# Equilibrium unfolding of a small bacterial cytochrome, cytochrome $c_{551}$ from *Pseudomonas aeruginosa*

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**Abstract The unfolding of the small cytochrome $c_{551}$ from the bacterium *Pseudomonas aeruginosa* has been characterized at equilibrium by circular dichroism (CD) and fluorescence spectroscopy. The process can be described by a two state mechanism and the thermodynamic stability of cytochrome $c_{551}$ is found to be smaller than that of the larger horse cytochrome *c* ($\Delta G_w = -8.2$ vs. $-9.7$ kcal/mol); we propose that this finding is related to the absence of an 'omega' loop in the bacterial cytochrome. Cytochrome $c_{551}$ loses most of its secondary structure at pH 1.5. The acid transition ($pK_A \approx 2$) is highly cooperative ($n \geq 2$); analysis of optical titrations and contact map suggests that (at least) His-16 (proximal $Fe^{3+}$ ligand) and Glu-70 are both involved in the acid transition. The role of selected hydrophobic, electrostatic and conformational contributions to the overall stability has been investigated by protein engineering. The equilibrium characterization of wild-type and mutant cytochrome $c_{551}$ supports the view that this small cytochrome is an interesting protein to analyze the thermodynamics and the kinetics of folding in comparison with the widely studied horse cytochrome *c*.**



*Key words:* Cytochrome *c*; Protein folding; Stability; Protein engineering

## 1. Introduction

Different amino acid sequences are known to be compatible with a common fold, as shown by comparison of the three-dimensional structure of different proteins from the same 'family'. Identification of residues and/or specific interactions necessary and sufficient to achieve the same characteristic fold [1,2] may be possible by comparing the folding pathway of proteins which share a common fold in spite of low sequence homology.

The class I *c*-type cytochromes belong to a large family of small monomeric heme proteins structurally and functionally extremely well characterized [3]; hence they represent an ideal protein to carry out comparative folding studies. In the past years, horse mitochondrial cytochrome *c* has been used in extensive equilibrium and kinetic studies, which provided basic information on its folding pathway [4–8], and led to the identification of intermediate states. Following this strategy, we have carried out a characterization of the unfolding of cytochrome $c_{551}$ (hereafter cyt $c_{551}$) from the bacterium *Pseudomonas aeruginosa* [9].

The three dimensional structure of cyt $c_{551}$ [10,11] shows that it is highly homologous to the mammalian protein [12], a major difference (Fig. 1) being the smaller size (82 residues compared to 104) due to the absence of the 'omega' loop [13] facing the heme propionates. This loop, present in the mammalian class I cytochromes [3], was proposed to be one of the 'cooperative units' of the folding process [5]. The primary structure of cyt $c_{551}$ shares only $\sim$30% homology with that of horse mitochondrial cyt *c* and its isoelectric point is very acidic ($pI = 4.7$) compared to 10.5–10.8 [3] for horse cyt *c*. A further point of interest is that cyt $c_{551}$ has only one histidine residue (His-16, coordinating the heme iron, Fig. 1) instead of the three residues present in the eukaryotic protein. This feature is attractive because folding kinetics could be studied at pH 7.0 without the miscoordination events observed for cyt *c* [4,8]; in addition to Met-61, coordinated on the distal side, cyt $c_{551}$ has one more methionine (Met-22) which, however, is unlikely to miscoordinate the metal during refolding, because of its topology. Finally, two Trp residues are present in cyt $c_{551}$: Trp-56, which is homologous to Trp-59 of horse cyt *c* and Trp-67 of *Rhodobacter capsulatus* cyt $c_2$ [3], which makes a hydrogen bond to one of the heme propionates, whereas Trp-77, located in the C-terminal helix and peculiar to cyt $c_{551}$, provides a second more distant fluorescent probe.

We report below the unfolding of wild-type cyt $c_{551}$ and compare its thermodynamic properties with those of the larger cytochromes. Moreover we have investigated the contribution of selected amino acids to the overall stability of cyt $c_{551}$ by protein engineering. Four site-directed mutants, all clustered in the region of the molecule facing the heme propionates in which no 'omega loop' is found in cyt $c_{551}$ (see Fig. 1), were expressed and characterized. First of all, Trp-56 was substituted by Phe (W56F) in order to reduce the hydrophobic side chain size and selectively eliminate one hydrogen bond (see above). Pro-58 in the proline rich stretch ($P_{58}I_{59}P_{60}M_{61}P_{62}P_{63}$) containing Met-61 (highlighted in Fig. 1), was substituted with Ala (P58A) in order to establish whether the rigidity of this stretch contributes to the stability of the folded protein, as suggested for the mammalian cytochromes [5]. The other two mutants (Ile-59 → Glu and Val-23 → Asp) were chosen because Ile-59 and Val-23 form a potentially important van der Waals tertiary contact in the native structure.

## 2. Materials and methods

### 2.1. Materials

Wild-type and mutant recombinant cyt $c_{551}$ from *Pseudomonas*

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*Abbreviations:* Gdn·HCl, guanidine hydrochloride; cyt *c*, cytochrome *c*; cyt $c_{551}$, cytochrome $c_{551}$; WT, wild type


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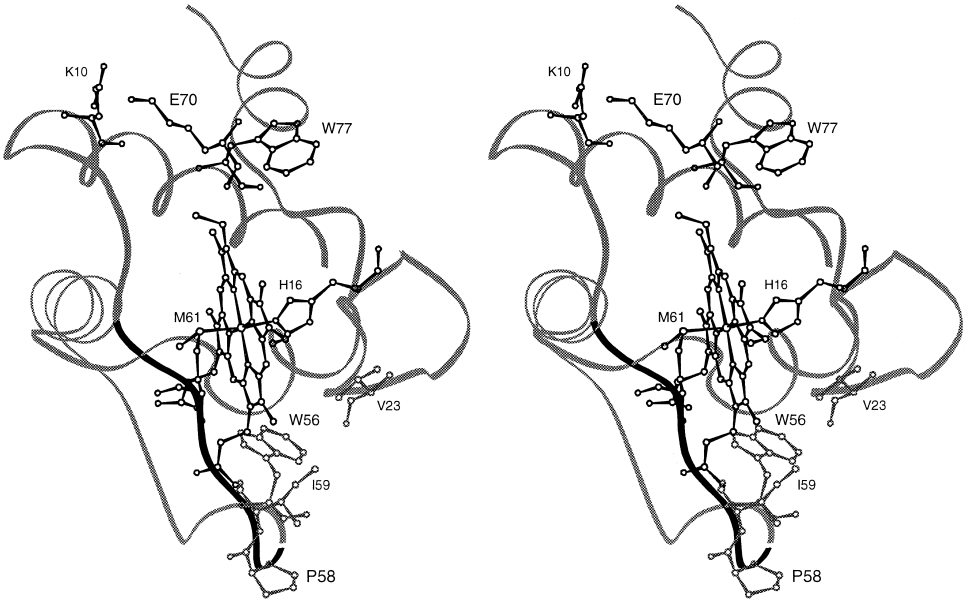


Fig. 1. Structure of cytochrome $c_{551}$ from *P. aeruginosa* (coordinate file, 351c, taken from the Brookhaven Protein Databank). In ball and stick are shown: (i) the residues which were mutated; (ii) the heme with its protein ligands (His-16 and Met-61); (iii) Trp-77, the only fluorescent probe in the mutant protein W56F; (iv) the ionic couple Lys-10/Glu-70 at the N- and C-terminal helices. Highlighted in black is the Pro-rich polypeptide stretch which provides a support for Met-61, the iron distal ligand.

*aeruginosa* were expressed and purified as described by Cutruzzolà et al. [9]. Unfolding experiments were carried out on the oxidized protein, as checked spectrophotometrically. All reagents were of analytical grade.

### 2.2. Spectroscopic measurements

Circular dichroism (CD) spectra were measured with a Jasco spectropolarimeter, Model J-500 A at 1 μM protein concentration. The results are expressed as mean residue ellipticity $[\theta] = 100\ \theta_{obs}(c \times l)^{-1}$, where $\theta_{obs}$ is the observed intensity, $c$ is the concentration in residue moles per liter, and $l$ is the light path in centimeters. The α-helical content was calculated from the ellipticity at 222 nm, according to Chen et al. [14]. All measurements were carried out at 10°C. Tryptophan fluorescence emission spectra were recorded between 300 and 400 nm using a SPEX 2000 single photon counting spectrofluorimeter (ISA Instruments). The excitation wavelength was 290 nm, the protein concentration 1 μM, and the cell length 1 cm.

The guanidine hydrochloride (Gdn·HCl) and urea induced unfolding of cyt $c_{551}$ were measured at pH 7 in 10 mM sodium phosphate buffer. In all experiments the protein had attained equilibrium within less than 2 min and unfolding was shown to be 100% reversible even after two hours incubation in Gdn·HCl (data not shown). Assuming a two-state mechanism, the urea and Gdn·HCl transitions were fitted with the equation proposed by Staniforth et al. [15]:

$$\Delta G_d = \Delta G_w + n\Delta G_{s,m}[D]/(K_{den.} + [D]) \quad (1)$$

where $\Delta G_w$ and $\Delta G_d$ are the free energy of folding in water and at a concentration d of denaturant, respectively; $n$ is the number of buried residues which becomes exposed upon denaturation – in some way analogous to m-values in a linear extrapolation method [16]; $\Delta G_{s,m}$ represents the maximum unfolding solvation free energy at infinite denaturant concentration (with a value of 0.775 kcal/mol in GuHCl and 1.2 kcal/mol in urea); $K_{den.}$ is an empirical constant that represents the concentration of denaturant at which half $\Delta G_{s,m}$ is achieved (with a value of 5.4 M in GuHCl and 25.25 M in urea). $\Delta G_d$ can be derived directly from the data considering that $\Delta G_d = -RT \ln (N/U)$ and $N/U = (S_N - S)/(S - S_U)$, where $S$ is the observed signal (CD or fluorescence), $S_N$ the signal of the native and $S_U$ that of the unfolded state.

Acid unfolding titrations were carried out recording the far-UV CD spectra after equilibrating the protein for 5 min in freshly prepared solutions at increasing HCl concentrations, from pH 7 to pH 0.5, in the absence of salt. Salt titrations at low pH (usually 1.3–1.5) were carried out equilibrating the protein for 5 min in solutions at the desired KCl concentration prepared by diluting with HCl at pH 1.5 a stock of KCl 6 M at the same pH.

Standard methods were used to calculate the apparent $pK_A$ and the Hill coefficient. Acid titrations were analysed using an equation taken from Tanford [17] where, for simplicity, unfolding is coupled to the protonation of residues occurring exclusively in the unfolded state. Under such conditions

$$\Delta G_d = \Delta G_w - RT \ln\{([H^+] + 10^{-pK_{A_i}})^{n_i} * ([H^+] + 10^{-pK_{A_j}})^{n_j}\}$$

where $n_i$ and $n_j$ are, respectively, the number of residues i and j which become protonated upon unfolding.

### 2.3. Contact map of cyt $c_{551}$ from P. aeruginosa

A 'contact map' of cyt $c_{551}$ was calculated using the PDB coordinates of the oxidized protein (351c, Brookhaven Protein Databank) and a program kindly given us by I.G. Badcoe (University of Bristol, UK) running on a Silicon Graphics Workstation. Any atom within 5 Å from a given protein residue or from the heme was taken to indicate a contact, providing a map idea of the surroundings of any residue/heme within the protein. Interactions were verified by looking more closely at the 3D structure of the protein.

## 3. Results and discussion

### 3.1. Gdn·HCl and urea denaturation of wild-type and mutant cyt $c_{551}$

The unfolding behavior and the thermodynamic stability of cyt $c_{551}$ at pH 7 and 10°C were characterized by Gdn·HCl and urea titrations. The CD signal at 222 nm was recorded in order to measure changes in α-helical content, which in the native protein is estimated to be 45%, in agreement with the 3D structure [10]. Trp fluorescence emission at 355 nm, which is completely quenched in the folded protein due to efficient Förster's energy transfer to the heme, was also measured. As shown in Fig. 2A, a single, highly cooperative transition between the native and unfolded states, with no detectable intermediates, is induced by both Gdn·HCl and urea. The unfolded protein has a very low ellipticity at 222 nm and a large

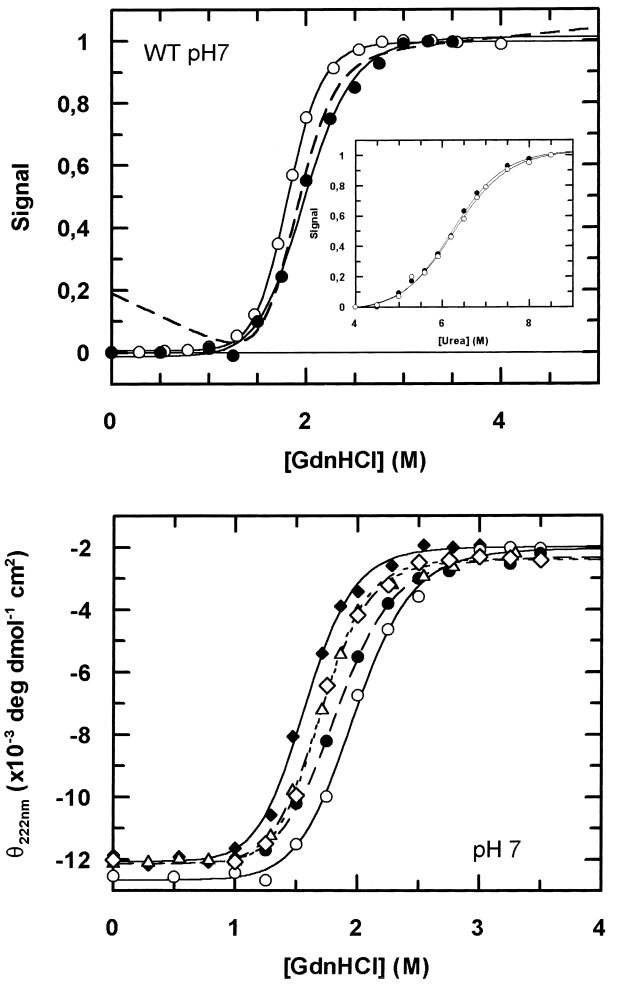


Fig. 2. Gdn·HCl titration of cytochrome $c_{551}$ and four mutants at pH 7 and 10°C. Top: Unfolding transition of the wild-type protein measured by Trp emission at 355 nm (○) and by far-UV CD at 222 nm (●); data were scaled taking the signal of the native state $S_N = 0$ and that of the totally unfolded protein $S_U = 1$. The inset shows, as a comparison, the urea titration of the same protein (see text). The change in CD signal occurring at low concentration of Gdn·HCl (dashed lines; see [27]) was corrected (solid lines) according to Santoro and Bolen [28] assuming a linear dependence of the signal of the native and unfolded species with denaturant: $S_N$ and $S_U$ are replaced by ($S_N$+$m_N$*[D]) and ($S_U$+$m_U$*[D]), respectively, in the equations described in Section 2.2. All the unfolding curves were fitted to the equation proposed by Staniforth et al. [16]. Bottom: Unfolding transitions of P58A (●), W56F (◇), I59E (△), V23D (◆) and wild-type (○). Lines superimposed on the data are the fitted curves.

fluorescence signal characteristic of a random coil, in accordance with that observed for eukaryotic cyt *c* [6].

The values of $\Delta G_w = -8.2 \pm 0.2$ kcal/mol and $C_m = 1.8$ M obtained for Gdn·HCl induced unfolding of WT cyt $c_{551}$ at pH 7 (Table 1) are smaller than those obtained for horse cyt *c* ($\Delta G_w = -9.7 \pm 1.1$ kcal/mol, $C_m = 2.77$ M; [7]). This difference is considered to be significant since it is in the range of stabilities of unfolding observed in the literature for different proteins and their mutants [18]; in addition our experimental error is fairly low. The reduced stability of cyt $c_{551}$ is probably due to the absence of the 20 residue 'omega' loop present in the eukaryotic class I cytochromes, or, in other words, to its smaller size. Table 1 also lists values of *n*, the number of buried residues which become exposed to solvent over the observed transition; this number is a reflection of the steepness of the transition. The value of 42 obtained for WT cyt $c_{551}$ is reasonable for an 82 residue protein, considering that on average 55–77% of the surface area of a protein becomes inaccessible upon folding [18], and that the smaller the protein, the smaller this fraction.

Unfolding of the four mutants, followed by far-UV CD (Fig. 2, panel B), again conforms to a single cooperative transition between two states; the values of $\Delta G_w$, *n* and $C_m$ are listed in Table 1. Fluorescence measurements are not shown for clarity reasons; however it is interesting to note that the emission of the W56F mutant is exactly half the intensity of all other proteins, showing that the two Trps of the WT and the other three mutants contribute equally to the overall fluorescence signal observed.

As outlined in Section 1, Trp-56 is within hydrogen-bonding distance from the heme propionate 17 [10]. Although Cutruzzolà et al. [9] reported this H-bond to be important in the modulation of the redox potential of cyt $c_{551}$, replacement of this residue by Phe (in the W56F mutant) has a marginal effect on the thermodynamic stability of the protein (Table 1). Recent data on the stability of cytochrome $c_2$ from the bacterium *Rb. capsulatus*, which has the largest 'omega' loop, indicate that substitution of the homologous Trp-67 with Tyr decreases the conformational stability of cyt $c_2$ by 2.5 kcal/mol [19]. Although these authors suggested that the primary role of this conserved Trp in cytochromes *c* is to provide a stabilizing hydrophobic core, our data seem to indicate that Trp-67 in *Rb. capsulatus* cyt $c_2$ may simply stabilize the 'omega' loop. Since there is no loop in cyt $c_{551}$, the homologous Trp-56 has no specific effect on stability.

Substitution of Pro-58 with Ala (P58A) was engineered to establish whether the rigidity of the proline rich stretch

Table 1

| | Gdn·HCl denaturation (pH 7) | | *n* | Acid denaturation | |
|---|---|---|---|---|---|
| | $C_m$ (M) | $\Delta G_w$ (kcal/mol) | | $\Delta G_w$ (kcal/mol) | p$K_A$ |
| WT | 1.8 | −8.2 ± 0.2 | 42 | −6.4 ± 0.2 | 1.9 |
| | 6.2$^U$ | −8 ± 0.1$^U$ | 37$^U$ | | |
| W56F | 1.5 | −7.5 ± 0.2 | 45 | −6.4 ± 0.2 | 1.9 |
| I59E | 1.5 | −7.6 ± 0.1 | 44 | −7.2 ± 0.2 | 2.2 |
| P58A | 1.5 | −6.3 ± 0.7 | 33 | −7.8 ± 0.2 | 2.0 |
| V23D | 1.5 | −6.8 ± 0.1 | 40 | −6.8 ± 0.2 | 2.2 |

Thermodynamic parameters derived from fitting the Gdn·HCl unfolding curves shown in Fig. 2 to the equation of Staniforth [16] ($^U$ = urea unfolding curves) and the acid unfolding curves shown in Fig. 3A to the equation of Tanford [17] (Section 2.2). The table shows, for each of the five proteins, the apparent stability of the folded state ($\Delta G_w$, kcal/mol) over the unfolded states in Gdn·HCl or acid. For the Gdn·HCl and urea data is also indicated the apparent number of residues which become exposed on unfolding during the N ↔ U transition, *n*, and the concentration of denaturant (in molarity) at the midpoint of this transition ($C_m$). The apparent p$K_A$s for the acid unfolding transitions (or midpoints) are also listed.

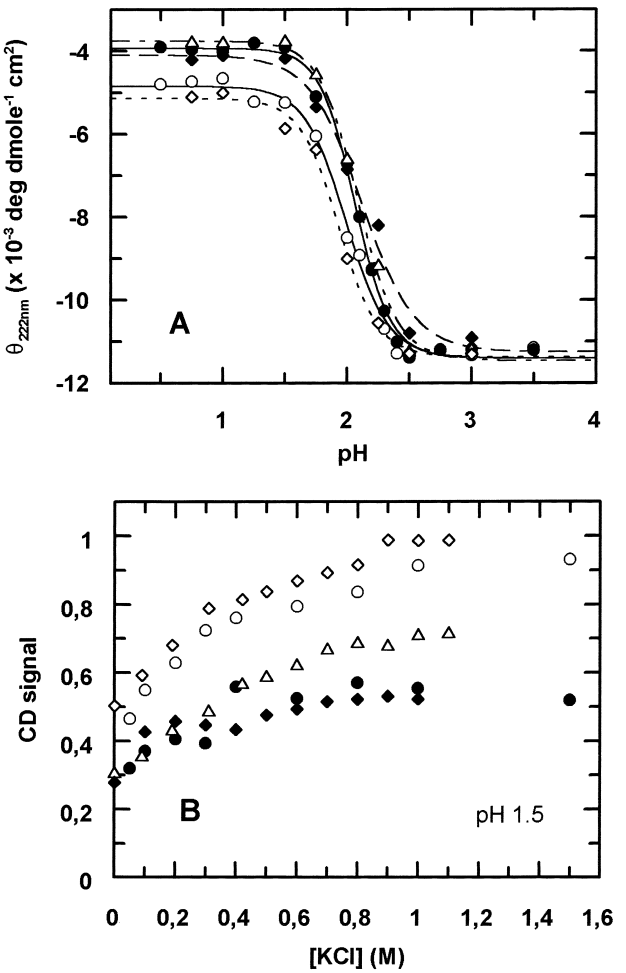


Fig. 3. Effect of acid and of salt on cytochrome $c_{551}$ and four mutants. A: Acid transition measured at increasing concentrations of HCl (in the absence of added salt) by far-UV CD at 222 nm, at 10°C, of wild-type (○), P58A (●), I59E (△), W56F (◇) and V23D (◆). Continuous (WT and P58A) and dashed (V23D, I59E, W56F) lines are fits to an equation from Tanford [17] (Section 2.2). B: KCl titration of the five proteins in HCl at pH 1.5 (acid unfolded state). The scale is such that a CD signal of 1 is equivalent to that of the native protein ($S_N$) and a signal of 0 equivalent to that observed for the totally unfolded protein ($S_U$), in Gdn·HCl. The ellipticities of the high salt species are therefore 50% for both V23D and P58A and 70% for I59E, relative to that of the native protein. Symbols are the same as in (A).

($P_{58}I_{59}P_{60}M_{61}P_{62}P_{63}$) containing the axial ligand Met-61 (Fig. 1) contributes to the stability of the folded protein. As shown in Table 1, P58A is significantly destabilized compared to WT (by 1.5–2.0 kcal/mol). However, since no significant change has been observed on the stability of P58A by alkaline titration of the 695 nm band (data not shown), a change in $-\Delta G_w$ for this mutant is more likely related to a greater stability of the unfolded state. More precisely, this mutation may enhance the conformational entropy of the U state: since prolines provide a constraint to the polypeptide backbone they reduce the number of states accessible to the unfolded chain [20,21].

In cyt $c_{551}$ Val-23 is engaged in a hydrophobic tertiary interaction with Ile-59 shielding the heme pocket from outside solvent (see Fig. 1). It was therefore expected that a non-conservative mutation of either of these residues should destabilize the protein. However, the Ile-59 to Glu mutation (I59E) has a smaller effect on $\Delta G_w$ than the Val-23 to Asp mutation (V23D) (Table 1). Although we have no solid explanation for this difference, it is possible that the side-chain of Glu, longer than that of Asp, may allow greater spatial mobility to the negative charge [22], therefore destabilizing the native state to a more limited extent.

### *3.2. pH titrations and salt effects*

By far UV CD measurements, a single cooperative transition is seen for the acid-induced unfolding of WT cyt $c_{551}$ at low salt (Fig. 3A). The apparent $pK_A = 1.9$ (Table 1) is somewhat lower than that determined for horse cyt $c$ ($pK_A = 2.5$ [6]). An acid-denatured state of cyt $c_{551}$, comparable to that obtained at pH 2 for horse cyt $c$ [6], is fully populated at pH = 1.5; its secondary structure content remains unchanged as the pH is lowered further. This acid-denatured state of cyt $c_{551}$ is different from a fully unfolded state (as obtained with 6 M Gdn·HCl), and contains a residual amount of secondary strucure (data not shown).

Inspection of the titration profile shows that more than one titratable group is involved in the acid denaturation, the calculated Hill coefficient being $\approx 2.3$, implying that at least 2 acidic groups must be involved. Several considerations can be made to assign these residues responsible for acid denaturation. First of all, acid titration of WT cyt $c_{551}$ followed by absorption at 695 nm (diagnostic of the Met–Fe bond in oxidized cyt $c$ [3]), indicates a change in Fe coordination low/high spin with a $pK_A = 2.1$ (and Hill coefficient $\sim 2.3$), most probably coupled to protonation of the proximal ligand His-16 (data not shown). Since far UV CD (Fig. 3) and 695 nm optical spectroscopy monitor an acid transition with the same $pK_A$ and the same degree of cooperativity, we conclude that cooperative acid unfolding is associated with His–Fe bond breaking and secondary structure loss.

In order to assign the other side-chains coupled to the acid denaturation of cyt $c_{551}$, we used the contact map (see Section 2.3) to identify internal electrostatic interactions. Glu-70, in the C-terminal helix, forms a salt bridge with Lys-10 in the N-terminal helix of the protein as seen in Fig. 1; this interaction is highly conserved among 6 different cyt $c_{551}$ [3]. Asp-19 forms another salt bridge with Lys-28. Thus our hypothesis is that protonation of His-16, Glu-70 and possibly Asp-19 are involved in the acid unfolding. Applying the equation from Tanford ([17], Section 2.2) which implies that unfolding is driven by the probability of a number $n_i$ and $n_j$ of groups to become protonated at the same denaturant concentration, a sufficiently good fit to the data (Fig. 3A), was obtained with $n_i + n_j = 2$. A contact map analysis could not be carried out on mammalian cyt $c$ since this protein's coordinates [23] are not yet available in the Brookhaven Protein Databank. A relevant observation, however, is that a conserved hydrophobic interaction between Leu-94 and Gly-6 has been proposed to be important in the formation of an early kinetic intermediate in horse cyt $c$ [7]. These two residues are located in the structural region between the C-terminal and N-terminal helices where Glu-70 and Lys-10 are found in cyt $c_{551}$.

On the other hand, the two heme propionates, HP-17 and HP-13, are unlikely to be involved in the acid unfolding transition. The former has a $pK_A = 6.2$ [3], well above the pH range of interest, and the latter ($pK_A = 3$ [24]), is unlikely to

be involved because it is highly solvent exposed and makes no interactions with the protein.

The acid-induced unfolding of the mutants, reported in Fig. 3A, also shows a single transition with apparent p$K_A$ between 1.9 and 2.2 (Table 1) and very much the same cooperativity ($n \sim 2$) as WT. However, inspection of the titration curves shows that the secondary structure content of the acid unfolded state of three mutants is somewhat smaller than WT (see asymptote in Fig. 3A). Fitting the acid transition as described above leads to an estimate of the stability of the native and acid unfolded states of the five cytochromes; the values of $-\Delta G_w$ are listed in Table 1. Examination of the data is consistent with the view that WT and W56F are the least unfolded at pH 1.5, since $-\Delta G_w$ for the native→acid unfolded state transition is smaller than for the other mutants.

It has been reported that the acid unfolded state of mammalian cyt *c* recovers most of its secondary structure by further increasing HCl or by addition of 0.5 M KCl [25,26]. WT cyt $c_{551}$ and W56F both regain secondary structure (up to 98%) by addition of KCl 1 M (Fig. 3B). The acid unfolded states of mutants P58A, I59E and V23D with a smaller α-helical content (Fig. 3A), recover significantly less secondary structure by KCl addition, and do not regain the α-helical content of their native states even at [KCl] > 1 M (Fig. 3B). No protein aggregation effect was observed since the same experiment (i.e. KCl titration of the acid unfolded state) carried out at 0.5, 1 and 5 μM protein concentration gave, after normalization, identical results.

### 3.3. Concluding remarks

The results presented in this study provide an example of a rationale for performing comparative folding studies on proteins which share a common fold in spite of low sequence homology. This approach provides information on the folding mechanism(s) of different proteins from the same class, and thereby allows to identify specific residues and/or interactions necessary and sufficient to achieve a characteristic fold. We have chosen to apply this comparative-folding approach to cyt *c*, and have started by characterizing the thermodynamic of unfolding of the small and acidic cyt $c_{551}$ from *P. aeruginosa* and four site-directed mutants.

We have shown by isothermal unfolding experiments that the thermodynamic stability of cyt $c_{551}$ is significantly lower (∼1.5–2.0 kcal/mol) than that of horse cyt *c*, an observation that we correlate to the absence of the 20 amino acid 'omega' loop, which shields the heme in the region of the propionates in horse cyt *c*. The significance of this loop for the stability of larger cytochromes *c* has been stressed by Caffrey and Cusanovich [19], who showed that Trp-67 plays an important role in stabilizing the core of this microdomain in cyt $c_2$ from *Rb. capsulatus*; accordingly, mutation of the homologous Trp-56 to Phe (W56F) in cyt $c_{551}$ has no effect on the overall stability of the protein (Table 1). Therefore we conclude that in the absence of the 'omega' loop, Trp-56 per se has no specific effects on the stability of cytochromes, reinforcing the view that the 'omega' loop makes a significant contribution to the overall stability of cytochrome *c* (and indeed was shown to be one of the folding domains [5]).

Analysis of the pH titrations as monitored by CD and optical spectroscopy and of a contact map of cyt $c_{551}$ allow to propose two (and possibly three) groups involved in the acid unfolding shown in Fig. 3A. The crucial observation is that the acid titration (p$K_A \sim 2$) is very cooperative, with a Hill coefficient ≥ 2. Our data support the hypothesis that His-16 (the proximal $Fe^{3+}$ ligand) and Glu-70 (engaged in a salt bridge with Lys-10 across the C-terminal and N-terminal helices) are both protonated upon unfolding. A step forward along this line will of course involve mutation of Glu-70. It is relevant that in this same region of cyt *c* an invariant hydrophobic interaction (between Gly-6 and Leu-94) is thought to be essential for the stability of the burst phase intermediate observed in the kinetic folding pathway of the mammalian protein [7]. We therefore believe that in two different cytocromes, characterized by the same fold, a crucial interaction, albeit of a different nature (electrostatic or hydrophobic), has been selected to stabilize the contacts in between the two terminal helices, a primary motif in the folding of cytochrome *c*.

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