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Review

Site-specific mutagenesis studies of cytochromes *c*

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Contents

0. Summary

Cytochromes *c* are among the best characterized proteins, which consequently make them attractive candidates for study by mutagenesis. Site-specific mutagenesis studies have been reported for five species of cytochromes *c*, including those from *Rhodobacter capsulatus*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, rat and horse. The effect of mutations to highly conserved residues on redox potential indicates that substitutions of the M80 axial heme ligand result in the greatest effect (i.e., > 200 mV) while other mutations generally have a small negative effect (i.e., < 60 mV). Denaturation of the mutants suggests that conformational stability is generally decreased upon substitution of conserved residues, with the largest observed destabilization being approx. 4 kcal/mol. As judged by the pK for the alkaline transition of the mutants, the stability of the Fe-S bond can be increased or de-

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creased by approx. 1 kcal/mol with the largest effects occurring when the mutated group is proximal to the heme. Electron transfer reactions between cytochrome *c* mutants and various physiological partners are generally not affected to a large degree, an observation that is consistent with their function in vivo. Structural characterization of several mutants by X-ray crystallography suggests that site-specific substitutions generally do not disrupt the overall conformation but result in small local and remote structural perturbations. NMR characterization of several mutants supports the lack of large structural changes but suggests that changes in the dynamic properties of mutants often occur. Taken together, these observations suggest that comprehensive study of equivalent mutations in a number of species is necessary to understand the determinants of cytochrome *c* structure and function as well as the determinants of evolutionary conservation.

1. Introduction

Class 1 *c*-type cytochromes are among the best characterized proteins. This structurally homologous family of proteins is found in both prokaryotes and eukaryotes and functions in a wide range of metabolic pathways. The amino acid sequences for over 200 species of cytochrome *c* are known and, from this sequence information, it is apparent that approx. 30% of the residues are highly conserved [1], with the remainder of the amino acid side chains variable, apparently to facilitate specific metabolic functions. Moreover, the cytochromes *c* exhibit well characterized and diverse biochemical properties such as oxidation-reduction potentials and electron transfer kinetics with physiological and non-physiological electron donors and acceptors [2]. Finally, there is extensive knowledge on the structural and dynamic properties of the cytochromes *c*. The X-ray structures are available for the eukaryotic species: yeast (*Saccharomyces cerevisiae*), rice, bonita, tuna, and horse [3–7]; and the prokaryotic species: *Rhodospirillum rubrum*, *Paracoccus denitrificans*, *Chlorobium thiosulfatophilum*, *Anacystis nidulans*, *Pseudomonas aeruginosa*, *Azobacter vinelandii*, and *Rhodobacter capsulatus* [8–14]. Furthermore, extensive NMR assignments have been reported for the cytochrome *c* from *Rb. capsulatus*, *P. aeruginosa*, yeast, and horse [15–19]. Together, the amino acid sequence, biochemical, functional, structural and dynamic differences among the cytochromes *c* make this class of proteins ideal for study by site-specific mutagenesis.

In this report, we will review single-site mutations of cytochromes *c* that have been characterized in vitro in order to better understand the class I *c*-type cytochromes and to facilitate the design and study of additional mutants. The reader is referred to Hampsey et al. [20] and Caffrey et al. [21] for discussion of the in vivo properties of single-site mutations in yeast iso-1 cytochrome *c* and *Rb. capsulatus* cytochrome c_2, respectively. To date, only yeast cytochrome *c* has been randomly mutagenized and screened for function in vivo [20,22,23], although similar approaches could be applied to other cytochromes *c* in the appropriate host. We will focus on mutations to highly conserved residues but, in addition, we will address mutations to nonconserved groups that exhibit interesting properties. The physical properties we have chosen to consider fit two criteria. First, they are informative probes of cytochrome *c* structure and function. Second, they are widely reported properties so that comparisons can be made between equivalent mutants in different species of cytochrome *c*. With these criteria in mind, we have chosen to focus the discussion on four in vitro properties of cytochromes *c* which include redox potential, overall conformational stability based on thermal or chemical denaturation, heme iron-methionine sulfur (Fe-S) bond stability based on the alkaline transition pK (pK_{alk}), and electron transfer kinetics. In biological electron transfer reactions, differences in redox potentials of electron transfer proteins provide the driving force or free energy for the reactions to occur. Interestingly, the redox potentials of the class I cytochrome *c* family vary from + 30 mV to + 480 mV [2]. Because the heme group and axial ligands are identical within this class of cytochromes, the observed diversity is solely due to the protein environment of the heme. Therefore, characterization of mutant redox potentials can lend insight into the determinants of redox potential. Moreover, mutants that exhibit unchanged redox potentials can often be considered to lack perturbation of the heme environment and hence redox potential is a sensitive probe of structural perturbations. The second property, overall conformational stability, is important because the relative stabilities of the oxidized and reduced states determine the redox potential. Furthermore, the presence of the hydrophobic heme group in the protein interior can be expected to have large, but as yet uncharacterized, effects on the stability of cytochrome *c*. Thus, mutational effects on conformational stability lend insight into the determinants of redox potential and the stability of heme proteins, as well as proteins in general. Moreover, large decreases in conformational stability of mutants can be indicative of structural changes. The third property, pK_{alk}, is a measure of the Fe-S bond stability. The alkaline transition is thought to reflect displacement of the M80 axial heme ligand by a lysine [24,25]. In yeast cytochrome *c*, the substituting lysine has been tentatively identified by mutagenesis studies as K79 [64]; however, this lysine is not conserved among prokaryotic cytochromes *c*, which also have an alkaline pK. Consequently, further mutations are necessary to

better characterize the identity of substituting ligands and determinants of the Fe-S bond stability. Those mutations affecting pK_{alk} should yield information on the structural changes which occur, as well as the strength of the Fe-S bond. The fourth property is the electron transfer rate constant between cytochrome *c* and various protein electron donors and acceptors. The electron transfer reactions of cytochromes *c* have been shown to be influenced by a number of factors including redox potential, electrostatics, surface topology, dynamics, and specific paths [2]. Site-specific mutagenesis studies can be directed at understanding the contribution of specific amino acids to each of these factors. In addition, we have considered the structural and dynamic information available for many of the mutations. Needless to say, structural information on the wild-type and mutant cytochromes is of great assistance for interpretation of mutational effects on in vitro properties (e.g., redox potential, conformational stability, pK_{alk}, or electron transfer reactions). Further, structural characterizations of the cytochrome *c* mutants test the notion that site-specific substitutions result in small localized structural perturbations. Finally, characterization of mutant cytochromes *c* by NMR lend insight into the structural and dynamic properties of mutant proteins in solution and can directly investigate the role of dynamics in cytochrome *c* function.

To date, single-site mutants of cytochromes *c* have been reported for four eukaryotic species and one prokaryotic species. For example, site-directed mutagenesis has been employed to generate mutants of yeast iso-1 and iso-2 cytochromes *c* [26,27], *D. melanogaster* (fruit fly) cytochrome *c* [28], rat cytochrome *c* [29], and *Rb. capsulatus* (purple phototrophic bacterium) cytochrome c_2 [21]. In the case of the eukaryotic cytochromes *c*, the resulting mutants were subsequently expressed in yeast strains lacking functional chromosomal copies of the wild-type cytochrome *c* genes. In the case of *Rb. capsulatus* cytochrome c_2, the resulting mutants were expressed in a *Rb. capsulatus* strain lacking a functional chromosomal copy of the cytochrome c_2 gene. In addition, random mutagenesis has been used to generate single-site mutants of yeast iso-1 cytochrome *c* [20,22,23]. As an alternative to genetic methods, chemical semi-synthesis has been used to generate variants of horse cytochrome *c*, which are the equivalent of site-specific mutations [30,31]. Thus, the methods for the generation and expression of cytochrome *c* mutants from a number of different species have been established.

Importantly, the X-ray crystal structures have been determined for three of the five species that have been mutated, including *Rb. capsulatus* cytochrome c_2 [14], yeast iso-1 cytochrome *c* [3], and horse cytochrome *c* [7,32]. Comparison of these structures, as well as those of other cytochrome *c* species mentioned in the introduction, indicates that the overall protein fold is very similar, the structural differences being primarily located within regions of amino acid insertions or deletions. In addition, extensive NMR assignments have been made for *Rb. capsulatus* cytochrome c_2 [15], yeast iso-1 cytochrome *c* [18], and horse cytochrome [19]. Consequently, the X-ray and NMR studies on the wild-type cytochromes facilitate study of the structural and dynamic properties of mutants.

2. Wild-type properties

Before considering the biochemical properties of the cytochrome *c* mutations in various species, it is useful to compare the properties of the wild-type species. In Fig. 1, the amino acid sequences of the five cytochrome *c* species that have been mutated to date are compared with residues homologous to horse cytochrome *c* boxed and the conserved residues reviewed here marked with an asterisk. With respect to horse

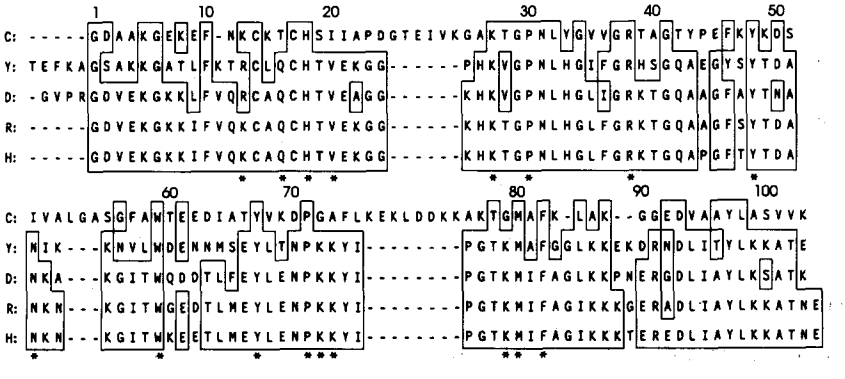

Fig. 1. Amino acid sequence homology between *Rb. capsulatus* (C), yeast iso-1 (Y), *D. melanogaster* (D), rat (R), and horse (H) cytochromes *c*. Numbering corresponds to that of horse; dashes represent deleted residues; boxed regions correspond to residues that are homologous to horse cytochrome *c*; asterisks indicate conserved residues that have been mutated. The sequences were taken from Ref. [1] for *Rb. capsulatus* and horse, from Ref. [33] for yeast iso-1, from Ref. [34] for *D. melanogaster*, and from Ref. [35] for rat.

cytochrome *c* (historically the best characterized), rat cytochrome *c* exhibits the highest degree of homology with no insertions or deletions and only six nonidentical residues. The cytochromes *c* from *D. melanogaster* and yeast exhibit less homology to horse cytochrome *c* (one insertion, one deletion, and 17 nonidentical residues in the case of *D. melanogaster*; one insertion, one deletion, and 37 nonidentical residues in the case of yeast). Cytochrome c_2 from *Rb. capsulatus* is clearly related to horse cytochrome *c* but is the most distant species (three insertions, three deletions and 60 nonidentical residues). Based on the amino acid sequence data, one might anticipate that those species that are most related by sequence homology would exhibit the most similar biochemical properties.

In Table 1, the redox potential, conformational stability, pK_{alk}, and electron transfer kinetic properties have been summarized for the five cytochromes *c* discussed above. The redox potentials of the four eukaryotic cytochromes are identical [28,29,38], but that of *Rb. capsulatus* cytochrome c_2 is 110 mV higher [36] suggesting that the heme environments of the eukaryotic species are similar to each other but different from that of *Rb. capsulatus*. Therefore, the redox potential appears to be only loosely correlated with amino acid sequence homology (cf. compare the redox potentials of horse, yeast and *Rb. capsulatus*). The overall conformational stabilities, as determined by urea or guanidine-HCl denaturation of the 220 nm circular dichroism signal (which reflects α helical content), are more diverse than the redox potentials, varying from 2.9 kcal/mol for yeast ferricytochrome *c* [39] to 7.5 kcal/mole in *Rb. capsulatus* ferrocytochrome *c* [36]. The pK_{alk} values are also diverse ranging from 8.5 for yeast cytochrome *c* [40] to 9.5 for rat cytochrome *c* [29], suggesting different degrees of stability for the Fe-S bond. The overall conformational and Fe-S bond stabilities are clearly not correlated with amino acid sequence homology (compare the pK_{alk} of horse, yeast and *Rb. capsulatus*). The electron transfer properties of different species of cytochromes *c* are somewhat more difficult to compare because reactions with common redox partners have not been widely reported. However, the second-order rate constants for photooxidation by detergent-solubilized *Rhodobacter sphaeroides* reaction centers under similar experimental conditions have been reported for three of the cytochrome *c* species. For example, at ionic strengths of 60 to 75 mM and pH 8.0, the second-order rate constant varies from $6 \cdot 10^7$ M^{-1} s^{-1} for yeast cytochrome *c* to $3 \cdot 10^8$ M^{-1} s^{-1} for horse cytochrome *c* [42]. This 5-fold difference in rate constant cannot be attributed to differences in redox potential and thus differences in the interaction domains (e.g., electrostatic or surface topology effects) must be responsible. Together, these observations indicate that there is little correlation between overall amino acid sequence homology and biochemical properties and point to the importance of site-specific mutagenesis as a method for understanding cytochrome *c* structure and function. Moreover, we can expect that the equivalent mutations in different species may not always have the same effect.

3. Site-specific mutant properties

For the purpose of this discussion, the differences in redox potential, conformational stability, and alkaline p*K* between wild-type and mutants will be reported in energetic terms. The conventions given below result in a positive parameter for destabilization and a negative value for stabilization of the mutant relative to wild-type. For redox potential differences, Eqn. 1 applies.

$$\Delta\Delta G_{redox} = -nF\Delta E'_0 \quad (1)$$

where $\Delta E'_0 = E'_0(\text{mutant}) - E'_0(\text{wt})$, F is Faraday's con-

Table 1
Properties of the wild-type cytochromes *c* that have been mutated

cyt	% AA [a] identity	E'_0 [b] (mV)	ΔG^*_u [c] (kcal/mol)	pK_{alk} [d]	rc_{k_2} [e] (M^{-1} s^{-1})	References
Rb. capsulatus	41	+370	4.4/7.5	8.9	$2 \cdot 10^8$	36,37
Yeast	65	+260	2.9/nr [f]	8.5	$6 \cdot 10^7$	38–40
D. melanogaster	84	+260	nr/nr	9.0	nr	28
Rat	94	+260	nr/nr	9.5	nr	29
Horse	100	+260	7.3/nr	9.1	$3 \cdot 10^8$	38,41,42

[a] Percent amino acid sequence identity relative to horse cytochrome *c*.
[b] Midpoint redox potential at pH 7.0 (ionic strength approx. 0.1 M).
[c] Conformational stability to urea or Gdn-HCl. First column corresponds to the oxidized state and the second column corresponds to the reduced state.
[d] Determined by the pH titration of the 695 nm absorbance band.
[e] Second order rate constant for photooxidation by detergent solubilized *Rb. sphaeroides* photosynthetic reaction centers at pH 8.0 and ionic strength 60–75 mM.
[f] Not reported.

stant, and $n = 1$. In the case of conformational stability, Eqn. 2 is used.

$$\Delta\Delta G_u^* = \Delta G_u^*(\text{wt}) - \Delta G_u^*(\text{mutant}) \quad (2)$$

where ΔG_u^* is the free energy of unfolding for the mutant or wild-type cytochrome. Finally, for changes in the alkaline pK, Eqn. 3 is applicable.

$$\Delta\Delta G_{alk} = -2.303RT\Delta pK_{alk} \quad (3)$$

where R is the gas constant, T is the temperature in degrees Kelvin, and $\Delta pK_{alk} = pK_{alk}(\text{mutant}) - pK_{alk}(\text{wt})$.

3.1. Redox potentials

In Table 2 the in vitro properties of site-specific mutations to highly conserved groups are compared. Note that residue numbering corresponds to that of horse cytochrome *c* for clarity. The largest effects on redox potential are those mutations to the M80 axial heme ligand. For example, the horse M80H and M80L mutants exhibit redox potentials that are destabilized by 5.3–7.1 kcal/mol in energetic terms [54]. In both cases, the observations are consistent with the redox potentials of natural variants possessing the analogous substitutions of the sixth heme ligand. For example, members of the class II cytochromes *c*, which lack the sixth heme ligand, exhibit redox potentials of −10 to +150 mV (i.e., ΔG_{redox} is destabilized 2.5–6.2 kcal/mole with respect to that of class I *c*-type cytochromes [2]). Moreover, members of the class III cytochromes *c*, which possess histidine as the sixth ligand, exhibit redox potentials of −100 to −300 mV (i.e., ΔG_{redox} is destabilized 8.3–12.9 kcal/mole with respect to class I *c*-type cytochromes [2]). Note that an even larger effect occurs in horse M80C in which the redox potential is lowered by approx. 650 mV [54]. In contrast to the M80 substitutions, substitution of the H18 axial ligand by arginine has been reported to have little effect on redox potential [46]. There are no natural variants of the histidine ligand and thus further characterization of the H18R mutant is required to determine whether arginine can serve as an axial heme ligand. It is important to note, however, that substitutions of the heme axial ligands may induce significant structural perturbations; consequently, structural characterization of these mutations is necessary to fully interpret their effects on redox potential.

Mutations to other highly conserved residues generally result in small, negative effects on redox potential. For example, the largest effects occur for substitutions at R38, Y48, Y67 and F82, which result in redox potentials that are decreased by less than 60 mV (i.e., destabilized by less than 1.4 kcal/mole) (Table 2). In these cases, the substituted groups are proximal to the heme. For example, R38 hydrogen bonds or charge pairs to the heme propionate-7, Y48 hydrogen bonds to the heme propionate-7 and Y67 and F82 are near the M80 axial heme ligand. Interestingly, substitution of conserved lysines 13 and 27 by acidic groups results in a small predictable effect on redox potential that is overcome at high ionic strength [43]. This effect of surface charge is due to favorable electrostatic interactions with the heme iron, which partially stabilize the ferric state and thus lower the redox potential. The electrostatic effects on redox potential suggest that it is important to consider buffer conditions (i.e., pH and ionic strength) when comparing mutant and wild-type redox potentials. In contrast, mutations to P30 and W59 have little effect on redox potential [28,48,69], suggesting that their heme environments have not been perturbed, a result that is somewhat surprising, since the carbonyl of P30 forms a hydrogen bond with the H18 axial heme ligand and the N1H of W59 hydrogen bonds to heme propionate-7 [3,7,14]. Finally, we note that for the two positions in which equivalent mutations have been generated in different species of cytochromes *c*, very similar effects on redox potential were observed. For example, the P30A mutants of *Rb. capsulatus*, *D. melanogaster*, and rat each resulted in a small effect on redox potential with $\Delta\Delta G_{redox}$ ranging from −0.05 to +0.18 kcal/mole [27,48]. Moreover, the Y67 mutants of *Rb. capsulatus*, rat and horse, each exhibited relatively large decreases in redox potential with $\Delta\Delta G_{redox}$ destabilized by 0.44–1.36 kcal/mole [29,30,36].

3.2. Overall conformational stabilities

In general, substitution of highly conserved residues results in decreased overall conformational stability (Table 2). For example, *Rb. capsulatus* P30A and W59Y (both redox states) are 2–3 kcal/mol less stable than wild-type [48,69], and yeast P71G (ferric state) is 1 kcal/mol less stable than wild-type [53]. The decreased conformational stability of the proline mutations could be due to the removal of structural constraints on the protein backbone; the decreased stability of the tryptophan mutant could result from the disruption of the hydrogen bond between the conserved tryptophan side chain and heme propionate-7. On the other hand, the mutated groups noted above are located in the protein interior [3,7,14] and thus the instability of the mutant may be due to the loss of hydrophobic or van der Waals interactions. For example, the substituting side chains of *Rb. capsulatus* P30A and W59Y and yeast P716 are less hydrophobic and smaller.

Surprisingly, *Rb. capsulatus* K13D and yeast K27Q, K27Y and K27W are 1–2 kcal/mol less stable than the wild-type [37,39]. Moreover, *Rb. capsulatus* K15E, a substitution to a nonconserved lysine that is proximal to K13, exhibits a similar 1 kcal/mol decrease in

Table 2
In vitro properties of mutations to conserved groups of cytochrome *c*

Residue [a]	Mutant [b]	$\Delta\Delta G_{redox}$ [c] (kcal/mol)	$\Delta\Delta G_u^*$ [d] (kcal/mol)	$\Delta\Delta G_{alk}$ (kcal/mol)	k_{et}/k'_{et} [f]	References
K13	c-K13D	+0.51 [g]	+1.3/nr [h]	+0.5 [g]	50 (k_2^{rc})	37,43,44
K15 [i]	c-K15D	+0.60 [g]	+1.3/nr	0.0	7.0 (k_2^{rc})	37,43,44
Q16	y-Q16K	nr	nr/nr	nr	2.4 (V_{max}^{ccp})	45
	y-Q16S	nr	nr/nr	nr	1.1 (V_{max}^{ccp})	45
H18	y-H18R	0	nr/nr	nr	nr	46
V20 [i]	y-V20C	nr	+1.1/nr	nr	nr	70
K27	c-K27E	+0.64 [g]	−0.2/nr	0.0	4.0 [g] (k_2^{rc})	37,43,44
	y-K27L	nr	−0.2/nr	nr	nr	39
	y-K27Q	nr	+1.1/nr	nr	1.2 (V_{max}^{ccp})	39,47
	y-K27W	nr	+2.0/nr	nr	0.9 (V_{max}^{ccp})	39,47
	y-K27Y	nr	+2.1/nr	nr	nr	39
P30	c-P30A	+0.18	+2.0/+2.1	0.0	1.0 (k_2^{rc})	48,49
	d-P30A	+0.02	+5.4/nr	+1.5	nr	28,65
	d-P30A	+0.09	nr/nr	+1.1	nr	28
	r-P30V	−0.05	nr/nr	+0.1	nr	28
R38	y-R38	+0.53	nr/nr	0.0	1.0 (V_{max}^{ccp})	50
	y-R38H	+0.62	nr/nr	+0.3	1.0 (V_{max}^{ccp})	50
	y-R38Q	+0.69	nr/nr	0.0	1.0 (V_{max}^{ccp})	50
	y-R38N	+0.78	nr/nr	0.0	1.0 (V_{max}^{ccp})	50
	y-R38L	+0.94	nr/nr	+0.5	1.0 (V_{max}^{ccp})	50
	y-R38A	+1.1	nr/nr	0.7	nr	50,67
Y48	y-Y48F	+0.50	nr/nr	+0.3	nr	51,67
N52	y-N52A	nr	−1.0/nr	nr	nr	52
	y-N52G	nr	+2.0/nr	nr	nr	52
	y-N52I	nr	−3.3/nr	nr	nr	52
W59	c-W59Y	−0.05	+2.5/+3.0	+1.4	0.9 (k_2^{rc})	69
Y67	c-Y67C	+0.44	+0.3/+4.1	0.0	1.1 (k_2^{rc})	36
	c-Y67F	+1.36	−0.4/+1.8	−2.3	1.1 (k_2^{rc})	36
	h-Y67F	+0.81	nr/nr	−2.3	nr	30
	r-Y67F	+0.81	+0.6/nr	−1.5	1.3 (V_{max}^{cco})	29,65
P71	y-P71G	nr	+1.2/nr	nr	nr	53
K72 [i]	y-K72A	+0.14	nr/nr	nr	nr	64
K73 [i]	y-K73M	nr	+1.0/nr	nr	nr	72
	y-K73Y	nr	+1.1/nr	nr	nr	72
	y-K73F	nr	+1.2/nr	nr	nr	72
	y-K73W	nr	+1.4/nr	nr	nr	72
K79	y-K79A	0	nr/nr	0	nr	64
M80	h-M80C	15.0	nr/nr	nr	nr	54
	h-M80H	+5.3	nr/nr	nr	nr	54
	h-M80L	+7.1	nr/nr	nr	nr	54
	h-M80A	+1.9	nr/nr	nr	nr	68
F82	y-F82A	+0.69	nr/nr	nr	nr	55
	y-F82G	+0.99	nr/nr	+1.1	5.0 (V_{max}^{ccp})	26,40,55
	y-F82I	+0.39	nr/nr	+1.8	nr	40,55
	y-F82L	+0.09	nr/nr	+1.8	nr	40,55
	y-F82S	+0.81	nr/nr	+1.1	1.4 (V_{max}^{ccp})	26,40,55
	y-F82Y	+0.23	+2.5/nr	+0.1	3.3 (V_{max}^{ccp})	26,40,55,66

stability [37]. In these cases, the lysines are located on the protein surface [3,7,14] and thus hydrophobic effects do not account for the mutant instabilities. The instability of *Rb. capsulatus* K13D and K15E has been attributed to unfavorable electrostatic interactions with the dipole of the helix in which they are located [37]. However, K27 is not located in a region of secondary structure [3,7,14], and thus the destabilization of yeast K27Q, K27Y and K27W is presumably due to perturbations of local structure, the specifics of which remain to be determined by X-ray crystallography or NMR studies. The instabilities of some mutations to charged groups, which are on the protein surface, point out the value of determining conformational stability on all mutants as a sensitive probe for structural changes.

Interestingly, the only reported substitutions that increase conformational stability are mutations to N52 and Y67. For example, the yeast N52A and N52I mutants increase stability by 1 and 3.3 kcal/mol, respectively, as opposed to the yeast N52G mutant which is 2 kcal/mol less stable than wild-type [52]. Due to the internal nature of N52, these effects have been attributed primarily to the hydrophobicity of the substituting group (i.e., alanine and isoleucine are more hydrophobic than asparagine; glycine is less hydrophobic than asparagine [56]). The *Rb. capsulatus* Y67F mutant is 0.4 kcal/mol more stable than the wild-type in the ferric state which can be attributed to the increased hydrophobicity of the substituting phenylalanine [29,36]. The conformational stability of ferrous *Rb. capsulatus* Y67F was shown to be decreased by 1.8 kcal/mol while the ferric form was stabilized by 0.4 kcal/mole [36]. Moreover, the stability of ferrous *Rb. capsulatus* Y67C was decreased 4 kcal/mol, as opposed to the oxidized state which was destabilized by only 0.3 kcal/mol [36]. Therefore, the conformational stability of different redox states can be affected to different degrees and in different directions. The decreased stability of the Y67 substitutions in the ferrous state has been attributed to disruption of a conserved hydrogen bonding network that occurs in the reduced but not oxidized cytochrome [36]. The significance of redox-dependent stabilities to other properties will be discussed below; nonetheless, it is important to determine conformational stability of mutants in both redox states, a practice that is not widely undertaken.

3.3. Fe-S bond stability

There are clearly no general trends for mutational effects on pK_{alk} (Table 2). For example, pK_{alk} is decreased by approx. 1 pH unit (i.e., the Fe-S bond is destabilized) in the eukaryotic P30, *Rb. capsulatus* W59, and many of the yeast F82 substitutions [28,49,55]. In contrast, the Y67F mutations in *Rb. capsulatus*, rat and horse exhibit pK_{alk} values that have been increased by greater than 1 pH unit (i.e., the Fe-S bond is stabilized by more than 1.4 kcal/mole) [29,30,36]. It appears that the largest effects on pK_{alk} occur when the mutated residue is in close proximity to the Fe-S bond. Moreover, the Fe-S bond appears to be stabilized upon increasing the hydrophobicity of its local environment. For example, the Y67F mutations increase hydrophobicity due to the more hydrophobic nature of the phenylalanine side chain and displacement of a conserved water molecule [29,36]. Finally, the equivalent mutations in different species can exhibit very different effects on pK_{alk}. For example, *Rb. capsulatus* P30A mutation exhibits no effect on pK_{alk} [49], in contrast to the P30A mutants of *D. melanogaster* and rat which exhibit pK_{alk} values that are destabilized by 1.1–1.4 kcal/mole [28]. The observations for the P30 mutants underscore the importance of characterizing a number of equivalent mutations in different species before drawing generalizations about the effect of a particular substitution.

3.4. Electron transfer properties

As is the case for comparing the wild-type electron transfer properties to one another, comparison of the mutant electron transfer properties is difficult because no standard assay has been used. However, in many cases either the second-order rate constant (k_2) or the

Notes to table 2:

[a] Numbering corresponds to that of horse cytochrome *c*.

[b] Lower case letters represent cytochromes *c* from: c, *Rb. capsulatus*; d, *D. melanogaster*; h, horse; r, rat; y, yeast.

[c] See Eqn. 1.

[d] See Eqn. 2. The first column corresponds to the oxidized state and the second column corresponds to the reduced state. Data were based on Gdn-HCl, urea or heat denaturation.

[e] See Eqn. 3.

[f] Ratio of wild-type to mutant second order (k_2) or maximal velocity (V_{max}) rate constants for electron transfer between cytochrome *c* and a protein electron donor or acceptor. Reactant partners correspond to rc, *Rb. sphaeroides* reaction center; cco, bovine cytochrome *c* oxidase; ccp, yeast cytochrome *c* peroxidase.

[g] Perturbation overcome at high ionic strength.

[h] nr = not reported.

[i] Nonconserved residue.

maximal velocity rate (V_{max}) have been determined for electron transfer between mutant cytochromes *c* and a protein electron donor or acceptor (e.g., photosynthetic reaction center, cytochrome *c* peroxidase, or cytochrome *c* oxidase). Note that k_2 typically is a measure of the product of a rapid binding equilibrium between donor and acceptor and the rate-limiting electron transfer step; while V_{max} is determined by the rate-limiting step after complex formation [2]. Normalizing for the effects of redox potential (which can be easily determined at the appropriate pH and buffer conditions), differences in the wild-type and mutant k_2 with a particular redox partner reflect perturbation of the electrostatic field and/or surface topology effects at the interaction domain (i.e., exposed heme edge in the case of cytochromes *c*); differences in the wild-type and mutant V_{max} with a particular redox partner reflect perturbation of the orientation and/or distance of the cytochrome *c* with respect to the other reactant, factors which are sensitive to the electrostatic field and/or surface topology at the interaction domain. Electrostatic and surface topology effects can be separated by determination of the ionic strength dependence of k_2 or V_{max}. It is useful to review the mutant electron transfer properties, but it is necessary to keep in mind the reaction partner, kinetic parameter reported, and the experimental conditions.

Interestingly, k_2 and V_{max} appear to be generally affected to surprisingly small degrees (Table 2). The largest effect occurs in *Rb. capsulatus* K13D for which k_2 is decreased by a factor of 40 relative to wild-type at low ionic strength [44]. Moreover, the *Rb. capsulatus* K15E mutant, which is a substitution to a nonconserved lysine but located in the cytochrome *c* interaction domain [14], exhibits a rate constant that is decreased by a factor of 20 at low ionic strength [44]. At high ionic strength, these effects were only partially overcome and thus structural perturbation of the interaction domain (i.e., changes in surface topology), as well as electrostatic factors, must be important in these mutants [44]. In contrast, *Rb. capsulatus* K27E exhibits a k_2 that is decreased by a factor of 3 at low ionic strength, an effect that is overcome at high ionic strength, and thus electrostatic effects predominate [44]. Significant effects on electron transfer kinetics have been observed for yeast F82G and F82Y which exhibit V_{max} that are decreased by factors of 5 and 3, respectively [26]. This decrease in V_{max} could be due in part to a decrease in the redox potential; however, the redox potential of yeast F82S is decreased by an amount similar to yeast F82G (35 vs. 43 mV), but V_{max} is only slightly affected. Moreover, the different R38 mutants exhibit similar decreases in redox potential but exhibit no measurable effect on V_{max} (Table 2). Thus, structural changes at the interaction domain, which in turn result in perturbation of the orientation and/or distance of the mutant cytochrome *c* with respect to the reactant partner, are responsible for the kinetic results. Interestingly, yeast Q16K results in a decreased V_{max} as opposed to yeast Q16S which exhibits no significant effect, suggesting that introduction of a positive charge at position 16 perturbs the orientation and/or distance of the cytochrome relative to the electron acceptor [45]. Surprisingly, mutations to P30, R38, W59 and Y67 exhibit only small effects on k_2 or V_{max} [29,36,49,50,69]. Together, the conformational stability data and kinetic results imply that mutations to conserved residues generally have larger effects on conformational stability than on electron transfer kinetics.

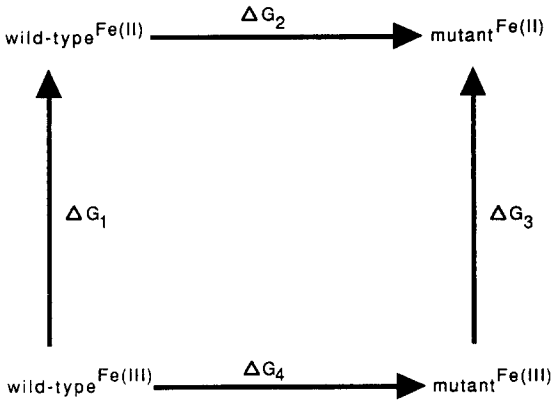

Fig. 2. Born-Haber thermodynamic cycle for mutagenic effects on redox potential.

4. Correlated properties

It is of interest to consider possible correlations between the various in vitro properties of the mutants. For example, redox potential is determined by the difference in the stability of the oxidized and reduced states. In the case of site-specific mutants, a Born-Haber thermodynamic cycle can be employed to describe this (Fig. 2). The free energies ΔG_1 and ΔG_3 represent the energy required to go from the oxidized state to the reduced state of the wild-type and mutant, respectively. These parameters are related to redox potential by the equation $\Delta G = -nF\Delta E$ and thus $(\Delta G_1 - \Delta G_3) = -nF(\Delta E_1 - \Delta E_3)$. The free energies ΔG_2 and ΔG_4 are the free energy differences due to the mutation in the oxidized and reduced states, respectively. Because the cycle is closed, $(\Delta G_1 - \Delta G_3)$ is equivalent to $(\Delta G_4 - \Delta G_2)$. Cutler et al. [50] have estimated the latter energy difference for yeast R38L by calculation of differences in the wild-type and mutant electrostatic free energies and found good correspondence between the observed redox potential differences and the calculated energy difference due to alteration of the local electrostatic field. As an alternative method to calculating ΔG_2 and ΔG_4, the confor-

Table 3
Correspondence between redox potential and conformational stability

cyt [a]	$\Delta G_1 - \Delta G_3$ [b] (kcal/mol)	$\Delta G_4 - \Delta G_2$ [c] (kcal/mol)
c-P30A	+0.12	+0.1
c-W59Y	~0.0	+0.5
c-Y67C	+0.4	+3.8
c-Y67F	+1.34	+2.2

[a] Notation as in Table 2.
[b] Calculated from $\Delta G_1 - \Delta G_3 = \Delta\Delta G_{redox}$ which was taken from Table 2.
[c] Calculated from $\Delta G_4 - \Delta G_2 = \Delta\Delta G_u^{red} - \Delta\Delta G_u^{ox}$ where $\Delta\Delta G_u^{red}$ and $\Delta\Delta G_u^{ox}$ were taken from Table 2.

mational stability estimated from denaturation studies can be used when this parameter has been determined in both redox states. In Table 3 we have given the $(\Delta G_1 - \Delta G_3)$ and $(\Delta G_4 - \Delta G_2)$ for the *Rb. capsulatus* mutants for which the conformational stability has been determined in both redox states. Comparison of $(\Delta G_1 - \Delta G_3)$ and $(\Delta G_4 - \Delta G_2)$ indicates that redox potential and conformational stability are correlated in *Rb. capsulatus* P30A, W59Y and Y67F within experimental error (±0.6 kcal/mol). In contrast, *Rb. capsulatus* Y67C exhibits values of $(\Delta G_1 - \Delta G_3)$ and $(\Delta G_4 - \Delta G_2)$ that are not within experimental error and thus Y67C conformational stability is not correlated with redox potential. Consequently, conformational stability appears to be generally correlated with redox potential (three out of four cases examined to date).

Examination of Table 2 suggests no apparent correlation between redox potential and pK_{alk}. This is somewhat surprising since pK_{alk} is related to the Fe-S bond strength; however, examination of the wild-type values in Table 1 suggests no correlation between the two parameters in different species of cytochromes *c*. There appears to be no correlation between redox potential and k_2, which is somewhat surprising based upon previous studies of cytochrome *c* reduction by *Clostridium pasteurianum* flavodoxin semiquinone, where redox potential was the dominant factor [2]. Consequently, in the case of the cytochrome *c* mutants studied to date, electrostatic and surface topology factors appear to be more important in their electron transfer reactions than small changes in redox potential. For those systems for which data are available (*Rb. capsulatus* K13D, K27E, P30A, W59Y, Y67C, Y67F, rat Y67F, and yeast F82Y), the change in ΔpK_{alk} qualitatively correlates with $\Delta\Delta G_u^*$ (oxidized) with two exceptions (*Rb. capsulatus* P30A and yeast F82Y). In both exceptions, there is a large decrease in conformational stability, but ΔpK_{alk} is near zero. The structural origin of the apparent anomaly remains to be determined. Conformational stability is clearly not correlated with k_2 or V_{max}, although in the case of *Rb. capsulatus* K13D, the decreased conformational stability may be indicative of structural perturbations that lead to an altered orientation and/or distance between reactants in the cytochrome c_2-reaction center complex [44]. Finally, pK_{alk} is clearly not correlated to k_2 or V_{max}. This is not too surprising because the experimental conditions for the referenced electron transfer reactions do not require rearrangement of the M80 ligand.

5. X-ray crystallography

A number of the mutant structures have been determined by X-ray crystallography, including yeast N52I [56], F82S [57], and F82G [58] ferrocytochromes *c*. These structures should be important to interpretation of the mutant properties described above. For example, X-ray structures of mutants can be used to distinguish between effects due to structural versus environmental changes. In the case of yeast N52I, the structure is very similar to that of the wild-type, but an internal water molecule, which is conserved among cytochromes *c*, is excluded [56]. Apparently, exclusion of the water molecule by disruption of a conserved hydrogen bonding network, of which N52 is part, results in increased local hydrophobicity which in turn is responsible for the increased conformational stability of N52I [56]. In the case of yeast F82S, the mutation results in both near and remote structural changes and increased exposure of the heme due to the decreased molecular volume of the serine side chain with respect to phenylalanine [57]. Presumably, the decreased redox potential of F82S [55] is due to decreased hydrophobicity of the heme and hence partial stabilization of the oxidized state [57]. Moreover, the decreased V_{max} [26] appears to be due to structural perturbation of the interaction domain [57]. In the case of yeast F82G, larger structural perturbation occurs [58] which is consistent with the larger effect on V_{max} (i.e., the surface topology of the F82G interaction domain has been perturbed to an extent that alters V_{max}). The decreased redox potential of F82G [55] has been attributed to decreased hydrophobicity of the heme as a result of the introduction of polar side chains into the heme environment and not increased solvent exposure as observed for yeast F82S [57]. The decreased pK_{alk} values of F82G [40] are consistent with decreased hydrophobicity near the Fe-S bond as described above.

6. NMR

NMR characterization of mutant cytochrome structural and dynamic properties is very useful for those cases in which the ^{1}H-NMR have been assigned (i.e., *Rb. capsulatus* [15], yeast [18] and horse [19]). For

example, chemical shift data can be used to identify amino acid residues whose environment has been perturbed and nuclear Overhauser effects can be used to identify perturbations of secondary and tertiary structure. In the case of *Rb. capsulatus* P30A ferrocytochrome c_2, these experiments have suggested that the overall conformation has not been perturbed but that local perturbations near the mutated site have occurred [48,59]. Interestingly, the hydrogen bond between the P30A carbonyl and the H18 N_π is clearly present, and thus the unique role of proline in orienting this bond is called into question [48,59]. A lack of structural change is consistent with the unchanged redox potential and pK_{alk} of *Rb. capsulatus* P30A [48,49]; however, the decreased conformational stability indicates that other factors such as increased dynamic fluctuation or solvent exposure occur in P30A [48,49]. For yeast Y48F the chemical shift and nuclear Overhauser effects suggest very little structural change; consequently, the 22 mV decrease in redox potential has been attributed to increased electron density of the propionate-7 carboxylate upon removal of the Y48 hydrogen bond [51]. Interestingly, larger structural perturbations appear to occur in the oxidized state [60], an observation that underscores the value of analyzing both redox states by X-ray crystallography and NMR. NMR characterization of yeast N52I has suggested that many small changes in structure occur in both redox states [71]. In the case of yeast F82S ferrocytochrome *c*, chemical shift data and nuclear Overhauser effects suggest that structural changes occur primarily in the vicinity of the mutated residue [60], which is in agreement with the X-ray data on the same redox state mentioned above [57].

Another important application of NMR spectroscopy to the study of cytochrome *c* mutants is the determination of proton exchange rates, which reflect the effects of protein dynamics on secondary and tertiary H-bonding. Based on the ^{15}NH-deuterium exchange rates of *Rb. capsulatus* cytochrome c_2, Gooley et al. [61] have recently demonstrated that a number of NH protons exchange faster in the oxidized state than the reduced state, with the largest differences of exchange rates corresponding to free energy differences of -3.5 kcal/mol. This value is very close to that of -3.1 kcal/mol calculated for the free energy difference in the conformational stabilities of the oxidized and reduced states of *Rb. capsulatus* cytochrome c_2 (Table 1). Thus, at least in this case, the decreased conformational stability of the oxidized state can be attributed to changes in H-bonding patterns. This type of analysis has been extended to the *Rb. capsulatus* P30A and Y67F mutants in the reduced state [62]. *Rb. capsulatus* Y67F, which exhibits a decreased redox potential of 59 mV (Table 2), exhibits increased NH proton exchange rates in a helical region whose exchange rates were previously shown to be sensitive to redox state in the wild-type. This observation led to the proposal that the H-bonding patterns of certain regions of the cytochrome are partial determinants of redox potential [62]. Moreover, the largest increases in the NH exchange rates of *Rb. capsulatus* P30A and Y67F as compared to wild-type correspond to free energy decreases of 2.6 and 1.6 kcal/mol, respectively; these decreases in free energy again are very similar to the observed decreases in the conformational stabilities of P30A and Y67F of 2.1 and 1.8 kcal/mol, respectively (Table 2). Thus, perturbation of H-bonding appears to be reflected in the conformational stability. Together, the X-ray crystallography and NMR studies promise to broaden our understanding of the determining factors in cytochrome *c* structure, as well as aid in the interpretation of mutant biochemical properties.

7. Conclusions

A large number of site-specific mutants from five species of cytochromes *c* have been characterized in vitro. In some cases, characterization has been extended to determination of the mutant structure by X-ray crystallography or mutant structural and dynamic properties by NMR. From these studies a number of generalizations can be drawn concerning redox potential, overall conformational stability, Fe-S bond stability, and electron transfer properties of cytochromes *c*. The generally small negative effect on redox potential suggests that, with the exception of the M80 heme ligand, highly conserved residues play limited roles in the determination of redox potential and the general decrease in redox potential suggests that the oxidized state of the mutants has been stabilized with respect to the reduced state. The first point, in retrospect, is not so surprising, since the nearly 500 mV variability in the redox potentials of class I *c*-type cytochromes must be due to the sum of many substitutions and not single-site substitutions. The second point is consistent with the redox potentials of model heme proteins with methionine-histidine ligation of the heme, which exhibit redox potentials near 0 mV [63]. Therefore, one apparent role of the protein moiety of cytochrome *c* is to destabilize the oxidized state with respect to the reduced state (i.e., increase redox potential) to a degree that is appropriate for electron transfer between physiological electron donors and acceptors. In contrast to redox potential effects, mutations generally decrease the overall conformational stability suggesting that many of the conserved residues play important roles in stability. Mutational effects on pK_{alk} suggest that the Fe-S bond can be either stabilized or destabilized, with the largest effects occurring when the mutated residue is proximal to the Fe-S bond.

Electron transfer reactions are affected to small degrees with the most significant effects occurring upon introduction of unfavorable electrostatic and/or surface topology interactions at the exposed heme edge. However, the lack of large effects on the electron transfer rate constants of the mutants in Table 2 suggests that none of the side chains mutated to date play critical roles in electron transfer, an observation that is consistent with their ability to function in vivo [20,21,27–29,39,50,52].

Together, the observations reviewed here indicate that: (i) conserved residues are not as important to cytochrome *c* structure and function as might have been expected, and thus full characterization of each mutant appears to be necessary to interpret the subtleties of the mutational effects and the determinants of evolutionary conservation; (ii) local perturbations can be transmitted to distant sites (e.g., yeast F82G and F82S), and thus structural characterizations by X-ray crystallography and NMR are important to interpretation of mutational effects on biochemical properties; (iii) the equivalent mutations in different species can exhibit very different properties (e.g., the pK_{alk} of *Rb. capsulatus*, *D. melanogaster*, and rat P30A), underscoring the importance of characterizing equivalent mutations in a number of species; (iv) relatively benign mutations can have surprising effects (e.g., the decreased conformational stability of *Rb. capsulatus* K13D and K15E), pointing out the value of thorough characterization of each mutant. It is anticipated that continued characterization of the present group of cytochrome *c* mutants and the generation and characterization of future mutants will greatly contribute to our knowledge of cytochrome *c* structure and function, as well as our knowledge of protein structure and function in general.

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