Relationship between Local and Global Stabilities of Proteins: Site-Directed Mutants and Chemically-Modified Derivatives of Cytochrome *c*†

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ABSTRACT: The methionine 80 sulfur–heme iron bond of rat cytochrome *c*, whose stability is decreased by mutating the phylogenetically invariant residue proline 30 to alanine and increased when tyrosine 67 is changed to phenylalanine, recovers its wild-type characteristics when both substitutions are performed on the same molecule. Titrations with urea, analyzed according to the heteropolymer theory [Alonso, D. O. V., & Dill, K. A. (1991) *Biochemistry 30,* 5974–5985], indicate that both single mutations increase the solvent exposure of hydrophobic groups in the unfolded state, while in the double mutant this conformational perturbation disappears. Similar increases in solvent exposure of hydrophobic groups are observed when the sulfur–iron bond of the wild-type protein is broken by alkylation of the methionine sulfur, by high pH, or by binding the heme iron with cyanide. The compensatory effects of the two single mutations do not extend to the overall stability of the protein. The added loss of conformational stability due to the single mutations amounts to 7.3 kcal/mol out of the 9 kcal/mol representing the overall free energy of stabilization of the native conformation of the wild-type protein. The folded conformation of the doubly mutated protein is only 2 kcal/mol less stable than that of the wild type. These results indicate that the double mutant protein is able to retain the essential folding pattern of cytochrome *c* and the thermodynamic stability of the methionine sulfur–heme iron bond, in spite of structural differences that weaken the overall stability of the molecule.

It is a tenet of protein chemistry that, for any polypeptide chain under a given set of environmental constraints, the stability of the native conformation is the result of all the noncovalent interactions between backbone and side-chain atoms and the covalent intrachain disulfide bonds. The noncovalent interactions are due to hydrophobic forces, dispersion forces, hydrogen bonds, and electrostatic and dipolar attractions and repulsions (Kauzmann, 1959; Creighton, 1984; Alber, 1989). In heme-IX proteins, to these interactions must be added those arising from the hydrophobic forces and van der Waals contacts involving the porphyrin atoms, the electrostatic and hydrogen-bonding energies arising from porphyrin propionyl groups, and the coordinative bonds to the heme iron atom.

Coordinative bonds between the metal and the protein side chains that act as its ligands exist in all metalloproteins. The distinctive feature of heme proteins is that their iron atoms are fixed in their positions by the chelating power of the porphyrin, which has been estimated at about 40 kcal/mol at room temperature (Falk, 1964). The remarkable strength of this equatorial ligation of the iron, which amounts to between one-third and one-half that of the covalent bonds found in proteins, is by far larger than that of its axial ligation by protein side chains, which is of the order of 5–10 kcal/mol.

Moreover, in cytochrome *c* the porphyrin is covalently bound to the protein by thioether bonds formed between two peripheral substituents, the vinyl side chains, and two polypeptide cysteinyl side chains [reviewed in (Margoliash and Schejter (1966)], so that, in terms of the energies involved, the whole metalloporphyrin moiety can be considered as integrated into the covalent structure of the protein. Two axial coordinative bonds complete the "closed crevice" (George & Lyster, 1958) structure of cytochrome *c*: a relatively weak bond between the iron and one of its axial ligands, the sulfur atom of methionine 80, on the "left side" (Figure 1A), and a stronger bond with the other axial ligand, the histidine 18 imidazole, on the "right side" (Figure 1B) (Takano & Dickerson, 1981a,b). These two amino acids form the *first tier* (Margoliash et al., 1990) of protein side chains interacting with the metal.

Studies of the ways in which the stability of a particular protein is altered by changes in primary structure resulting from mutations, or chemical modification of specific residues, are usually limited to overall conformation energies. In a few cases, specific disulfide bonds have been also investigated (Goldenberg & Creighton, 1986); however, the energies of specific bonds are usually deduced from global stability changes (Kellis et al., 1988), an approach of arguably limited validity. The relatively low stability of the sulfur–heme iron bond of cytochrome *c* in the ferric state, which can be reversibly broken by exogenous ligands, moderate increases in temperature, or changes in pH (Margoliash & Schejter, 1966), presents a unique opportunity to investigate the effects of variations in primary structure on the stability of this specific bond and on the contribution of the latter to the overall stability of the protein.

We have previously described the effects of site-directed mutations at two phylogenetically invariant residues of cytochrome *c* belonging to the *second tier* (Margoliash et al., 1990) of iron-related side chains: on the right side of the heme (Figure 1B) proline 30, whose peptide-chain carbonyl

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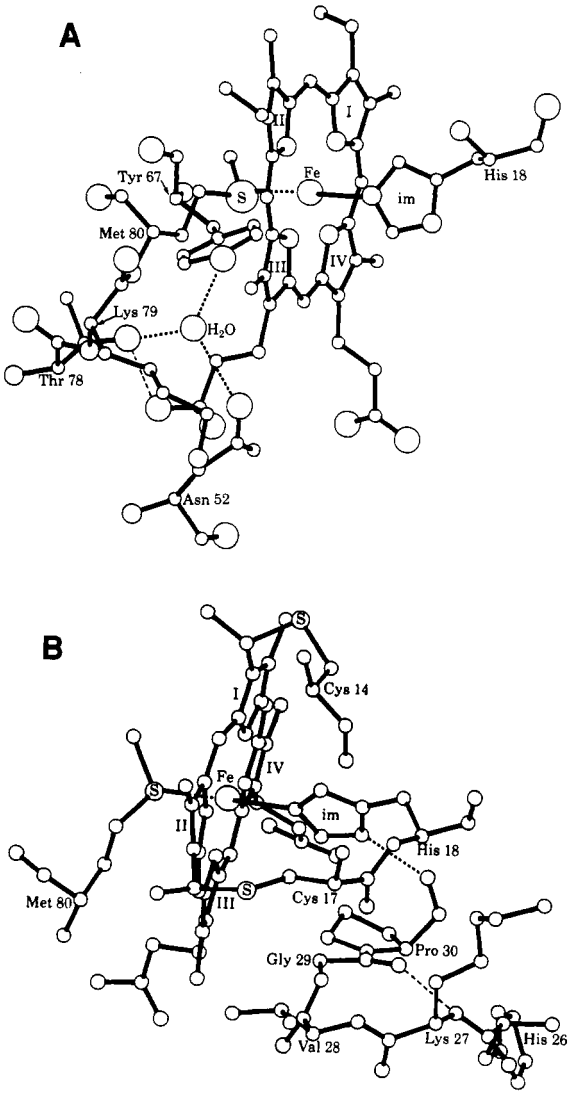


FIGURE 1: Diagrams of the structure of cytochrome *c* in the regions important to the two mutations studied. Fe indicates the heme iron atom, im shows the imidazole side chain of histidine 18, and S is the sulfur atom of methionine 80. These are the axial ligands of the heme iron atom, from the "right" and the "left" sides of the protein molecule, respectively. I, II, III, and IV indicate the corresponding pyrrole rings of the heme. The amino acid residues are indicated in three-letter code placed near their α-carbon atoms. (A) Part of the "left" side of the protein relevant to the effects of the Y67F substitution. The molecule is viewed from the "front", defined as the area containing the solvent-accessible edge of the heme plane, containing pyrrole rings II and III. The heme plane is slightly titled to the left, so that the imidazole axial ligand on the right side has moved closer to the viewer than the sulfur axial ligand on the other side of the heme plane. H_2O marks the internal water molecule hydrogen-bonded (dotted lines) to the side chains of asparagine 52, tyrosine 67, and threonine 78. (B) Part of the "right" side of the protein relevant to the effects of the P30A and P30V substitutions. The two S notations, other than that marking the sulfur of methionine 80, represent the sulfur atoms of cysteines 14 and 17, to which the vinyl side chains of the heme are covalently bonded. The molecule is rotated so that the heme plane is viewed from the "top front", slightly tilted to the left, with the imidazole axial ligand on the right side closer to the viewer than the sulfur axial ligand on the left side. The imidazole axial ligand is hydrogen-bonded (dotted line) from its imino nitrogen to the backbone carbonyl of proline 30. This proline is preceded by a γ bend consisting of residues 27, 28, and 29, hydrogen-bonded (dotted line) between the carbonyl of the last residue and the amide nitrogen of the first. The more usual hydrogen bond between the carbonyl of the first residue and the amide nitrogen of the last is absent. Residues 27, 28, and 29 are lysine, threonine, and glycine in rat cytochrome *c* and lysine, valine, and glycine in tuna cytochrome, the protein whose structure (Takano & Dickerson, 1981a,b) was employed in producing the diagrams depicted here.

is hydrogen-bonded to the pyrrolic nitrogen of histidine 18 (Takano & Dickerson, 1981a,b), was replaced by alanine and by valine (P30A and P30V)[1] (Koshy et al., 1990), while on the left side tyrosine 67, which is located close to the methionine 80 side chain (Figure 1A), was substituted by phenylalanine (Y67F) (Luntz et al., 1989).

These two mutations had diametrically opposed, as well as quantitatively different, local and global effects. In the proline 30 to alanine or valine mutants of rat cytochrome *c* (RN*c*-P30A-II or RN*c*-P30V-II) and in the proline 30 to alanine mutant of the *Drosophila melanogaster* protein (DM*c*-P30A), the methionine 80 sulfur–iron bond was weaker than in the wild-type cytochromes by 1.5 kcal/mol, as judged by the effect of pH (Theorell & Akesson, 1941; Davis et al., 1974) and temperature (Schejter & George, 1965) on the 695-nm spectral band of the ferric protein, the spectral feature representing the sulfur–iron bond. These mutations also weakened the overall conformational stability of the protein but to a distinctly lesser extent (Koshy et al., 1990). Conversely, in the tyrosine 67 to phenylalanine mutant of the rat protein (RN*c*-Y67F-II), the sulfur–iron bond was stronger than in the wild type by 1.5–2 kcal/mol, while the overall stability of the protein at high urea concentrations increased by 0.6 kcal/mol (Luntz et al., 1989). Thus, in both cases, the global effects exerted on the protein appeared to be smaller than the local effects on the sulfur–iron bond, suggesting the existence of mechanisms that partly compensate for stability changes in the tertiary structure brought about by the mutations.

Recent investigations of the effects of double mutations on the conformational stabilities of various proteins have produced a striking result: rather than the expected cooperativity of the mutations, additive effects were observed in several instances, including compensatory effects by which certain mutations restored to the proteins overall stabilities that had been changed by previous mutations (Hurle et al., 1986; Shortle & Meeker, 1986; Matsumura et al., 1986; Wetzel et al., 1988; Sandberg & Terwilliger, 1989, 1991; Wells, 1991). It appeared of interest, therefore, to investigate whether the opposite effects of the two mutations of cytochrome *c* described above would be compensated in a protein containing substitutions at both these sites. To this end, rat cytochrome *c* was mutated at residues 30 and 67 to alanine and phenylalanine, respectively. The present article describes the properties of the doubly mutated protein, RN*c*-P30A/Y67F-II. Studies of the stability of the sulfur–iron bond showed that the local effects of the single mutations compensated each other in the doubly mutated protein. The effects of urea on cytochrome *c* in its native state, and in states in which the closed crevice structure is destroyed, were analyzed on the basis of a thermodynamic model (Tanford, 1970; Becktel & Schellman, 1987; Alonso & Dill, 1991). This analysis indicated that disturbing the native closed crevice results in solvent exposure of hydrophobic groups and lowered overall conformational stability. While similar effects were caused by the single mutations, in the double mutant the overall stability

[1] Abbreviations: gdn, guanidine; pK_{acid}, midpoint of acid ionization; pK_{alk}, midpoint of alkaline ionization; di-CM-*c*, di(met-65-sulfur,met-80-sulfur) carbamoylcytochrome *c* from horse; P30A and P30V, Pro-30 mutations to Ala-30 and Val-30, respectively; Y67F, Tyr-67 mutation to Phe; RN*c*-II, recombinant wild-type rat cytochrome *c*; RN*c*-P30A-II and RN*c*-P30V-II, recombinant rat cytochrome *c* carrying the P30A and P30V mutations, RN*c*-Y67F-II, recombinant cytochrome *c* carrying the Y67F mutation; RN*c*-P30A/Y67F-II, recombinant cytochrome *c* carrying the P30A and Y67F mutations; II, material in chromatographic fraction II, in which the N-terminal amino acid is not acetylated.

was only partly restored, even though the hydrophobic groups which had been exposed were again shielded from the solvent.

EXPERIMENTAL PROCEDURES

Preparative Procedures. The cytochromes *c* of horse, guanaco, great grey kangaroo, mink, Adelie penguin, pigeon, and chicken were isolated from heart, general skeletal, or flight muscles and purified as described by Margoliash and Walasek (1963) and Brautigan et al. (1978). The horse protein was alkylated by treatment with bromoacetamide in the presence of 0.1 M cyanide to produce the dicarbamoylmethylated derivative at methionines 65 and 80 (di-CM-*c*) (Schejter & Aviram, 1970). The procedures for site-directed mutagenesis, recombinant DNA manipulation, culture of the transfected yeast cells, and isolation and purification of recombinant rat cytochrome *c* and its mutants have been described elsewhere (Koshy et al., 1990; Luntz et al., 1989). As previously reported, two chromatographic fractions, I and II, representing N-terminally acetylated and nonacetylated rat proteins, respectively, were obtained for each protein. Both were completely trimethylated at lysine 72. The present study was carried out with fraction II material, which constituted about 75% of the total yield.

The doubly mutated protein, RN*c*-P30A/Y67F-II, was spectroscopically similar to the recombinant wild-type rat cytochrome *c*, RN*c*-II, except for the following differences: (a) the optical spectrum of the ferric form was slightly blue-shifted, the visible and Soret maximum being located at 528 and 408 nm instead of the normal positions of 530 and 410 nm, respectively; (b) in the ferrous form, the ratio of the absorbancies at the α- and β-band maxima was 1.62 instead of the normal ratio of 1.78–1.82 found in wild-type cytochromes *c*; and (c) the isosbestic point of the Soret bands of the oxidized and reduced cytochromes *c* was in its normal position at 410 nm but because of the shift of the oxidized band it was not at the peak of the latter, as is usually the case, but at a slightly longer wavelength.

Analytical Methods. Optical spectroscopic observations were made with Hitachi 557 and Cary 14 spectrophotometers. Fluorescent emission spectra of cytochrome *c* solutions obtained upon excitation at 280 nm were recorded with an Aminco SLM8000 spectrofluorometer. Reduction potentials were measured by the method of mixtures, as described by Margalit and Schejter (1973). Alkaline titrations were performed by dissolving ferricytochrome *c* in solutions of 0.1 M glycine–NaOH buffers, and acid titrations were performed by adding very small amounts of dilute HCl to solutions of the protein in 5 mM sodium phosphate buffer, pH 6.5. The pH values were read directly in the cuvettes, using a combined glass microelectrode, and the spectra were recorded.

Denaturant titrations of the changes in fluorescent emission were performed by dissolving the cytochromes in concentrated urea (ultrapure, Sigma Chemical Co., St. Louis) or neutralized guanidine hydrochloride solutions, containing 0.1 M sodium–potassium phosphate buffer, pH 7.0, and subsequently diluting the solution. The concentrations of urea and guanidine were estimated from refractometric readings (Pace, 1986).

Analysis of Spectroscopic Titrations at 695 nm. The 695-nm band of ferricytochrome *c* characterizes the ligation of the low-spin iron by thioether sulfur (Schechter & Saludjian, 1967; Schejter et al., 1991), and the loss of this band accompanies the cleavage of the methionine 80 sulfur–iron bond (Wooten et al., 1985). At equilibrium, the spectroscopic change can be treated as the two-state process represented by

$$\overset{\overset{X}{\frown}}{(\text{Met})\text{S}\text{–}\text{Fe}^{3+}\text{–Im(His)}} \rightleftarrows \overset{\overset{\text{Met}}{\frown}}{\text{X}\text{–}\text{Fe}^{3+}\text{–Im(His)}} \qquad (1)$$

where X stands for a ligand of unknown identity (Dickerson et al., 1971; Pettigrew et al., 1976; Morishima et al., 1977; Brautigan et al., 1977; Bosshard, 1981; Moore & Pettigrew, 1990) or for an empty coordination position (Aviram & Krauss, 1974; Schejter & Plotkin, 1988).

Thermal Titrations. The 695-nm band of ferric cytochrome *c* loses intensity as the temperature is raised (Schejter & George, 1965). Titrations of this thermal effect were performed in cuvettes thermostated by liquid circulation and the temperatures were read in the cuvette with a Cole-Parmer 8505-45 Type K thermocouple thermometer. Absorbance measurements for the initial state of eq 1 were made at 15 °C. The solutions were then heated by increments of approximately 3 °C, thermal equilibrium was determined by reading for 1 min, absorbance measurements were made after thermal equilibrium was reached, and constancy in the absorbance readings was checked for 1 min.

Analysis of the Spectrofluorometric Titrations with Denaturants. The changes observed in the fluorescent emission at 350 nm, upon excitation at 280 nm, represent unfolding of the protein molecule (Tsong, 1975) and were treated as two-state denaturation equilibria (Pace, 1986). Fluorescent emission values, F, were determined at various urea or guanidine concentrations, with F_0 and F_∞ indicating the values of F in the absence of and after saturation with denaturant. The equilibrium constants between the two states were defined as

$$K = (F - F_0)/(F_\infty - F) \qquad (2)$$

from which the free energy changes, ΔG_D, can be estimated using

$$\Delta G = -RT \ln K \qquad (3)$$

The urea titrations were analyzed as described by Pace (1986) using

$$\Delta G_D = \Delta G_{D,aq} - m[\text{denaturant}] \qquad (4)$$

in which $\Delta G_{D,aq}$ is the free energy change for the unfolding process extrapolated to 0 M denaturant. Equations 2–4 can be combined into the following form:

$$-RT \ln [(F - F_0)/(F_\infty - F)] = \Delta G_{D,aq} - m[\text{denaturant}] \qquad (5)$$

Since values for F_∞ could not always be reached, even for the highest possible concentrations of denaturant, the data were fitted to eq 5 by nonlinear regression analysis, using software (Fig.P, Version 5) purchased from Biosoft (Cambridge, UK).

Molecular Graphics. These were examined employing the Evans and Sutherland PS 390 display system.

RESULTS

Titrations of the Alkaline Ionization. In the moderately alkaline ionization the magnetic low-spin state of the iron and the visible and Soret bands are only marginally affected, but the band at 695 nm disappears (Theorell & Akesson, 1941). While kinetic experiments showed that this ionization also involved a conformation change (Davis et al., 1974) and NMR experiments demonstrated that the methionine 80 sulfur–iron bond was broken (Wooten et al., 1981), the small changes

Table I: Effects of Mutations on the pH and Temperature-Dependent Equilibria of the Cytochrome *c* Crevice Structure and on the Reduction Potential of the Protein

protein	acid p*K*	alkaline p*K*	$T_{1/2}$ (°C)	E_0 (mV)
RN*c*-II	2.8	9.6	60	+259
RN*c*-P30A-II	4.0	8.5	51	+258
RN*c*-Y67F-II	2.1	10.7	90	+224
RN*c*-P30A/Y67F-II	2.6	9.5	62	+236

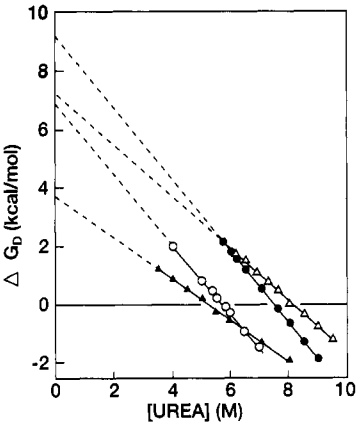


FIGURE 2: Fluorometer urea titrations of recombinent wild-type singly and doubly mutated rat ferricytochrome *c* (3–5 μM) in 0.1 M phosphate buffer, pH 7.0. Excitation wavelength, 280 nm; emission wavelength, 350 nm. The straight lines are drawn using values of the intercept, $\Delta G_{D,aq}$, and slope *m* obtained by nonlinear regression fitting of the experimental data to eq 5. RN*c*-II (●); RN*c*-P30A/Y67F-II (○); RN*c*-P30A-II (▲); RN*C*-Y67F-II (△).

in ultraviolet circular dichroism indicated that the conformation changes were of a minor nature (Myer, 1968).

Table I lists the observed midpoints of this ionization, pK_{alk}, measured for rat cytochrome *c* and its singly and doubly mutated homologues. It is immediately apparent that the opposite alterations of pK_{alk}, caused by the P30A and Y67F mutations, compensate each other almost exactly in the doubly mutant.

Titrations of the Acidic Ionization. In acid solutions, the magnetic and optical spectroscopic properties of ferric cytochrome *c* change, indicating the transition of the iron to the paramagnetic state, which has been classically interpreted as due to changes in the ligation of the metal (Theorell & Akesson, 1941). The actual nature of this change has been widely discussed, and it has become clear that at low pH there is a complicated equilibrium between diverse molecular forms of the protein (Dyson & Beatty, 1982; Moore & Pettigrew, 1990). One of the structural changes that occurs involves the cleavage of the sulfur–iron bond (Wooten et al., 1981).

The observed midpoints of the transition, pK_{acid}, for all the proteins studied are listed in Table I. It is again evident that the opposite effects of the single mutations are compensated in the doubly mutated protein.

Thermal Titrations. The thermal titration of RN*c*-P30A/Y67F-II was completed without the interference of precipitation. The midpoint of the thermal titration was reached at 62 °C (Table I), which is close to the value of 60 °C observed for the wild-type protein (Luntz et al., 1989). Thus, the 30 °C increase in thermal stability of the sulfur–iron bond produced by the Y67F mutation (Luntz et al., 1989), and the 9 °C decrease effected by the P30A mutation (Koshy et al., 1990) were apparently compensated by the mutation at the other site in each of the singly mutated proteins (see Figure 2).

Table II: Parameters for the Unfolding of Natural, Site-Directed Mutants and Chemically-Modified Cytochromes *c* in Urea or Guanidine Solutions

cytochrome	$\Delta G_{D,aq}$ (kcal/mol)	*m* (kcal·l·mol^{-2})	$[urea]_{1/2}$ (M) or $[guanidine]_{1/2}$ (M)
horse	8.73	1.14	7.67
horse (gdn)	8.10	2.79	2.90
chicken	9.51	1.21	7.84
guanaco	9.13	1.15	7.92
kangaroo	9.41	1.22	7.68
mink	9.07	1.25	7.25
penguin	8.41	1.06	7.94
pigeon	8.25	1.11	7.44
horse di-CM*c*	4.31	0.66	6.53
horse di-CM*c* (gdn)	3.89	1.51	2.57
horse, pH 10.5	6.60	0.91	7.22
horse, CN complex	5.26	0.71	7.40
RN*c*-II	9.15	1.21	7.60
RN*c*-P30A-II	3.76	0.70	5.38
RN*c*-Y67F-II	7.19	0.86	8.33
RN*c*-P30A/Y67F-II	6.74	1.16	5.81

Titrations of the Effect of Denaturants on Fluorescence. The cytochromes *c* were studied at concentrations of 3–5 μM in 0.1 M phosphate, pH 7.0, in solutions containing increasing concentrations of denaturants. The cuvettes were irradiated at 280 nm, and the emitted fluorescence was recorded between 320 and 380 nm. The emitted radiation had maximal intensity at 350 nm, and the intensities observed at this wavelength, expressed in arbitrary units, were used for the calculations. In the urea titrations, the end point was usually reached at 8.5–9 M urea, but there were occasional exceptions in which the end was not reached even at 10 M urea. Thus, it was necessary to assign the end points of these titrations by fitting the data to eq 5 with a nonlinear regression analysis (see Experimental Procedures). For uniformity, this procedure was used for all the titrations.

The two parameters that characterize eqs 4 or 5, $\Delta G_{D,aq}$ and *m*, are given in Table II for all the titrations, together with the concentrations of denaturant at the midpoints of the titrations, $[denaturant]_{1/2}$. To categorize the differences between the wild-type cytochromes *c* and the artificial mutants, similar titrations were performed on a variety of cytochrome *c* listed in Table II. The values of $\Delta G_{D,aq}$ were found to vary between 8.25 and 9.51 kcal/mol, while those of *m* varied between 1.06 and 1.25 kcal·l·mol^{-2} and those of $[urea]_{1/2}$ varied between 7.25 and 7.94 M. These results indicate a highly conserved uniformity in the behavior toward urea of a group of mammalian and avian cytochromes *c*, some of which vary in their amino acid sequences by over 10% (Borden & Margoliash, 1976).

Two types of changes of the cytochrome *c* protein were found to alter this pattern significantly, treatments that destroyed the native crevice structure and the single-site mutations (Table II). The former included (a) chemical modification of the horse protein to di-CM-*c*, which decreased by 40% the value of the slope, *m*, and by a similar extent the value of $\Delta G_{D,aq}$, resulting in a $[urea]_{1/2}$ value decreased by 1.1 kcal/mol; (b) alkalinization to pH 10.5, which decreased *m* by 20% and $\Delta G_{D,aq}$ by 25% but $[urea]_{1/2}$ by only 5%; and (c) complexation with cyanide, which resulted in values of *m* and $\Delta G_{D,aq}$ lower by 40% but only 4% lower in $[urea]_{1/2}$.

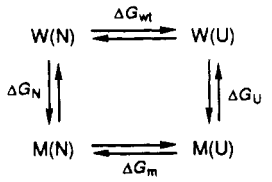
The urea titrations of the wild-type and mutant rat cytochrome *c* are shown in Figure 2. The wild-type protein exhibits values of $\Delta G_{D,aq}$, *m*, and $[urea]_{1/2}$ similar to those for the cytochrome *c* of the various species examined. The single mutants, RN*c*-Y67F-II and RN*c*-P30A-II, had slopes 29% and 42% lower than that of the wild-type rat protein,

respectively. However, while for the Y67F mutant the value of $\Delta G_{D,aq}$ was decreased by only 22%, the corresponding value for the P30A mutant was decreased b 59%. The overall result of these effects of the mutations on the behavior of cytochrome *c* in urea solutions was that in the case of RN*c*-Y67F-II there was an actual increase of $[urea]_{1/2}$ of 0.7 kcal/mol, while for RNc-P30A-II there was a decrease of 2.2 kcal/mol.

The effect of the double mutation on the slope, *m*, was to restore its original value almost entirely. However, since $\Delta G_{D,aq}$ was still 2.4 kcal/mol less for the double mutant than for the wild-type protein, its $[urea]_{1/2}$ was also 1.8 kcal/mol lower.

DISCUSSION

Comparative studies of the stabilities of wild-type proteins and their mutants are frequently analyzed using thermodynamic cycles of the form



(6)

where W and M stand for the wild-type protein and its mutant, respectively, N represents the native state, and U is the unfolded state. The two horizontal reactions are experimentally observable through thermal or denaturant titrations, and the free energy changes ΔG_{wt} and ΔG_m can be calculated; the vertical reactions represent the mutations but can equally represent other changes of the primary structure, such as by chemical modification. On the basis of cycles of this type, it is often claimed that mutations have weakened or strengthened the overall stability of a protein by the extent of $\Delta G_{wt} - \Delta G_m$ (Ackers & Smith, 1985).

Using these cycles is tantamount to assuming that the effects of the structural changes are exactly the same in the two vertical reactions, namely, that similar free energy changes would accompany the transformation of the wild-type protein into its mutant, whether they were performed in the native or in the unfolded state of the protein. This, in turn, implies assuming that substitution of one side chain for another in the highly structured environment of the folded state of a protein will have the same energetic consequences as performing the same substitution in the much less restricted environment of its unfolded state, as has been pointed out by Becktel and Schellman (1987) and by Alber (1989). Furthermore, the vertical reactions cannot be performed under conditions that allow measurement of their thermodynamic parameters. In spite of such difficulties, the assumptions just discussed appear to have been justified (Horovitz, 1987; Wells, 1991) in cases in which multiple mutations resulted in additive strengthening or weakening of conformational stabilities (Matsumura et al., 1986; Sandberg & Terwilliger, 1989). As already mentioned, the local effects of mutations are usually studied by observing changes of overall conformational stabilities, from which the magnitudes of the local changes are deduced (Kellis et al., 1988). The present work has the advantage that the reversible breaking of a well-defined bond is observable sprectroscopically, allowing independent measurement of the effect of primary structure changes on this bond and on the stability of the protein as a whole.

Alkaline and Acid Ionizations. The midpoint of the alkaline ionization varies for the cytochromes *c* of different species from pH 8.4 to pH 10.4 (Brautigan et al., 1977). For most it is at pH 9.4. The P30A mutation of the rat protein lowered the observed pK by 1.1 pH units, while the Y67F mutation raised the pK by 1.2 pH units (see Table I). As stated above, the alkaline ionization involves two reactions: a fast deprotonation with a pK of 11.0, followed by a slower displacement of the methionine 80 side chain from iron coordination (Davis et al., 1974; Wooten et al., 1981).

Preliminary pH-jump studies have shown that the mechanism of this ionization remains unchanged in the singly mutated proteins (T. L. Luntz, E. A. E. Garber, A. Schejter, and E. Margoliash, unpublished results). Furthermore, the P30A and P30V mutations do not involve ionizable side chains, and the possibility that a tyrosine side chain would ionize with an observed p*K* below 11.2 in the wild-type cytochrome *c* has been eliminated by NMR studies (Boswell et al., 1983). Thus, the most plausible explanation of the observed effects of the two single mutations is that these were the consequences of changes in the iron ligation equilibrium and not on the proton affinity of the ionizing group.

The smallest structural change that can be visualized for the process represented by eq 1 is a rotation of the methionine 80 side chain around its β–γ C–C bond. Molecular graphic observations show that this rotation places the methionine-80 methyl group in a position that overlaps almost exactly with that of the internal water molecule (see Figure 1A) held by hydrogen bonds to the side chains of tyrosine 67, asparagine 52, and threonine 78 (Takano & Dickerson, 1981a,b). This leads to the suggestion that cleavage of the sulfur–iron bond and expulsion of this water molecule from its intramolecular position are linked processes. Thus, the alkaline ionization should result in the liberation of these three side chains from their unfavorable positions in the protein interior and a corresponding entropy increase; the latter has been shown experimentally (Davis et al., 1974). Also in keeping with this proposal is that other processes in which the methionine 80 sulfur is diplaced from iron coordination, such as the binding of exogenous ligands (George & Tsou, 1952; George et al., 1967) or warming the molecule to moderate temperatures (Schejter and George, 1965), are driven by entropy changes of similar magnitudes.

The hypothesis that links these processes to the proposed changes in the region of this internal water molecule also explains the higher stability of the iron–sulfur bond in RN*c*-Y67F-II. The removal of the phenolic hydroxyl eliminates one of the three hydrogen bonds required to hold the water molecule in the protein interior (Rashin et al., 1986), also liberating the asparagine 52 and threonine 78 side chains from their unfavorable internal positions, which, in turn, should result in a less favorable contribution to the ionization-triggered equilibrium (Luntz et al., 1989).

It is important to note that this proposal does not imply a direct effect of the mutation on the sulfur–iron bond itself, just like the weakening of the same bond caused by P30A and P30V substitutions could also be due to an indirect effect, transmitted along or across the protein chain. Indeed, these mutations are in a region of the protein of minimal mobility and solvent accessibility (Northrup et al., 1980a,b) and they involve the replacement of a side chain known to interfere with the rotational freedom of the adjacent residue, which in this case is part of a sharp γ bend with an unusual hydrogen-bonding pattern (Koshy et al., 1990) (see Figure 1B). Alternatively, the mutation might have weakened the hydrogen bond in which the carbonyl of the mutated side chain is the acceptor; this, in turn, could have altered the position of the

histidine 18 imidazole ring relative to the porphyrin and weakened the back-donating ability of the iron through a trans effect (Koshy et al., 1990).

The preceding arguments lead to the conclusion that the methionine 80 sulfur–iron bond is specifically weakened by the mutations at position 30 and strengthened by the mutation at position 67. Whatever the mechanisms underlying these effects, the double mutation appears to directly compensate the two single mutations, restoring the bond to its original strength.

An analogous explanation applies to the effect of the double mutation on the complex mechanism of the acid ionization. Since none of the side-chain substitutions involve groups that ionize in that pH range, it appears that the single mutations caused opposite changes on the sulfur–iron bond that were reflected in higher observed pK values for the proline 30 mutants (Koshy et al., 1990) and a lower pK for the tyrosine 67 mutant (Luntz et al., 1989). The return of the acid pK to a value close to that of the wild-type protein in the double mutant protein shows that the effects of the single mutations are additive and, being of opposite signs, compensate each other. It is of particular interest that, while the mutated sites are structurally distant, thermodynamically they are linked through the bond on which the additivity of stabilizing and destabilizing effects were observed.

Thermal Titrations of the 695-nm Band. The 695-nm band disappears (Schejter & George, 1965) at temperatures considerably lower than those at which the protein denatures thermally, as determined calorimetrically (Privalov & Khetchinashvili, 1974). That this is caused by the cleavage of the sulfur–iron bond is supported by the correlation between the midpoint temperatures of thermal titrations of cytochromes c of different species and the pK values of their alkaline ionizations (Osheroff et al., 1980). Therefore, additivity of the effects of mutation on the alkaline ionization of the protein should be similarly observed with respect to temperature-induced changes in the 695-nm band. Previous studies (see Table I) had indicated that the midpoint of the thermal titration was 30 °C higher for RNc-Y67F-II than for the wild-type protein (Luntz et al., 1989) and 9 °C lower for RNc-P30A-II (Koshy et al., 1990). In the present study, the thermal titration midpoint for RNc-P30A/Y67F-II was only 2 °C higher for RNc-II, showing the expected additive effect of the double mutation on the thermal stability of the iron–sulfur bond.

Effects of Denaturants. Experiments on the effects of denaturants on proteins are commonly analyzed by assuming a two-state equilibrium between the native state, N, and the denatured or unfolded state, U (Tanford, 1970). In the present study, we adopt the two-state thermodynamic model (Tanford, 1970; Schellman, 1978, 1987; Becktel & Schellman, 1987) in its statistical thermodynamic formulation, the heteropolymer theory (Dill & Shortle, 1991), recently developed and discussed in detail by Alonso and Dill (1991). This offers a theoretical basis for the well-substantiated observation (Ahmad & Bigelow, 1986; Knapp & Pace, 1974; Santoro & Bolen, 1988; Shortle & Meeker, 1986) that the free energy of the unfolding process depends linearly on the concentration of the denaturant, as expressed by eq. 4. Since the use of extrapolation procedures from the high concentrations of denaturant at which the unfolding phenomena are actually observed to the pure aqueous solvent is open to criticism (Hurle et al., 1986; Kellis et al., 1988), the model is especially important because of the theoretical justification that it provides for such an extrapolation.

In the case of cytochrome c, linearity was observed by McLendon and Smith (1978), in the titrations of seven different wild-type cytochromes c with guanidine hydrochloride, from which we calculate average values of $\Delta G_{D,aq} = 7.77$ kcal/mol and of the slope $m = 2.98 \pm 0.39$ kcal·l·mol^{-2}. In the present study, linearity was found for the urea titrations of eight different cytochromes c. The average $\Delta G_{D,aq}$ for this set of mammalian and avian proteins was 8.96 kcal/mol, with a standard deviation of 0.45 kcal/mol; the average slope, m, was 1.17 ± 0.06 kcal·l·mol^{-2}. Thus, both parameters are properties highly conserved in the phylogenetic descent of the cytochrome c molecule.

While $\Delta G_{D,aq}$ clearly represents the equilibrium between the native and unfolded states in pure water, the physical meaning of m is much less obvious. In the thermodynamic model, m is interpreted as being a function of ΔA, a parameter that represents the increase in the exposure of the nonpolar residues to the solvent when the protein passes from the folded to the unfolded state (Tanford, 1970; Schellman, 1978, 1987; Becktel & Schellman, 1987; Ahmad & Bigelow, 1986; Alonso & Dill, 1991). Since m depends strongly on the amino acid composition of the protein, its value should be conserved in a group of homologous proteins within the limits of the changes in composition.

In its present version, the heteropolymer theory may not be highly predictive for cytochrome c, because it does not take into account (Alonso & Dill, 1991) the presence of electrostatic contributions, which are large (Margoliash & Schejter, 1966), or of the heme group. Nevertheless, calculations of m for various cytochromes c based on the theory of Alonso and Dill (1991) were on average no more than 15% higher than the experimental values determined by McLendon and Smith (1978).

To further justify our use of the heteropolymer model, two additional checks were performed by comparing the titrations of horse cytochrome c and di-CM-c with urea and guanidine. First, the extrapolations to zero concentration of the two denaturants resulted in values of $\Delta G_{D,aq}$ that differed by only 0.5 kcal/mol, within the experimental error typical of denaturant titrations; this indicates that, for cytochrome c, $\Delta G_{D,aq}$ behaves as a thermodynamic function of state, independently of the nature of the denaturing agent (Santoro & Bolen, 1988). A second check was based on the theoretical prediction (Alonso & Dill, 1991) that the ratio of the slopes in guanidine and urea should be given by

$$\frac{m(\text{gdn})}{m(\text{urea})} = 3.02 - 0.31[\text{gdn}]_{1/2} \qquad (7)$$

From the data of Table II, the theoretical ratio of the slopes for the horse protein can be estimated as 2.12, while the experimental value was 2.45; for horse di-CM-c, the theoretical ratio is 2.22 and the experimental value is 2.29.

In the light of the strongly conserved value of m in the wild-type cytochromes c, the finding that five different processes listed in Table II, the two single mutations, the chemical modification of the methionine 80 side chain, the ligation of the iron by cyanide, and the alkalinization to pH 10.5, caused such large and similar changes in m emerges as a surprising result. No less striking is the fact that, in the double mutant, the value of m returns to its normal range (Table II). Large effects of single mutations on m of about 30% were previously reported for three of the 11 mutants of staphylococcal nuclease studied by Shortle and Meeker (1986). While these extensive deviations were attributed to the existence of specific interactions in the unfolded state (Shor-

tle & Meeker, 1986), Alonso and Dill (1991) argued that the relatively large influence of the latter could be due to the intrinsic instability of the protein being studied. This does not apply to our case, because the cytochromes *c* of higher eukaryotes are very stable proteins (Margoliash & Schejter, 1966; Moore & Pettigrew, 1990), and another explanation seems to be warranted.

The value of *m* according to the heteropolymer theory depends on only one parameter that is directly related to the structure of the protein, ΔA, which is proportional to the difference in the number of internal hydrophobic residues in the N and U states of the protein. Clearly, as already pointed out by Alonso and Dill (1991), neither single residue mutations nor chemical modifications could have caused the changes in *m* exhibited by several of the proteins examined above, unless the primary structure changes alter considerably the conformation of the protein. Although the manifold of conformations that constitute the U state includes many that preserve structured domains (Dill & Shortle, 1991), for a protein with the small chain length and the amino acid composition of cytochrome *c* the heteropolymer theory predicts that in the U state there is almost total exposure of hydrophobic residues to the solvent (Alonso & Dill, 1991). Hence, whatever may be the changes brought about by mutations or chemical modifications of cytochrome *c*, the effects revealed by the urea titrations should reflect changes mainly in the conformation of the N state.

In RN*c*-P30A-II, the disruption of the rigid and solvent-inaccessible right-side domain (Northrup et al., 1980a,b) can be easily visualized as causing increased exposure of hydrophobic residues, with the consequent decrease of ΔA. Furthermore, the extrapolation to 0 M urea leads to a value of $\Delta G_{D,aq}$ that is 5.4 kcal/mol lower than in the wild-type protein. This indicates that the effect of the mutation on the protein in the native conformation was distinctly larger than the local destabilization of the sulfur–iron bond, which amounted to 1.5–2 kcal/mol. However, since the overall stability is 9 kcal/mol (Table II), while that of the sulfur–iron bond is about 3 kcal/mol (Margoliash & Schejter, 1966), the relative effect of the mutation on the latter was much larger than on the former. Consequently, the cleavage of the sulfur–iron bond was more than 90% completed in 2 M urea while the protein still retained 85% of its native helical content (Koshy et al., 1990).

In RN*c*-Y67F-II, it is reasonable to expect that the hypothesized rearrangement of the left-bottom quadrant of the molecule created a cavity that was filled partly by the solvent in the N state, again decreasing ΔA. This explanation is also borne out by the fact that similar effects on *m* were observed when the protein was titrated at alkaline pH or chemically modified at the methionine 80 sulfur or when the iron was bound to cyanide. In all these instances, the methionine side chain is displaced from its normal position and must cause a reorganization of the protein chain in the left side of the molecule.

It was earlier stated (Luntz et al., 1989), on the basis of a comparison of the titration curves at high concentrations of urea, that RN*c*-Y67F-II was slightly more stable than RN*c*-II. Since linear extrapolations of urea titration data to zero denaturant concentration are theoretically justified (Alonso & Dill, 1991), this procedure was applied to RN*c*-Y67F-II, resulting in $\Delta G_{D,aq}$ = 7.19 kcal/mol, 2 kcal/mol less than for RN*c*-II (Table II). Thus, since the sulfur–iron bond also contributes to the overall conformational stability of the protein, its strengthening by about 1.5 kcal/mol corresponding to a pK_{alk} increase of 1.1 units (Table I) must have been accompanied by the destabilization of other noncovalent bonds by at least 3.5 kcal/mol.

With respect to the double mutant protein, apparently contradictory results were obtained in that the wild-type value of *m*, which had been strongly decreased by the single mutations, was recovered in the double mutant, but the overall stability of the protein was not. The overall effects of the single mutations were clearly not additive in the double mutant, which was only 2.4 kcal/mol less stable than RN*c*-II, while the summed effects of the single mutations should have amounted to a destabilization of 7.4 kcal/mol. Thus, the thermodynamic additivity of the mutations was limited to their localized effects on the sulfur–iron bond and did not extend to the global stability of the protein. The local domain of the closed crevice has spectroscopic and thermodynamic properties in RN*c*-Y67F/P30A-II which are essentially identical to those in RN*c*-II, indicating a high degree of structural similarity. However, the modified overall structure must be different from that of the wild type, since its general conformational stability is distinctly weaker. It appears, then, that it is the highly conserved "cytochrome *c* fold" that characterizes the protein (Margoliash, 1972) that is maintained, including the distribution of hydrophobic side chains between the protein surface and its interior, while the interactions between internal side chains may undergo rearrangements that the protein as a whole is able to tolerate without losing its functional efficiency.

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