Mutagenesis of histidine 26 demonstrates the importance of loop-loop and loop-protein interactions for the function of iso-1-cytochrome *c*

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Abstract

In yeast iso-1-cytochrome *c*, the side chain of histidine 26 (His26) attaches omega loop A to the main body of the protein by forming a hydrogen bond to the backbone atom carbonyl of glutamic acid 44. The His26 side chain also forms a stabilizing intra-loop interaction through a hydrogen bond to the backbone amide of asparagine 31. To investigate the importance of loop-protein attachment and intra-loop interactions to the structure and function of this protein, a series of site-directed and random-directed mutations were produced at His26. Yeast strains expressing these variant proteins were analyzed for their ability to grow on non-fermentable carbon sources and for their intracellular production of cytochrome *c*. While the data show that mutations at His26 lead to slightly decreased intracellular amounts of cytochrome *c*, the level of cytochrome *c* function is decreased more. The data suggest that cytochrome *c* reductase binding is affected more than cytochrome *c* oxidase or lactate dehydrogenase binding. We propose that mutations at this residue increase loop mobility, which, in turn, decreases the protein's ability to bind redox partners.

Keywords: loop dynamics; loop replacement; loop swap; omega loop; random mutagenesis; secondary structure; site-directed mutagenesis; yeast

We are interested in understanding the role that nonregular secondary structures, loops and turns, play in protein structure, folding, stability, and function. In particular, we have been focusing on omega (Ω -) loops, nonregular secondary structure segments that are six or more residues long and whose endpoints are close in space (Leszczynski, 1986). When these structures were first described, it was observed that they occur at the protein surface, an observation that suggested that they may play a role in function. It was also observed that they are compact, suggesting that they may form independent subdomains or substructures or that they may participate in protein folding (Leszczynski, 1986; Fetrow, 1995).

Since the original description of Ω -loops, the role that specific loops play in the function of certain proteins and protein families, including the immunoglobulins (Jones et al., 1986; Tramontano & Lesk, 1992), the TIM barrel proteins (Pompliano et al., 1990), proteases (Hedstrom et al., 1992), nucleases (Allemann et al., 1991; Poole et al., 1991; Raines et al., 1995), and dihydrofolate reductase (Li et al., 1992; Gekko et al., 1993; Falzone et al., 1994), among

others (Pan et al., 1993; First & Fersht, 1993; DiBella & Scheraga, 1996; Liang et al., 1996), has been studied, but their role in overall protein stability and folding has not been as well studied (Chou et al., 1992; Scheerlinck et al., 1992; Urfer & Kirschner, 1992; Hardy et al., 1994; Helms & Wetzel, 1995). Recently, it has been suggested that loop replacement or modification is a good method of protein engineering (ElHawrani et al., 1994; Fetrow, 1995; Shortle & Sondek, 1995); therefore, an understanding of loop-loop and loop-protein interactions is essential to our ability to design and engineer stable proteins for specific purposes.

Iso-1-cytochrome *c* from the yeast *Saccharomyces cerevisiae* contains four Ω -loops: loop A, residues 18–32; B, residues 34–45; C, residues 40–54; and D, residues 70–84 (Fetrow et al., 1989, Fig. 1). It has been shown by NMR spectroscopy that some amide protons in these Ω -loops are protected from exchange with deuterons on a time scale that allows calculation of an exchange rate, an observation that prompted Pielak and coworkers to suggest that several of these Ω -loops are indeed independent substructures (Marmorino et al., 1993). Further proton exchange experiments on horse cytochrome *c* at low denaturant concentrations showed that there are four substructures in this molecule and that complete Ω -loops comprise at least part of three of these substructures (Bai et al., 1995; Bai & Englander, 1996). According to the Gibbs free ener-

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Fig. 1. **A:** A ribbon structure of oxidized iso-1-cytochrome *c* (C102T) (Berghuis & Brayer, 1992). The substructures identified by the proton exchange experiments of Englander and coworkers (Bai et al., 1995) are colored as follows: substructure I (N- and C-terminal helices, residues 3–13 and 88–103, respectively), black; substructure II (60's helix, residues 60–69, and Ω -loop A, residues 18–32), dark gray; substructure III (loops B and C, residues 34–44 and 40–54, respectively), light gray; substructure IV (loop D, residues 70–84), white. The side chains of His26, heme ligands His18 and Met80, and the heme group are shown as ball and stick models. **B:** A closer view of His26 from yeast iso-1-cytochrome *c* and its two hydrogen bonds (dashed lines with heavy atom distances indicated) to the carbonyl oxygen of glutamic acid 44 and the backbone nitrogen of asparagine 31. Residues 26, 31, and 44 are shown in black; the backbone of loop A is medium gray; the loop C backbone is light gray; the heme is dark gray. **C:** Comparison of the structures of histidine and asparagine residues (gray). The atoms that are able to participate in hydrogen bonds are shown in black.

gies calculated from these data, the most stable substructure, substructure I, is composed of the cytochrome *c* N- and C-terminal helices. The second most stable substructure, II, is comprised of the 60's helix and Ω -loop A. Loops B and C, the overlapping Ω -loops, make up substructure III, while the least stable substructure, IV, is loop D (Bai et al., 1995; Bai & Englander, 1996). The locations of the analogous substructures in iso-1-cytochrome *c* are shown in Figure 1.

Because of the identification of its component Ω -loops as substructures, cytochrome *c* is the ideal protein in which to study the importance of loop-loop and loop-protein interactions. Iso-1-cytochrome *c* from the yeast *Saccharomyces cerevisiae* is especially appropriate because of the ease with which site-directed and random-directed amino acid mutations can be produced (Pielak et al., 1985; Hampsey et al., 1986; Fetrow et al., 1989; Auld & Pielak, 1991; Fumo et al., 1995) and the facility with which the protein can be purified (Sherman et al., 1968). In this facultative aerobe, growth curves in media containing nonfermentable carbon sources can identify functional properties of variant iso-1-cytochromes *c* (Ernst et al., 1985; Fetrow et al., 1989) and low temperature spectroscopy of intact yeast cells grown on fermentable carbon sources (Elliott & Margoliash, 1971; Cottrell et al., 1975; Schweingruber et al., 1977) can detect structural properties of the protein in vivo; consequently, folded, but nonfunctional, proteins can be identified without resorting to protein purification. Further, a number of crystal and solution structures of eukaryotic cytochromes *c* have been solved (Tanaka et al., 1975; Takano & Dickerson, 1981; Ochi et al., 1983; Bushnell et al., 1990; Louie & Brayer, 1990; Murphy et al., 1992; Qi et al., 1994; Baistrocchi et al., 1996; Qi et al., 1996; Banci et al., 1997) and many mutant proteins have been crystallized and their structures solved (Hampsey et al., 1986; Louie et al., 1988; Langen et al., 1992; Murphy et al., 1993; Berghuis et al., 1994; Lo et al., 1995). Thus, in yeast cytochrome *c*, amino acid changes in loop-loop and loop-protein interactions can be produced easily; the effect of these mutations on the structure and the function of the protein can be studied both in vivo and in vitro.

To study loop substructure properties and loop-protein interactions, loop A in iso-1-cytochrome *c* was replaced with homologous loops from other cytochromes *c* (Fetrow et al., 1989; Murphy et al., 1993; Fetrow et al., 1997). The results from these loop A replacement mutations suggest that this loop does indeed act as an independent substructure; however, all replacements affect overall protein stability.

C14, a yeast strain encoding RepA4, one of these variants, expresses a hybrid cytochrome *c* in which the protein is folded properly, but is completely nonfunctional on lactate medium (Fetrow et al., 1989; Fetrow et al., 1997). The RepA4 protein contains loop A from the *Pseudomonas aeruginosa* cytochrome *c*551, rather than the wild-type loop A (Table 1). During analysis of strain C14, two independent colonies that grew much more rapidly than C14 were isolated. The *cyc1* locus of each of these strains was sequenced and each contained a single nucleotide change. Although the single change was different in the two strains, the result was a lysine-to-asparagine mutation in the *P. aeruginosa* loop of the hybrid iso-1-cytochrome *c* (T.S. Cardillo, J.S. Fetrow, and F. Sherman, unpubl. obs.).

Molecular modeling was used to compare the structure of loop A from iso-1-cytochrome *c* to loop A from cytochrome *c*551. The ends of these homologous loops are structurally similar (Fig. 2), although the loop lengths are different (compare wild type and

Table 1. Summary of strain names and sequences of strains containing mutations in loop A and at His26 in yeast iso-1-cytochrome *c*

Str ^a	Prot ^b	Loop A sequence									
Control strains											
C93	WT (C102T)	18					26	29	32		
C15	[Del Cyt c]	His-Thr-Val-Glu-Lys-Gly-Gly-Pro- His -Lys-Val-Gly-Pro-Asn-Leu									
C3	[Del Loop A]	His-Thr-Val-Glu-						-Gly-Pro-Asn-Leu			
C14	RepA4 (<i>P. aerug.</i> loop A)	18					23	24	25	29	32
		His-Ala-Ile-Asp-					Thr- Lys -Met-Val-Gly-Pro-Asn-Leu				
5R3-SM	Spontaneous mutant (<i>P. aerug.</i> loop A)	His-Ala-Ile-Asp-					Thr- Asn -Met-Val-Gly-Pro-Asn-Leu				
Strains containing site-directed mutations at Lys23 in RepA4 loop replacement											
C7	RepA4;K23S;C102	His-Ala-Ile-Asp-					Thr- Ser -Met-Val-Gly-Pro-Asn-Leu				
C16	RepA4;K23Q;C102	His-Ala-Ile-Asp-					Thr- Gln -Met-Val-Gly-Pro-Asn-Leu				
C24	RepA4;K23D;C102	His-Ala-Ile-Asp-					Thr- Asp -Met-Val-Gly-Pro-Asn-Leu				
C71	RepA4;K23H;C102	His-Ala-Ile-Asp-					Thr- His -Met-Val-Gly-Pro-Asn-Leu				
C72	RepA4;K23N;C102	His-Ala-Ile-Asp-					Thr- Asn -Met-Val-Gly-Pro-Asn-Leu				
C73	RepA4;K23A	His-Ala-Ile-Asp-					Thr- Ala -Met-Val-Gly-Pro-Asn-Leu				
Strains containing directed, random mutations at His26											
Str ^a	Prot ^b	Sequence ^c		Str ^a	Prot ^b	Sequence ^c					
5R1	H26C	Cys	TGC	5R43	H26L	Leu	TTG				
5R4	H26V	Val	GTT	5R47	H26S	Ser	TCT				
5R5	H26W	Trp	TGG	5R49	H26D	Asp	GAC				
5R25	H26G	Gly	GGG	5R50	H26A	Ala	GCG				
5R28	H26I	Ile	ATT	5R53*	H26M;A7E	Met	ATG				
5R29	H26F	Phe	TTT								

^a Str is the yeast strain name expressing the protein.^b Prot is the protein name. All variant proteins, produced by either site-directed or random-directed mutagenesis, are expressed from the *cyc1* locus in the chromosome (see Methods).^c Sequence is the protein and DNA sequence at the position analogous to His26 in the directed, random mutants. An asterisk (*) indicates that the strain contained a second site mutation.

RepA4 in Table 2). Upon superposition of the conserved loop ends, the lysine in the *P. aeruginosa* loop that spontaneously mutates to an asparagine is structurally homologous to histidine 26 (His26) in iso-1-cytochrome *c* (Fig. 2). In the iso-1-cytochrome *c* crystal structure (Louie & Brayer, 1990), this histidine side chain forms two hydrogen bonds, one loop-protein hydrogen bond, His26:NE2 to Glu44:O, and one intra-loop hydrogen bond, His26:ND1 to Asn31:N (Fig. 1B). Both the native histidine and the spontaneously mutated asparagine side chains can act as a donor and acceptor in the formation of hydrogen bonds (Fig. 1C). This observation prompted us to hypothesize that these two hydrogen bonds are critical for function, but not folding, of cytochrome *c*.

His26 is highly conserved in the 106 sequences of eukaryotic cytochromes *c* published thus far (Pettigrew & Moore, 1987; Pielak et al., 1995). Histidine is found in 92 organisms, asparagine in 12 organisms, and serine and threonine each in one organism (Table 2). All are capable of acting as both hydrogen bond donors and acceptors. The most commonly occurring residues, histidine and asparagine, are similar, but not isosteric, in the location of the donor and acceptor atoms (Fig. 1C). Furthermore, the two hydrogen bonds from residue 26 are maintained in eight of the nine

wild-type cytochromes *c* from five different eukaryotic organisms whose structures have been solved by X-ray crystallography. Iso-2-cytochrome *c* from yeast is the single exception. In this protein, asparagine is found at position 26 and its side chain forms a hydrogen bond with the backbone amide of asparagine 31. In the crystal structure, the distance to the carbonyl oxygen of residue 44 is slightly long for an optimal hydrogen bond—about 3.39 Å (Murphy et al., 1992). Even so, the Asn26 side chain is oriented in the proper direction, suggesting that a weak hydrogen bond interaction is present.

In another loop replacement mutant protein (RepA2), His26 is also replaced by an asparagine. In the crystal structure of this protein, the backbone of loop A is unchanged from the wild type, but the backbone of the adjacent loop C is slightly distorted. Both hydrogen bonds to the asparagine side chain are thus maintained (Murphy et al., 1993). These observations further support the suggestion that a hydrogen bonding, but not charged, residue is critical at position 26 for eukaryotic cytochrome *c* function.

To test our hypothesis, we performed directed, random mutagenesis of His26 in iso-1-cytochrome *c* and site-directed mutagenesis of the analogous lysine in the RepA4 loop replacement protein.

Fig. 2. The backbone of loop A from *P. aeruginosa* cytochrome *c*551 (white) superimposed on the backbone of loop A from iso-1-cytochrome *c* (light gray). The residues at the loop ends (backbone atoms of His18, Pro30, and Asn31 from iso-1 and His16, Pro25, and Ala26 from cytochrome *c*551) were used for the superposition. The side chain of His26 from iso-1-cytochrome *c* and the homologous lysine in cytochrome *c*551 are shown as black ball and stick models and labeled. The side chains of the conserved His18 (16) and Pro31 (25) (*P. aeruginosa* residue numbers in parentheses) are also shown.

The function of yeast strains containing these mutants was studied by performing growth curves on a nonfermentable carbon source. The amount of mutant protein present within the cell was analyzed by low temperature spectroscopy of intact yeast cells. Temperature sensitivity of these strains was also analyzed. The production of these mutations and the *in vivo* results are the first step in understanding how loop-loop and loop-protein hydrogen bonds affect

Table 2. Sequence of the residue analogous to histidine 26 from 106 eukaryotic cytochromes *c*

Organism	Res26
<i>Saccharomyces cerevisiae</i> iso-1 (and 91 other eukaryotes sequenced)	His
<i>Saccharomyces cerevisiae</i> iso-2	Asn
<i>Candida glabrata</i>	Asn
<i>Schizosaccharomyces pombe</i>	Asn
<i>Neurospora crassa</i>	Asn
<i>Humicola lanuginosa</i>	Asn
<i>Ustilago sphaerogena</i>	Asn
<i>Crithidia oncopelti</i>	Asn
<i>Crithidia fasciculata</i>	Asn
<i>Euglena gracilis</i>	Asn
<i>Euglena viridis</i>	Asn
<i>Arabidopsis thaliana</i>	Asn
<i>Stellaria longipes</i>	Asn
<i>Tetrahymena pyriformis</i>	Ser
<i>Caenorhabditis elegans</i>	Thr

the substructure stability of the loop and the overall stability of the protein.

Results

Yeast strains produced by random, directed mutagenesis and by spontaneous mutations

Random, directed mutagenesis allows us, in a single experiment, to screen for all possible amino acid substitutions at a given position that allows production of a functional cytochrome *c* (Fumo et al., 1995; Mulligan-Pullyblank et al., 1996). The resulting substitutions provide an ideal measure of the functionally allowed residues at the randomized position in iso-1-cytochrome *c*. C14, a strain expressing RepA4, a nonfunctional cytochrome *c* containing a loop A replacement from *P. aeruginosa* cytochrome *c*551 at the *cyc1* locus (Table 1), was transformed with an oligonucleotide designed for two purposes: (1) to replace the RepA4 *P. aeruginosa* loop with the wild-type iso-1-cytochrome *c* loop, and (2) to randomize the codon encoding residue 26. Upon transformation, any cell that takes up and integrates a randomized oligonucleotide will, by design, replace the *P. aeruginosa* loop A in the hybrid RepA4 protein with the native yeast loop A. The protein thus produced will be wild-type iso-1-cytochrome *c*, except at residue 26. If the randomized codon at position 26 encodes a residue that produces a functional cytochrome *c*, the yeast colony producing the functional protein will grow above the background lawn of cells that express a nonfunctional cytochrome *c*. Such colonies can be easily selected and the *cyc1* locus sequenced. The net result is a series of yeast strains, each producing iso-1-cytochrome *c* with mutations at residue 26.

Three transformations with random oligonucleotides were accomplished and 112 transformants expressing a functional cytochrome *c* were selected and subcloned. All but one of the transformants exhibited the correct phenotype (leu⁺, ura⁻, trp⁻, his⁻) on selective media plates. The strain with the incorrect phenotype was not further considered.

Upon sequencing the *cyc1* locus of 34 transformants, we discovered something that we had not previously observed in directed, random mutagenesis experiments: Approximately 22% of the transformants had not integrated the randomized oligonucleotide and thus, still expressed a hybrid cytochrome *c* molecule with its replacement loop A from *P. aeruginosa*. Upon comparison of the *cyc1* sequence of these strains to the parent RepA4 strain, it was discovered that each exhibited a single nucleotide modification. Eight of these strains contained an AAG to AAT nucleotide change and four contained an AAG to AAC change, but both resulted in mutation of lysine to asparagine at the position homologous to His26. This is similar to the spontaneous mutations that we had observed previously (see Introduction). In directed, random mutagenesis of C14, this spontaneous mutation occurs with amazingly high frequency. For comparison, one spontaneously mutated (SM) strain, 5R3-SM, was chosen as representative (Table 1) and its properties are presented here. All SM strains tested behaved in a similar fashion.

Each of the other 22 (66%) sequenced transformants had properly integrated the oligonucleotide, resulting in a re-replacement of the wild-type iso-1-cytochrome *c* loop for the *P. aeruginosa* loop, with randomization of the codon at position 26. Eleven different amino acid substitutions were identified, including one with a second site change (5R strains in Table 1). The amino acids found

at position 26 cover the range from charged to hydrophobic to hydrophilic. Because of the functional selection necessary for directed, random mutagenesis, all of these amino acids produce at least a minimally functional cytochrome *c*. Clearly, the dual hydrogen bond donor and acceptor in the side chain of position 26 is not required for minimal function of these single site variants.

Directed mutagenesis of lysine in *P. aeruginosa* loop in RepA4

We then asked if another amino acid besides asparagine in the RepA4 loop could result in a functional protein. Directed, random mutagenesis of the lysine in the RepA4 protein was thus performed; however, only transformants containing the previously observed lysine to asparagine change were obtained. Consequently, site-directed mutagenesis of the lysine to serine, glutamine, aspartic acid, histidine, or alanine in RepA4 was performed (C-strains in Table 1). To demonstrate that the lysine-to-asparagine change is the cause of the gain of functionality in the spontaneous-mutant strains, the lysine was also explicitly mutated to an asparagine to produce strain C72. The strain names, protein names, and sequences of the loop A region of the mutant cytochromes *c* are shown in Table 1.

Function of cytochrome *c* protein in mutant yeast strains

To quantify the *in vivo* functionality of the variant cytochrome *c* proteins in yeast strains produced by site-directed and random-directed methods, we monitored strain growth in liquid lactate medium (Fig. 3; Table 3). Three of the RepA4 strains, C7, C16, and C24, did not grow on lactate (Table 3). Either cytochrome *c* is not produced in these strains, or it is produced, but is nonfunctional. Strain C72, in which asparagine replaces the Lys23 in RepA4, does grow in lactate medium, demonstrating that the lysine-to-asparagine spontaneous mutation in RepA4 is the direct cause of the gain in functionality. The other two RepA4 strains, C71 and C73, with histidine and alanine replacing the lysine, respectively, also barely grew in lactate medium (Table 3). Thus, asparagine, histidine, or alanine at the position analogous to His26 will produce a minimally functional cytochrome *c* in RepA4 variants containing the *P. aeruginosa* loop. Histidine and asparagine side chains can both act as hydrogen bond donors and acceptors (Fig. 1C), but alanine cannot.

Yeast strains expressing native cytochrome *c* with the native loop A and single mutations at position 26 exhibit a range of growth in lactate medium (Fig. 3, Table 3). Strikingly, no strain exhibits greater than 70% of the level of growth found in the wild-type protein. 5R53, whose cytochrome *c* is H26M (but which also contains a second site mutation), barely grows in lactate even though 5R53 contains the wild-type yeast loop A, except for the single site changes. Its growth is virtually indistinguishable from 5R3-SM containing a spontaneous mutation in the *P. aeruginosa* loop hybrid, but this may be attributable to the second site mutation (A7E). The other 5R strains containing a single mutation at His26 show a range of functionality from 21 (H26A) to 67% (H26S). These data show that most any His26 mutation will dramatically decrease the growth of strains containing the variant cytochromes *c*. This decrease in growth could be due to a decrease in the functionality of the cytochrome *c* or to a decrease in the amount of folded cytochrome *c* present in the cell.

Fig. 3. Representative growth curves of yeast strains grown on liquid lactate medium, a nonfermentable carbon source. Growth on this carbon source is a measure of the functionality of the cytochrome *c* in isogenic strains. A summary of all data can be found in Table 3. The curves through the data points have no theoretical significance. **A:** Yeast strains derived from strain C14, which express the hybrid protein with the *P. aeruginosa* loop A. The cytochrome *c* in these strains contain site-directed mutations of Lys23, which is structurally analogous to His26 (see Fig. 2). **B:** Representative growth curves of the 5R yeast strains containing directed, random mutations at His26 in iso-1-cytochrome *c*.

Amount of folded cytochrome *c* present in mutant yeast strains

Low temperature spectroscopy of intact yeast cells provides a quantitative measure of how much holo-cytochrome *c* is produced within the cell (Elliott & Margoliash, 1971; Cottrell et al., 1975; Schweingruber et al., 1977). Holo-cytochrome *c* is defined as a cytochrome *c* polypeptide that has entered the mitochondria, has been covalently linked to a heme group, and has folded properly. The heme α/β band is produced as a result of a π to π^* transition in the heme group (Moore & Pettigrew, 1990) and the presence of this absorbance at 550 nm is an indication that the protein has folded into a cytochrome *c*-like conformation around the heme. The α/β absorbance peak is intense and can be observed in intact yeast cells at low temperatures. Thus, low temperature difference spectroscopy was performed to quantify the amount of intracellular, folded cytochrome *c* and to determine if the decrease in function of the His26 mutants was due to a corresponding decrease in the amount of intracellular holo-cytochrome *c*.

Table 3. Summary of function, structure, and temperature sensitivity assays

Yeast strain	Protein name	Relative growth (lac) ^a (%)	Relative amt cytc ^b (%)	Temp Sens ^c	
				YPL	YPG
C93	Wild type	100	100	WT	WT
C15	Del Cyt	0	0	NG	NG
C3	DelA	0	0	NG	NG
C14	RepA4	0	46	NG	NG
C7	RepA4;K23S;C102	0	0	ND	ND
C16	RepA4;K23Q;C102	0	0	ND	ND
C24	RepA4;K23D;C102	0	0	ND	ND
C71	RepA4;K23H;C102	7	10	ND	ND
C72	RepA4;K23N;C102	5	15	ND	ND
C73	RepA4;K23A	2	18	ND	ND
5R3-SM	RepA4;K23N	5	26	TS	PG/TS
5R4	H26V	42	74	WT	NWT
5R5	H26W	34	38	WT	NWT
5R50	H26A	21	58	WT	NWT
5R28	H26I	43	84	WT	SG
5R29	H26F	23	50	WT	SG
5R25	H26G	49	59	STS	SG
5R1	H26C	36	71	STS	PG
5R47	H26S	67	64	NWT	PG
5R43	H26L	29	47	SG,TS	PG
5R49	H26D	42	58	NWT	BG
5R53	H26M;A7E*	1	40	NG	NG

^aRelative growth (lac) is the level of growth of each yeast strain at 30 °C, as determined by growth curves in liquid lactate medium, normalized to the growth of the wild-type strain (C93, 100%) and the *cyc1* deletion strain (C15, 0%). As the strains are isogenic, except at the *cyc1* locus, this number represents the relative function of the cytochrome *c* protein present in the strain.

^bRelative amt cytc is the amount of holo-cytochrome *c* present in the yeast cell at 30 °C, as determined by low temperature difference spectroscopy, normalized to the amount of protein present in the wild-type strain (C93, 100%) and the *cyc1* deletion strain (C15, 0%).

^cTemperature sensitivity of cells grown on lactate (YPL) and glycerol (YPG) nonfermentable carbon sources at ambient, 30 °C, and 37 °C. WT indicates that strain growth was indistinguishable from wild type (C93); NG indicates that no growth was detectable in this assay at any temperature; TS indicates that the strain grew like wild type at ambient and 30 °C, but did not grow at 37 °C; STS indicates that the strain grew like wild type at ambient and 30 °C, but grew less well than wild type at 37 °C; PG indicates that the strain grew poorly at all temperatures; SG indicates that the strain grew slowly in days 1 and 2, but did reach close to wild-type levels by day 3; NWT indicates that the strain grew somewhat less well than wild type at all temperatures, but not as poorly as the SG or PG strains; BG indicates that barely any growth was visible at any temperature on any day; ND, not determined.

Strains B7711 (Fetrow et al., 1989) and C14 (Fetrow et al., 1997), both of which express RepA4, the loop replacement protein containing the *P. aeruginosa* loop A, do not grow on nonfermentable carbon sources, but do produce some (35–50% of wild type) folded cytochrome *c*. 5R3-SM and C72, both of which differ from C14 by the single lysine-to-asparagine change, produce slightly less (25–30%) folded cytochrome *c* (Table 3), but the protein produced in these strains is now functional enough to allow the yeast to grow on nonfermentable carbon sources.

Strains expressing variants in which the lysine in the replacement *P. aeruginosa* loop of RepA4 was mutated were also analyzed by low temperature spectroscopy (Fig. 4). Strain C72, which encodes RepA4;K23N, produces a very small but detectable (15%) amount of holo-cytochrome *c*. Likewise, strains C71 and C73 encoding RepA4;K23H and RepA4;K23A also produce small but detectable amounts of protein (Table 3). All other strains derived from RepA4 with single-site replacement of the lysine do not produce any holo-cytochrome *c* that is visible by low temperature spectroscopy of intact yeast cells (Table 3).

On the other hand, all strains generated by directed, random mutagenesis of His26 produce at least 35% of the normal level of folded cytochrome *c* (Fig. 4; Table 3). 5R5 and 5R53, which

express H26W and H26M, respectively, produce about 40% of the normal level of protein. 5R43 produces 47%. All other 5R strains containing His26 mutations to cysteine, valine, leucine, glycine, isoleucine, phenylalanine, serine, aspartic acid, or alanine, produce 50–85% of the wild-type level of protein in the cell. Previously, it has been shown that the growth of strains expressing wild-type iso-1-cytochrome *c* at 50% of the normal protein level are quite similar to the growth of wild type (Schweingruber et al., 1979; Ernst et al., 1985; Fetrow et al., 1989). These experiments demonstrate that while His26 mutations do cause some decrease in the amount of cellular cytochrome *c*, the decrease in protein does not account for the decrease in function observed during growth in lactate medium.

Temperature sensitivity of His26 mutants

The temperature sensitivity of yeast strains containing the His26 random mutations was tested on lactate (YPL) and glycerol (YPG) nonfermentable carbon sources. As a control, all strains were tested on the fermentable carbon source, dextrose (YPD). Small quantities of cells were plated on each of these media and grown at room temperature (approximately 25 °C), 30 °C, and 37 °C. Cell growth was compared over the course of three days.

Fig. 4. Representative low temperature spectra of intact yeast cells. Strains were grown on rich dextrose medium, so, if it is produced, folded cytochrome *c* will be visible even if it is nonfunctional. A summary of all data can be found in Table 3. **A:** Strains derived from C14 containing site-directed mutations at Lys23 in the *P. aeruginosa* guest loop. **B:** 5R strains containing directed, random mutations at His26 in iso-1-cytochrome *c*.

As expected, the wild-type strain, C93, grew well at all three temperatures, on all three days, on all three media. The two negative control strains, C15 containing a deletion of cytochrome *c* and C3 expressing a cytochrome *c* loop A deletion (Table 2), did grow on YPD, but did not grow on YPL or YPG at any temperature (Table 3). C15 and C3 did not grow as well as C93 on YPD at any temperature, however (data not shown). C14, the original loop replacement strain, did not grow at any temperature on solid lactate or glycerol media. All strains expressing the spontaneous mutation of lysine to asparagine, as exemplified by 5R3-SM, did not grow as well on YPL at 37°C as at 25°C or 30°C, thus these were labeled as “TS” or “temperature sensitive.” On YPG, the spontaneous mutants grew very poorly at all temperatures and were labeled as “PG” or “poor grower.” They grew even more poorly at 37°C and were thus also labeled as TS (Table 3).

The temperature sensitivity of strains created by directed, random mutagenesis is shown in Table 3. Two observations can be made from these data. First, on lactate, only one strain (5R43) is temperature sensitive and two (5R25, 5R1) are slightly tempera-

ture sensitive. Most of the strains look very similar to wild type on YPL plates. On the surface, these results would seem to contradict those obtained from the growth curves in lactate medium. There are two explanations for this apparent contradiction. First, the media for the growth curves and plate assays are different. For the growth curves, a synthetic lactate medium, which contains no peptone and little yeast extract, is used. For the plate assays, both peptone and yeast extract are found in the medium. The liquid synthetic media is, thus, a much more stringent environment. Second, because the culture density is measured, the growth curves are a more quantitative assay. Differences between strains can be observed more clearly in the growth curves than in the plate assay. The plate assays are apparently crude enough that differences in growth cannot be observed until strain growth is below 40–50% of wild type.

The second, rather striking, observation that can be made from the data presented in Table 3 is that almost all of the strains grow less well on glycerol than the wild-type strain, even though their growth on lactate is often indistinguishable from that of wild type in the same assay. These data provide evidence that mutagenesis of His26 has a more significant effect on the metabolism of glycerol than on the metabolism of lactate.

Discussion

Previously, we observed that mutant yeast strains expressing the RepA4 protein have a strong tendency to produce a spontaneous mutation of an asparagine for a lysine in one of the replacement loop residues (J.S. Fetrow, T.S. Cardillo, & F. Sherman, unpubl. obs.). Superposition of the RepA4 loop, which comes from *P. aeruginosa* cytochrome *c*551, onto the native yeast loop shows that the mutable lysine is structurally analogous to His26 (Fig. 2). Furthermore, His26 is highly conserved among eukaryotic cytochromes *c* (Table 1). Thus, site-directed and random-directed mutagenesis were used to identify the importance of His26 to the in vivo structure and function of yeast iso-1-cytochrome *c*. The data show that mutations at this residue decrease both the amount and function of holo-cytochrome *c* (Table 3). The data further suggest that, while both lactate and glycerol metabolism are affected by His26 mutations, glycerol metabolism is more severely affected.

Causes for the decrease in intracellular cytochrome *c*

In the strains containing single mutations at His26, the amount of intracellular holo-cytochrome *c* decreases to 34–84%. What causes the decrease in the amount of intracellular protein? The most obvious explanation is that the decrease is caused by an increase in intracellular proteolysis, which itself could be caused by a general decrease in protein stability. The T_m of iso-1-cytochrome *c* is 53°C (Cohen & Pielak, 1994; Fetrow et al., 1997). The T_m of H26V from yeast iso-1-cytochrome *c* is about 47°C when determined by far-UV circular dichroism spectroscopy (D.J. Wiland & J.S. Fetrow, unpubl. obs.). At first glance, it would seem unlikely that such a small decrease in the T_m could cause a significant increase in intracellular proteolysis at 30°C.

Our work on other iso-1-cytochrome *c* loop variants shows that a 5° drop in the T_m can cause a decrease of 20% in the amount of intracellular protein in strains grown at 30°C (Fetrow et al., 1997). Analysis of single site mutations at the adjacent lysine 27 shows that a T_m of 37°C (16°C less than wild type) is found in several proteins with only 50% of the normal cytochrome *c* levels (Das

et al., 1988; Hickey et al., 1988), although the T_m might not be directly correlated because these proteins were carboxymethylated at cysteine 102 so that they could be studied *in vitro*. These data suggest that the decreased stability of H26V could cause some increase in intracellular proteolysis, resulting in a decrease in the amount of observed protein. On the other hand, there is not always a direct correlation between the amount of intracellular iso-1-cytochrome *c* and the protein's T_m . Other work on loop replacements shows that a T_m of 38–39 °C (15 °C less than wild type) can yield protein levels of 53 to 71% (Fetrow et al., 1997).

If a decrease in protein stability causes the decrease in intracellular cytochrome *c* levels in the 5R strains, one would expect this effect to be exacerbated at higher temperatures. However, the data presented in Table 3 show that none of the strains is particularly temperature sensitive, except those expressing H26L, H26C, or H26G. Cysteine might interfere by virtue of its ability to form disulfide bonds, and thus, cause dimerization of the protein. Glycine, by virtue of its lack of a β -carbon, could destabilize the protein and make the backbone more susceptible to proteolysis. The other mutant strains, however, do not appear to be temperature sensitive. Thus, thermal stability does not appear to be the direct cause of the decrease in cytochrome *c* levels.

Other factors could play a role. For instance, the protein levels could decrease if the protein folded more slowly than wild type, making the polypeptide chain more susceptible to proteolysis during folding. However, a non-native histidine ligand can bind the heme iron during cytochrome *c* folding, thus slowing the folding of the wild-type protein (Elöve et al., 1994). Recent work on the related H33N;H39K variant of iso-2-cytochrome *c* shows that replacement of these histidines actually increases the folding rate (Pierce & Nall, 1997). Thus, it is unlikely that replacement of His26 would slow folding.

One explanation that is compatible with, but not proven by, the current data is that the removal of the loop-loop and loop-protein hydrogen bonds (Fig. 1) causes the loop to become more mobile, possibly resulting in an increase in intracellular proteolysis. The resulting motion could be hinge-like, with the loop remaining as a stable structure, or the motion could be the result of a general increase in the "floppiness" of the loop. Hydrogen exchange studies at low denaturant concentrations suggest that loop A is a stable substructure (Bai et al., 1995; Bai & Englander, 1996); thus, the mobility increase would most likely be a hinge motion of the complete substructure, unless the His26-to-Asn31 hydrogen was crucial for the internal loop structure. To probe these possibilities further, we plan to perform hydrogen exchange and NMR relaxation studies on one or more of the variant proteins presented in this study.

Structure and function correlations

Does the decrease in mitochondrial cytochrome *c* account for the decreased function *in vivo*? A plot of the relative function versus the relative amount of holo-cytochrome *c* *in vivo* shows that the decrease in intracellular cytochrome *c* does not adequately account for the decreased function (Fig. 5). Other researchers have shown that the growth of strains expressing wild-type cytochrome *c* at 50% of the normal level are virtually indistinguishable from strains expressing wild-type strains (the X in Fig. 5) (Schweingruber et al., 1979; Ernst et al., 1985; Fetrow et al., 1989). Thus, the decrease in intracellular cytochrome *c* is not the direct cause of the decrease in function; therefore, mutations at His26 must also disrupt the function of iso-1-cytochrome *c*.

Fig. 5. A plot of relative amount of intracellular, holo-cytochrome *c* vs. the level of growth on lactate medium for the 5R strains, a spontaneous mutation strain (RepA4-SM), and the control strains. Data points are labeled with the one-letter code of the amino acid found at position 26. The X indicates the amount of function in lactate medium of strains producing wild-type iso-1-cytochrome *c*, but only producing it at 50% of the wild-type level (Schweingruber et al., 1979; Ernst et al., 1985; Fetrow et al., 1989). Data are taken from Table 3.

This decrease in the function could be caused by a disruption in the binding of cytochrome *c* to its redox partners or by interference with the electron transfer process itself. Several studies have shown that the adjacent Lys27 is protected from modification when cytochrome *c* is bound to its redox partners, particularly to cytochrome *c* reductase (reviewed in Pettigrew & Moore, 1987). However, several changes at Lys27 showed no effect on the kinetics of iso-1-cytochrome *c* oxidation or reduction by cytochrome *c* peroxidase, cytochrome *b*₅, cytochrome *b*₂, or cytochrome *c* oxidase (Das et al., 1988), suggesting that mutations at Lys27 (and possibly the adjacent His26) should not have much effect on electron transfer itself.

Alternatively, the His26 mutations could have loosened the heme pocket, causing a change in the redox potential and a decrease in the rate of electron transfer. *In vitro* studies on the His26 variant of rat cytochrome *c* suggest that the heme is more accessible to water in this variant protein (Qin et al., 1995). However, replacement of loop A with the homologous loop from cytochrome *c*2 caused only a 19 mV drop in the redox potential (Murphy et al., 1993). It is unlikely that a single site mutation that is not adjacent to the heme could have as significant an effect as replacement of an entire loop. Thus, a change in redox potential is not a likely cause of the decrease in function.

The temperature sensitivity data in Table 3 suggest that the growth of yeast strains containing His26 variants is worse on glycerol than on lactate. These results would suggest that the interaction of cytochrome *c* with cytochrome *c* reductase is disrupted more in the 5R strains than the interaction of cytochrome *c* with cytochrome *c* oxidase. If the mutations had affected the interaction with the oxidase or cytochrome *c* peroxidase, then growth on both glycerol and lactate would be affected similarly because all electrons that are passed to cytochrome must ultimately be passed to cytochrome *c* oxidase or peroxidase (Pettigrew & Moore, 1987). Electrons generated by metabolism of glycerol must pass through cytochrome *c* reductase; whereas, a portion of the electrons produced by metabolism of lactate are passed to cytochrome *c* via cytochrome *b*₂ (lactate dehydrogenase).

Taken together, our data suggest that mutation of His26 affects the interaction between cytochrome *c* and its redox partners, cytochrome *c* reductase in particular. As mentioned above, modification studies implicate Lys27 in reductase binding (Pettigrew & Moore, 1987). These data are also consistent with a model where the loss of His26 hydrogen bonds in the single-site variants causes an increase in the motion of loop A, producing interference with the binding of cytochrome *c* to cytochrome *c* reductase, with less interference in binding to lactate dehydrogenase and cytochrome *c* oxidase.

This interpretation is also consistent with the results obtained on the RepA4 loop replacement variants (Table 3). RepA4 is non-functional, but still expresses 46% of the normal intracellular level of holo-cytochrome *c* (Fetrow et al., 1997). RepA4 strains that produce a minimally functional cytochrome *c* (C71, C72, C73, 5R3-SM) contain less folded protein (Table 3). Clearly, the minimal functionality of the cytochromes *c* found in these strains is not caused by an increase in the protein levels. In strains C72 and C73, replacement of Asn or His for Lys in the loop at the position analogous to His26 could allow the appropriate hydrogen bonds to form (compare the structures of Asn and His side chains in Fig. 1C), thus producing a slightly more functional cytochrome *c*. Recent work on a His26 variant of rat cytochrome *c* has led to the hypothesis that replacement of His26 causes an increase in the mobility of parts of loop A and C (Qin et al., 1995). In accord with this interpretation, we suggest that His26 replacement increases the mobility of loop A, thus interfering with redox partner, particularly reductase, binding.

Conclusions

Mutations at His26 in iso-1-cytochrome *c* lead to decreased intracellular cytochrome *c* levels. Furthermore, His26 mutations lead to a significant decrease in the cytochrome *c* function in vivo. The amount of folded cytochrome *c* does not correlate with the level of function of the mutant proteins in vivo. The data are in accord with the proposal that mutations at position 26, with the concomitant loss of a loop-protein and a loop-loop hydrogen bond, increase the mobility of the loop (Qin et al., 1995). This, in turn, decreases redox partner binding. Our data suggest that binding to cytochrome *c* reductase is affected more by these mutations than binding to cytochrome *c* oxidase or to lactate dehydrogenase.

Materials and methods

Yeast strains and nomenclature

All yeast strains were derived from strain B7684 whose genotype is *MATa*, *cyc1-814::URA3*, *cyc7-68::CYH2*, *his3-ΔE1*, *ura3-52*, *leu2-3*, 112, *trp1-289*, *can1-100*, *cyh2*. Yeast strains used in this study were derived from this strain by one of two procedures: site-directed mutagenesis and random, directed mutagenesis (see below). Strains produced by site-directed mutagenesis are designated by a "C" followed by the strain number and they are of the same genotype as B7684, except that the mutagenesis protocol causes an insertion of *LEU2* upstream of *CYC1* and the replacement of *URA3* by the exact mutation at the *CYC1* locus, as previously described (Mulligan-Pullyblank et al., 1996). C93 is the standard, "wild-type" yeast strain used in these studies and other yeast strains used in this study are isogenic, except at the *CYC1*

locus. Diploid strains are denoted by a small d following the strain number, as C93d.

Strains produced by directed, random mutagenesis are designated as 5R strains followed by the strain number, as for example 5R1. These strains are derived from strain C14, which is ultimately derived from B7684, as described below. In the course of directed, random mutagenesis, several "spontaneous mutation" strains were obtained (see Results). These strains were identified only after sequencing and had already been assigned 5R strain numbers. To differentiate these strains from the ones that had incorporated the randomized oligonucleotide, an "-SM" (spontaneous mutation) designation was placed after the strain name, as in 5R3-SM. The 5R strains are all isogenic with C14, except at the *CYC1* locus. All strains, both C-strains and 5R-strains, are lacking the iso-2-cytochrome *c* isozyme.

Variant proteins are named by the wild-type amino acid name (in one-letter code) followed by the residue number followed by the variant amino acid; thus, H26A contains an alanine for histidine substitution at residue 26. Double or triple amino acid replacements are denoted as single mutations separated by a semi-colon, as in H26Q;K27N. Proteins with mutations in the *P. aeruginosa* loop A (derived from RepA4) contain the RepA4 designation in the name and residues in the loop are numbered consecutively from His18, the first loop residue. (Loop residue numbering in RepA4 is specified in Table 2.)

The standard eukaryotic sequence alignment for numbering yeast cytochrome *c* sequences is used; thus, yeast cytochrome *c* extends from threonine -5 to glutamic acid 103. All but five of the cytochromes *c* used in this study, including the "wild-type" protein found in strain C93, contain threonine rather than a cysteine at position 102 and are thus C102T, though this is not explicitly stated in the protein name. This amino acid substitution makes in vitro analysis easier by preventing dimerization of the protein; the C102T protein has been shown to exhibit characteristics similar to those of the wild-type protein (Cutler et al., 1987). The five proteins with a cysteine at position 102 are marked in Table 2 and the protein name explicitly defines them as being C102. C6 is the yeast strain producing wild-type iso-1-cytochrome *c* with a cysteine at position 102.

Site-directed mutagenesis of iso-1-cytochrome *c*

To assess accurately the effect of the His26 mutations on the structure and function of the protein in vivo, a single copy of the mutant *cyc1* gene must be inserted into the yeast chromosome at the *CYC1* locus. This mutant gene is then under the normal metabolic regulatory mechanisms and the results obtained from in vivo studies are more consistent than those obtained by expressing the mutation on a plasmid (J.S. Fetrow, unpubl. obs.). To create the C-strains used in this study, a previously described mutagenesis and integration protocol was used (Mulligan-Pullyblank et al., 1996). Briefly, a plasmid encoding iso-1-cytochrome *c* (Fetrow et al., 1989) was subject to standard mutagenesis protocols (Kunkel et al., 1987). Mutagenized plasmids were cut with *MluI* and the small fragment was ligated into plasmid pYC31 (Mulligan-Pullyblank et al., 1996). Plasmids were then cut with *BamHI* and the digestion mix was transformed into B7684 (Mulligan-Pullyblank et al., 1996). B7684 is *leu*⁻ and *URA*⁺. When the *BamHI* fragment containing the mutagenized *cyc1* gene is properly integrated into the yeast chromosome, it becomes *LEU*⁺ and *ura*⁻. Strains containing the mutant *cyc1* gene are subcloned and checked on

selective media plates. The *CYC1* locus of each strain was completely sequenced at least twice using the dye-deoxy sequencing protocol (Applied Biosystems) on a PCR amplified product, as previously described (Mulligan-Pullyblank et al., 1996).

Random, directed mutagenesis of His26 in iso-1-cytochrome c

To replace His26 with all possible amino acids that produce a functional cytochrome *c* in a single experiment, random-directed mutagenesis (Fumo et al., 1995) of this residue was performed. The starting strain for this procedure is C14 (Table 2), a strain that was produced by site-directed mutagenesis (see above) and that expresses a loop A replacement cytochrome *c* that folds, but is nonfunctional (Fetrow et al., 1997). This strain is transformed by standard lithium acetate procedures (Ito et al., 1983) with an oligonucleotide that encodes the wild-type yeast loop A, but is randomized at the codon for His26:

5' CGT CTA CAA TGT CAC ACA GTG GAA AAA
GGC GGT CCC NNN AAA GTA GGT CCA AAC
TTG CAT GGT ATC 3'.

Underlined bases indicate changes from the wild-type loop sequence. This oligonucleotide was synthesized on an Applied Biosystems DNA Model 373A synthesizer. For randomization of all three bases in the His26 codon, equal quantities of all four nucleotides were combined in the reaction mix.

Yeast cells transformed with 60 μ g of this oligonucleotide were plated on YPD (1% yeast extract, 2% peptone, 2% dextrose) media and grown at 30 °C for 4–6 days. After two days, cells containing a functional cytochrome *c* grow as larger colonies over the background lawn of cells containing a nonfunctional cytochrome *c*. These colonies are picked, subcloned on YPG (1% yeast extract, 2% peptone, 3% glycerol) plates, and checked for phenotype by plating on other selective media plates. The *CYC1* locus of each strain was completely sequenced at least twice using the dye-deoxy sequencing protocol (Applied Biosystems) on a PCR-amplified product, as described above.

Growth curves in lactate medium

To test the level of function of the mutant cytochromes *c* within the cell, yeast strains were grown at 30 °C in synthetic liquid lactate medium. A functional cytochrome *c* is required for growth in this nonfermentable medium and the amount of growth is a measure of the level of functionality of the cytochromes *c* in isogenic strains; thus, the cell growth was observed by monitoring culture turbidity over a period of 30 h. To counteract possible interference from any background mutations in other genes (Schweingruber et al., 1977), haploid yeast were first made diploid by crossing with strain B2111, which contains a *CYC1* deletion (Fetrow et al., 1989). Growth curves were performed as previously described (Mulligan-Pullyblank et al., 1996). The percentage of growth for each mutant strain was determined by comparison to the growth of strains C93 (wild type) and C15 (*cyc1* deletion) at 30 h ($(\text{Density}_{\text{mutant}} - \text{Density}_{\text{C15}}) / (\text{Density}_{\text{C93}} - \text{Density}_{\text{C15}})$). Repeated experiments have shown the error to be approximately 3–10%. Reported results are the average of at least three experiments.

Whole cell low temperature difference spectroscopy

To determine the amount of cytochrome *c* present in whole yeast cells, low temperature difference spectroscopy was performed (Sherman & Slonimski, 1964; Cottrell et al., 1975). We have not been successful in obtaining consistent results using a paste of cells and normalizing the quantity of cytochrome *c* by measuring the ratio of cytochrome *c* to cytochrome *b* (Linske-O'Connell et al., 1995); therefore, we quantitate the number of cells in the cuvette prior to spectroscopic measurements and perform the experiment as previously described (Mulligan-Pullyblank et al., 1996). Briefly, the culture density is determined and a known quantity of cells is divided into two portions. One aliquot is oxidized with potassium ferricyanide and the other is reduced with dithionite. Saturated sucrose is added to each and the cells are frozen in liquid nitrogen. A difference, visible absorption spectrum from 500 to 700 nm is then collected. The absorbance at 548 nm, which is proportional to the amount of holo-cytochrome *c* present in the cell, is recorded. The relative amount of protein in the cell was determined by comparison to the amount of protein in an equal quantity of cells of strains C93 and C15 ($(\text{Absorbance}_{\text{mutant}} - \text{Absorbance}_{\text{C15}}) / (\text{Absorbance}_{\text{C93}} - \text{Absorbance}_{\text{C15}})$). Results from repeated experiments show that the error in these measurements is 5–12%. Reported results are the average from at least three experiments.

Temperature sensitivity analysis

Yeast strains were grown in 1 mL liquid YPD medium overnight. Seventy microliters of the culture were placed in an Eppendorf tube and centrifuged. Culture supernatant was removed, the cells resuspended in 180 μ L sterile distilled water and centrifuged again. The cell pellet was washed twice with sterile distilled water and finally resuspended in 1 mL sterile water. Five microliters of this suspension were plated on three plates: YPD, YPG, and YPL (1% yeast extract, 2% peptone, 1% lactate). One of each plate was then grown at room temperature, at 30 °C, and at 37 °C. In our laboratory, room temperature is approximately 25 °C. The relative growth was recorded for three days following plating. Growth was scored on a scale of 0–3, with the wild-type strain (C93) being scored as 3 and the cytochrome *c* deletion strain (C15) being scored as 0. Numerical scores were then used to establish the classifications described in Table 3.

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References

- Allemann RK, Presnell SR, Benner SA. 1991. A hybrid of bovine pancreatic ribonuclease and human angiogenin: An external loop as a module controlling substrate specificity. *Protein Eng* 4:831–835.
- Auld DS, Pielak GJ. 1991. Constraints on amino acid substitutions in the N-terminal helix of cytochrome *c* explored by random mutagenesis. *Biochemistry* 30:8684–8690.
- Bai Y, Englander SW. 1996. Future directions in folding: The multi-state nature of protein structure. *Proteins Struct Funct Genet* 24:145–151.
- Bai Y, Sosnick TR, Mayne L, Englander SW. 1995. Protein folding intermediates: Native-state hydrogen exchange. *Science* 269:192–197.
- Baistrocchi P, Banci L, Vertini I, Turano P, Bren KL, Gray HB. 1996. Three-dimensional solution structure of *Saccharomyces cerevisiae* reduced iso-1-cytochrome *c*. *Biochemistry* 35:13788–13796.

- Banci L, Bertini I, Bren KL, Gray HB, Sompornpisut P, Turano P. 1997. Solution structure of oxidized *Saccharomyces cerevisiae* iso-1-cytochrome *c*. *Biochemistry* 36:8992–9001.
- Berghuis AM, Brayer GD. 1992. Oxidation state dependent conformational changes in cytochrome *c*. *J Mol Biol* 223:959–976.
- Berghuis AM, Guillemette JG, Smith M, Brayer GD. 1994. Mutation of tyrosine-67 to phenylalanine in cytochrome *c* significantly alters the local heme environment. *J Mol Biol* 235:1326–1341.
- Bushnell GW, Louie GV, Brayer GD. 1990. High resolution three-dimensional structure of horse heart cytochrome *c*. *J Mol Biol* 214:585–595.
- Chou K-C, Maggiora GM, Scheraga HA. 1992. Role of loop-helix interactions in stabilizing four-helix bundle proteins. *Proc Natl Acad Sci USA* 89:7315–7319.
- Cohen DS, Pielak GJ. 1994. Stability of yeast iso-1-ferricytochrome *c* as a function of pH and temperature. *Protein Sci* 3:1253–1260.
- Cottrell SF, Rabinowitz M, Getz GS. 1975. Cytochrome synthesis in synchronous cultures of the yeast, *Saccharomyces cerevisiae*. *J Biol Chem* 250:4087–4094.
- Cutler RL, Pielak GJ, Mauk AG, Smith M. 1987. Replacement of cysteine-107 of *Saccharomyces cerevisiae* iso-1-cytochrome *c* with threonine: Improved stability of the mutant protein. *Protein Eng* 1:95–99.
- Das G, Hickey DR, Principio L, Conklin KT, Short J, Miller JR, McLendon G, Sherman F. 1988. Replacements of lysine 32 in yeast cytochrome *c*: Effects on the binding and reactivity with physiological partners. *J Biol Chem* 263:18290–18297.
- DiBella EE, Scheraga HA. 1996. The role of the insertion loop and tryptophan 148 in the activity of thrombin. *Biochemistry* 35:4427–4433.
- ElHawrani AS, Foreton KM, Sessions RB, Clarke AR, Holbrook JJ. 1994. Engineering surface loops of proteins—A preferred strategy for obtaining new enzyme function. *Trends Biotechnol* 12:207–211.
- Elliott WB, Margoliash E. 1971. High resolution low temperature spectroscopy of cytochrome *c*. In: Grove EL, Perkins AJ, eds. *Developments in applied spectroscopy*. New York: Plenum Press. pp 125–140.
- Elöve GA, Bhuyan AK, Roder H. 1994. Kinetic mechanism of cytochrome *c* folding: Involvement of the heme and its ligands. *Biochemistry* 33:6925–6935.
- Ernst JF, Hampsey DM, Stewart JW, Rackovsky S, Goldstein D, Sherman F. 1985. Substitutions of proline 76 in yeast iso-1-cytochrome *c*: Analysis of residues compatible and incompatible with folding requirements. *J Biol Chem* 260:13225–13236.
- Falzone CJ, Wright PE, Benkovic SJ. 1994. Dynamics of a flexible loop in dihydrofolate reductase from *Escherichia coli* and its implication for catalysis. *Biochemistry* 33:439–442.
- Fetrow JS. 1995. Omega loops: Nonregular secondary structures significant in protein function and stability. *FASEB J* 9:708–717.
- Fetrow JS, Cardillo TS, Sherman F. 1989. Deletions and replacements of omega loops in yeast iso-1-cytochrome *c*. *Protein Struct Funct Genet* 6:372–381.
- Fetrow JS, Horner SR, Oehrl W, Schaak DL, Boose TL, Burton RE. 1997. Analysis of the structure and stability of loop A replacements in yeast iso-1-cytochrome *c*. *Protein Sci* 6:195–208.
- First EA, Fersht AR. 1993. Mutational and kinetic analysis of a mobile loop in tyrosyl-tRNA synthetase. *Biochemistry* 32:13658–13663.
- Fumo G, Spitzer JS, Fetrow JS. 1995. A method of directed random mutagenesis of the yeast chromosome shows that iso-1-cytochrome *c* heme ligand His18 is essential. *Gene* 164:33–39.
- Gekko K, Yamagami K, Kunori Y, Ichihara S, Kodama M, Iwakura M. 1993. Effects of point mutation in a flexible loop on the stability and enzymatic function of *Escherichia coli* dihydrofolate reductase. *J Biochem (Tokyo)* 113:74–80.
- Hampsey DM, Das G, Sherman F. 1986. Amino acid replacements in yeast iso-1-cytochrome *c*. *J Biol Chem* 261:3259–3271.
- Hardy F, Vriend G, van der Vinne B, Frigerio F, Grandi G, Venema G, Eijssink VGH. 1994. The effect of engineering surface loops on the thermal stability of *Bacillus subtilis* neutral protease. *Protein Eng* 7:425–430.
- Hedstrom L, Szilagyi L, Rutter W. 1992. Converting trypsin to chymotrypsin: The role of surface loops. *Science* 255:1249–1253.
- Helms L, Wetzel R. 1995. Destabilizing loop swaps in the CDRs of an immunoglobulin V_H domain. *Protein Sci* 4:2073–2081.
- Hickey DR, McLendon G, Sherman F. 1988. Thermodynamic stabilities of yeast iso-1-cytochromes *c* having amino acid substitutions for lysine 32. *J Biol Chem* 263:18298–18305.
- Ito H, Fukuda Y, Murata K, Kimura A. 1983. Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* 153:163–168.
- Jones PT, Dear PH, Foote J, Neuberger MS, Winter G. 1986. Replacing the complementarity-determining regions in human antibody with those from a mouse. *Nature* 321:522–525.
- Kunkel TA, Roberts JD, Zakour RA. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Meth Enzymol* 154:367–382.
- Langen R, Brayer GD, Berghuis AM, McLendon G, Sherman F, Warshel A. 1992. Effect of the Asn52 → Ile mutations on the redox potential of yeast cytochrome *c*. *J Mol Biol* 224:589–600.
- Leszczynski JSF. 1986. Loops: A novel class of protein secondary structure [PhD thesis]. The Pennsylvania State University College of Medicine.
- Li L, Falzone CJ, Wright PE, Benkovic SJ. 1992. Functional role of a mobile loop of *Escherichia coli* dihydrofolate reductase in transition-state stabilization. *Biochemistry* 31:7826–7833.
- Liang H, Petros AM, Meadows RP, Yoon HS, Egan DA, Walter K, Holzman TF, Robins T, Fesik SW. 1996. Solution structure of the DNA-binding domain of a human papillomavirus E2 protein: Evidence for flexible DNA-binding regions. *Biochemistry* 35:2095–2103.
- Linske-O'Connell LI, McLendon G, Sherman F. 1995. Site specific combinations of stabilizing and destabilizing amino acid replacements in yeast cytochrome *c*: *In vivo* and *in vitro* effects. *Biochemistry* 34:7103–7112.
- Lo TP, Murphy MEP, Guillemette JG, Smith M, Brayer GD. 1995. Replacements in a conserved leucine cluster in the hydrophobic heme pocket of cytochrome *c*. *Protein Sci* 4:198–208.
- Louie GV, Brayer GD. 1990. High-resolution refinement of yeast iso-1-cytochrome *c* and comparisons with other eukaryotic cytochromes *c*. *J Mol Biol* 214:527–555.
- Louie GV, Pielak GJ, Smith M, Brayer GD. 1988. Role of phenylalanine-82 in yeast iso-1-cytochrome *c* and remote conformational changes induced by a serine at this position. *Biochemistry* 27:7870–7876.
- Marmorino JL, Auld DS, Betz SF, Doyle DF, Young GB, Pielak GJ. 1993. Amide proton exchange rates of oxidized and reduced *Saccharomyces cerevisiae* iso-1-cytochrome *c*. *Protein Sci* 2:1966–1974.
- Moore GR, Pettigrew GW. 1990. *Cytochromes c: Evolutionary, structural and physicochemical aspects*. Berlin: Springer-Verlag.
- Mulligan-Pullyblank P, Spitzer JS, Gilden BM, Fetrow JS. 1996. Loop replacement and random mutagenesis of omega loop D, residues 70–84, in iso-1-cytochrome *c*. *J Biol Chem* 271:8633–8645.
- Murphy MEP, Fetrow JS, Burton RE, Brayer GD. 1993. The structure and function of omega loop A replacements in cytochrome *c*. *Protein Sci* 2:1429–1440.
- Murphy MEP, Nall BT, Brayer GD. 1992. Structure determination and analysis of yeast iso-2-cytochrome *c* and a composite mutant protein. *J Mol Biol* 227:160–176.
- Ochi H, Hata V, Tanaka N, Kakudo M, Sakurai T, Aihara S, Morita Y. 1983. Structure of rice ferricytochrome *c* at 2.0 Å resolution. *J Mol Biol* 166:407–418.
- Pan Q-W, Tanase S, Fukumoto Y, Nagashima F, Rhee S, Rogers PH, Arnone A, Morino Y. 1993. Functional roles of valine 37 and glycine 38 in the mobile loop of porcine cytosolic aspartate aminotransferase. *J Biochem* 268:24758–24765.
- Pettigrew GW, Moore GR. 1987. *Cytochromes c biological aspects*. Berlin: Springer-Verlag.
- Pielak GJ, Auld DS, Beasley JR, Betz SF, Cohen DS, Doyle DF, Finger SA, Fredericks ZL, Hilgen-Willis S, Saunders AJ, Trojak SK. 1995. Protein thermal denaturation, side-chain models, and evolution: Amino acid substitutions at a conserved helix-helix interface. *Biochemistry* 34:3268–3276.
- Pielak GJ, Mauk AG, Smith M. 1985. Site-directed mutagenesis of cytochrome *c* shows that an invariant Phe is not essential for function. *Nature* 313:152–154.
- Pierce MM, Nall BT. 1997. Fast folding of cytochrome *c*. *Protein Sci* 6:618–627.
- Pompliano DL, Peyman A, Knowles JR. 1990. Stabilization of a reaction intermediate as a catalytic device: Definition of the functional role of the flexible loop in triosephosphate isomerase. *Biochemistry* 29:3186–3194.
- Poole LB, Loveys DA, Hale SP, Gerlt JA, Stanczyk SM, Bolton PH. 1991. Deletion of the Ω-loop in the active site of *Staphylococcal nuclease*. I. Effect on catalysis and stability. *Biochemistry* 30:3621–3627.
- Qi PX, Beckman RA, Wand AJ. 1996. Solution structure of horse heart ferricytochrome *c* and detection of redox-related structural changes by high-resolution ¹H NMR. *Biochemistry* 35:12275–12286.
- Qi PX, DiStefano DL, Wand AJ. 1994. Solution structure of horse heart ferrocyanochrome *c* determined by high-resolution NMR and restrained simulated annealing. *Biochemistry* 33:6408–6417.
- Qin W, Sanishvili R, Plotkin B, Schejter A, Margoliash E. 1995. The role of histidines 26 and 33 in the structural stabilization of cytochrome *c*. *Biochim Biophys Acta* 1252:87–94.
- Raines RT, Toscano MP, Nierengarten DM, Ha JH, Auerbach R. 1995. Replacing a surface loop endows ribonuclease A with angiogenic activity. *J Biol Chem* 270:17180–17184.
- Scheerlinck J-PY, Lasters I, Claessens M, DeMaeyer M, Pio F, Delhaise P, Wodak SJ. 1992. Recurrent αβ loops structures in TIM barrel motifs show a distinct pattern of conserved structural features. *Protein Struct Funct Genet* 12:299–313.

- Schweingruber ME, Sherman F, Stewart JW. 1977. Altered absorption spectra of iso-1-cytochrome *c* from mutants of yeast. *J Biol Chem* 252:6577–6580.
- Schweingruber ME, Stewart JW, Sherman F. 1979. Primary site and second site revertants of missense mutants of the evolutionarily invariant tryptophan 64 in iso-1-cytochrome *c* from yeast. *J Biol Chem* 254:4132–4143.
- Sherman F, Slonimski PP. 1964. Respiration-deficient mutants of yeast. II: Biochemistry. *Biochim Biophys Acta* 90:1–15.
- Sherman F, Stewart JW, Parker JH, Inhaber E, Shipman NA, Putterman GJ, Gardisly RL, Margoliash E. 1968. The mutational alteration of the primary structure of yeast iso-1-cytochrome *c*. *J Biol Chem* 243:5446–5456.
- Shortle D, Sondek J. 1995. The emerging role for insertions and deletions in protein engineering. *Curr Opin Biotechnol* 6:382–393.
- Takano T, Dickerson RE. 1981. Conformation change of cytochrome *c*. II: Ferricytochrome *c* refinement at 1.8 Å and comparison with the ferrocyclochrome structure. *J Mol Biol* 153:95–115.
- Tanaka N, Yamane T, Tsukihara T, Ashida T, Kakudo M. 1975. The crystal structure of bonito (katsuo) ferrocyclochrome *c* at 2.3 Å resolution. II. Structure and function. *J Biochem (Tokyo)* 77:147–162.
- Tramontano A, Lesk AM. 1992. Common features of the conformations of antigen-binding loops in immunoglobulins and application to modeling loop conformations. *Proteins Struct Funct Genet* 13:231–245.
- Urfer R, Kirschner K. 1992. The importance of surface loops for stabilizing an eightfold $\beta\alpha$ barrel protein. *Protein Sci* 1:31–45.