Site Specific Combinations of Stabilizing and Destabilizing Amino Acid Replacements in Yeast Cytochrome *c*: *In Vivo* and *in Vitro* Effects

Lisa I. Linske-O'Connell,[†,§] Fred Sherman,[†] and George McLendon*[,†,‡]

Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, and Department of Chemistry, Princeton University, Princeton, New Jersey 08544

Received July 13, 1994; Revised Manuscript Received February 6, 1995[⊗]

ABSTRACT: Oligonucleotide-directed mutagenesis *in vivo* was used to create destabilizing site specific changes at position 6 in iso-1-cytochrome *c* in *Saccharomyces cerevisiae*. These changes were made in combination with the stabilizing N52I substitution. The resulting proteins showed that a variety of forces and interactions are responsible for these destabilizations. Increasing side chain size was the strongest predictor of decreases in cytochrome *c* levels *in vivo*. With intermediate size replacements, increasing hydrophobicity correlated with the proteins' thermostability. Some differences in protein levels *in vivo* could not be explained by side chain size and hydrophobicity alone. Therefore, specific interactions of individual amino acids may also be involved. The N52I-stabilizing mutation tended to increase the protein levels to the same degree relative to the amino acid at position 6. These stabilized cytochromes had an increased specific activity when compared to the series with the original N52. Strains with these altered cytochromes *c* showed temperature sensitivities for protein levels and function. Thermodynamic measurements *in vivo* of the WT (C102A), N52I, G6A, G6A N52I, and G6S N52I correlated with the *in vivo* data. The variant G6A N52I showed additivity (Wells, 1990) of the $C_{m's}$ and $\Delta\Delta G$'s of unfolding for guanidine hydrochloride denaturation.

The relationship between amino acid sequence and protein structure is not well understood. One way of investigating this relationship is to study the forces that stabilize or destabilize a protein. Since most single-amino acid changes have little effect on the structure and stability of a protein (Pakula & Sauer, 1989; Hampsey *et al.*, 1986, 1988), valuable information can be gained by systematically studying changes at structurally important sites. Combinations of stabilizing and destabilizing changes in a single protein can be used to investigate the effects and possible interactions.

Cytochrome *c* is well suited for these kinds of structural studies. It has a known crystal structure (Louie *et al.*, 1988; Louie & Brayer, 1989). There is also a wealth of phylogenic and mutational information (Hampsey *et al.*, 1986, 1988). The relationship between protein levels *in vivo* and protein stability *in vitro* has been established at least within a specific set of replacements at position 52 (L. Linske-O'Connell *et al.*, accompanying manuscript). Previous amino acid replacements in iso-1-cytochrome *c* from the yeast *Saccharomyces cerevisiae* have been shown to cause a wide range of protein stabiilty effects. The majority appears functionally neutral. Some substitutions adversely affect the function or structure of the protein. Several changes actually increase the thermodynamic stability (Das *et al.*, 1989; Hickey *et al.*, 1991a, 1992; L. Linske-O'Connell *et al.*, accompanying manuscript). This work involves changes at two sites (Figure 1). Alterations at position 6 (mammalian numbering system) cause a decrease in stability. The N52I replacement used in this work acts as a "global suppressor" of other inactivating missense mutants (*e.g.*, G6S) through an increase in thermostability. (Das *et al.*, 1989; Hickey *et al.*, 1991a, 1992).

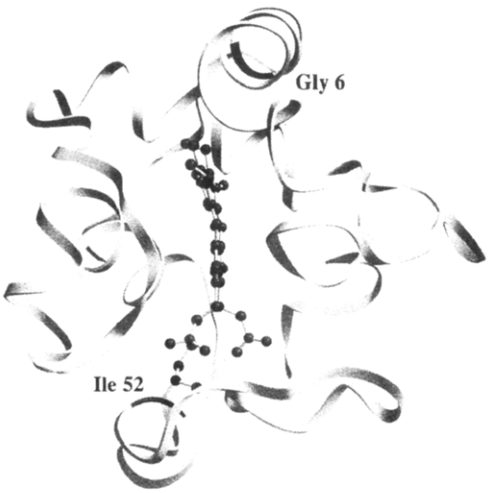

FIGURE 1: Secondary structure of cytochrome *c*. A ribbon drawing of the secondary structure and heme group of iso-1-cytochrome *c* is shown. The positions of G6 and N52I are darkened on the ribbon. The isoleucine side chain and the heme group are shown in a ball and stick configuration.

The evolutionarily conserved G6 is the fourth residue of the N-terminal helix in cytochrome *c*. It interacts with L94 in the C-terminal helix where the two intersect at a 90° angle (Louie *et al.*, 1988; Louie & Brayer, 1989). The formation and interaction of these two helices is one of the earliest detectable refolding events *in vitro* after guanidine hydrochloride denaturation (Roder *et al.*, 1988; Elove *et al.*, 1992). Earlier studies have shown that G6 substituions result in a marked decrease in cytochrome *c* levels (Hampsey *et al.*, 1986, 1988; Berroteran & Hampsey, 1991: Auld & Pielak,

[†] University of Rochester School of Medicine and Dentistry.
[‡] Princeton University.
[§] Current address: ABL, NCI-FCRDC, P.O. Box B, Frederick, MD 21702-1201.
[⊗] Abstract published in *Advance ACS Abstracts,* May 15, 1995.

0006-2960/95/0434-7103$09.00/0

1991). These destabilizing replacements of G6 are probably the result of structural distortions caused by side chains larger than the glycine proton (Hampsey *et al.*, 1986, 1988).

The N52I replacement was originally recovered as a "global suppressor" (Shortle & Lin, 1985) of G29S, H33P (Das *et al.*, 1989), and G6D (Berroteran & Hampsey, 1991). As a global suppressor, N52I was able to restore function to these nonfunctional variants by increasing the overall stability of the protein. X-ray crystallography has shown that G6 is far removed in three-dimensional space as well as in primary sequence. Thus, this site probably does not interact with position 52. In the native protein, asparagine 52 is involved in a hydrogen bond network in the hydrophobic interior that includes Y67, T78, a heme propionic group, and an interior water molecule. When N52I is introduced into a wild type amino acid sequence, there is a dramatic 17 °C thermostabilization (Das *et al.*, 1989; Hickey *et al.*, 1991a, 1992). Crystal structure analysis shows a loss of the interior water molecule and a change in the hydrogen-bonding network (Hickey *et al.*, 1991a, 1992). This stabilization reflects the hydrophobicity of the residue at this site (L. Linske-O'Connell *et al.*, accompanying manuscript).

To facilitate future studies of the isolated proteins, a C102A mutation has been made in iso-1-cytochrome *c*. In the wild type protein, this C-terminal penultimate amino acid is the only free cysteine residue. It has been shown to cause dimerization of the protein at increased concentrations. Studies of the C102A and C102S variants showed that they were about as stable as the normal cytochrome *c* (Hickey *et al.*, 1991a, 1992). Unless specifically noted, all strains discussed contain this replacement.

Direct transformation of yeast with oligonucleotides is a convenient way to recover functional mutants of cytochrome *c* (Moerschell *et al.*, 1988, 1991). Two series of position 6-destabilizing mutants of iso-1-cytochrome *c*, with and without the N52I-stabilizing mutation, were made using synthetic oligonucleotides transformed directly into the yeast *S. cerevisiae* (Figure 2). With this method, functional variants are selected from the 20 amino acid replacements possible at a selected site in the protein.

EXPERIMENTAL PROCEDURES

In a previous study (Moerschell *et al.*, 1990), iso-1-cytochromes *c* with all 20 amino acid replacements at a specific site were generated with 20 different oligonucleotides. This study uses oligonucleotides with mixed codons at the site of interest to generate functional variants of cytochrome *c* (Figure 2, Table 1). Transforming yeast directly with synthetic oligonucleotides can result in the recovery of amino acid replacements at a selected locus in cytochrome *c*. This procedure produces isogenic strains with a single copy of the altered cytochrome *c* at the correct chromosomal position. Therefore, these altered cytochromes *c* are under the same regulation as the wild type cytochrome *c*. This allows accurate quantification *in vivo* of the functions and protein levels of these mutant proteins.

Strain Construction. Two yeast strains were derived from B-7528 (*MAT***a** *cyc1-31 cyc7-67 ura3-52 lys5-10*; Moerschell *et al.*, 1988). Strain B-8163 was made with the allele *cyc1-1031* allele (Figure 2A). The changes were made by site-directed mutagenesis (Kunkel *et al.*, 1987) of plasmid pAB458. The nonfunctional genes were substituted at the *CYC1* locus as previously described (Fetrow *et al.*, 1989). These two strains contained a low-reversion frame shift and stop lesion at position 6. To facilitate the future study of isolated proteins, both strains had the free cysteine at position 102 changed to an alanine. (The C102A replacement is analogous to the residue found in iso-2-cytochrome *c* at that position.) The *cyc1-1027* allele contained the wild type N52. The N52I-stabilizing mutation was made in *cyc1-1031*. As a additional control, B-8176, a strain with *cyc1-1154* (a frame shift and stop mutation at position 6 with wild type N52 C102), was constructed.

Oligonucleotide-Directed Transformation. The method of Yamamoto *et al.* (1992) was used for transformation. A series of four degenerate oligonucleotides (Figure 2B) was constructed to correct the frame shift and stop while inserting all 20 amino acids at codon 6 in B-8163 and B-8291. The strains containing the frame shift and stop lesions were transformed with oligonucleotides listed in Figure 2. The transformations were done at 30 °C using 100 μg of oligonucleotide with $\sim 5 \times 10^6$ cells. After 4 days, random transformants were picked and streaked for single colonies. The strains were put into groups defined by cytochrome *c* levels *in vivo* as determined by visual low-temperature spectra (Hickey *et al.*, 1991b) and growth characteristics on nonfermentable carbon sources. DNA from representative strains in each group was amplified by PCR at the CYC1 locus. The 5′ region of the gene was sequenced. Oligonucleotides encoding position 6 amino acids not recovered in the first experiment were made. Transformations were incubated at 30 °C for a longer period to recover slower growing strains. To select for thermolabile mutants of cytochrome *c*, these transformations were also incubated at room temperature. The resulting strains were characterized and grouped as above. Table 1 lists the position 6 amino acid replacements with their codons, strain numbers, and allele numbers. Multiple isolates of the same variant cytochromes *c* had visual spectral levels within experimental error. This strategy was able to recover cytochromes *c* with less than 1% of normal levels. Strains with complete deficiencies in cytochrome *c* are not recovered.

Protein Levels in Vivo. A low-temperature (−196 °C) scanning spectrophotometer was used to measure the relative absorbance of cytochromes *c* and *b* in a constant amount of cells in each of the strains. The method used was as previously described (Hickey *et al.*, 1991b; accompanying paper) with the following exceptions. Haploid strains were grown on a medium containing 1% sucrose for 4 days. The cytochrome *c* peak was at 546 mμ for the N52 mutants and 548 mμ for the mutants containing N52I. Cytochrome *b* (558 mμ) is very consistent from strain to strain. It was used as an internal standard. A cytochrome *c*/*b* ratio was taken. This ratio was normalized to that of control strains containing the C102A cytochrome *c*. The *c*/*b* ratios of mother strains B-8163 and B-8291 (devoid of cytochrome *c* due to the frame shift stop lesion) were subtracted from each series of mutants. The strain with the normal G6 N52 C102A was set at 100%. This way a direct measurement of the cellular cytochrome *c* can be done in intact cells. Very low levels fo cytochrome *c* were detectable. Small changes in the cellular levels matched changes in the temperature sensitive growth pattern. The relative levels of the visually estimated cytochromes *c* matched those measured with the scanning spectrometer (unpublished results).

A

```
 -3          1          5                        10                   52   102
-Phe-Lys-Ala-Gly-Ser-Ala-Lys-Lys-Gly-Ala-Thr-Leu-Phe-Lys-Thr-Arg-Cys -Asn- -Cys-
 TTC AAG GCC GGT TCT GCT AAG AAA GGT GCT ACA CTT TTC AAG ACT AGA TGT  AAT   TGT  CYC1

                                                                   -Asn- -Ala-
 TTC AAG GCC GGT TCT GCT AAG TAA ΔGT GCT ACA CTT TTC AAG ACT AGA TGT  AAT   GCT  cyc1-1027

                                                                   -Ile- -Ala-
 TTC AAG GCC GGT TCT GCT AAG TAA ΔGT GCT ACA CTT TTC AAG ACT AGA TGT  ATT   GCT  cyc1-1031

     AAG GCC GGT TCT GCT AAG AAA NNN GCT ACA CTT TTC AAG ACT AGA              Oligonucleotide
```

Codon 6

B

Oligonucleotide	*Codon 6*	*Possible Amino Acids*	*Recovered Amino Acids*	
			cyc1-1027	*cyc1-1031*
Experiment 1, 30°C				
OL90.3112	**NWN**	V, D, N, L, I, Q, E, H, M, F, K, Y	V	V, D, N,L, I, Q, E, F K, Y
OL90.1101	**HGR**	W,R	None	W, R
OL90.1110	**VCN**	A, T, P	A	A, T, P
OL90.2111	**DGY**	G, S, C	G, S	G, S,C
Experiment 2, 30°C				
OL90.194	CAC	H	None	H
OL90.195	ATG	M	None	M
OL90.196	TGT	C	None	
OL90.197	**HWN**	N, L, I, Q, H, M, F, K, Y	I, Q	
OL90.198	GAN	D, E	None	
OL90.199	**MCN**	T, P	T	
Experiment 3, 22°C				
OL90.194	CAC	H	H	
OL90.195	ATG	M	M	
OL90.196	TGT	C	C	
OL90.197	**HWN**	N, L, I, Q, H, M, F, K, Y	N, I, M, Y	
OL90.198	GAN	D, E	D, E	
OL90.1101	**HGR**	W, R	None	

FIGURE 2: Site specific oligonucleotide-directed mutagenesis *in vivo*. (A) The amino acid and nucleotide sequences of the normal 5′ region and amino acids at positions 52 and 102 of the normal *CYC1* gene. The corresponding nucleotide sequences of the *cyc1-1027* and *cyc1-1031* alleles and the general sequence of the synthetic oligonucleotides are presented below the normal sequence. (B) The oligonucleotides used to encode all 20 amino acids at position 6 of iso-1-cytochrome *c*. The mixed oligonucleotide sequences used at position 6 are shown. The amino acids encoded by each oligonucleotide are represented by their one letter code (N = G, A, T, or C; M = A or C; R = A or G; W = A or T; Y = C or T; V = A, C, or G; H = A, C, or T; D = A, G, or T). The amino acids recovered in each of the three experiments are listed.

Temperature Sensitive Protein Levels in Vivo. Strains were grown for 4 days on 1% sucrose media at 30 and 37 °C. Strains grown at room temperature were left for 5 days to insure complete derepression at this suboptimal temperature. In a "blind" study, the relative percent cytochrome *c* was visually estimated by low-temperature spectroscopy.

Temperature Sensitive Growth. The haploid strains were plated on a medium containing glycerol as the nonfermentable carbon source. Growth on glycerol is dependent on about 1% functioning cytochrome *c*. The plates were incubated at the standard temperature of 30 °C for 4 days. Thermostability of the mutant cytochromes *c* was tested at an elevated 37 °C. Strains grown at the suboptimal room temperature grew slightly less well than at 30 °C. Growth was rated by colony size. The normal strain grown at 30 °C on the nonselective medium, YPD, was arbitrarily rated as a 4. Growth on selective media containing glycerol as the carbon source gave a relative growth of 2 for the strains containing the normal iso-1-cytochrome *c* (C102A). The *CYC1* genes of some of the slower growing strains were resequenced after growth to insure no undetected reversion had taken place. Similar growth studies were performed with diploid strains. This was done to eliminate possible recessive mutations that were inadvertently caused by the transformation process. These diploids were constructed by crossing the haploid strains to B-7682 (*MATα cycl-31 cyc7-67 his1 trp2-1*), a cytochrome c deficient strain. The diploid series did not manifest possible recessive mutations that would affect growth. The diploid strains gave a similar growth pattern to the haploids, except with very low level mutants. These low-level diploids tended to grow less well, possibly due to a gene dosage effect.

Reagents. Taq polymerase was obtained from Cetus for PCR reactions. The Stratagene Sequencing kit was used for

Table 1: Amino Acid Replacements at Position 6 with the Codon for That Change, Strain Number, and Allele Number for (A) the Normal N52 C102A Series and (B) the Stabilized N52I C102A Series

(A) N52 C102A				(B) N52I C102A			
pos 6	codon	strain no.	allele no.	pos 6	codon 6	strain no.	allele no.
fss[a]	TAAΔGT	B-8163	*cyc1-1027*	fss[a]	TAAΔGT	B-8291	*cyc1-1031*
G	GGC	B-8297	*CYC-1112*	G	GGC	B-8292	*CYC-1126*
A	GCT	B-8298	*CYC-1113*	A	GCC	B-8293	*CYC-1127*
S	AGC	B-8299	*CYC-1114*	S	AGT	B-8294	*CYC-1128*
C	TGT	B-8309	*CYC-1115*	C	TGT	B-8318	*CYC-1129*
T	ACT	B-8300	*CYC-1116*	T	ACC	B-8295	*CYC-1130*
D	GAT	B-8310	*CYC-1118*	D	GAC	B-8320	*CYC-1133*
P	nr[b]			P	CCG	B-8319	*CYC-1131*
N	AAT	B-8311	*CYC-1119*	N	AAC	B-8321	*CYC-1134*
V	GTT	B-8301	*CYC-1117*	V	GTC	B-8296	*CYC-1132*
E	GAA	B-8314	*CYC-1122*	E	GAG	B-8325	*CYC-1138*
Q	CAA	B-8313	*CYC-1121*	Q	CAG	B-8324	*CYC-1137*
H	CAC	B-8315	*CYC-1123*	H	CAC	B-8326	*CYC-1139*
L	nr			L	CTT	B-8322	*CYC-1135*
I	ATA	B-8312	*CYC-1120*	I	ATT	B-8323	*CYC-1136*
M	ATG	B-8316	*CYC-1124*	M	ATG	B-8327	*CYC-1140*
K	nr			K	AAG	B-8329	*CYC-1142*
R	nr			R	AGA	B-8332	*CYC-1145*
F	nr			F	TTT	B-8328	*CYC-1141*
Y	TAC	B-8317	*CYC-1125*	Y	TAT	B-8330	*CYC-1143*
W	nr			W	TGG	B-8331	*CYC-1144*

[a] fss—frame shift and stop at codons 5 and 6. [b] nr—not recovered.

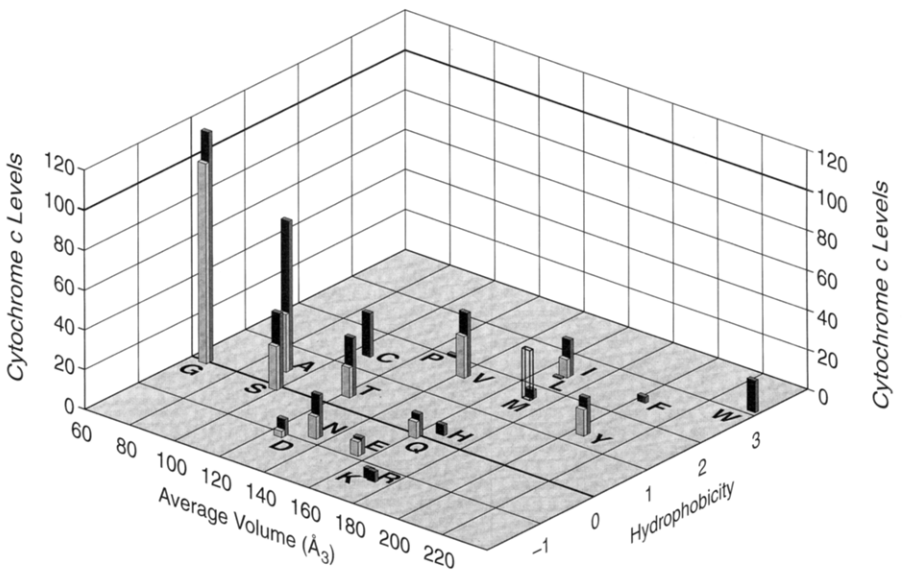

FIGURE 3: Cellular cytochrome *c* levels. Protein levels of iso-1-cytochrome *c in vivo* in N52 (gray bars) and N52I (solid bars) strains having various replacements of G6 are shown. The percent cytochrome *c* levels are plotted against the average amino acid side chain volume and hydrophobicity, using the values presented in Table 2.

sequencing reactions. All other reagents were from standard suppliers.

Protein Isolation and Thermodynamic Studies. The methods used are the same as Linske-O'Connell *et al.* (accompanying paper) with the following exceptions. The strains containing G6A, G6A N52I, and G6S N52I were grown at 22 °C for 2 days to increase the yield. These variants were isolated on ice. Proteins that contained changes at position 6 larger than serine were so unstable that they denatured during isolation even at decreased temperatures. Calculations for the guanidine hydrochloride denaturation are based on a two-state unfolding. The microcalorimetric data from G6S N52I are the result of a single run. Subsequent runs were not repeatable. Therefore, accurate measurements cannot be made.

RESULTS

Using mixed oligonucleotides to directly transform the *CYC1* locus is a convenient way of recovering all functional single-site variants of iso-1-cytochrome *c*. This method results in a stable single chromosomal copy of the substituted gene. The mutant protein is under the same control as the wild type cytochrome *c*. The resulting isogenic strains can be accurately compared for protein levels and function of their cytochromes *c in vivo*.

To insure that the C102A replacement had no gross effect on the cellular protein levels, control strains containing G6, G6S, and G6C were made with the wild type C102. These replacements were chosen to cover a range of expression levels *in vivo* from 20% to 100% cytochrome *c*. Their visual spectra were indistinguishable from those of the strains recovered with the C102 replacement (unpublished results).

Table 2: Properties of Strains with Iso-1-cytochromes *c* with Position 6 Replacements[a]

			relative % cytochrome *c*		growth on glycerol medium			
pos 6	avg vol (Å^3)	hydrophobicity	N52	N52I	N52	N52I	N52	N52I
G	66	0	100	115	++	+++	++	++
A	92	0.42	29	75	+	+++	0	++
S	99	−0.05	22	38	±	+++	0	++
C	106	1.34	**	22	±	++	0	0
T	122	0.35	15	29	0	+++	0	++
D	125	−1.05	3	8	0	+++	0	+
P	129	1.98	*	1	0	++	0	±
N	135	−0.82	11	21	0	+++	0	++
V	142	1.66	21	32	0	+++	0	++
E	155	−0.87	7	8	0	+++	0	+
Q	161	−0.03	8	11	0	+++	0	+
H	167	0.18	**	5	0	+++	0	±
L	168	2.32	*	0.5	0	++	0	0
I	169	2.46	9	18	0	+++	0	++
M	171	1.68	25[a]	4	0	+++	0	+
K	171	−1.35	*	5	0	++	0	0
R	173	−1.37	*	5	0	+	0	0
F	203	2.44	*	3	0	+++	0	0
Y	204	1.31	13	18	0	+++	0	++
W	238	3.07	*	16	0	+	0	0

[a] The amino acid at position 6 is shown by the single letter code. The average volume values are from Richards (1977) except arginine, which is from Zamyatnin (1972). The hydrophobicity values, based on the amino acid Δg_{tr} (octanol to water), are from Fauchere and Pliska (1983). * = not recovered. ** = highly revertable. [b] Low cytochrome *b*.

Table 3: Guanidine Hydrochloride Denaturation[a]

cytochrome *c* (C102A)	ΔG_U (kcal/mol)	m_g (kcal L/mol^2)	C_m (M)	$\Delta\Delta C_m$ (M)	$\Delta\Delta G_U$ (kcal/mol)
N52	5.86	−4.36	1.34		
G6A	2.36	−5.40	0.44	−0.90	−3.50
N52I	9.96	−4.77	2.09	0.75	4.10
G6A, N52I	5.85	−4.78	1.22	−0.12	−0.01
G6S, N52I	5.55	−4.75	1.17	−0.17	−0.31

[a] Each protein is considered to have an estimated error of ±0.03 M for the C_m and ±5% for the ΔG_u. These values were estimated from replicate determinations of the wild type and N52I proteins as outlined in Linske-O'Connell *et al.* (accompanying manuscript).

Table 4: Differential Scanning Microcalorimetric Data

cytochrome *c* (C102A)	T_m (°C)	ΔH (kcal/mol)	ΔS (cal/mol K)	ΔG (kcal/mol)	$\Delta\Delta G$ (kcal/mol)
N52	49.2	78.8	244	4.03	
N52I	62.2	101.3	301	7.17	3.14
G6A, N52I	47.4	64.7	202	2.77	−1.26

This supports the thermodynamic data (Hickey *et al.*, 1991a) which shows the C102A change does not cause a large change in thermostability in cytochrome *c*. No other effect on the cellular protein level can be attributed to that change. All other strains discussed contain the C102A replacement.

Strains containing the variant cytochromes *c* were assayed for protein levels and function *in vivo* (Table 1, Figures 3−5). Six of the amino acid replacements in the N52 background were not recovered and presumed nonfunctional (G6P, G6L, G6F, G6K, G6W, and G6R). Strains with the replacements G6C and G6H tended to revert on nonselective media. They were, therefore, deleted from further study. The G6M variant had a cytochrome *b* level that was lower than those of the other strains studied. It gave an artificially high cytochrome *c*/*b* ratio. The cause for this is unknown. When calculated with an average cytochrome *b* level, the ratio was in keeping with the other results.

In all cases measured, the position 6 variants in the normal N52 background gave lower cytochrome *c* levels or were not recovered (and thus presumably lower) compared with strains containing the stabilizing N52I (Figure 3, Table 2). The relative pattern of the N52 mutants was similar to that of their stabilized equivalents. In general, the strains containing the N52I-stabilizing replacement had a similar increase (between 3% and 15%) in cytochrome *c* level when compared to their unstabilized counterparts. This similar pattern suggests the additivity of the stabilizing effect of N52I and the destabilizing effects at position 6. To test temperature sensitivity *in vivo*, the strains were grown at 22, 30, and 37 °C (Figure 4). Proteins near their T_m would be expected to have different degrees of temperature sensitivity. The proteins stable enough to be isolated had T_m's higher than the growth conditions (Table 4). The levels *in vivo* were minimally affected by increasing temperature. Proteins with intermediate cellular levels had a varied response as would be expected if the cellular cytochrome *c* levels were affected by stability. The cytochromes *c* with large amino acids in the unstabilized N52 series showed little response to temperature indicating their T_m values were lower than the growth conditions.

In the strains with normal N52 background, the ability to grow on glycerol medium sharply decreased with increasing side chain size at the normal 30 °C incubation temperature (Figure 5, Table 2). At 37 °C, the only variant that could still function contained the wild type glycine at position 6. The strains that contained the N52I global suppressor were able to function with all 20 amino acids at 30 °C. Increasing the temperature to 37 °C decreased relative growth for all G6 substitutions. Strains that contained lower levels of cytochrome *c* at 30 °C were more profoundly affected at the increased temperature. Generally, the relative growth correlated with the amounts of cytochrome *c* within each of the two position 52 series. For these position 6 variants, the specific activity (as defined by growth on glycerol plates versus the levels of cytochrome *c in vivo*) of the stabilized variants was higher than that in the strains with the wild type N52. This includes strains that had very low levels of the protein *in vivo*.

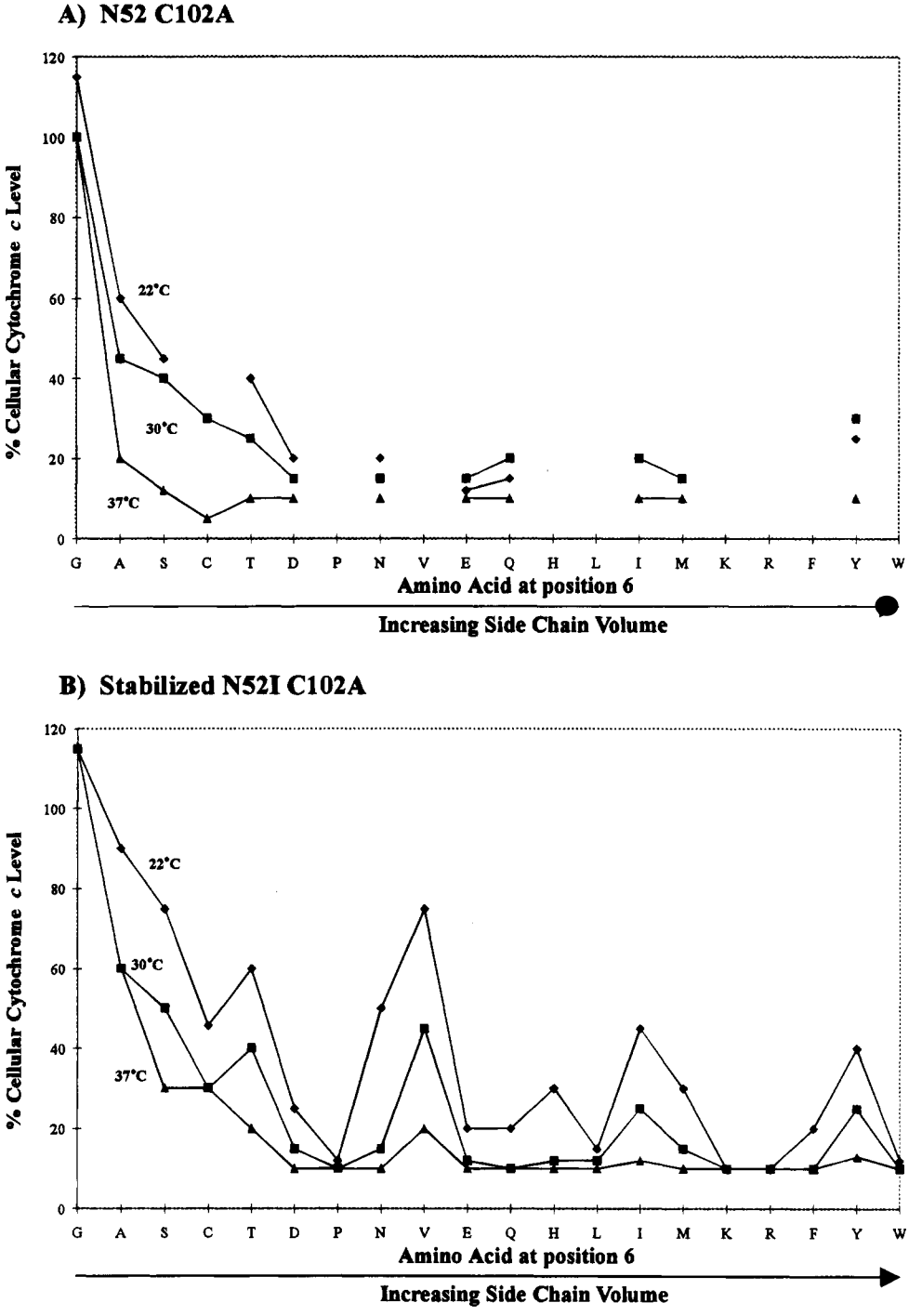

FIGURE 4: Temperature sensitive cytochrome *c* levels *in vivo*. The temperature sensitivity of the cytochrome *c* protein levels *in vivo* for each of the position 6 variants by growth at 22, 30, and 37 °C is shown. The approximate cytochrome *c* levels are plotted against the amino acid volume at position 6. The amino acids are in increasing order by average side chain volume. (A) Position 6 replacements with the normal N52. (B) Position 6 replacements with the stabilizing N52I replacement.

Thermodynamic studies were carried out with proteins stable enough for isolation. Guanidine hydrochloride denaturation and differential scanning microcalorimetry (Tables 3 and 4, Figures 6 and 7) show the decrease in cellular protein levels is related to the stability of the protein *in vitro*. G6A shows a very large decrease in stability as measured by the C_m and $\Delta\Delta G_U$ of unfolding in guanidine hydrochloride. The addition of a single methyl group to this site causes a decrease in stability by more than one-half of the free energy of the entire protein. The added bulk of a serine residue is so destabilizing it causes the protein to denature during isolation. The G6A N52I replacement has a C_m and T_m just slightly less than that of the normal G6 N52 protein. The G6S N52I cytochrome *c* has a C_m slightly lower than that of the normal protein but is thermally so labile that microcalorimetric measurement is unreproducible. Chemical denaturation profiles (Figure 6) show these double replacements have characteristics from both G6-destabilizing replacements and the stabilizing N52I. G6A N42I and G6S N52I have slopes from the pre-unfolding base lines that differ from that of the normal N52. The prefolding base line and m_g (slope from the ΔG versus concentration of guanidine hydrochloride in the transition zone) are similar to those seen with the N52I replacement. The transition range is smaller

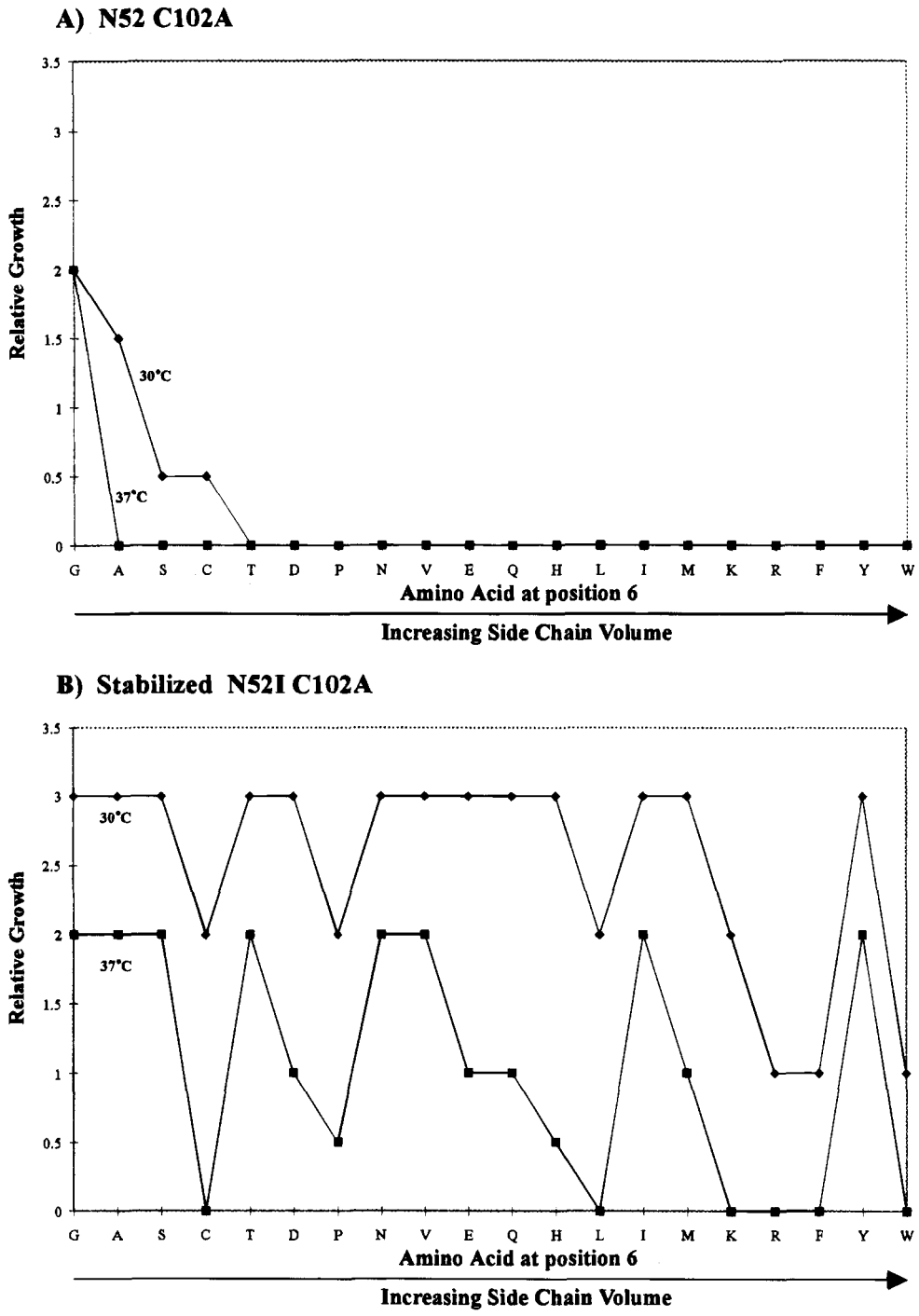

FIGURE 5: Temperature sensitive growth. The temperature sensitive function of the position 6 cytochrome *c* variants as measured by growth is shown. Strains containing these replacements were grown on glycerol as a nonfermentable carbon source at 30 and 37 °C. Relative growth is plotted against increasing side chain size as represented by the average amino acid volume (see methods section for details.). (A) Position 6 replacements with the normal N52. (B) Position 6 replacements with the stabilizing N52I replacement.

as is seen with the G6A replacement. Previous work at position 52 shows that the thermodynamic data from guanidine hydrochloride and microcalorimetry give related results. While the microcalorimetric work and cellular levels indicate that the double replacements are less stable than the normal N52, the $\Delta\Delta G_U$ values are plotted for the different methods (see figures in L. Linske-O'Connell *et al.*, accompanying manuscript, the double replacements have higher chemical denaturation free energies than would be predicted. It is possible that the methods used to determine the free energy values from guanidine hydrochloride denaturation are biased by the detection method and/or the analysis method. Unfolding is monitored by an increase in the fluorescence of W59 (Tsong, 1974). Structurally, this residue is in the same hydrophobic pocket as N52. With a stabilizing replacement in the area that is being monitored, a local conformational change at position 6 may not be quantitatively measured. If so, then the protein unfolding is no longer a two-state process, and the free energy value becomes overestimated.

The results show that the N52I replacement acts as a global suppressor to overcome destabilization from replacements at position 6. Among the available replacements, only G6A N52I can be analyzed for additivity (Wells, 1991). The increase in C_m seen in the N52I variant and the decrease in

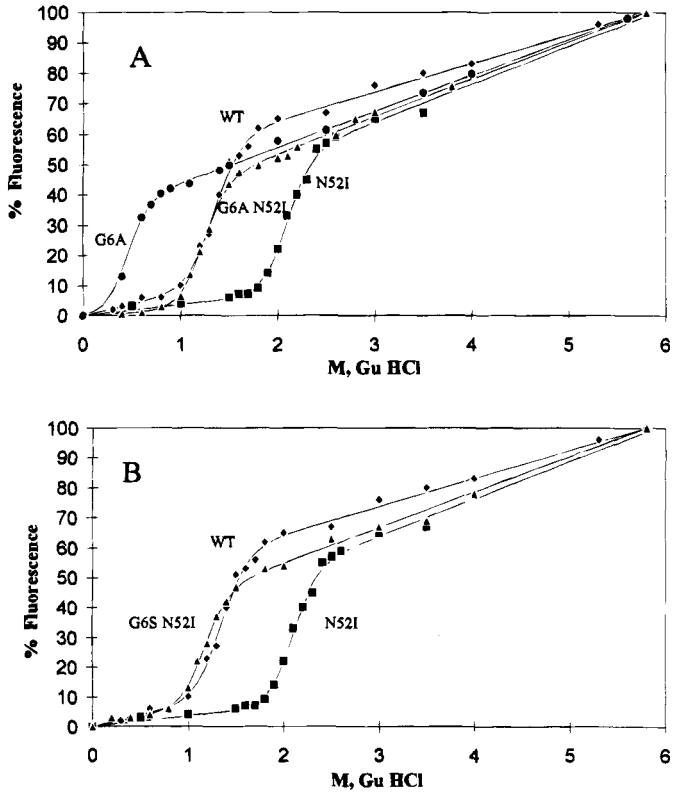

FIGURE 6: Guanidine hydrochloride denaturation. The relative fluorescence of unfolding for (A) 7A (●), G6A N52I (▲), N52 (◆), and N52I (■) and (B) G6S N52I (▲), N52 (◆), and N52I (■) versus the concentration of guanidine hydrochloride in 0.1 M potassium phosphate, pH 7.0, at room temperature is plotted. Theoretical best fits were calculated using nonlinear least squares as outlined in Linske-O'Connell *et al.* (accompanying manuscript).

the G6A variant add up to give the slight destabilization seen with both together. The $\Delta\Delta G_U$ values are additive within the accuracy of the method.

DISCUSSION

A series of past experiments showed the need for a systematic study of the interaction of the destabilizing changes at position 6 and the stabilizing changes at position 52. The global supression of a change at position 52 was first shown by Petrochilo and Verdiere (1977). An unidentified change at position 52 was able to restore activity to a G6S variant of iso-2-cytochrome *c*. Later genetic work by Berroteran and Hampsey (1991) was able to show that strains containing N52I overcome a G6S mutation in iso-1-cytochrome *c*. The double mutant exhibited a growth rate and a cellular level of protein about the same as wild type strains. Strain containing N52I alone had cellular protein levels that were about 20% greater than that of the wild type protein.

Later work recovered a limited series of substitutions at position 6 using random mutagenesis of positions 6 and 10 in iso-1-cytochrome *c* (Auld & Pielak, 1991). Six single changes at position 6 were identified, G6S, G6D, G6M, G6L, G6F, and G6K. These changes resulted in a temperature sensitive phenotype or loss of function of the protein. Other changes at position 6 were identified as deleterious in combination with changes at position 10. This work showed that side chain volume at position 6 affected the protein and basic amino acids were not tolerated. Refolding studies on horse cytochrome *c* showed the structural importance of position 6 by identifing the interaction between the N-terminal (containing G6) and C-terminal helices as one of the earliest events in the protein-folding pathway (Roder *et al.*, 1988; Elove *et al.*, 1992). Thermodynamic work on the isolated protein in the Sherman and McLendon labs showed that the single-site N52I change had a dramatic effect on the protein stability. When the N52I protein was compared to the wild type iso-1-cytochrome *c* in thermodenaturation experiments, the T_m of unfolding increased by 17 °C (Das *et al.*, 1989).

Experiments with a series of stabilized position 52 proteins show increased levels *in vivo* and correlate with increased stability *in vitro Das et al.*, 1989; Hickey *et al.*, 1991a, 1992; L. Linske-O'Connell *et al.*, accompanying manuscript). This relationship has been shown to depend on protein denaturation and subsequent proteolysis in the mitochondria by pulse-chase experiments of N52 and N52I cytochromes *c* (Pearce and Sherman, submitted for publication). The comparison of the three most stable replacements, G6A, G6S N52I, and G6A N52I, with the two controls, N52 and N52I, also shows a relationship between stability *in vitro* and the cellular protein levels (Figure 7). The hypothesis that this relationship is true for all the position 6 variants is supported by the fact that protein levels *in vivo* are sensitive to temperature (Figure 4) to different degrees as reflected by their theoretical T_m values *in vivo*. Since the G6 replacements span a broad range of levels from 0% to 100%, they can provide a more detailed analysis of the relationship between the physical stability of cytochrome *c* and the cellular turnover in pulse-chase analysis.

For the double replacements, G6A N52I and G6S N52I, the $\Delta\Delta G_U$ values for chemical denaturation were higher than what would be expected from microcalorimetric measurements and cellular protein levels (Figure 7). This, possibly, can be explained by a local stabilization due to the N52I

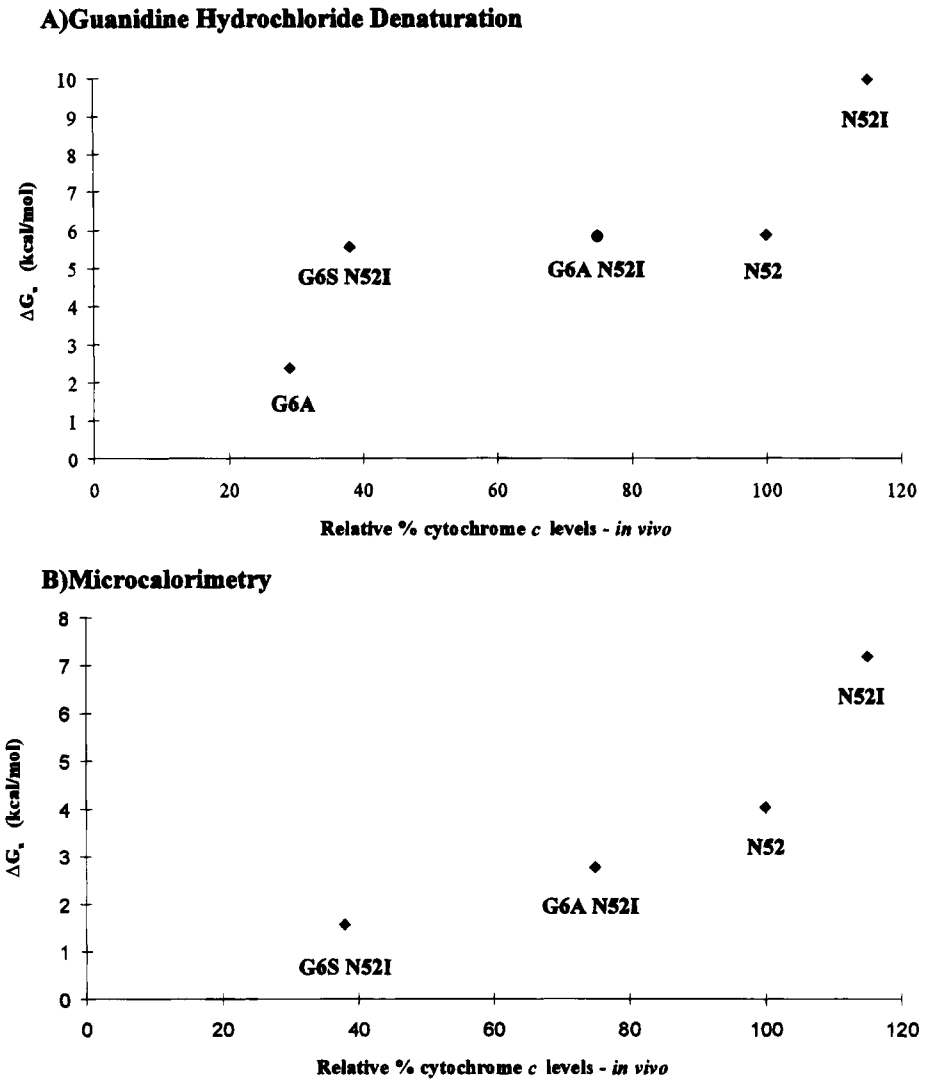

FIGURE 7: Thermodynamic parameters of position 6 replacements versus cellular protein levels. (a) Guanidine hydrochloride denaturation in 0.1 M potassium phosphate, pH 7.0. (B) Microcalorimetry in 50 mM potassium acetate, pH 4.6.

replacement near the tryptophan that is followed during denaturation. The denaturation profiles of the double mutants look very similar to that of N52I except they are shifted to a lower T_m. The combination of a stabilizing change in tbe hydrophobic core and a destabilizing change at an area where the N-terminal and C-terminal helices interact could indicate an interesting change in the folding profile of the protein.

As shown above, cellular protein levels of cytochrome *c* can track thermodynamic changes in cytochrome *c* replacements. Thus, low-temperature spectra of isogenic strains predicted the thermodynamics for protein unfolding and thereby indicate possible forces affecting the proteins too unstable for measurement *in vitro*. Destabilization can be correlated with the increasing side chain size at position 6 in both the N52 and N52I series. The relationship between cytochrome *c* levels *in vivo* and substitutions at position 6 is not a simple one. There are multiple forces that affect the stability of position 6 variants, including side chain size, hydrophobicity, charge, and specific side chain interactions such as hydrogen bonding and aromatic side chain stacking. Among these effects, increasing side chain size most strongly correlated with decreasing protein levels *in vivo*. The addition of a single methyl group in the G6A mutants led to a 70% and 25% decrease in observed protein levels within the N52 and N52I backgrounds, respectively. Serine, the next largest amino acid with an additional hydroxyl group, caused even further destabilization. With larger substitutions, increasing hydrophobicity correlated with increased levels of cytochrome *c* (*e.g.*, G6V, G6T, and G6M). Substitutions with a charged group, G6D, G6E, G6R, and G6K, resulted in decreased levels. Proteins containing the aromatic amino acids, especially tyrosine, were surprisingly stable for residues with such large bulky side chains. This could be due to their hydrophobic nature. Since aromatic residues can stabilize through the "weak polar interactions" (Burley & Petsko, 1985, 1988) made by aromatic packing, it is possible that the aromatic position 6 mutants are interacting with the evolutionarily conserved F10 and Y97. Three residues, G6P, G6L, and G6C, gave inconsistently lowered levels based on their side chain size and hydrophobicity when compared to the rest of the data. These decreased levels would be expected for proline, which is known to disrupt α-helical structure due to thee lack of an α-amine. Leucine is very similar to isoleucine in side chain size and hydrophobicity. The G6L mutation resulted in low cytochrome *c* levels with N52I and unrecoverable activity in the N52 background. A specific side chain interaction such as a hydrogen bond may account for this difference. G6C may

have a lower than expected level due to cysteine's reactiveness in biosynthetic processes unrelated to protein stability.

Within each of the position 52 series, the growth characteristics of the mutant cytochromes *c* correlates well with the protein levels *in vivo* (Figure 5). Strains in the series with N52I grew better than the wild type N52 strains with the same change. Thus, the global suppressor, N52I, was able to restore function to very destabilized mutants in iso-1-cytochrome *c* (*e.g*, G6P N52I). Interestingly, when two mutants with differences at position 52 but similar protein levels *in vivo* were compared (*e.g.*, G6A and G6T N52I or G6V and G6N N52I), the cytochromes *c* with the stabilizing N52I change showed higher specific activity measured by growth on the nonfermentable carbon source, glycerol.

These series of amino acid replacements will enable the relationship between thermostability and proteolytic degradation of cytochromes *c* in the intermitochondrial space to be further investigated. The double replacements, G6A N52I and G6S N52I, may provide interesting variations in the study of protein folding of cytochrome *c*.

REFERENCES

Auld, D. S., & Pielak, G. J. (1991) *Biochemistry 30*, 8684−8690.

Berroteran, R. W., & Hampsey, D. M. (1991) *Arch. Biochem. Biophys. 288*, 261−269.

Burley, S. K., & Petsko, G. A. (1985) *Science 229*, 23−28.

Burley, S. K., & Petsko, G. A. (1988) *Adv. Protein Chem. 39*, 125−189.

Das, G., Hickey, D. R., McLendon, D., McLendon, G., & Sherman, F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 496−499.

Dixon, M., Hill, R., & Keilin, D. (1931) *Proc. R. Soc. London 109*, 29−34.

Elove, G. A., Chaffotte, A. F., Roder, H., & Goldberg, M. (1992) *Biochemistry 31*, 6876−6883.

Fauchere, J.-L., & Pliska, V. (1983) *Eur. J. Med. Chem. Chim. Ther. 18*, 369−375.

Fetrow, J. S., Cardillo, T. S., & Sherman, F. (1989) Proteins: *Struct., Funct., Genet. 6*, 372−381.

Hampsey, D. M., Das, G., & Sherman, F. (1986) *J. Biol. Chem. 261*, 3259−3271.

Hampsey, D. M., Das, G., & Sherman, F. (1988) *FEBS Lett. 231*, 275−283.

Hickey, D. R., Berghuis, A. M., Lafond, G., Jaeger, J. A., Cardillo, T. S., McLendon, D., Das, G., Sherman, F., Brayer, G. D., & McLendon, G. (1991a) *J. Biol. Chem. 226*, 11686−11694.

Hickey, D. R., Jayaraman, K., Goodhue, C. T., Shah, J., Clements, J. M., Tsunasawa, S., & Sherman, F. (1991b) *Gene 105*, 73−81.

Hickey, D. R., McLendon, G., & Sherman, F. (1992) *Stability of Protein Pharmaceuticals: In Vivo Pathways of Degradation and Strategies for Protein Stabilization* (Ahern, T. M., & Manning, M. C., Eds.) Plenum, New York.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.

Louie, G. V., & Brayer, G. D. (1990) *J. Mol. Biol. 214*, 527−555.

Louie, G. V., Hutcheon, W. L. B., & Bryaer, G. D. (1988) *J. Mol. Biol. 199*, 295−314.

Moerschell, R. P., Tsunasawa, S., & Sherman, F. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 524−528.

Moerschell, R. P., Hosokawa, Y., Tsunasawa, S., & Sherman, F. (1990) *J. Biol. Chem. 265*, 19638−19643.

Moerschell, R. P., Das, G., & Sherman, F. (1991) *Methods Enzymol.* 194, 362−369.

Montonaga, K., Misaka, E., Nakajima, E., Ueda., S., & Nakanishi, K. (1965) *J. Biochem. (Tokyo)* **57**, 22−28.

Pakula, A. A., & Sauer, R. T. (1989) *Annu. Rev. Genet.* 423, 289−310.

Petrochilo, E., & Verdiere, J. (1977) *Biochem. Biophys. Res. Commun. 79*, 364−371.

Richards, F. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 151−176.

Roder, H., Elgrove, G. A., & Englander, S. W. (1988) *Nature 335*, 700−704.

Sherman, F. (1991) *Methods Enzymol. 194*, 3−21.

Sherman, F., & Slonimski, P. P. (1964) *Biochem. Biophys. Acta 90*, 1−15.

Shortle, D., & Lin, B. (1985) *Genetics 110*, 539−555.

Tsong, T. Y. (1974) *J. Biol. Chem. 249*, 1988−1990.

Wells, J. A. (1990) *Biochemistry 29*, 8509−8517.

Yamamoto, T., Moerschell, R. P., Wakem, L. P., Ferguson, D., & Sherman, F. (1992) *Yeast 8*, 935−948.

Zamyatnin, A. A. (1972) *Prog. Biophys. Mol. Biol. 24*, 107−123.

BI9415661