Lysines in the Amino-Terminal α-Helix Are Important to the Stability of *Rhodobacter capsulatus* Cytochrome c_2[†]

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ABSTRACT: The protein stabilities of wild type and four site-directed mutants of *Rhodobacter capsulatus* cytochrome c_2 have been characterized. The integrity of the cytochrome c_2 iron–sulfur environment was ascertained by titration of the 696-nm absorbance band with alkali, and the conformational stability was determined by titration of the 220-nm circular dichroism signal with Gdn-HCl. Analysis of the alkaline transition pK value of K12D (lysine-12 substituted by aspartate) indicated that the K12D iron–sulfur environment was destabilized by 0.6 kcal/mol relative to the wild-type cytochrome c_2 at low ionic strength. In contrast, the alkaline transition pK values of K14E (lysine-14 substituted by glutamate), K32E (lysine-32 substituted by glutamate), and K14E/K32E (lysines-14 and -32 substituted by glutamates) were indistinguishable from the wild type, indicating that these substitutions have no effect on the stability of the iron–sulfur environment. Gdn-HCl denaturation of K12D and K14E indicated that both these mutations decreased conformational stability by 1.3 kcal/mol. In contrast, mutant K32E exhibited a small stabilizing effect of 0.2 kcal/mol. Gdn-HCl denaturation of K14E/K32E indicated that this mutation decreased conformational stability by 1.3 kcal/mol, which is consistent with the additive effects of the single charge mutations at positions 14 and 32. The conformational instability of mutants possessing negative charges at position 12 or 14 is best explained by their positioning at the carboxy-terminal region of the amino-terminal α-helix of *R. capsulatus* cytochrome c_2. Accordingly, introduction of negatively charged groups into this region appears to destabilize cytochrome c_2 through energetically unfavorable interactions with the dipole of the amino-terminal helix. It is suggested that differences between the stabilities of the amino-terminal α-helices of *R. capsulatus* cytochrome c_2 and horse cytochrome c may account for their differences in stability to Gdn-HCl denaturation.

Protein structures are stabilized by a variety of intramolecular interactions including hydrophobic, electrostatic, van der Waals, and hydrogen bonds. Electrostatic interactions could include ion–ion, ion–dipole, and dipole–dipole interactions. For example, comparisons of the amino acid sequences of thermophilic and mesophilic ferredoxins have indicated that ionic interactions can be important to the stability of these proteins (Perutz, 1978). Site-directed mutagenesis of a number of charged residues in the λ repressor (Hecht et al., 1983) and T4 lysozyme (Alber et al., 1988) has substantiated this hypothesis. On the basis of the relative positions of charged residues in naturally occurring α-helices (Chou & Fasman, 1974) and the effects of changing charge distributions on model peptides (Shoemaker et al., 1987), charge distribution is thought to be an important factor in the stability of α-helices through energetically favorable interactions with the helix dipole. Further, destabilization of an individual α-helix has been shown to destabilize the entire α-helical structure in ribonuclease S (Mitchinson & Baldwin, 1986). Site-directed mutagenesis studies of T4 lysozyme have confirmed the effect of the stability of the α-helix dipole on the global stability of the protein. For example, substitution of aspartic acid for neutral residues located in the carboxy-terminal region of two different helices of T4 lysozyme resulted in increased thermostability in both mutants (Nicholson et al., 1988).

Recently, we have developed a genetic system to study electron-transfer, redox potential, and stability properties of *Rhodobacter capsulatus* cytochrome c_2 (Caffrey, 1991; Caffrey & Cusanovich, 1991; Caffrey et al., 1991). In the present study, the stability properties of four variants of *R. capsulatus* cytochrome c_2 lysines have been characterized. Mutations K12D[1] (lysine-12 to aspartate), K14E (lysine-14 to glutamate), and K32E (lysine-32 to glutamate) result in changing the sign of the charge of a surface group from +1 to −1 at neutral pH. Mutant K14E/K32E (lysines-14 and -32 to glutamates) results in changing the sign of the charge at two positions simultaneously, a net charge change of 4 at neutral pH. On the basis of structural (Salemme et al., 1973), kinetic (Rickle & Cusanovich, 1979; Hall et al., 1987), and chemical protection studies (Bosshard et al., 1987) of cytochromes c, *R. capsulatus* lysines-12 and -32, or their equivalents in other species, are thought to play important roles in cytochrome c electron-transfer reactions. We have previously demonstrated that each of the lysine mutations has little measurable effect on the intracellular concentration of cytochrome c_2 and thus no effect on cytochrome c_2 processing (i.e., heme attachment) and stability in vivo (Caffrey, 1991). Moreover, the four mutations were functional in vivo, demonstrating that each was structurally competent to cycle electrons between cytochrome c_2 and its physiological partners (Caffrey, 1991). Further, we have demonstrated that the four charge mutations exhibit little effect on redox potential, indicating that their heme environments have not been significantly perturbed relative to the wild-type cytochrome c_2 (Caffrey & Cusanovich, 1991). In what follows, we compare the stability of *R. capsulatus* cytochrome c_2 charge mutants to that of the wild-type protein. The integrity of the iron–sulfur

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[1] Abbreviations: wt, *R. capsulatus* wild-type cytochrome c_2; mut, mutant; K12D, K14E, and K32E, lysines-12, -14, or -32 substituted with aspartate or glutamate; K14E/K32E, lysines-14 and -32 substituted by glutamates; Gdn-HCl, guanidine hydrochloride.

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environment was ascertained by titration of the 696-nm absorbance band. Conformational stability was assessed by Gdn-HCl denaturation. We present evidence that introduction of negative charges into the carboxy terminus of the amino-terminal α-helix destabilizes the conformational stability of *R. capsulatus* cytochrome c_2 through energetically unfavorable interactions with the amino-terminal α-helix dipole.

Materials and Methods

Mutagenesis procedures and protein isolations have been described elsewhere (Caffrey, 1991; Caffrey et al., 1991). The purified charge mutants exhibited no spectral differences from the wild-type cytochrome in the visible and ultraviolet regions (Caffrey & Cusanovich, 1991).

The alkaline transition pK values were determined by titrating the 696-nm absorbance with 0.3 NaOH. Absorbance intensities were calculated by the method of Kaminsky et al. (1973). Experimental conditions were 100 μM cytochrome, 1 mM potassium phosphate, and 300 μM $K_3Fe(CN)_6$. Absorption spectra were taken on a Hewlett Packard 8452A spectrophotometer. In all determinations, the Hill slopes were 1.0 ± 0.1. From replicate analyses, errors in the calculated pK values are estimated to be ±0.05 pH unit.

Gdn-HCl (United States Biochemical, ultrapure) denaturation was examined by the change in the circular dichroism signal at 220 nm, the local minima attributed to helical structure. Spectra were taken on an Aviv circular dichroism spectropolarimeter Model 60 DS using a 1-cm sample cell. Experimental conditions were 2.5 μM ferricytochrome c_2 in 20 mM Tris-HCl (pH 7.5)/40 mM NaCl at 25 °C. Samples were preequilibriated for 30 min in denaturant at 25 °C before analysis. Estimates of ΔG_u^* (free energy of unfolding in the absence of denaturant), m (cooperativity of unfolding), and C_m (midpoint concentration of denaturant required to unfold half of the protein) were obtained as previously outlined (Knapp & Pace, 1974; Schellman, 1978). In this analysis, a two-state equilibrium is assumed with K_u (the equilibrium constant of unfolding) being defined as $(X_{obs} - X_i)/(X_f - X_{obs})$ where X_{obs}, X_i, and X_f are the observed, initial, and final circular dichroism signals, respectively. The parameters ΔG_u^* and m are estimated from linear regressions of ΔG_u (free energy of unfolding) versus [Gdn-HCl] using the equations $\Delta G_u = -RT \ln K_u$ and $\Delta G_u = \Delta G_u^* - m$[Gdn-HCl]. By definition at C_m, $\Delta G_u = 0$ and thus $C_m = \Delta G_u^*/m$. Linear regressions of ΔG_u versus [Gdn-HCl] yielded correlation coefficients >0.98, supporting the analysis as a two-state equilibrium. From replicate analyses, the uncertainties of m, C_m, and ΔG_u^* were estimated to be ±0.2 kcal·L/mol^2, ±0.1 mol/L, and ±0.3 kcal/mol, respectively. The parameter $\Delta\Delta G_u'$ is estimated from the difference in wild-type and mutant ΔG_u values at a Gdn-HCl concentration midway between their C_m values [i.e., $\Delta\Delta G_u'$ = mutant $\Delta G_u(C_m')$ − wt $\Delta G_u(C_m')$ where C_m' = (mutant C_m + wt C_m)/2 (Caffrey et al., 1991)]. On the basis of errors in m and C_m, the uncertainty of $\Delta\Delta G_u'$ is estimated to be ±0.1 kcal/mol.

Results

The stability of the cytochrome c iron–sulfur environment can be ascertained by titration of the 696-nm absorbance with alkali (Pettigrew et al., 1978). This absorbance is thought to reflect the environment and strength of the heme iron–methionine sulfur bond, and a decrease in the pK value of the "alkaline transition" would reflect destabilization (Schechter & Saludjian, 1967). In Figure 1A, titrations of the *R. capsulatus* wild-type and mutant ferricytochromes c_2 at low ionic strength are presented. As can be seen, mutants K14E, K32E, and K14E/K32E exhibit pK values that are indistinguishable from the wild-type value of 8.90. Thus, substitution of these lysines does not measurably affect the *R. capsulatus* cytochrome c_2 iron–sulfur bond environment. In contrast, the pk value of K12D is 8.50, which is significantly less than the wild-type value. The free energy difference in the pH region near the pK values can be estimated by the equation $\Delta\Delta G_{alk} = -2.3RT(pK_{mutant} - pK_{wt})$. In the case of K12D, $\Delta\Delta G_{alk}$ is −0.55 kcal/mol, and thus it appears that substitution of lysine-12 by aspartate has resulted in destabilization of the *R. capsulatus* cytochrome c_2 iron–sulfur bond environment. However, it is important to note that these determinations were at low ioic strength (<0.005 M). In Figure 1B, titrations of the wild type and K12D at 0.10 M ionic strength are presented. Interestingly, at the higher ionic strength, K12D exhibits an alkaline transition pK that is indistinguishable from the wild-type value of 9.05. Therefore, it appears that the instability of K12D at low ionic strength is due to unfavorable interactions between the aspartate side chain and other charged groups.

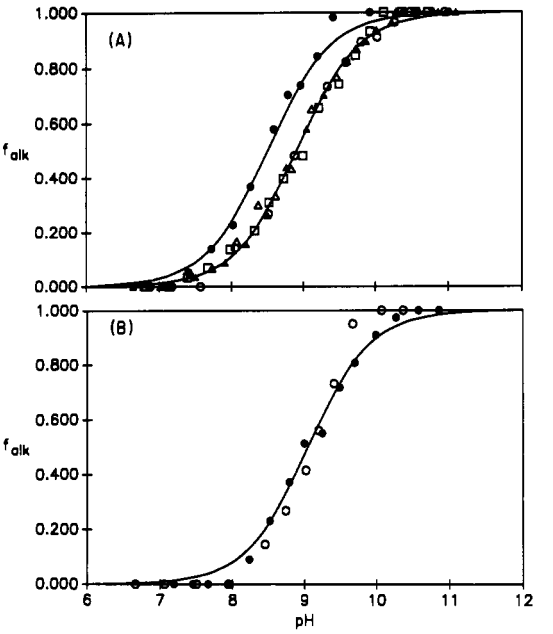

FIGURE 1: Titration of the 696-nm absorbance of wt (○), K12D (●), K14E (△), K32E (▲), and K14E/K32E (□) ferricytochromes c_2 at 23 °C. The parameter f_{alk} corresponds to the fraction in the alkaline state. Curves represent theoretical f_{alk} values with n = 1 for pK_{alk} = 8.50 and 8.90, respectively, in (A) and pK_{alk} = 9.05 in (B). Buffer conditions were (A) 1 mM PO_4 and 300 μM $K_3Fe(CN)_6$ and (B) 1 mM PO_4, 100 mM NaCl, and 300 μM $K_3Fe(CN)_6$.

The conformational stability of cytochrome c can be determined by titration of the 220-nm circular dichroism signal, which primarily reflects α-helical structure, with Gdn-HCl (Knapp & Pace, 1974; Hickey et al., 1988). In Figure 2, the fraction of unfolded protein (f_u) has been plotted as a function of [Gdn-HCl] for wild-type and charge mutant cytochromes. Linear regressions of the data yield the parameters ΔG_u^*, m, and C_m given in Table I. In addition, the previously determined values for Gdn-HCl denaturation of horse cytochrome c have been included for reference. One method to compare the stability of a mutant to the wild-type protein is to subtract the wild-type ΔG_u^* from the mutant ΔG_u^* to yield the parameter $\Delta\Delta G_u^*$. However, this approach is extremely sensitive to experimental error because ΔG_u^* is determined by extrapolating the measured data to zero Gdn-HCl concentration. Thus, a very small change in the slope (m) can lead to a relatively large difference in ΔG_u^*. An alternative method of comparing mutant stability to wild-type stability is to

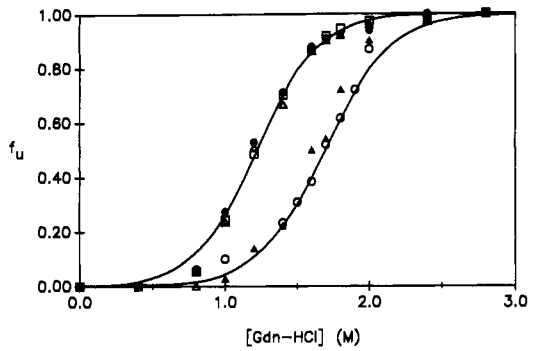

FIGURE 2: Fraction of unfolding (f_u) as a function of [Gdn-HCl] for wt (○), K12D (●), K14E (△), K32E (▲), and K14E/K32E (□) ferricytochromes c_2 at pH 7.5. The parameter f_u is defined by $(X_{obs} - X_i)/(X_f - X_{obs})$ where X_{obs}, X_i, and X_f are the observed, initial, and final circular dichroism signals at 220 nm, respectively. The curves correspond to the fits of the wild-type and K12D data. Buffer conditions were 20 mM Tris-HCl (pH 7.5) and 40 mM NaCl at 25 °C.

Table I: Stability Properties of the *R. capsulatus* Wild-Type and Charge Mutant Cytochromes c_2 and Horse Cytochrome *c*, Based upon Their Denaturation by Gdn-HCl[a]

cyt	C_m[b]	m[c]	ΔG_u^*[d]	$\Delta\Delta G_u^*$[e]	C_m'[f]	$\Delta\Delta G_u'$[g]
wt	1.7	2.7	4.4			
K12D	1.2	2.8	3.3	−1.2	1.5	−1.3
K14E	1.2	2.8	3.5	−1.0	1.5	−1.3
K32E	1.6	3.0	4.8	+0.4	1.7	+0.2
K14E/K32E	1.2	3.0	3.6	−0.9	1.5	−1.3
horse[h]	2.4	3.0	7.3	+2.8	2.1	+2.1

[a] Experimental conditions for the *R. capsulatus* cytochromes c_2 were pH 7.5 at 25 °C. Experimental conditions for horse cytochrome *c* were pH 6.5 at 25 °C. [b] Units are moles per liter. [c] Units are kilocalories liter per mole squared. [d] Units are kilocalories per mole. [e] $\Delta\Delta G_u^*$ = mut ΔG_u^* − wt ΔG_u^*. [f] C_m' = (mut C_m + wt C_m)/2. [g] $\Delta\Delta G_u'$ = mut $\Delta G_u'$ − wt $\Delta G_u'$ at [Gdn-HCl] = C_m'. [h] Taken from Knapp and Pace (1974). $\Delta\Delta G_u^*$, C_m', and $\Delta\Delta G_u'$ were determined with respect to the *R. capsulatus* wt cytochrome c_2 values.

subtract the wild-type ΔG_u from the mutant ΔG_u at a concentration midway between their C_m values (Caffrey et al., 1991). This parameter, which we call $\Delta\Delta G_u'$, has the advantage of minimizing errors in $\Delta\Delta G_u'$ with respect to $\Delta\Delta G_u^*$ if the *m* values are similar. $\Delta\Delta G_u'$ minimizes these errors because it is estimated in the [Gdn-HCl] region in which K_u is directly determined and not extrapolated over a large concentration region. Therefore, in the present analysis, we use $\Delta\Delta G_u'$ to assess mutational effects on the conformational stability of cytochrome c_2. Note that a similar approach has been previously used in the analysis of the stabilities of subtilisin variants (Kellis et al., 1988).

From comparison of the wild-type and mutant $\Delta\Delta G_u'$ values, substitution of lysine-12 by aspartate (K12D) results in 1.3 kcal/mol of instability, and substitution of lysine-14 by glutamate (K14E) results in 1.3 kcal/mol of instability. In contrast, substitution of lysine-32 by glutamate (K32E) results in 0.2 kcal/mol of stabilization. In the case of the double charge mutant K14/K32E, the cytochrome has been destabilized by 1.3 kcal/mol. Comparing the $\Delta\Delta G_u^*$ yields similar results but with greater uncertainties (Table I). On the basis of the data presented, it appears that changing the sign of the charge at positions 12 or 14 destabilizes cytochrome c_2 by approximately 1.3 kcal/mol. Changing the sign of the charge at position 32 results in a small amount of stabilization (approximately 0.2 kcal/mol) and establishes that the instability of mutants at positions 12 and 14 is not the result of changes in the net protein charge. Finally, the stability of the double charge mutant K14E/K32E (1.3 kcal/mol) is close to that of the sum of the single charge mutants K14E and K32E (1.0 kcal/mol), suggesting no strong synergism.

Discussion

The results presented here establish that charge substitutions in different regions of *R. capsulatus* cytochrome c_2 have different effects on the stability of the iron–sulfur environment and α-helices. For example, substitution of lysine-12 by aspartate (K12D) resulted in decreasing both the stability of the iron–sulfur bond environment and the conformational stability. On the other hand, substitution of lysine-14 by glutamate (K14E) or lysines-14 and -32 simultaneously by glutamates (K14E/K32E) resulted in no change in the stability of the iron–sulfur bond environment but significant destabilization of the conformational stability. Finally, substitution of lysine-32 by glutamate resulted in little change in either the stability of the iron–sulfur bond environment or the conformational stability. Decreases in the stabilities of charge-substituted mutants suggest that stabilizing electrostatic interactions have been disrupted or that destabilizing electrostatic interactions have been introduced. To interpret the instabilities of K12D, K14E, and K14E/K32E, it is useful to consider the location of the mutated group with respect to its ionic neighbors and ionic strength effects. Examination of the *R. capsulatus* cytochrome c_2 structure determined by X-ray crystallography (Benning et al., 1991) reveals that lysine-12 could be involved in an ionic interaction with the side chain of glutamate-9 with the N_e to O_d distance being approximately 3 Å. Consequently, introduction of a negatively charged side chain at this position could disrupt a stabilizing ion–ion interaction or introduce a destabilizing charge repulsion interaction. In the case of K12D, the alkaline transition data (Figure 1A,B) appear to support the charge repulsion model. Accordingly, the charge repulsion disrupts local secondary and tertiary structure which is in turn propagated to the sequentially and spatially distant iron–sulfur bond environment. In contrast to the positioning of lysine-12, the side chains of lysines-14 and -32 possess no possible ionic contacts of less than 9 Å. Therefore, the absence of perturbation of the K14E, K32E, and K14E/K32E alkaline transition p*K* values (Figure 1A) is consistent with the absence of stabilizing ionic interactions occurring between these residues and acidic residues.

In contrast to the alkaline transition determinations, the Gdn-HCl denaturations were determined at high ionic strengths because Gdn-HCl is a strong electrolyte. At ionic strengths greater than 1 M, ion–ion interactions can be expected to be minimized due to the large concentration of counterions present. Consequently, the conformational instabilities of K12D, K14E, and K14E/K32E require an alternative explanation. Ion–dipole and dipole–dipole interactions are known to be less sensitive to ionic strength than ion–ion interactions (Koppenol, 1980). For example, in model peptides, ion–dipole interactions appear to persist at 1 M NaCl (Marqusee & Baldwin, 1987). In *R. capsulatus* cytochrome c_2, lysines-12 and -14 occur in the carboxy-terminal region of the amino-terminal α-helix (Gooley et al., 1990; Benning et al., 1991). In contrast, lysine-32 occurs in a region of nonregular secondary structure (Benning et al., 1991). In the case of lysines-12 and -14 but not lysine-32, energetically favorable interactions between the side chain and helix dipole are possible. According to this model, introduction of negatively charged side chains would lead to an unstable amino-terminal helix which in turn destabilizes the other regions of secondary structure.

An alternative model is to consider that lysines-12 and -14 contribute to the magnitude and orientation of the amino-terminal helix dipole and that the resulting dipole forms en-

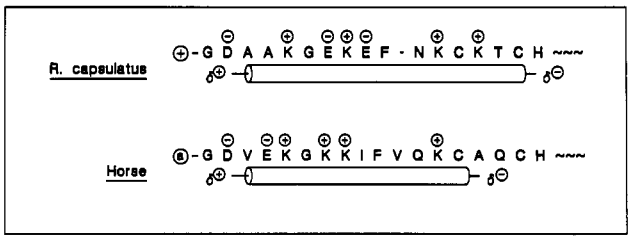

FIGURE 3: Amino acid sequences of the amino-terminal region of *R. capsulatus* cytochrome c_2 and horse cytochrome *c*. Helical regions are denoted by cylinders. The partial charges due to the helix dipole and the expected charges of side chains at neutral pH are included.

ergetically favorable dipole–ion or dipole–dipole interactions. For example, the amino-terminal helix could interact with the heme group which possesses a formal charge of +1 in the oxidized state. Alternatively, energetically favorable interactions could occur between the dipole of the amino-terminal helix and other dipoles of the protein (e.g., α-helix or heme group). Nonetheless, the dipole of the amino-terminal helix appears to be important to the conformational stability of *R. capsulatus* cytochrome c_2.

In Figure 3, the amino acid sequences of the *R. capsulatus* wild-type cytochrome c_2 and horse cytochrome *c* amino-terminal regions are presented. In addition, the α-helices within these regions have been denoted on the basis of NMR studies (Feng et al., 1989; Gooley et al., 1990). First, note that in the *R. capsulatus* wild-type sequence, the charge distribution is not oriented to maximally stabilize the amino-terminal α-helix dipole. The sequence ends with two favorable basic groups (lysines-12 and -14) but possesses no favorable charge orientation (i.e., acidic side chains) in the amino-terminal region. Moreover, the charge distribution can be seen to decrease the helix dipole and affect its orientation due to the presence of the basic groups at positions 12 and 14. In contrast to *R. capsulatus* cytochrome c_2, the homologous horse cytochrome *c* sequence begins with a favorable acidic group followed by basic groups that may be expected to stabilize the helix dipole by energetically favorable ion–dipole interactions. On the basis of NH exchange studies, the amino-terminal helix of *R. capsulatus* cytochrome c_2 has been demonstrated to be at least 2.5 kcal/mol less stable than the equivalent helix of horse cytochrome *c* (Gooley et al., 1990; Wand et al., 1986). This value is in excellent agreement with the differences in stability of 2.1–2.8 kcal/mol, based on Gdn-HCl titrations (Table I). Consequently, an unfavorable charge distribution for the amino-terminal helix dipole of *R. capsulatus* cytochrome c_2 may be primarily responsible for the difference in stability to Gdn-HCl between horse ferricytochrome *c* and *R. capsulatus* ferricytochrome c_2.

Structural characterizations of the *R. capsulatus* charge mutants by X-ray crystallography and two-dimensional NMR techniques are presently underway. It is anticipated that these studies as well as future mutations to other groups within the α-helices of *R. capsulatus* cytochrome c_2 will further elucidate the reasons for the decreased stability of the mutations at positions 12 and 14. Nonetheless, the present study has demonstrated that substitution of charged residues with residues of the opposite sign can result in a significant degree of instability and that stabilization of the dipole of α-helices may be an important determinant in cytochrome *c* stability.

Registry No. C_2, 9035-43-2; lysine, 56-87-1.

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