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Thermodynamics of Loop Formation in the Denatured State of *Rhodopseudomonas palustris* Cytochrome *c'*: Scaling Exponents and the Reconciliation Problem

K. Sudhindra Rao, Franco O. Tzul, Arwen K. Christian, Tia N. Gordon, and Bruce E. Bowler*

Department of Chemistry & Biochemistry, Center for Biomolecular Structure & Dynamics, University of Montana, Missoula, Montana, 59812, USA

Abstract

The observation that denatured proteins yield scaling exponents, ν , consistent with random coil behavior and yet can also have pockets of residual or non-random structure has been termed the “reconciliation problem”. To provide greater insight into the denatured state of a foldable sequence, we have measured histidine-heme loop formation equilibria in the denatured state of a class II *c*-type cytochrome, cytochrome *c'* from *Rhodopseudomonas palustris*. We have prepared a series of variants that provide His-heme loop stabilities, $pK_{\text{loop}}(\text{His})$, for loop sizes ranging from 10 to 111 residues at intervals of 7 to 11 residues along the sequence of the protein. We observe a scaling exponent for loop formation, ν_3 , of 2.5 ± 0.3 . Theoretical values for ν_3 range from 1.8 to 2.4, thus the observed ν_3 is consistent with random coil behavior. However, in contrast to data for loop formation as a function of loop size obtained with peptides of homogeneous sequence, we observe considerable scatter about the linear dependence of loop stability on loop size. Thus, foldable sequences behave very differently from homogeneous peptide sequences. The observed scatter suggests that there is considerable variation in the conformational properties along the backbone of a foldable sequence, consistent with alternating compact and extended regions. With regard to the “reconciliation problem”, it is evident that a scaling exponent consistent with a random coil is necessary but not sufficient to demonstrate random coil behavior.

Keywords

Denatured states; Loop formation; Random coil; Scaling exponents; Reconciliation problem

Introduction

The role of the denatured state in directing the early stages of protein folding has been widely debated.^{1–6} Non-random structure in the denatured state has the potential to limit the search required to fold a protein by providing nucleation sites from which growth of the native structure can proceed. NMR studies provided the first compelling evidence for residual structure in the denatured state.^{7–12} Fluorescence resonance energy transfer (FRET) methods have yielded additional evidence that denatured states can be compact with non-random structure.^{13–17} On the other hand, measurements of the radius of gyration (R_g) or hydration

*To whom correspondence should be addressed. Phone (406) 243-6114. Fax (406) 243-4227. bruce.bowler@umontana.edu.

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(R_h) of denatured proteins by small-angle X-ray scattering (SAXS),^{18,19} NMR²⁰ and viscometric^{21–23} methods give a scaling exponent, ν , of ~ 0.6 , consistent with a random coil.⁴ The evidence that proteins both have residual or non-random structure and behave as random coils has been dubbed the “reconciliation problem”.^{4,19} A study by Fitzkee and Rose,²⁴ however, has shown that the scaling properties of a disordered protein are not necessarily sensitive to the presence of local structure. Thus, experimental data that is sensitive to local residual structure in a disordered protein and can provide a measure of the scaling exponent for a disordered protein is essential to better understand the basis of the “reconciliation problem”.

Measurement of loop formation for loops of different sizes within a polypeptide provides scaling exponents for disordered polypeptides.^{25–27} Loop formation should also be sensitive to pockets of residual or non-random structure which cause local variations in the degree of extension of the polypeptide chain or its flexibility. Regions with and without residual structure in a denatured protein might be expected to cause significant scatter about the log-log plot of the equilibrium or rate constant for loop formation versus loop size. Existing kinetic and equilibrium data for loop formation suffer from two problems. The first of these is that much of the kinetic data has been obtained with homopolymers or alternating co-polymers.^{25–27} Thus, if there is non-random structure, it will be the same throughout the sequence and not detectable as scatter about the dependence of rate versus loop size. Secondly, kinetic and equilibrium data on loops formed in the denatured state of naturally-occurring proteins is often limited to a single data point.^{28–30} In the best circumstances, four data points have been used to determining a scaling exponent.^{31,32} Thus, it has been difficult to assess scatter about the log-log plot of rate or equilibrium constants versus loop size for foldable protein sequences.

To provide a more complete dependence of loop formation data on loop size, we have applied our denatured state histidine-heme loop formation method^{31,32} to cytochrome *c'* (Cyt*c'*) from *Rhodospseudomonas palustris*. This class II *c*-type cytochrome is a four helix bundle protein with the heme attached through Cys 113, Cys 116 and His 117 near the C-terminus of the polypeptide chain (Fig. 1).³³ Histidine-heme loops formed from the N-terminal side of the heme in the denatured state are not subject to a heme excluded volume effect as with loops formed from the C-terminal side of the heme.³⁵ Thus, a larger dynamic range of loop sizes for denatured state loop equilibria can be measured than for our previous studies with yeast iso-1-cytochrome *c*.^{31,32} We present denatured state equilibrium loop formation data for 13 variants of Cyt*c'* which have a single histidine besides the native state heme ligand, His 117. We observe significant scatter about the log-log plot, consistent with regions of non-random structure alternating with regions of more random structure in the denatured state of this protein.

Results and Discussion

His-heme loop formation in the denatured state

The scaling of the dimension of a given type of polymer with length is commonly used to classify its conformational properties. Scaling exponents for a polymer can be obtained by radius of gyration (R_g) measurements or by determining the probability of forming closed loops of different sizes for a particular polymer. The Jacobson-Stockmayer equation^{36,37} is used to evaluate loop equilibria as a function of loop size for polymers, assuming a Gaussian distribution for the end-to-end distance of the chain (Eq 1), where ν_3 is the scaling exponent, N is the number

$$\Delta S_{\text{loop}} = -\nu_3 R \ln(N) + R \ln \left((3/2\pi C_n \ell^2)^{\nu_3} V_i \right) \quad (1)$$

of monomers in the loop, R is the gas constant, C_n is Flory's characteristic ratio, ℓ is the distance between monomers and V_i is the volume within which the ends of the chain must be constrained for the loop to form. For a freely jointed random coil, ν_3 is 1.5. For a random coil with excluded volume values of 1.8 to 2.4 are expected.^{38–40}

We use His-heme binding in the denatured state of *c*-type cytochromes (heme is covalently bound through a CXXCH heme attachment motif) to monitor loop formation. Since histidine must be deprotonated to bind to the heme, His-heme loop formation equilibria can be evaluated with a simple pH titration (Fig. 2). We monitor a change in the spin-state of the Fe^{3+} -heme when the loop forms, as the weak field ligand, H_2O , is replaced with the strong field imidazole ligand from the side chain of a histidine (Fig. 2). This spin-state change is readily monitored through a blue shift in the heme Soret band near 400 nm as the Fe^{3+} -heme goes from the low spin (loop formed) to the high spin (loop broken) state. The data can readily be fit to a modified form of the Henderson-Hasselbalch equation (Eq 6, Materials and Methods) which yields an apparent pK_a , $\text{pK}_a(\text{obs})$, and the number of protons, n , released upon loop formation. A lower $\text{pK}_a(\text{obs})$ indicates a more stable loop (i.e., higher concentration of protons – lower pH – required to break the His-heme bond). The loop formation equilibrium in Figure 2 can be broken down into a two step process (Eq 2).

Thus, the overall equilibrium, $\text{pK}_a(\text{obs})$, can be taken as the sum of the histidine protonation equilibrium, $\text{pK}_a(\text{HisH}^+)$, and the equilibrium for loop formation via binding of a fully deprotonated histidine to the heme ($\text{pK}_{\text{loop}}(\text{His})$, Eq 3).

$$\text{pK}_a(\text{obs}) = \text{pK}_a(\text{HisH}^+) + \text{pK}_{\text{loop}}(\text{His}) \quad (3)$$

In our studies on loop formation in the denatured state of iso-1-cytochrome *c*, we have measured $\text{pK}_a(\text{HisH}^+)$ for histidines at positions that differ widely in local electrostatics and for concentrations of gdnHCl ranging from 3 M to 6 M. We find that $\text{pK}_a(\text{HisH}^+) = 6.6 \pm 0.1$ irrespective of gdnHCl concentration or local electrostatics. Therefore, in concentrated gdnHCl solutions, the intrinsic pK_a of the imidazole side chain of histidine appears to be insensitive to local sequence. Thus, we typically subtract $\text{pK}_a(\text{HisH}^+)$ from $\text{pK}_a(\text{obs})$ to obtain $\text{pK}_{\text{loop}}(\text{His})$.

If we assume random coil behavior (i.e. loop formation is completely entropic) and that the enthalpy of forming the Fe^{3+} -imidazole bond is the same for all His-heme loops, then we can equate the free energy of His-heme binding [$\Delta G_{\text{loop}}(\text{His}) = 2.3RT\text{pK}_{\text{loop}}(\text{His})$] to $-T \Delta S_{\text{loop}}$ for a random coil (Eq 1). Thus, for a random coil, the dependence of $\text{pK}_{\text{loop}}(\text{His})$ on the log of loop size, $\text{Log}(N)$ should be linear with a slope equal to the scaling exponent, ν_3 (Eq 4, $\text{pK}_{\text{loop}}(\text{His})_{\text{ref}}$ and N_{ref} are the $\text{pK}_{\text{loop}}(\text{His})$ and loop size, N , for an arbitrary reference loop).^{31,32}

$$\text{pK}_{\text{loop}}(\text{His}) = [\text{pK}_{\text{loop}}(\text{His})_{\text{ref}} - \text{Log}(N_{\text{ref}})] + \nu_3 \text{Log}(N) \quad (4)$$

Variant design

Cytc' from *R. palustris* has a 5-coordinate heme with His117 providing the sole axial ligand.³⁴ Leu12 sits above the vacant coordination site. There are no histidines besides His117 in the

sequence of the wild type protein. The N-terminal glutamine of wild type Cyt_c' cyclizes to form pyroglutamate during expression from *E. coli*, yielding a mixture of glutamine and pyroglutamate at the N-terminus.⁴¹ Thus, we use a Q1A variant⁴¹ as our pseudo wild-type (pWT) in this work. Sites chosen for mutation to histidine were all solvent-exposed to minimize stability effects on the native structure (Table 1). The set of variants can form loops in the denatured state ranging from 10 to 111 residues in length.

Equilibrium unfolding of Cyt_c' variants

GdnHCl denaturation was carried out for the pWT and all variants at pH 6.5. Typical data are shown in Fig. 3 and the thermodynamic parameters (ΔG_u^0 (H₂O), the free energy of unfolding in the absence of denaturant; m , the rate of change of the free energy of unfolding, ΔG_u , as a function of denaturant concentration; C_m , the concentration of gdnHCl at the unfolding midpoint) for all variants are collected in Table 2. All variants are less stable than the pWT. For five of the variants, K97H, A66H, D58H, K31H and D3H, the substitutions are in non-helical regions. The remaining eight variants have substitutions in helices. Of these, Lys and Ala to His mutations (K13H, K20H, K39H, K49H, E73H, K84H, A91H and A104H) might be expected to be destabilizing to the native state since the helical propensities of alanine and lysine are considerably higher than that of histidine.^{43,44} Except for the E73H variant, which is at the end of a short helix, most of the variants in this latter group have substitutions that are about one turn into the helix. The K13H and K84H variants have substitutions near the center of a helix, where the effects of decreased helix propensity are expected to be most pronounced.⁴⁴ Inspection of the ΔG_u^0 (H₂O) values in Table 2, however, does not show a clear correlation with helix position or propensity. Several of the mutated residues make electrostatic contacts of 5 Å or less, including Lys13 (5 ± 2 Å to Glu17), Lys20 (3.4 ± 0.4 Å to Glu17) and Lys49 (4.4 ± 0.4 Å to Asp46). At pH 6.5, the charge on a surface exposed His will only be partial, so some loss in stability due to less favorable electrostatics is possible. The side chain of Ala66 is within 4 to 6 Å of the guanidyl and amino groups of Arg121 and Lys124, respectively. Thus, the A66H substitution could introduce unfavorable electrostatic repulsion. However, the correlation with native state electrostatics is not qualitatively obvious from the ΔG_u^0 (H₂O) values in Table 2.

Although there are a few outliers, there is a trend of increasing stability, judged either by C_m or ΔG_u^0 (H₂O), as the size of the histidine-heme loop that can form in the denatured state increases from 10 (A104H variant) to 111 (D3H variant). This trend suggests that the relative stabilities of the variants have a denatured state component, since the smallest His-heme loops will stabilize the denatured state the most.⁴⁵

In our studies on yeast iso-1-cytochrome *c* variants which form denatured state histidine-heme loops, we have noted that the m -value decreases as denatured state loop size increases out to a loop size of ~70 and then begins to increase slightly.⁴⁶ The data in Table 2 are consistent with this earlier observation. The current data and our previous results suggest that moderate sized loops in the denatured state lead to a decrease in the solvent exposed surface area of the denatured state. The K20H variant is a prominent outlier. Its m -value is the lowest among the pWT and 13 histidine variants, which suggests that it may unfold via a partially unfolded intermediate, perhaps involving His 20-heme ligation.^{46,47} The loss of the Lys20 to Glu17 electrostatic contact may also play a role in the stability behavior of this variant.

Equilibrium loop formation in the denatured state

As is evident from the equilibrium unfolding data in Fig. 3 and Table 2, pWT Cyt_c' and all of the variants are fully unfolded in 3 M gdnHCl, the conditions we have used to measure His-heme loop formation in the denatured state of Cyt_c'. For denatured state loops smaller than ~80 residues, loop formation is monophasic and can readily be fit to a modified form of the

Henderson-Hasselbalch equation (Eq 6, Materials and Methods; Fig. 4). The number of protons, n , released is always close to one (Table 3), as expected, since loop formation requires ionization of one proton from the histidine when it binds to the heme forming the loop. In general, as the size of the loop becomes larger, the $pK_a(\text{obs})$ increases. Thus, loop formation is less favorable for larger loops, consistent with the greater loss in entropy expected when a larger loop closes. However, close inspection of the data shows that the increase in $pK_a(\text{obs})$ with loop size is not smooth.

pWT Cyt c' also shows a high spin to low spin transition as pH increases yielding a $pK_a(\text{obs})$ of ~ 7.3 (Table 3, cFig. 4), even though there is no histidine in the sequence which can form a loop in the denatured state. Based on our previous work on denatured state loop formation with yeast iso-1-cytochrome variants, this high spin to low spin transition is likely due to lysine binding to the heme at higher pH.⁴⁸ Effectively, this $pK_a(\text{obs})$ of ~ 7.3 sets an upper limit on the dynamic range of His-heme loop formation measurements in the denatured state of Cyt c' .

For denatured state loops larger than ~ 80 residues, the loop formation curves broaden and become biphasic. The data either do not fit well to the modified form of the Henderson-Hasselbalch equation or give n (number of protons released) significantly less than 1. We have previously observed similar behavior for larger denatured state loops with iso-1-cytochrome c .³² For these larger loops, the His-heme binding is too weak to push the spin-state transition to completion. Thus, at higher pH, the lysine side chains that produce the spin-state transition in pWT Cyt c' complete the spin-state transition. The participation of two ionizable ligands with different intrinsic pK_a 's produces the biphasic spin state transition. A biphasic spin-state transition for the K13H variant is shown in Fig. 5. The fit to Eq 7 (Materials and Methods) yields $pK_{\text{loop}}(\text{His})$, $pK_a(\text{HisH}^+)$, and the pK for Lys-heme loop formation for a fully ionized lysine, $pK_{\text{loop}}(\text{Lys})$. These parameters are collected in Table 4 for the four variants which show biphasic loop formation.

In Table 4, it is evident that $pK_a(\text{HisH}^+)$ is similar for all four variants. Averaged across all four variants, we obtain $pK_a(\text{HisH}^+) = 6.7 \pm 0.2$. This value compares well to $pK_a(\text{HisH}^+) = 6.6 \pm 0.1$ observed for variants of iso-1-cytochrome c .³²

Comparison of His-heme loop stability, $pK_{\text{loop}}(\text{His})$, for these four variants shows that $pK_{\text{loop}}(\text{His})$ becomes less negative as the loop size increases (Table 4), indicating that loop formation is less favorable as the size of the loop increases. Thus, the same trend is observed as with the $pK_a(\text{obs})$ values for shorter loops in Table 3. Closer inspection of the $pK_{\text{loop}}(\text{His})$ data shows that loop stability does not decrease smoothly as loop size increases, also in agreement with the behavior of $pK_a(\text{obs})$ for the variants with shorter loops.

To put loop stability on the same scale for all variants, we convert the $pK_a(\text{obs})$ data for the variants in Table 3 into $pK_{\text{loop}}(\text{His})$ using Eq 2 and the $pK_a(\text{HisH}^+)$ observed for the variants in Table 4. A plot of the loop stability, $pK_{\text{loop}}(\text{His})$, versus the logarithm of loop size, $\text{Log}(N)$, is given in Fig. 6. There is a strong correlation between loop stability and loop size ($R^2 = 0.87$), as expected, since larger loops will be entropically less favorable. But, it is evident that there is considerable scatter about the best fit line.

Properties of a denatured protein and the reconciliation problem

The dependence of $pK_{\text{loop}}(\text{His})$ on $\text{Log}(N)$ yields a scaling exponent, v_3 , of 2.5 ± 0.3 in 3 M gdnHCl. This value is close to the range of 1.8 to 2.4 expected for a random coil with excluded volume based on theoretical treatments.^{38–40} Values of v_3 derived from the kinetics of loop formation as a function of loop size for both peptides^{25,27,49} and DNA^{50,51} mostly range from 1.5 to 2.1. The value we observe here is, within error, similar to these values. Previously, we have observed $v_3 \sim 4$ in 3 M gdnHCl for loop formation in the denatured state of iso-1-

cytochrome *c*, which decreases to $v_3 \sim 2$ in 6 M gdnHCl.^{31,32} The high scaling exponent observed for iso-1-cytochrome *c* in 3 M gdnHCl was attributable to residual structure in smaller loops in the denatured state which melted out in 6 M gdnHCl.³²

With iso-1-cytochrome *c*, $pK_a(\text{obs})$ reached a minimum value (maximal stability) for a loop size near 35 and remained essentially constant (or increased – depending on gdnHCl concentration) for smaller loop sizes when the histidines involved in loop formation were on the C-terminal side of the site of heme attachment. This result suggested that chain stiffness was a factor for loop sizes out to 35 residues.^{31,32} Later results on iso-1-cytochrome *c* variants with histidines N-terminal to the site of heme attachment indicated that an excluded volume effect due to the heme played an important role in the leveling out of $pK_a(\text{obs})$ for loop sizes <35 residues when the histidines were on the C-terminal side of the heme.³⁵ This heme excluded volume effect occurs because the main chain must wrap around the heme to form a loop when the histidines are on the C-terminal side of the heme.³⁵ The data for the iso-1-cytochrome *c* variants with histidines on the N-terminal side of the heme, such that the main chain does not have to wrap around the heme to form a loop, suggested that the onset of chain stiffness effects might occur for loops in the range of 10 to 15 residues in length. However, the available range of loop sizes was too small for this conclusion to be certain. Since class II *c*-type cytochromes, like Cyt c' , have the heme attached near the C-terminal end of the polypeptide chain, we now have an extensive loop size range with which to evaluate the onset of chain stiffness, without the interference of a heme excluded volume effect. From Fig. 6, we can see that onset of chain stiffness occurs for loops of 10 to 15 residues. This result is consistent with theoretical predictions⁵² and with measurements of rates of loop formation.^{25–27,49}

While v_3 for the broad range of loop sizes we have measured for Cyt c' is close to the value expected for a random coil with excluded volume, it is evident from the data that there is a high degree of scatter about the best linear fit to the data (Fig. 6). Deviations from the line are as much as 0.4 to 0.7 log units, a factor of 2.5 to 5 in the equilibrium constant for His-heme loop formation, $K_{\text{loop}}(\text{His})$. Deviations of this magnitude are seen for the E73H, K20H and D3H variants. Data for the kinetics of loop formation with synthetic peptides show minimal deviation from the best fit line.^{25–27,49} This observation indicates that the denatured states of naturally-occurring foldable sequences behave very differently from the simple homopolymers or alternating co-polymers that have been used as models for denatured states of proteins. The scatter in the favorability of loop formation, $pK_{\text{loop}}(\text{His})$, may reflect an alternation along the main chain between compact regions having residual structure or having ϕ, ψ angles in the α or turn region of the Ramachandran plot and more disordered regions having a more extended polypeptide backbone. Although theoretical studies on ϕ, ψ preferences of amino acids differ quantitatively, there is agreement that these preferences are context dependent.^{53,54} Thus, sequence-dependent variation in chain extension provides a reasonable explanation for the deviation from perfect random coil behavior evident in the denatured state of Cyt c' . In fact, thermodynamic modeling of protein denatured states indicates much greater variability in local stability for natural-occurring foldable sequences than for random sequences.⁵⁵ With regard to local sequence preferences, the program AGADIR⁵⁶ predicts strong helical propensity for residues 73 to 93 of Cyt c' . The similarity of $pK_{\text{loop}}(\text{His})$ for histidines at positions 73 and 84 to that for a histidine at position 91 (Fig. 6) may in part be due to the propensity of the sequence following residue 91, in the longer loops formed by histidines 84 and 73, to adopt helical ϕ, ψ angles. This possibility is in line with thermodynamic modeling of protein denatured states, which suggest that sequence dependent preferences for helical structure may be important for efficient protein folding.⁵⁵ It must be kept in mind that FRET experiments on the denatured state of Cyt c' , indicate that its denatured state is compact.¹³ Thus, it is also possible that the heterogeneous behavior of the denatured state of Cyt c' is caused in part by long-range residual structure as observed for lysozyme with NMR methods¹⁰ and for iso-1-cytochrome *c* with FRET methods.¹⁵

Interestingly, it appears that the scatter due to such an alternation of regions having and lacking non-random structure averages to yield a scaling factor near to that expected for a random coil with excluded volume. Thus, a bulk observable, the scaling factor (v_3), appears to be insensitive to local residual structure in a polypeptide chain. Fitzkee and Rose in their Monte Carlo simulation of protein denatured states with rigid segments come to the same conclusion.²⁴ Thus, measurement of a scaling exponent alone appears to be insufficient to demonstrate random coil behavior.

The observed scatter in loop propensity about the average loop stability versus $\text{Log}(N)$ dependence in Figure 6 also suggests that the lip of the folding funnel does not have uniform features. Some early contacts will be favored by as much as 10-fold for similar loop sizes as can be seen in the comparison of the $\text{pK}_{\text{loop}}(\text{His})$ values for the E73H ($N = 41$) and A66H ($N = 48$) variants in Table 3. Thus, the details of sequence appear to be able to encode contact preferences that will act to guide the folding process even early in folding near the top of the folding funnel.

Conclusion

We have carried out measurements of loop stability as a function of loop size in the denatured state of Cyt c' . By obtaining measurements of loop stability at loop size intervals of 7 to 11 residues along the entire length of the main chain of this protein, we have been able to obtain information on the variability of the conformational properties along the chain, not available with other methods. The significant scatter about the linear dependence of loop stability, $\text{pK}_{\text{loop}}(\text{His})$, versus the log of loop size, $\text{Log}(N)$, is consistent with an alternation of regions of non-random structure with regions of more random structure. Interestingly, the scaling exponent, v_3 , still yields a value of 2.5 ± 0.3 which is consistent with a random coil. This observation provides an experimental demonstration that scaling properties of polymers are insufficient, in isolation, to designate the behavior of a polymer as that of a random coil. Data which can provide insight into local behavior along the polypeptide chain are also essential.

Materials and Methods

Mutagenesis

Mutagenesis was carried out using the QuikChange II PCR-based mutagenesis kit (Stratagene). Primers were designed using Stratagene's primer design program, available on the web (<http://www.stratagene.com/sdmdesigner/default.aspx>). The pETcp vector containing the pWT (Q1A) gene for Cyt c' (obtained from Jay Winkler at Caltech) was used as a template for mutagenesis.⁴¹ The only modification made to the standard Stratagene PCR protocol was to change the annealing temperature from 55 °C to 68 °C (the same as the extension temperature) to compensate for the high GC content of the Cyt c' gene. PCR product was transformed into the TG-1 *Escherichia coli* cell line and DNA was extracted from overnight cultures using the Wizard Plus DNA miniprep kit (Promega). Mutations were confirmed by DNA sequencing at the Murdock DNA Sequencing Facility at The University of Montana.

Protein expression and purification

All Cyt c' variants in the pETcp vector were expressed from Novagen BL21-DE3 cells that were co-transformed with the pEC86 vector.⁵⁷ The pEC86 vector (provided by Linda Thöny-Meyer, Eidgenössische Technische Hochschule, Zürich) carries the *E. coli* cytochrome *c* maturation genes, *ccmABCDEFGH*, required for covalent attachment of heme to *c*-type cytochromes in the periplasm of *E. coli*. Typically, single colonies were used to inoculate five 10 mL Terrific broth cultures containing 34 µg/mL of chloramphenicol (pEC86) and 100 µg/mL of ampicillin (pETcp) and the colonies grown overnight at 37 °C. The cultures producing

the pinkest *E. coli* cells were used to inoculate 1 L cultures of Terrific broth containing ampicillin and chloramphenicol, as above. When cultures reached an OD₆₀₀ of ~1.2, IPTG was added to 100 μ M concentration and the cultures grown at 37 °C with shaking at 200 rpm. Harvested cell pellets were stored at –80 °C. Expression levels from cultures not induced with IPTG indicated that induction with IPTG was not critical.

Two different lysis and crude purification procedures were used. In the first, thawed cells were homogenized in lysis buffer (~4 mL/g cells: 20% sucrose, 30 mM Tris pH 8, 1 mM EDTA with fresh PMSF added to 2 mM) using a glass homogenizer. Lysozyme (5 mg/g cells), RNase and DNase (both at 0.5 mg/g cells) were added and the ice-cold cell suspension was passed through a French Press twice. The cell lysate was cleared by centrifugation and adjusted to pH 6. Precipitate was removed by centrifugation and the lysate diluted to an ionic strength of ~5 mM with deionized water and readjusted to pH 6. Following centrifugation to remove solids, the supernatant was loaded onto ~100 mL of CM sepharose resin equilibrated to 10 mM sodium phosphate buffer, pH 6. A 500 mL linear gradient from 0 to 200 mM NaCl in 10 mM sodium phosphate, pH 6, was used to elute the protein. Typical yields at this point were 10 mg/L of culture.

In the second procedure, lysis involved a combination of three freeze (–80 °C)/thaw (4 °C) cycles and osmotic shock.⁵⁸ Cell pellets were resuspended in lukewarm lysis buffer (30% sucrose, 1 mM EDTA, 30 mM Tris-HCl, pH 8.0) on an orbital shaker at room temperature for 20 minutes at 200 rpm. Approximately 75 mL of lysis buffer per liter of culture were used for cell pellet resuspension. The resuspended pellet was then centrifuged at 5500 rpm for 10 min. The sucrose buffer containing most of the protein was then poured off and PMSF protease inhibitor was added to 1 mM. The resulting pellet was then resuspended in equal volumes of cold 5 mM MgSO₄ solution if the pellet still had color. If the pellet was beige to white in color then purification continued on the “sucrose cut” only. The 5 mM MgSO₄ resuspension was then centrifuged at 8500 rpm for 1 hour. Supernatant was then collected and PMSF protease inhibitor was added to 1 mM.

The crude protein extract was diluted to 5 mM ionic strength with cold deionized water before CM sepharose purification. The pH of the crude protein extract was adjusted to pH 5.0 with concentrated acetic acid and the suspension was centrifuged for 30 min at 10,000 rpm to remove precipitates that may have developed after pH adjustment. The cleared lysate was immediately top loaded onto a CM sepharose column pre-equilibrated with CM buffer A (5 mM NaOAc, pH 5.0). Protein was eluted with a 500 mL linear salt gradient from 0 to 500 mM NaCl using CM buffer B (5 mM NaOAc, 500 mM NaCl, pH 5.0).

Final purification was by HPLC. Protein was exchanged in low salt HPLC Buffer A (10 mM sodium phosphate, pH 6.0) via centrifuge ultrafiltration. Protein was then loaded onto a Waters AP-1 ProteinPak SP-8HR cation exchange column and eluted with HPLC Buffer B (10 mM sodium phosphate, pH 6.0, 500 mM NaCl) using the following gradient: 0–10 min. 0% B, 10–11 min. increase linearly to 4.5% B, from 21–31 min. increase linearly to 5.1% B and hold at 5.1 % B for 20 minute, from 51–71 min. increase linearly to 12% B, 71–72 min. increase to 100% B and hold for 15 min., 87–88 min. decrease to 0% B and hold for 15 min. This gradient reliably removed a persistent impurity near 9100 m/e in the MALDI-TOF mass spectrum. All HPLC purified Cytc’ variants used in experiments gave a single molecular ion in the MALDI-TOF mass spectrum that corresponded to the expected mass of the variant within the mass accuracy of the instrument (Applied Biosystems Voyager – DE PRO Biospectrometry Workstation; ~10 amu at 10,000 molecular weight).

All proteins were in the oxidized form (Fe(III)-heme) as isolated. All experiments were carried out on the oxidized form of the protein. Protein concentrations were determined using $\epsilon_{398} =$

85,000 M⁻¹ cm⁻¹ for the Fe(III) protein at pH 7.0 in 100 mM sodium phosphate buffer,⁵⁹ using a Beckman Coulter DU 800 Spectrophotometer.

Thermodynamic stability measurements

Global protein stability measurements of all variants were done at 25 °C as a function of gdnHCl concentration using an Applied Photophysics Chirscan circular dichroism spectrometer coupled to a Hamilton MICROLAB 500 Titrator using methods described previously.⁶⁰ Data were acquired at pH 6.5 in the presence of 20 mM MES, 40 mM NaCl as buffer. Plots of ellipticity at 222 nm (θ_{222} , corrected for background at 250 nm) versus gdnHCl concentration were fitted to a six parameter equation (Eq 5) that evaluates the slope and intercept of the native (m_N , θ_N) and denatured state (m_D , θ_D) baselines and uses a linear free energy relationship ($\Delta G_u = \Delta G_u^0(\text{H}_2\text{O}) - m[\text{gdnHCl}]$) and a two-state assumption to evaluate ΔG_u in the transition region.⁶¹ The fits to the data provide the free energy of unfolding in the absence of denaturant,

$$\theta_{222} = \frac{(\theta_N + m_N[\text{gdnHCl}]) + (\theta_D + m_D[\text{gdnHCl}]) e^{(m[\text{gdnHCl}] - \Delta G_u^0(\text{H}_2\text{O}))/RT}}{1 + e^{(m[\text{gdnHCl}] - \Delta G_u^0(\text{H}_2\text{O}))/RT}} \quad (5)$$

$\Delta G_u^0(\text{H}_2\text{O})$, and the m -value. Reported parameters are the average and standard deviation of at least three independent trials.

Equilibrium loop formation in the denatured state

Equilibrium loop formation in the denatured state (3 M gdnHCl, 5 mM Na₂HPO₄, 15 mM NaCl) was monitored through pH titrations using a Beckman Coulter DU 800 Spectrophotometer. All titrations were done at 3 μ M protein concentration and at room temperature, 22 ± 1 °C. Spectra from 350 to 450 nm were acquired at each pH. Some titrations were carried out in 3 M gdnHCl, 5 mM Na₂HPO₄, 15 mM NaCl, as described previously (pWT, K31H, D3H and A104H).^{31,32} Absorbance at 398 nm (A_{398} , after subtracting absorbance at 450 nm as baseline) versus pH were fit to a modified form of the Henderson-Hasselbalch equation (Eq 6, A_{LS} is the absorbance of the low spin heme at 398 nm when the loop is formed at high pH and A_{HS} is the absorbance at 398 nm of the high spin form of the heme when the loop is broken at low pH) allowing extraction of the apparent pK_a for loop formation, $pK_a(\text{obs})$, and the number of protons, n , involved in the process. For the remaining variants, pH titrations in 3 M gdnHCl,

$$A_{398} = \frac{A_{LS} + A_{HS} 10^{n(pK_a(\text{obs}) - \text{pH})}}{1 + 10^{n(pK_a(\text{obs}) - \text{pH})}} \quad (6)$$

5 mM Na₂HPO₄, 15 mM NaCl were carried out in a semimicro quartz cuvette suitable for use with a magnetic stirrer (Hellma Cat. #: 109.004F). After preparation of an initial 1.05 mL sample, the pH was adjusted by adding 1 μ L of aqueous HCl or NaOH, 2 μ L 3x protein/buffer (9 μ M protein, 15 mM Na₂HPO₄, 45 mM NaCl) and 3 μ L of 6.0 M gdnHCl. The pH was measured with an Accumet calomel combination microelectrode (Fisher Cat. #: 13-620-95). The spectrum at each pH was measured from 450 to 350 nm with a Beckman Coulter DU 800 Spectrophotometer. For these data, absorbance at 450 nm as a function of pH was fit to a second order polynomial to yield a smoothed background that was then subtracted from the absorbance at 398 nm prior to fitting the data to the modified form of the Henderson-Hasselbalch equation as described above. Reported parameters for all variants are the average and standard deviation of at least three independent trials.

For variants which gave biphasic plots of absorbance at 398 nm, A_{398} , versus pH for denatured state titrations, a model involving two competing heme ligands with different pK_a 's (histidine and lysine) was used to fit the data (Eq 7).³² In fitting the data to this equation, the

$$A_{398} = \frac{A_{HS} + A_{LS} \left(\left(\frac{10^{-pK_{loop}(\text{His})}}{1 + 10^{pK_a(\text{HisH}^+) - \text{pH}}} \right) + \left(\frac{10^{-pK_{loop}(\text{Lys})}}{1 + 10^{pK_a(\text{LysH}^+) - \text{pH}}} \right) \right)}{1 + \left(\frac{10^{-pK_{loop}(\text{His})}}{1 + 10^{pK_a(\text{HisH}^+) - \text{pH}}} \right) + \left(\frac{10^{-pK_{loop}(\text{Lys})}}{1 + 10^{pK_a(\text{LysH}^+) - \text{pH}}} \right)} \quad (7)$$

intrinsic pK_a of the lysine, $pK_a(\text{LysH}^+)$, was set to 10.5. The intrinsic pK_a of the histidine, $pK_a(\text{HisH}^+)$, is obtained from the fit, as are the stabilities of the His-heme loop, $pK_{loop}(\text{His})$, and the Lys-heme loop, $pK_{loop}(\text{Lys})$. A_{HS} and A_{LS} are the absorbance at 398 nm of the high spin state with the loop broken and the low spin state with the loop formed, respectively.

AGADIR calculations

The sequence of pWT Cyt c ' was used as input for the web-based implementation of AGADIR (<http://agadir.crg.es/>).⁵⁶ The default parameters (5 °C, pH 7, $\mu = 0.1$) were used in the calculation. Helix 3 (residues 75 to 95) is predicted to be >15% helix from residues 75 to 92 and ~35% helix from residues 81–85. The percent helix of all other parts of the protein is predicted to be less than 10%.

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Abbreviations used

Cytc'	cytochrome c '
format for indicating mutations	single letter abbreviation for original amino acid followed by position number followed by one letter abbreviation for new amino acid, for example, D3H indicates that the aspartate at position 3 has been replaced with histidine
pWT	pseudo-wild type, contains the mutation Q1A relative to the naturally occurring Cyt c '
gdnHCl	guanidine hydrochloride
C_m	midpoint of guanidine hydrochloride unfolding titration
ΔG^0	$u(\text{H}_2\text{O})$, free energy of unfolding extrapolated to zero denaturant concentration
m	slope of the dependence of free energy of unfolding on guanidine hydrochloride concentration
$pK_{loop}(\text{His})$	stability of a histidine-heme loop in the denatured state

ν_3

scaling exponent for loop formation

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Fig. 1. Structure of *R. palustris* Cytc' (1A7V)³⁴ showing the positions of histidine substitutions. The heme is shown in blue and red (iron atom). The His117 axial ligand and the residue blocking the 6th coordination site, Leu12, are shown in green. The positions of the residues mutated in each variant are shown with gray spheres. Mutations are indicated with standard notation (see abbreviation footnote) next to each sphere.

Fig. 2.
Schematic representation of His-heme loop formation in the denatured state of Cyt c'.

Fig. 3. GdnHCl denaturation monitored by circular dichroism at 222 nm. Data were acquired at pH 6.5 and 25 °C. Plots of ellipticity at 222 nm, θ_{222} , versus gdnHCl concentration are shown for pWT ($\circ$) Cyt c' and the A104H ($\square$) and D58H (Δ) variants of Cyt c'. Solid curves are nonlinear least squares fits to a linear free energy relationship, as described in Materials and Methods (Eq 5), yielding the stability, $\Delta G^{\circ}_{\text{u}}(\text{H}_2\text{O})$, and the m -value. Thermodynamic parameters are collected in Table 2.

Fig. 4.

Denatured state His-heme loop formation as a function of pH in 3 M gdnHCl at 22 ± 1 °C. Plots of absorbance at 398 nm, A_{398} , versus pH are shown for pWT ($\circ$) Cytc' and the A104H ($\square$) and D58H (Δ) variants of Cytc'. Solid curves are nonlinear least squares fits to a modified form of the Henderson-Hasselbalch equation (Eq 6, Materials and Methods) yielding $pK_a(\text{obs})$ and n , the number of protons released upon loop formation. Parameters are collected in Table 3.

Fig. 5. Biphasic denatured state loop formation as a function of pH in 3 M gdnHCl at 22 ± 1 °C. The low pH phase is due to His-heme binding. The higher pH phase is attributable to Lys-heme binding. Plot of absorbance at 398 nm, A_{398} , versus pH is shown for the K13H ($\diamond$) variant of Cytc'. The solid curve is a nonlinear least squares fit to a model involving two possible heme ligands with different intrinsic pK_a 's, Eq 7 in Materials and Methods. Thermodynamic parameters from the fit are collected in Table 4.

Fig. 6.

Plot of loop stability, $pK_{\text{loop}}(\text{His})$, versus the logarithm of loop size, $\text{Log}(N)$. All data were collected in 3 M gdnHCl at 22 ± 1 °C. The data points are shown as open circles with errors bars at one standard deviation. For most variants, the error is less than the size of the symbol. The solid line is a fit of the data to a linear equation, yielding a scaling exponent, $v_3 = 2.5 \pm 0.3$.

Table 1

Cytc' variants

Cytc' variant	Loop size (N)	% Solvent Accessibility ^a
A104H	10	70 ± 1
K97H	17	75 ± 4
A91H	23	74 ± 1
K84H	30	68 ± 3
E73H	41	72 ± 1
A66H	48	49 ± 4
D58H	56	72 ± 14
K49H	65	58 ± 1
K39H	75	83 ± 8
K31H	83	100
K20H	94	72 ± 1
K13H	101	79 ± 2
D3H	111	65 ± 13

^aSolvent accessibility was calculated with the web program, GETAREA 1.0, <http://curie.utmb.edu/getarea.html>,⁴² using the Protein Data Bank file, 1A7V.pdb, as input.³⁴ The reported values are the average for the two Cytc' molecules in the unit cell.

Table 2

Thermodynamic parameters for gdnHCl unfolding of Cyt^c variants at pH 6.5 and 25 °C.

Cyt ^c variant	$\Delta G^{\circ'}_{\text{u}}(\text{H}_2\text{O}),^a$ kcal/mol	$m,^a$ kcal/(mol×M)	$C_{\text{m}},^b$ M
A104H	6.08 ± 0.26	5.59 ± 0.12	1.09 ± 0.05
K97H	6.80 ± 0.09	5.28 ± 0.07	1.29 ± 0.01
A91H	7.07 ± 0.09	5.75 ± 0.20	1.23 ± 0.03
K84H	5.89 ± 0.34	5.32 ± 0.32	1.11 ± 0.01
E73H	5.76 ± 0.13	5.08 ± 0.14	1.13 ± 0.01
A66H	6.79 ± 0.09	4.88 ± 0.06	1.39 ± 0.01
D58H	6.72 ± 0.09	4.80 ± 0.11	1.40 ± 0.02
K49H	6.75 ± 0.10	4.66 ± 0.07	1.45 ± 0.01
K39H	6.91 ± 0.15	4.70 ± 0.10	1.47 ± 0.01
K31H	7.41 ± 0.29	5.04 ± 0.20	1.47 ± 0.03
K20H	5.89 ± 0.13	4.17 ± 0.09	1.41 ± 0.01
K13H	8.17 ± 0.11	4.91 ± 0.07	1.66 ± 0.01
D3H	7.41 ± 0.17	5.08 ± 0.15	1.46 ± 0.05
pWT	8.77 ± 0.19	5.12 ± 0.10	1.71 ± 0.04

^a Average of at least three trials. The error is the larger of the standard deviation of the three trials or the average of the standard error for that parameter for all fits.

^b Average and standard deviation of at least three trials.

Table 3

Thermodynamic parameters at 22 ± 1 °C in 3 M gdnHCl for monophasic loop formation in the denatured state of Cyt_c variants^a

Cyt _c variant ^b	pK _a (obs)	n ^c	pK _{loop} (His) ^d
A104H (10)	4.12 ± 0.07	1.23 ± 0.21	-2.57 ± 0.07
K97H (17)	4.42 ± 0.07	0.97 ± 0.06	-2.27 ± 0.07
A91H (23)	5.08 ± 0.05	1.07 ± 0.03	-1.61 ± 0.05
K84H (30)	4.93 ± 0.04	0.97 ± 0.10	-1.76 ± 0.04
E73H (41)	5.07 ± 0.04	1.02 ± 0.10	-1.62 ± 0.04
A66H (48)	5.92 ± 0.09	1.04 ± 0.04	-0.77 ± 0.09
D58H (56)	5.68 ± 0.04	1.05 ± 0.03	-1.01 ± 0.04
K49H (65)	6.19 ± 0.02	1.01 ± 0.04	-0.50 ± 0.02
K31H (83)	6.18 ± 0.07	0.91 ± 0.08	-0.51 ± 0.07
pWT	7.30 ± 0.05	0.97 ± 0.16	—

^a All parameters are the average and standard deviation of at least three trials.

^b Loop size is given in brackets.

^c Number of protons released upon loop formation.

^d Calculated using Eq 3 and pK_a(HisH⁺) = 6.7, the average from the data in Table 4.

Table 4

Thermodynamic parameters at 22 ± 1 °C in 3 M gdnHCl for biphasic loop formation in the denatured state of Cyt^c variants^a

Cyt ^c variant ^b	pK _{loop} (His)	pK _a (HisH ⁺)	pK _{loop} (Lys) ^c
K39H (75)	-0.81 ± 0.05	6.83 ± 0.09	-2.57 ± 0.14
K20H (94)	-0.77 ± 0.04	6.81 ± 0.05	-3.25 ± 0.17
K13H (101)	-0.22 ± 0.06	6.72 ± 0.09	-3.12 ± 0.07
D3H (111)	0.53 ± 0.15	6.4 ± 0.3	-3.47 ± 0.06

^a All parameters are the average and standard deviation of at least three trials.

^b Loop size is given in brackets.

^c The intrinsic pK_a for the lysine which binds to the heme in the denatured state at higher pH was assumed to be 10.5.