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Dramatic thermostabilization of yeast iso-1-cytochrome *c* by an asparagine → isoleucine replacement at position 57

(protein structure/*Saccharomyces cerevisiae*/cytochrome *c*/protein folding/mutagenesis)

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ABSTRACT **Two *Saccharomyces cerevisiae* yeast mutants, *cyc1-73* and *cyc1-190*, contain nonfunctional and presumably unstable forms of iso-1-cytochrome *c* due to Gly-34 → Ser and His-38 → Pro replacements, respectively. Second-site reversions that produced Asn-57 → Ile replacements at least partially restored function, presumably by alleviating the instability of these two altered iso-1-cytochromes *c*. Introduction of the Ile-57 replacement by site-directed mutagenesis in an otherwise normal protein resulted in a 17°C increase in the transition temperature (T_m), corresponding to over a 2-fold increase in the free energy change ($\Delta G°$) for thermal unfolding.**

The native conformation and stability of cytochrome *c* are determined by numerous noncovalent interactions between the polypeptide chain residues and between the amino acids and the covalently bound heme group. Thus, individual amino acid residues each contribute to the overall stability of the protein. Evolutionary selection presumably optimizes protein structure with respect to function and stability so that "random" amino acid replacements generally are neutral or detrimental. However, certain amino acid replacements at specific sites can enhance stability. While general methods for stabilizing proteins are not yet known, genetic selection and screening (1–5) and rational approaches (6–14) have resulted in producing mutant proteins with slightly increased stability. We have combined genetic selection with oligonucleotide-directed mutagenesis to produce an altered iso-1-cytochrome *c* with an unusually large increase in thermal stability. Reversion of two missense mutants resulted in second-site replacements of Asn-57 by Ile, presumably by stabilizing the altered iso-1-cytochromes *c*. Introduction of the Ile-57 replacement in an otherwise normal sequence caused a 17°C increase in the transition temperature (T_m), corresponding to a greater than 2-fold increase in the free energy change of thermal unfolding: $\Delta G°$ for the normal iso-1-cytochrome *c* and the Ile-57 mutant protein were equal to 3.8 and 8.0 kcal/mol (1 kcal = 4.18 kJ), respectively, at 25°C.

MATERIAL AND METHODS

Genetic Nomenclature and Yeast Strains. The symbols *CYC1* and *CYC1*$^+$ denote, respectively, any functional allele and the wild-type allele encoding iso-1-cytochrome *c* in the yeast *Saccharomyces cerevisiae*. The symbols *cyc1-73* and *cyc1-190* denote two mutants having nonfunctional iso-1-cytochrome *c* at approximately 70% of the wild-type level as measured by low-temperature spectroscopy (15). *CYC1-73-A*, *CYC1-73-B*, etc. and *CYC-190-A*, *CYC1-190-B*, etc. are functional intragenic revertants derived *in vivo* from the mutants of *cyc1-73* and *cyc1-190*, respectively. *CYC1-811* is a functional variant constructed by site-directed mutagenesis as described below. The genetic techniques and the media used with the *cyc1* mutants, including procedures for mutagenic treatments, reversion, and testing of revertants, have been described earlier (15, 16). The change of Asn-57 to Ile in iso-1-cytochrome *c* was accomplished by the site-directed mutagenesis method of Kunkel *et al.* (17). *Escherichia coli* strain BW313 (*dut ung thi-1 relA spoT1*/F′*lysA*) (18) was transformed with yeast shuttle plasmid pAB458 containing a *Bam*HI–*Hin*dIII fragment encompassing the *CYC1*$^+$ gene (J. Fetrow, T. Cardillo, and F.S., unpublished results). Upon infection of the BW313 strain bearing pAB458 with helper phage R408 (19), a single-stranded DNA form of the plasmid containing the *CYC1* fragment was produced and used as template. A 21-residue oligonucleotide (5′-ACAGATGC-CATTATCAAGAAA-3′) synthesized with Applied Biosystems DNA synthesizer model 380A was used as the mutagenic primer. After completion of primer extension and detection of the desired change by DNA sequencing, the plasmid containing the change was used to transform yeast strain B-6748. Integration of the plasmid was carried out by the method of Holzschu *et al.* (20). The resultant yeast strain, B-7626, contains a single chromosomal copy of the altered allele, *CYC1-811*, that encodes the [Ile^{57}]iso-1-cytochrome *c*. The DNA sequence of the *CYC1-811* mutation was further verified by cloning and sequencing the relevant chromosomal region, using the methods of Hampsey *et al.* (15).

Protein Preparation and Thermal Denaturation. The normal [Asn^{57}]- and the mutant [Ile^{57}]iso-1-cytochromes *c* were extracted from commercial bakers' yeast and the laboratory strain B-7626 carrying the allele *CYC1-811*, respectively. To prevent dimerization through Cys-107 disulfide bridges, both proteins were chemically blocked with methyl methanethiosulfonate (21–23). The [Ile^{57}]-, SCH_3-modified protein, referred to here as [Ile^{57}]Cyt-SCH_3, was further purified by gel filtration chromatography. The monomeric purity of these chemically modified proteins was verified by Laemmli gel electrophoresis after reaction with $CuSO_4$ (23).

The proteins were thermally denatured in 100 mM sodium phosphate, pH 6.0, and the transitions were monitored by the absorbance change at 287 nm (23). The resulting absorbance versus temperature data were evaluated for van't Hoff enthalpy and entropy changes for thermal unfolding at the midpoint temperature—i.e., ΔH°_m and ΔS°_m, respectively. The temperature at the midpoint of the transition, T_m, was also calculated.

RESULTS

Revertants. Sherman *et al.* (16, 24) and Hampsey *et al.* (15, 25) have characterized numerous *cyc1* mutants lacking iso-

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Abbreviations: [Asn^{57}]Cyt-SCH_3 and [Ile^{57}]Cyt-SCH_3, the [Asn^{57}]- and [Ile^{57}]iso-1-cytochromes *c*, respectively, chemically modified at Cys-107 with methyl methanethiosulfonate.

1-cytochrome *c* or containing nonfunctional forms. Furthermore, revertants containing at least partially functional iso-1-cytochrome *c* have been selected on lactate medium and the alterations have been characterized by protein analysis of the revertant iso-1-cytochromes *c* or by DNA sequencing of the revertant *CYC1* genes (reviewed in ref. 25). Most revertants contain single amino acid replacements that restore function by corrections within the original codon. Many examples include single replacements of amino acids whose codons differ from nonsense codons by single base-pair changes, thus demonstrating that the site was compatible with amino acid replacements having a wide range of properties. On rare occasions, revertants can arise by mutation at a second site while retaining the original mutation. For example, replacements of Trp-64 by Cys, Gly, or Ser were restored by a second-site Ser-45 → Phe mutation, probably by the function of the Trp-64 side chain being supplied by the sterically similar Phe-45 side chain (26).

The *cyc1-73* mutant contains a minimally functional form of iso-1-cytochrome *c* due to a Gly-34 → Ser replacement (15, 25). We have characterized eight UV-induced revertants by DNA sequencing of the relevant region within *Eco*RI–*Hin*dIII fragments (Table 1). All but one of the revertants contained the wild-type sequence, a Ser-34 → Gly reversion; the other mutant, *CYC1-73-A*, retained the original Ser-34 change but contained a second-site Asn-57 → Ile replacement (Table 1; Fig. 1).

A second missense mutant, *cyc1-190*, contains a His-38 → Pro change that produces a nonfunctional iso-1-cytochrome *c* (15, 25). Analysis of 14 revertants arising spontaneously or induced by various mutagens (Table 1) revealed functional replacements at the original site (Pro-38 → Leu; Pro-38 → Ser; and Pro-38 → Ala) and one second-site revertant, *CYC1-190-G*, containing an Asn-57 → Ile replacement (Table 1; Fig. 1). Thus, the Ile-57 replacement appears to stabilize both the Ser-34 and Pro-38 iso-1-cytochrome *c* even though there are no obvious interactions between the residues at position 57 and those at 34 and 38 (27).

The Ile-57 Protein. Because the Ile-57 second-site replacement served as a "global" suppressor (2) for two different missense mutations, we decided to construct and investigate an iso-1-cytochrome *c* having only an Asn-57 → Ile replacement. The mutation, *CYC1-811*, was constructed *in vitro* by site-directed mutagenesis, and properties of the corresponding yeast strain were examined. Low-temperature (−196°C) spectroscopic examination indicated that the level of iso-1-cytochrome *c* in the Ile-57 strain was normal, whereas growth on lactate medium indicated that the function of the iso-1-cytochrome *c* was normal or near normal *in vivo*. The [Ile^{57}]iso-1-cytochrome *c* was isolated and its -SCH_3 derivative was prepared for stability studies. Modification of Cys-107 with methyl methanethiosulfonate prevents dimerization that occurs by disulfide linkage and allows the examination of thermal unfolding without this additional complexity (22, 23). Thermal denaturation results are presented in Fig. 2, where the relative absorbance as a function of temperature is given for the normal [Asn^{57}]Cyt-SCH_3 and the mutant [Ile^{57}]Cyt-SCH_3 proteins.

Each protein unfolds in a single cooperative transition with upward-sloping baselines. The unfolding transformations were also shown to be reversible (results not presented). The proteins differ, however, in two ways: first, the T_m is 17°C higher for [Ile^{57}]Cyt-SCH_3 than for the [Asn^{57}]Cyt-SCH_3 blocked normal protein. Second, the cooperativity of the transition increases. Fig. 2 shows that the transition for the altered [Ile^{57}]Cyt-SCH_3 protein occurs over a narrower temperature range than does the transition for the normal [Asn^{57}]Cyt-SCH_3 protein. This increased cooperativity of unfolding of [Ile^{57}]Cyt-SCH_3 is reflected in the significantly larger transition enthalpy and entropy changes listed in Table 2 for the altered protein. Semilogarithmic plots of the unfolding equilibrium constant versus reciprocal temperature are linear for both proteins over their transition temperature regions, providing estimates of the enthalpy and entropy at T_m, ΔH°_m and ΔS°_m. To evaluate ΔG°, a value of the change in heat capacity at constant pressure, ΔC_p, is required. This was obtained from a plot of ΔH°_m versus T_m for eight different altered forms of iso-1-cytochrome *c* (23). This value was further verified by measurements of ΔH°_m versus T_m at various concentrations of added guanidine hydrochloride (0–0.3 M). Both approaches gave a value of $\Delta C_p \approx 2.0 \pm 0.2$ kcal/mol·K. Thus, the free energy changes for thermal

Table 1. Revertants of the *cyc1-73* and *cyc1-190* mutants

Normal				Mutant				Revertant			
Amino acid	Codon	Allele		Amino acid	Codon	Allele		Amino acid	Codon	Allele	Mutagen*
Gly-34	GGT	*CYC1*$^+$	→	Ser-34	AGT	*cyc1-73*	→	Gly-34	GGT	*CYC1-73-B*	UV
										CYC1-73-C	UV
										CYC1-73-E	UV
										CYC1-73-F	UV
										CYC1-73-G	UV
										CYC1-73-H	UV
										CYC1-73-I	UV
Gly-34	GGT	*CYC1*$^+$	→	Ser-34	AGT	*cyc1-73*	→	Ser-34	AGT	*CYC1-73-A*	UV
Asn-57	AAT			Asn-57	AAT			Ile-57	ATT		
His-38	CAT	*CYC1*$^+$	→	Pro-38	CCT	*cyc1-190*	→	Leu-38	CTT	*CYC1-190-A*	None
										CYC1-190-C	UV
										CYC1-190-H	UV
										CYC1-190-K	NA
										CYC1-190-L	EMS
								Ser-38	TCT	*CYC1-190-B*	UV
										CYC1-190-E	UV
										CYC1-190-F	UV
										CYC1-190-I	EMS
										CYC1-190-J	EMS
								Ala-38	GCT	*CYC1-190-D*	DES
His-38	CAT	*CYC1*$^+$	→	Pro-38	CCT	*cyc1-190*	→	Pro-38	CCT	*CYC1-190-G*	UV
Asn-57	AAT			Asn-57	AAT			Ile-57	ATT		

*Mutagen abbreviations: UV, ultraviolet light; NA, nitrous acid; EMS, ethyl methanesulfonate; DES, diethyl sulfate.

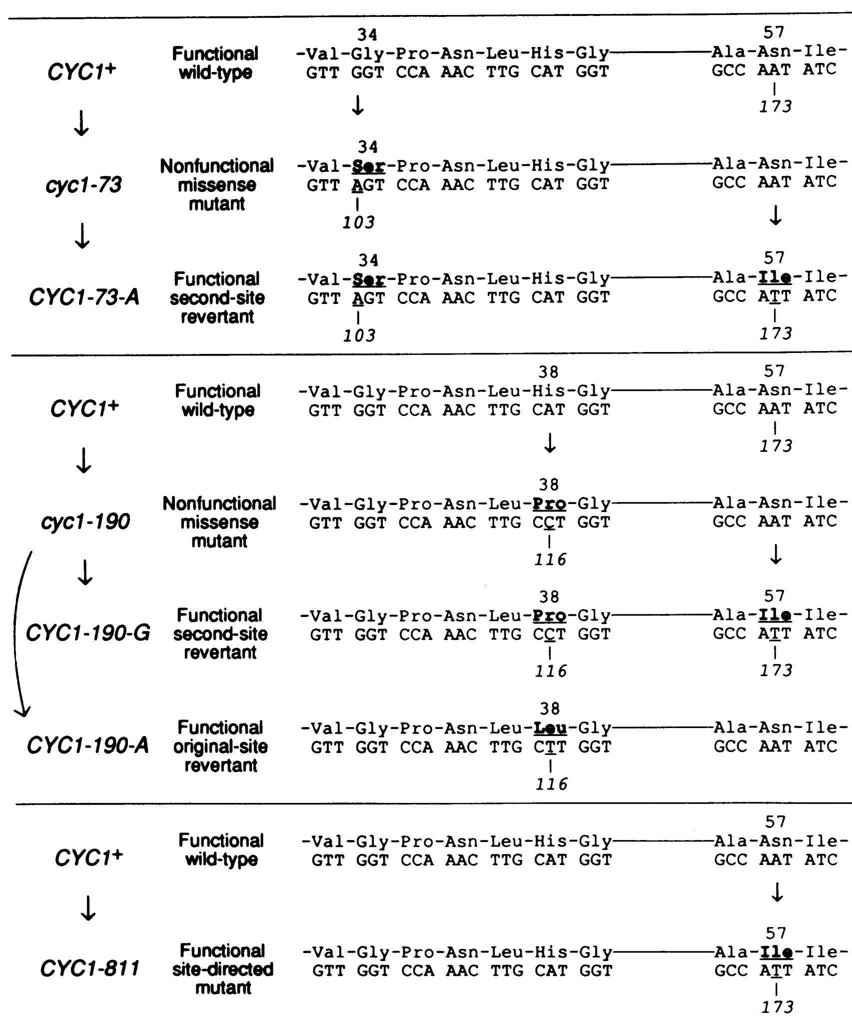

FIG. 1. Mutational pathways leading to the formation of functional and nonfunctional forms of iso-1-cytochrome c. The amino acids and the nucleotides are numbered beginning with, respectively, the amino-terminal threonine and the A of the ATG initiator codon.

unfolding (ΔG°) for [Asn^{57}]Cyt-SCH_3 and [Ile^{57}]Cyt-SCH_3 were extrapolated from their T_m values to 25°C by using the values of ΔH°_m, ΔS°_m, and ΔC_p as listed in Table 1. ΔG° at 25°C for [Ile^{57}]Cyt-SCH_3 (8.0 kcal/mol), given in Table 2, is over twice as large as ΔG° for the normal [Asn^{57}]Cyt-SCH_3 protein (3.8 kcal/mol).

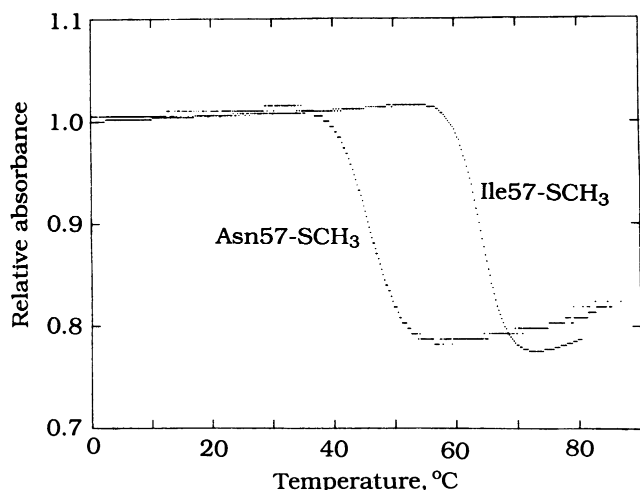

FIG. 2. Relative absorbance at 287 nm versus temperature for the [Asn^{57}]Cyt-SCH_3 and [Ile^{57}]Cyt-SCH_3 proteins. The relative absorbance is the absorbance at any temperature divided by the initial absorbance at 0°C.

DISCUSSION

Previously, Hampsey et al. (15, 25) reported that replacement of Gly-34 in iso-1-cytochrome c with Ser, Asn, or Asp caused instability *in vitro*, as inferred from lability during extractions, and caused diminished function *in vivo*, as measured by less growth in lactate medium. The loss of function by the replacements of Gly-34 was attributed to the inability of the protein to accommodate a side chain larger than the glycine proton, thereby altering the bond angles at position 34 (15, 25). Similarly, iso-1-cytochromes c are functional with the

Table 2. Thermodynamic values for thermal unfolding of the normal [Asn^{57}]Cyt-SCH_3 and the mutant [Ile^{57}]Cyt-SCH_3 proteins

Protein	T_m, °C	ΔH°_m, kcal/mol*	ΔS°_m, cal/mol·K*	ΔG° at 25°C, kcal/mol†
[Asn^{57}]Cyt-SCH_3	46.5 ± 1.0	79 ± 5	247 ± 14	3.8
[Ile^{57}]Cyt-SCH_3	63.7 ± 1.0	109 ± 9	323 ± 27	8.0

*ΔH°_m and ΔS°_m are the average values obtained by van't Hoff analysis of two unfolding experiments using independently prepared protein samples.

†ΔG° at 25°C was calculated by using the presented ΔH° and ΔS° values with change in heat capacity ΔC_p. $\Delta C_p \approx 2$ kcal/mol·K was estimated from a plot of T_m versus ΔH°_m for eight different altered forms of iso-1-cytochrome c.

normal His-38 and the Leu-38, Thr-38, Ser-38, and Ala-38 replacements but unstable and nonfunctional with a Pro-38 replacement (Table 1; refs. 16 and 26). Presumably, proline abolishes function by distorting the protein backbone structure (15, 25). The partial restoration of function for both mutations *cycl-73* (Gly-34 → Ser) and *cycl-190* (His-38 → Pro) by the second-site change Asn-57 → Ile (Fig. 1) implies that Ile-57 stabilizes altered iso-1-cytochrome *c* in a general way and that the Ile-57 replacement can be considered a "global" suppressor (2). The results of thermal stability measurements *in vitro* are consistent with this view in that the Ile-57 replacement alone dramatically stabilizes iso-1-cytochrome *c*. The T_m and ΔG° at 25°C rose from 47°C and 3.8 kcal/mol, respectively, for the normal [Asn^{57}]Cyt-SCH_3 protein, to 64°C and 8.0 kcal/mol, respectively, for the mutant [Ile^{57}]Cyt-SCH_3 protein. We note that this perturbation free energy, $\Delta\Delta G^\circ$, can be deduced from the following approximate thermodynamic relationship suggested by Schellman (4): $\Delta\Delta G^\circ = (\Delta H^\circ_m)(\Delta T/T^\circ_m)$. When $\Delta T = 17$°C, $T_m = 319.5$ K, and $\Delta H^\circ_m = 79$ kcal/mol, then $\Delta\Delta G^\circ = 4.2$ kcal/mol, which is in good agreement with observation.

The forces which stabilize proteins act simultaneously on every residue, resulting in a small net stabilization. Because only total protein thermodynamics can be measured, the contribution of single amino acids to overall protein stability is not easily evaluated. Thus, prediction of the effect of amino acid substitutions on stability and the choice of residues for alteration to obtain maximal thermostability are, at present, difficult tasks. The data reported here show that genetic selection can uncover potentially significant stabilizing residues. Alteration of such sites by themselves can be profitably undertaken to obtain thermostable proteins.

Many proteins exhibit decreased stability after alteration of individual residues (23, 28). Some of the previous approaches used to obtain thermostable proteins are as follows: (*i*) introduction of disulfide bonds (9, 11, 13), (*ii*) exploitation of the difference in configurational backbone entropy between native and denatured states (14, 29), and (*iii*) alteration of specific residues to increase α-helical stability (7, 8, 30). Because application of the first stabilization method requires the proper geometry and separation between the linking Cys residues, its use is not general. The latter two methods generally yield only small T_m increases. In contrast, the work described here demonstrates that single-residue changes involving only noncovalent contacts can significantly increase the thermal stability of a protein. To our knowledge, no other single amino acid replacements produce T_m or ΔG° increments as large as those reported here, including those found in bacteriophage T4 lysozyme (14), the amino-terminal domain of the bacteriophage λ repressor (8, 31), the serine protease subtilisin (5), or the α subunit of tryptophan synthase (32).

Asn-57 is located within a short helical segment. Between the amide group of Asn-57 and the heme propionate side chain a water molecule forms a bridge by two hydrogen bonds (27); replacement of Asn-57 with Ile abolishes this side-chain hydrogen-bond interaction. Although the data are insufficient to reveal the reasons for this dramatic thermostabilization, one reasonable explanation may be that Asn-57, a helix-breaking residue, is replaced by Ile, a helix-forming residue (33). The amino acids that can arise from Asn-57 by single base-pair substitutions include Tyr, Ser, Thr, Asp, Ile, Lys, and His; of these, Ile, Lys, and His are clearly better helix formers than Asn (33). In addition, of these three replacements, Ile is the one most frequently found in the middle of an α-helix (34), such as at position 57 in iso-1-cytochrome *c*. Another possible reason for the high thermostability may simply be the greater hydrophobicity of Ile relative to Asn. The difference in free energy changes on transferring the Ile and Asn side chains from ethanol to H_2O is 3 kcal/mol (35), a difference that could account for approximately 70% of the increase in ΔG°. These hypotheses may be tested by examining iso-1-cytochromes *c* with other amino acid replacements at position 57 and other sites.

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