at temperatures that correspond to the onset of thermal denaturation (Fig. 2), a process that is accompanied by a rapid change in the heat capacity of cytochrome *c* and that is observed at similar temperatures in microcalorimetric experiments (30). Because of this, the temperature, $t_{1/2}$, at which the equilibrium reaches its midpoint ($\Delta G = 0$) could not be determined directly, but was calculated from the values of ΔH and ΔS, and are listed in Table 2, together with the values of ΔH, ΔS, and ΔG (the latter at 25°C).

These results clearly indicate that the mutation has the effect of making the transition to the X-Fe state more unfavorable, as shown by the significantly higher midpoint temperature. This is mainly the result of an increase in ΔH, of 5 kcal/mol, partly mitigated by an accompanying increase in ΔS, of 11 entrophy units.

The Effect of Urea. The loss of the 695-nm band induced by urea (23) was analyzed (31) in terms of a two-state system that also can be represented formally by Eq. 1. No significant differences were found between the wild-type protein and the tyrosine-67 to phenylalanine (Y67F) mutant: the band was 50% reduced in intensity at 7.2 M urea for RNc-II and 7.3 M urea for RNc(Y67F)-II.

To determine the effects of urea on the general conformations of the wild-type and mutant proteins, ultraviolet circular dichroic spectra were recorded at various urea concentrations, and the fractions of unfolded and folded protein were calculated from the mean residue ellipticities at 220 nm. An analysis of these results in terms of a two-state system (31) encountered the difficulties of extrapolation over exceedingly wide ranges of urea concentration, and, since the differences in stability between the two proteins were small, a modified procedure was adopted (32). Using this procedure, RNc(Y67F)-II was found to be more resistant than RNc-II to denaturation by urea by 0.6 kcal/mol at urea concentrations between 5 and 8 M.

Table 2. Thermodynamic parameters for the thermal equilibrium between Fe-S and Fe-X forms of wild-type and mutant rat cytochromes *c*

Cytochrome *c*	ΔG (25°C), kcal/mol	ΔH, kcal/mol	ΔS, entropy units	$t_{1/2}$, °C
RNc-II	1.2	11.4	34.2	60
RNc(Y67F)-II	2.7	16.4	45.2	90

Structural Analysis by NMR. Preliminary assignments of several resonances of the ^{1}H NMR spectrum of ferric RNc(Y67F)-II were made based upon the one-dimensional nuclear Overhauser effect and 2D-NOESY experiments. It was observed that the connectivities of the alanine-15 ($C_\alpha H$), threonine-19 ($C_\alpha H$), and phenylalanine-82 (m- and p-protons) were similar to those observed with RNc-II, indicating no significant alterations in the structure of the protein in the vicinity of these residues located on the right, the top front and the top left regions of the protein. In contrast, a leucine methyl group at −2.99 ppm, tentatively assigned to residue 68, shows a strong connectivity with the heme methyl group at position 5 at 10.1 ppm (Fig. 3). In all other cytochromes *c* we have examined, RNc-II, rat, horse, tuna, *Drosophila*, and *Candida krusei*, the leucine-68 methyl resonance at −2.7 ppm displays a strong connectivity with the heme methyl group at position 1, at approximately 6.9 ppm, and none with the heme methyl group at position 5. For the nuclear Overhauser effect to be observed between leucine-68 and the heme methyl group at position 5, the corresponding protons must be within 5 Å of each other. This implies a reorganization of the protein in the lower left quadrant, which a molecular graphics examination indicated can be achieved by swinging the side chain of residue 67 downwards and allowing that of leucine-68 to occupy the space generated by the loss of the internal molecule of water (see *Discussion*).

DISCUSSION

From crystallographic evidence, it has been proposed that tyrosine-67 acts as donor in a hydrogen bond with the iron-bound methionine-80 sulfur (1, 2). The results described above show that the substitution of phenylalanine for this tyrosine makes the cleavage of the iron-sulfur bond more endothermic and endergonic, indicating that the tyrosine–sulfur hydrogen bond is unlikely to play a significant role in stabilizing the iron–sulfur bond. The side chain of tyrosine-67 is also hydrogen-bonded to a water molecule (Fig. 1) buried inside the protein, and the water is also hydrogen-bonded to the side chains of asparagine-52 and threonine-78 (1, 2). As shown below, this is an important function of tyrosine-67.

The effects of the tyrosine-67 to phenylalanine mutation are striking, since they yield stabilization to the cytochrome *c* structure even though a group involved in intramolecular binding has been eliminated. Indeed, the tyrosine-67 to phenylalanine mutation increased the stability of the Fe-S bond by 2 to 3 kT. At 25°C, ΔG for the equilibrium described by Eq. 1 is larger by 1.7 kcal/mol for the mutant protein, the pK value of the alkaline ionization is 1.2 pH units higher in the mutant, corresponding to an increase of 1.7 kcal/mol in ΔG, and the pK value of the acid heme-linked ionization is 0.7 unit lower in RNc(Y67F)-II than in RNc-II, which is equivalent to an increase in the stability of the Fe-S bond of 1.0 kcal/mol. Furthermore, the circular dichroic titrations indicate that the protein has become more resistant to denaturation by urea, by a similar extent.

It has been estimated that creation of a cavity of the size of a water molecule should destabilize a protein structure by 1–2 kT (33); and it has been shown experimentally that creation of a cavity of the size of a methylene group destabilizes the resistance of an enzyme to denaturation by urea by 1.1 kcal/mol (32). Thus, the elimination of the

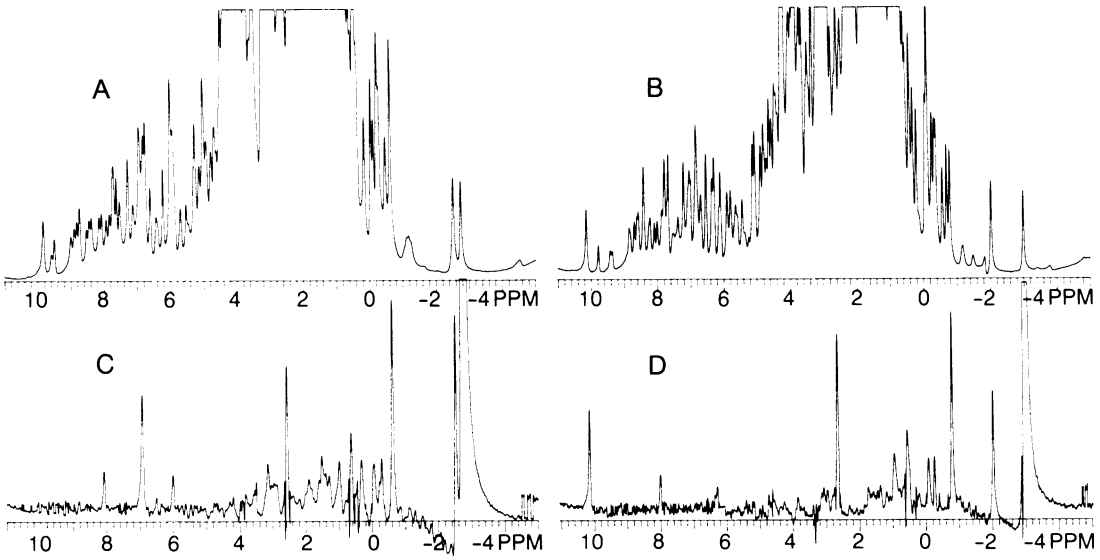

FIG. 3. NOESY spectra were taken of 4.2 mM RNc-II or 4.8 mM RNc(Y67F)-II ferricytochromes *c*, in 20 mM potassium phosphate/0.5 M KCl, pH 6.8, with a mixing time of 100 ms. The first Fourier transforms of the RNc-II (*A*) and RNc(Y67F)-II (*B*) cytochrome *c* NOESY data. (*C*) Trace through the RNc-II 2D-NOESY data depicting the connectivities of leucine-68. (*D*) Trace through RNc(Y67F)-II 2D-NOESY data showing the connectivities associated with the methyl resonance at −2.99 ppm, tentatively identified as leucine-68.

hydroxyl group from tyrosine-67, considered only in terms of the cavity created, would have been expected to destabilize cytochrome *c* by 1–2 kcal/mol, rather than stabilize it by a similar extent. Were it not for the presence of the water molecule, each of the three side chains bound to it would most likely have oriented toward the surface of the protein rather than to its interior, resulting in some energetic cost. Using Janin's criteria (34), the free energy of transfer of the three side chains from the surface to the protein interior should amount to 1.1 kcal/mol, and the free energy of folding of the wild-type protein chain must include a destabilizing contribution of this magnitude. The transfer of the water molecule from the bulk water to the interior is compensated by the formation of three hydrogen bonds (35), but there is an entropy loss of about 2 kcal/mol (36), also unfavorable, bringing the total destabilization energy up to about 3 kcal/mol.

Good predictions of the number of water molecules filling protein cavities can be made if it is assumed that a water molecule can be buried only when at least three hydrogen bonds are formed (33). Accordingly, the buried water molecule cannot be held in the RNc(Y67F)-II mutant, where only two hydrogen bonds can be formed, and the structure of the protein domain within which the water molecule is buried in RNc-II is likely to be different in the mutant. Preliminary evidence supporting this assumption was found in the 2D-NOESY spectrum of RNc(Y67F)-II, in which connectivities that do not exist in RNc-II were clearly observed between the methyl protons of heme methyl group at position 5 and leucine-68.

In the absence of an internal water molecule and its orienting effect on three protein side chains, the destabilization contribution to the native folding of RNc-II, discussed above, cannot exist in RNc(Y67F)-II. Consequently, ΔG for the equilibrium represented by Eq. 1 should be less favorable for the mutant than for the wild-type protein, as observed. Quantitatively, the experimental values of 1.1–1.7 kcal/mol measured for the differences in ΔG for the various processes is gratifyingly close to the tentative estimate of 3 kcal/mol that follows from the above discussion. An additional consequence of the postulated absence of the water molecule in the mutant protein is that the left side of the heme may have become more hydrophobic, as suggested by the NMR data. This region is part of the enzymic interaction domain (37), and the increased hydrophobicity may facilitate the access of the uncharged hydrophobic reductant employed in the polarographic assay, *N,N,N′,N′*-tetramethylphenylenediamine, resulting in the observed increase in V_{max} of the reaction with cytochrome oxidase. An obvious prediction is that other hydrophobic replacements of tyrosine-67 that diminish or eliminate the possibility of capturing a water molecule in the protein interior, as well as mutations of asparagine-52 and threonine-78 to side chains devoid of hydrogen-bonding capacity, should have similar effects on the protein.

It is also interesting to compare the properties of RNc(Y67F)-II with those of *Euglena gracilis* cytochrome *c*, that has a phenylalanine at position 67 but also many additional changes (6). In certain respects, the two proteins show analogous differences of behavior when compared to the wild-type mammalian cytochromes *c*, such as RNc-II or the horse protein (7). For example, the *Euglena* protein has a higher pK value for the alkaline heme-linked ionization, 10.0, and a lower redox potential, 244 mV. However, it is clearly a weaker structure, since it becomes high-spin above 55°C, is autooxidizable, and binds carbon monoxide; none of these properties were observed for RNc(Y67F)-II.

Finally, it is not obvious why the evolutionary process should have maintained the space-filling structure to the left side of the front surface of cytochrome *c*, which involves the internal water molecule and its hydrogen-bonded side chains, when a more stable arrangement was readily available, as in the tyrosine-67 to phenylalanine mutant protein. One may suggest that the relative instability of the enzymic interaction domain on the surface of cytochrome *c* imparted by that structure provides some functional advantage that is not obvious in the steady-state kinetic assays employed so far, which are rate-limited by parameters other than the electron transfer itself or the protein conformation changes that allow the electron transfer to take place.

Note Added in Proof. A mutant yeast iso-1-cytochrome *c* described by Das *et al.* (38), in which asparagine-52 (vertebrate cytochrome *c* numbering) is replaced by isoleucine, has the increased stability shown by the RNc(Y67F)-II mutant, as predicted from the proposed internal water molecule elimination mechanism.

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