Mol Gen Genet (1995) 249:155–161

ORIGINAL PAPER

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Enhanced stability in vivo of a thermodynamically stable mutant form of yeast iso-1-cytochrome *c*

Received: 29 March 1995 / Accepted: 20 June 1995

Abstract Previous work has established that the N57I amino acid replacement in iso-1-cytochrome *c* from the yeast *Saccharomyces cerevisiae* causes an unprecedented increase in thermodynamic stability of the protein in vitro, whereas the N57G replacement diminishes stability. Spectrophotometric measurements of intact cells revealed that the N57I iso-1-cytochrome *c* is present at higher than normal levels in vivo. Although iso-1-cytochrome *c* turnover is negligible during aerobic growth, transfer of fully derepressed, aerobically grown cells to anaerobic growth conditions leads to reduction in the levels of all of the cytochromes. Pulse-chase experiments carried out under these anaerobic conditions demonstrated that the N57I iso-1-cytochrome *c* has a longer half-life than the normal protein. This is the first report of enhanced stability in vivo of a mutant form of a protein that has an enhanced thermodynamic stability in vitro. Although the N57I protein concentration is higher than the normal level, reduced growth in lactate medium indicated that the specific activity of this iso-1-cytochrome *c* in vivo is diminished relative to wild-type. On the other hand, the level of the thermodynamically labile N57G iso-1-cytochrome *c* was below normal. The in vivo levels of the N57I and N57G iso-1-cytochrome *c* suggest that proteins in the mitochondrial intermembrane space can be subjected to degradation, and that this degradation may play a role in controlling their normal levels.

Key words Cytochrome *c* · Protein stability · Protein degradation · Mitochondria · *Saccharomyces cerevisiae*

Communicated by D. Y. Thomas

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Introduction

The control of gene expression can involve modulation of both the rate of synthesis, which is determined by transcription and translation levels, and the rate of degradation, which determines the half-life of the protein. Although the steady-state level of iso-1-cytochrome *c* in the yeast *Saccharomyces cerevisiae* is primarily dependent on the rate of transcription, in this study we demonstrate that the level is also influenced by its rate of degradation. Mutant forms of iso-1-cytochrome *c* can be present at either above or below the normal cellular level, depending on their relative in vivo stabilities.

Iso-1-cytochrome *c* has been extensively studied, and the levels and functional changes associated with numerous amino acid replacements are known (Hampsey et al. 1986, 1988; Hickey et al. 1992). Almost all of the altered iso-1-cytochromes *c* are less stable than or as stable as, the normal protein; this holds for those having various replacements for the normal residues K32 (Das et al. 1988; Hickey et al. 1988); P76 (Ernst et al. 1985; Ramdas et al. 1986) and F87 (Pielak et al. 1985; Liang et al. 1988; Louie et al. 1988; Louie and Brayer 1989), and those having certain multiple replacements (Dumont et al. 1990; Liggins et al. 1994). G11S, G33S, and H38P are three replacements of particular interest that result in nonfunctional iso-1-cytochrome *c* (Hampsey et al. 1986, 1988) and whose function is restored by the single amino acid replacement N57I (Das et al. 1989; Berroteran and Hampsey 1991). Thus the N57I replacement serves as a "global suppressor" (Shortle and Lin 1985) counteracting unrelated defects at three different sites in the molecule. Furthermore, an unprecedented increase in thermodynamic stability was observed when the N57I replacement was introduced into iso-1-cytochrome *c* by site-directed mutagenesis. Examination of the N57I, N57A, and normal iso-1-cytochrome *c* revealed a high correlation between

differences in free energy changes and transfer free energies, suggesting that hydrophobic interactions are the main factor in the enhanced stability of the N57I iso-1-cytochrome *c* (Hickey et al. 1991a; Linske-O'Connell et al. 1995). On the other hand, the N57G replacement greatly reduced thermodynamic stability. The enhanced and diminished thermodynamic stabilities in vitro of the N57I and N57G iso-1-cytochrome *c*, respectively, provide an opportunity to investigate properties of such proteins in vivo. Although the turnover of iso-1-cytochrome *c* was found to be negligible during aerobic growth, transfer of fully derepressed cells to anaerobic conditions and glucose medium resulted in the rapid depletion of all cytochromes. Pulse-chase experiments conducted under these conditions allowed us to determine the half-lives of normal and mutant forms of iso-1-cytochrome *c*. We show that N57I iso-1-cytochrome *c* occurs at higher than normal levels in yeast, and that this higher level is due to an increased half-life in vivo. The thermodynamically labile N57G iso-1-cytochrome *c* was found to be present below in normal concentration. However, the specific activity in vivo, as measured by growth in lactate medium, a nonfermentable medium, was below normal for all mutants, including the N57I mutant that contains an above normal level of iso-1-cytochrome *c*.

Materials 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 *S. cerevisiae*. The major haploid strains used in this study have been described by Hickey et al. (1991a) and are listed in Table 1. The diploid strains constructed from these haploid strains are also presented in Table 1.

Low-temperature spectrophotometric recordings of intact cells

The levels of cytochromes $a{\cdot}a_3$, *b*, *c* and c_1 were estimated by absorbancy recordings of intact cells at −196° C, using an Aviv model 14 spectrophotometer as described by Hickey et al. (1991b). Cells transferred from the aerobic, derepressed state to anaerobic conditions were collected at various times by centrifugation, blotted dry, mounted on a sample holder having a 1 mm path length, frozen in liquid nitrogen, and immersed in liquid nitrogen within an unsilvered Dewar flask aligned in the spectrophotometer.

For comparative determinations of cytochrome *c* levels in strains with different amino acid substitutions, yeast were grown for 3 days at 30° C on plates containing ethanol medium (Sherman 1991). Cells were scraped from the plates, blotted dry, and similarly prepared for spectrophotometric measurements. The relative intracellular amounts of iso-1-cytochrome *c* were estimated from the heights of the c_z band after baseline corrections.

Anaerobic growth

Studies of the fall in cytochrome levels during anaerobic growth were carried out with yeast first grown to stationary phase in ethanol medium (Sherman 1991) and subsequently inoculated into high-glucose medium (1% Difco-yeast extract, 2% Difco-peptone and 10% glucose) to a density of 4×10^7 cells per ml. This medium was previously made oxygen-free by flushing the system for approximately 12 h with nitrogen that-was purified with an Oxiclear gas purifier (Lab Clear); the medium was subsequently maintained anaerobic throughout the experiment with a constant flow of nitrogen gas.

Pulse-chase labelling of cells and immunoprecipitation of cytochrome *c*

Pulse-chase experiments were conducted with yeast first grown aerobically under derepressed conditions and subsequently transferred to an anaerobic growth condition. Cells grown to stationary phase in 600 ml of ethanol medium were washed twice and resusp-

Table 1 Properties of strains and altered iso-1-cytochrome *c*

Diploid strains	Haploid strains MATa × MATα[a]	Pertinent genotype	Replacements	ΔΔG^0 (kcal/mol)[b]	Level of isol-1[c]	Growth curves[d]
B-8419	B-7553 × B-2350	*CYC1*$^+$ × *CYC1-239-N*	None	–	100%	A
B-8420	B-7553 × B-3947	*CYC1*$^+$ × *cyc1-Δ*	None	0.0[e]	50%	B
B-8421	B-7907 × B-3947	*cyc1-899* × *cyc1-Δ*	N57I C107A	6.2	70%	C
B-8423	B-7725 × B-3947	*cyc1-833* × *cyc1-Δ*	N57A C107A	3.9	50%	D
B-8422	B-7906 × B-3947	*cyc1-898* × *cyc1-Δ*	N57G C107A	0.9	30%	E
B-8424	B-7626 × B-3947	*cyc1-811* × *cyc1-Δ*	N57I	4.3[e]	55%	F
B-8225	B-7706 × B-3947	*cyc1-820* × *cyc1-Δ*	C107A	2.9	50%	G

[a] The isogenic MATa haploid strains, the altered iso-1-cytochromes *c* and their stabilities (ΔΔG^0) have been described by Hickey et al. (1991a). *cyc1-Δ* denotes a deletion encompassing the *CYC1* locus and flanking regions. *CYC1-239-N* contains a normal iso-1-cytochrome *c*. The diploid strains were constructed by crossing each of the isogenic haploid strains to B-3947 (*cyc1-Δ*). The diploid strains B-8419 and B-8420 are, respectively, homozygous and heterozygous for *CYC1*, and contain, respectively, 100% and 50% of the normal diploid level of iso-1-cytochrome *c*

[b] The ΔΔG^0 values are from Hickey et al. (1991a)

[c] The levels of iso-1-cytochrome *c* were estimated from the spectrophotometric recordings shown in Fig. 1 and are expressed as percentages of the normal diploid level estimated for strain B-8419

[d] The growth curves are shown in Fig. 4

[e] Value derived from iso-1-cytochrome *c* with the C107 residue modified by methyl methanethiosulfonate. Guanidine denaturation studies demonstrated that the modified C107 iso-1-cytochrome *c* is about 1 kcal/mol less stable than the C107A and unmodified protein (Hickey et al. 1991a)

ended to a final volume of 40 ml in semisynthetic sulfate free medium (Reid 1983), lacking yeast extract and containing 2% ethanol. The cells were subsequently incubated at 30° C for 40 min. [^{35}S]methionine (1300 Ci/mmole, Amersham) was added to a final concentration of 0.125 Ci/ml, and the cells were labelled for 10 min, followed by a chase period in a solution of methionine at a final concentration of 30 mM. Washed cells were then inoculated to a final density of 4×10^7 per ml into the anaerobic system described above. The effectiveness of the addition of unlabeled methionine in terminating labeling was confirmed by demonstrating that trichloroacetic acid-precipitable counts did not increase in the cultures during the chase period. Because there is no cytochrome *c* synthesis during the anaerobic chase period, reutilization of the radiolabel does not have to be considered.

The procedure for breaking cells followed that of Yaffe and Shatz (1984). After various periods of anaerobic incubation, 40 ml portions of the culture were removed, the cells were washed and resuspended in 20 ml water, and the suspension was added to an equal volume of an ice-cold solution of 0.4 N NaOH and 17% 2-mercaptoethanol. After 10 min on ice, 2 ml of 100% trichloroacetic acid was added to precipitate the cells. After 10 min on ice, samples were centrifuged at $15000 \times g$ for 10 min, the supernatant removed and 10 ml acetone was added. Samples were re-centrifuged at $15000 \times g$ for 10 min. The pellets were rinsed once more in acetone, allowed to dry, and solubilized by boiling for 5 min in a solution of 2% SDS, 0.15 M TRIS-HCl, pH 7.5, 1 mM EDTA, 5% 2-mercaptoethanol and 2 mM phenylmethylsulfonyl fluoride. These solubilized samples were centrifuged at $15000 \times g$ for 5 min and the pellet was discarded.

Immunoprecipitation was carried out as described by Dumont et al. (1990). Each sample was mixed with 10 ml TNET (1% Triton X-100, 0.14 M NaCl, 1 mM EDTA, and 50 mM TRIS-HCl, pH 8.0). The sample was then mixed with 0.45 ml serum and incubated with shaking at 4° C overnight. A fixed, 10% suspension of protein-A coated *Staphylococcus aureus* cells was added and the samples were incubated with shaking at room temperature for 1 h, followed by centrifugation at $1000 \times g$ for 15 min. The cells and bound antigen were washed three times in TNET, once in a solution of 0.1% SDS, 10 mM TRIS-HCl, pH 8.0, 2 mM EDTA, 0.025% sodium azide, then once more in TNET. A total of 0.5 ml of 2 × loading buffer for SDS polyacrylamide gel electrophoresis (4% SDS, 0.125 M TRIS-HCl, pH 6.8, 20% glycerol, 10% 2-mercaptoethanol, and 0.002% bromophenol blue) was added to each sample, and the mixture was incubated in boiling water for 5 min. Portions of the supernatant were loaded onto a 10% SDS polyacrylamide gel optimized for separation of small fragments (Schagger and von Jagow 1987). The antibodies used for immunoprecipitation were prepared essentially as described by Matner and Sherman (1982).

Growth curves

Growth of cells in 10 ml of liquid lactate medium (Sherman et al. 1974) was determined in 125 ml side-arm flasks that were vigorously shaken at 30° C using the conditions previously described (Schweingruber et al. 1979). Culture turbidity was measured with a Klett-Summerson colorimeter containing a No. 2 (560–650 nm) light filter. More accurate cell densities were determined with a Coulter counter (Model ZM, with a 256 Coulter channelizer) and are expressed as cells per ml.

Results

Levels of iso-1-cytochrome *c* in vivo

Studies in vivo were carried out with a series of isogenic diploid strains, listed in Table 1, that were constructed by crossing each of the isogenic haploid strains to the haploid strain B-3947 lacking iso-1-cytochrome *c*. More consistent results can be obtained with the diploid series because interfering recessive mutations, possibly arising during manipulation of the haploid strains, are not manifested in the diploid strains. In addition to replacements of N57, the haploid series contained iso-1-cytochrome *c* with a C107A replacement, which was originally introduced to prevent dimerization in vitro, and is required for convenient measurement of thermodynamic parameters (Hickey et al. 1991a).

The relative absorption spectra of the cytochromes in an isogenic series of yeast mutants having various amino acid substitutions at positions 57 and 107 are compared in Fig. 1. Curve A corresponds to a diploid *CYC1*$^+$/*CYC1*$^+$ homozygous strain, whereas curve B corresponds to the *CYC1*$^+$/*cyc1-Δ* heterozygous strain; the heights of the C_α peaks in curves A and B, represent convenient 100% and 50% standards, respectively, for comparison of cytochrome *c* levels. Curve C, corresponding to the *cyc1-899*/*cyc1-Δ* heterozygous strain having the N57I C107A replacement (Table 1),

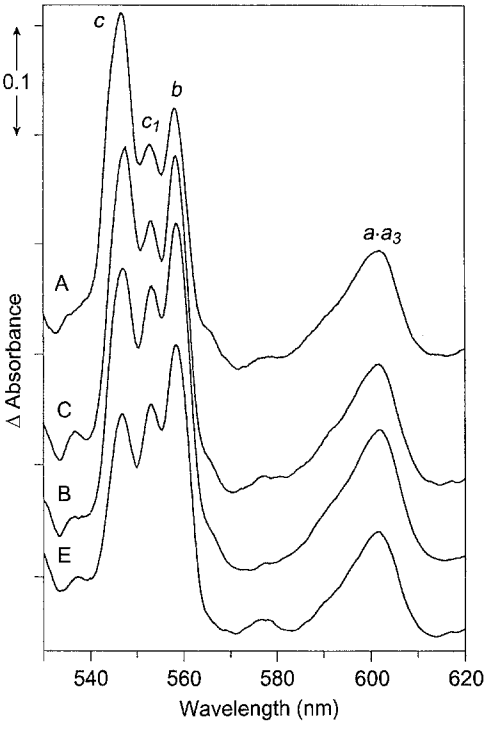

Fig. 1 Low-temperature (−196° C) spectrophotometric recordings from a series of isogenic and heterozygous diploid strains. The α-peaks of cytochromes $a \cdot a_3$, *b*, c_1 and *c* are located, respectively, at 602.5, 558.5, 553.3 and 547.3 nm. The diploid strains were constructed by crossing the two series of isogenic haploid strains described in Table 1. The following strains are shown: curve A, 100% standard (B-8419); curve B, 50% standard (B-8420); curve C, N57I C102A (B-8421); and curve E, N57G C102A (B-8422). Because the B-8421–B-8225 diploid strains are heterozygous, they should be compared to the normal heterozygous strain B-8420

has a c_α peak higher than that for the *CYC1*$^+$/*cyc1-Δ* standard, indicating that *cyc1-899* produces an above normal level of cytochrome *c* in vivo. Furthermore, quantitative analysis of peak heights indicates that *cyc1-899/cyc1-Δ* strains contain approximately 70% of the normal *CYC1*$^+$/*CYC1*$^+$ amount (Table 1); thus, the *cyc1-899* allele is producing approximately 140% of the normal *CYC1*$^+$ level. Similar analyses established that the single N57I replacement caused a slight increase in the amount of iso-1-cytochrome *c*, whereas the single C107A replacement and the double N57A C107A replacements did not produce any observable change (Table 1). The *cyc1-898/cyc1-Δ* heterozygous strain having the N57G C107A replacement (Curve E) contained approximately 30% of the diploid level Table 1), which is below the expected 50% level. These observations were made not only in the diploid strains presented in Fig. 1, but also in the corresponding haploid strains (data not presented). These levels reflect the previously reported thermostabilities of each protein (Hickey et al. 1991a; Linske-O'Connel 1995), in that an increased or decreased thermostability relative to wild type, leads to an increase or decrease in the in vivo level, respectively. As expected the amounts of cytochromes $a{\cdot}a_3$, *b*, and c_1, remained approximately normal in these diploid and haploid strains.

Cytochrome levels under repressed conditions

Because the N57I C107A iso-1-cytochrome *c* is more stable than the wild-type in vitro, the simplest explanation for the increased level in vivo is diminished degradation. Attempts to examine turnover by pulse-chase experiments on actively growing cells under derepressed conditions revealed long half-lives that were technically difficult to quantitate. However, half-lives could be measured by pulse-chase experiments after transferring fully derepressed cells to a highly repressive anaerobic condition with a high glucose concentration in the medium (see below). This switch from obligatory aerobic respiration to obligatory anaerobic fermentation halts further synthesis of iso-1-cytochrome *c* and many other mitochondrial proteins (Jayaraman et al. 1966; Chapman and Bartley 1968). The fall in the levels of all cytochromes was observed when fully derepressed cells were transferred to repressive conditions, i.e., anaerobic growth in high glucose medium, as seen in Fig. 2. In fact, levels of all of the cytochromes diminished more rapidly than could be accounted for by simple dilution of growing cells. These results, and the fact that transcription ceases under these repressive conditions, indicated that the cytochromes were being degraded.

Although there was a slight difference in the cytochrome levels between the wild-type and N57I C107A strains at time zero, when the yeast were transferred from aerobic to anaerobic conditions, the decrease in levels of cytochromes *b*, c_1 and $a{\cdot}a_3$ proceeded at similar rates. It is significant that the rate of depletion of iso-1-cytochrome *c* was greater in the normal strain (Fig. 2A) than in the N57I C107A mutant (Fig. 2B).

Degradation of iso-1-cytochrome *c* in vivo

Isogenic strains containing wild-type and N57I C107A iso-1-cytochrome *c*, respectively, grew identically under anaerobic conditions (Fig. 3A). Turnover of iso-1-cytochrome *c* in both strains is indicated in Fig. 3B and 3C. Most importantly, within the 24 h period of the measurements, the wild-type iso-1-cytochrome *c* (Fig. 3B) underwent a more rapid degradation than the N57I C102A iso-1-cytochrome *c* (Fig. 3C). Clearly, wild-type iso-1-cytochrome *c* was degraded within 16 h after shifting from aerobic to anaerobic conditions, whereas, the N57I C102A iso-1-cytochrome *c* still persisted 24 h after the transfer. Thus, by comparing the

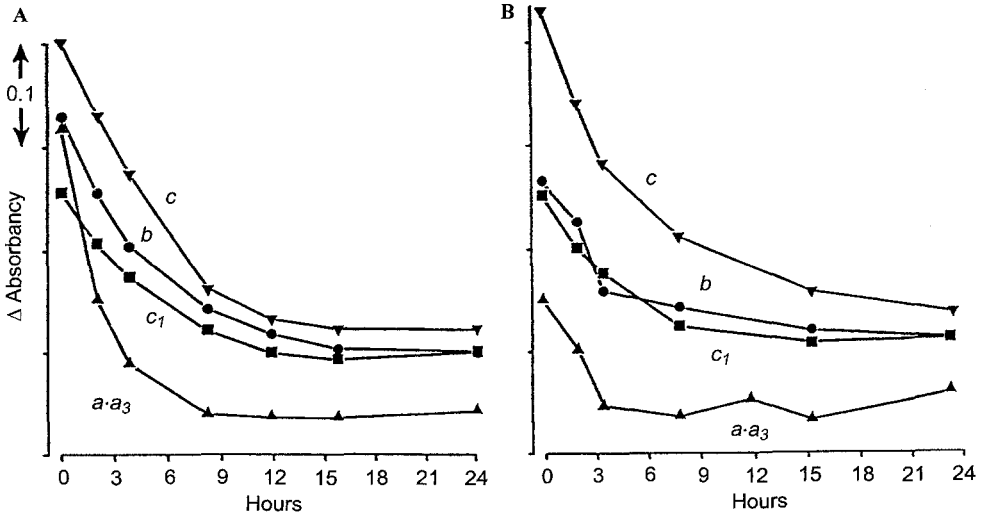

Fig. 2A, B The relative amounts of the cytochromes $a{\cdot}a_3$, *b*, c_1 and *c* after various periods of anaerobic growth. **A** Normal strain B-7553. **B** N57I C102A mutant strain B-7907

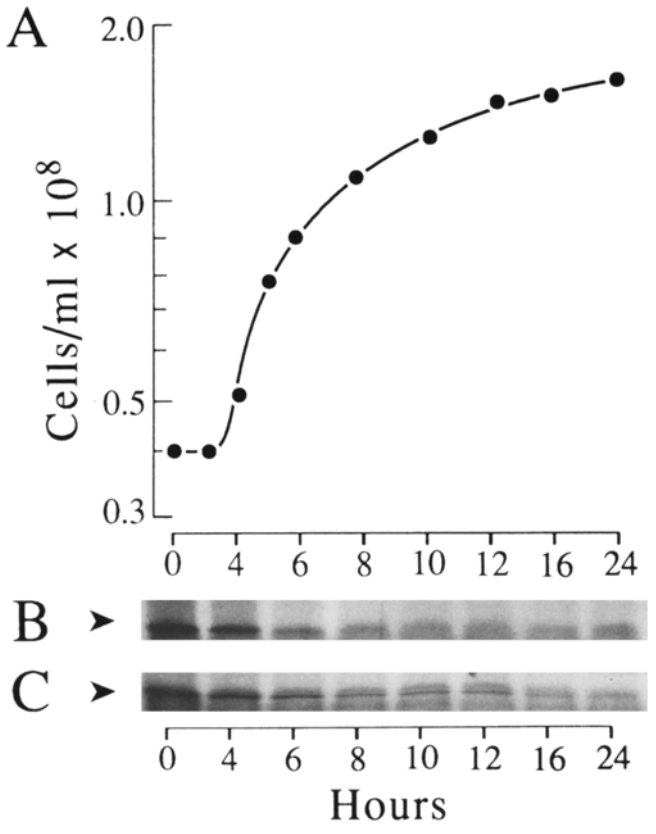

Fig. 3A–C Anaerobic growth of the normal strain B-7553, and the N57I C102A mutant strain B-7907, with the corresponding levels of [^{35}S]methionine-labelled iso-1-cytochrome *c*. **A** Anaerobic growth curve of the normal strain B-7553; an identical growth curve was obtained with the N57I C102A mutant strain B-7907. For **B** (B-7553) and **C** (B-7907), total immunoprecipitated iso-1-cytochrome *c* was extracted from cells grown for the corresponding times indicated in **A**. The *arrows* indicate the positions of iso-1-cytochrome *c*

amounts of labeled protein in the pulse chase experiment at various time points (Fig. 3B and 3C), and by following the decrease in level of iso-1-cytochrome *c* (Fig. 2A and 2B), we can conclude that wild-type iso-1-cytochrome *c* has a shorter half life than the N57I C102A iso-1-cytochrome *c*. Under the shift from aerobic to anaerobic conditions, the wild-type iso-1-cytochrome *c* had a half life of less than 6 h, whereas the N57I C102A iso-1-cytochrome *c* had a half life of approximately 7.5 h.

Activities of iso-1-cytochrome *c* in vivo

The overall in vivo functional activities of mutant iso-1-cytochrome *c* were assessed by comparing the growth of isogenic diploid strains in lactate medium (Fig. 4), a medium that is particularly suitable for revealing cytochrome *c* activity (Sherman et al. 1974). All mutants with single or double replacements of iso-1-cytochrome *c* exhibited diminished growth. Most

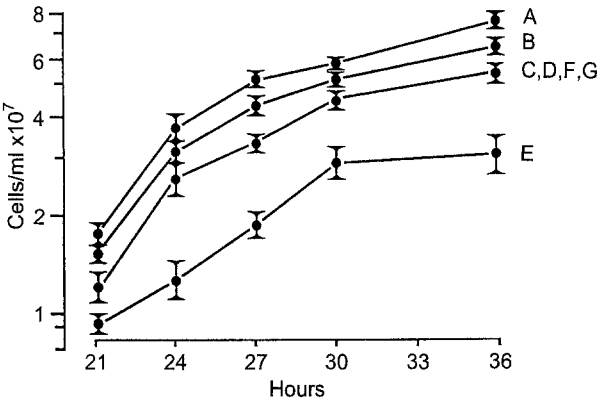

Fig. 4 Time course of growth in lactate medium of the isogenic and heterozygous diploid strains described in Table 1. A 100% standard (B-8419); B, 50% standard (B-8420); C, N57I C102A (B-8421); D, N57I C102A (B-8423); E, N57G C102A (B-8422); F, N57I (B-8424); and G, C102A (B-8425). The late logarithmic and early stationary phase, shown in the figure, is the most sensitive phase of growth for revealing differences

importantly, the mutant with N57I C107A iso-1-cytochrome *c* grew at a lower rate than the normal strain in lactate medium, even though the mutant contained a larger amount of iso-1-cytochrome *c* in vivo, and even though this N57I C107A iso-1-cytochrome *c* was more stable in vitro.

Discussion

In this paper, we have shown that the in vivo level of yeast iso-1-cytochrome *c* is related to its in vitro thermodynamic stability. The N57I replacement considerably enhances thermodynamic stability (Table 1; Hickey et al. 1991a) and causes an approximately 1.5-fold increase in the in vivo protein level (Table 1; Fig. 1). An increased level of N57I iso-1-cytochrome *c* was also reported by Berroteran and Hampsey (1991). On the other hand, the N57G replacement diminished both the thermodynamic stability in vitro and steady-state concentration in vivo (Table 1; Fig. 1). Pulse-chase experiments established that the enhanced level of the N57I C107A iso-1-cytochrome *c* in vivo was due to diminished degradation. Although it is well known that certain mutationally altered and presumably labile proteins, such as the N57G and numerous other iso-1-cytochromes *c* are more rapidly degraded in vivo (Goldberg et al. 1976), enhanced levels of thermodynamically stable proteins have not been previously reported. Our findings constitute the first report of a correlation between in vivo level and thermodynamic stability in vitro. The results with the N57I, N57 and N57G iso-1-cytochrome *c* suggest that proteins with above and below normal stability are degraded and that this degradation plays a role in determining their normal steady-state levels. It would be of interest to examine the cellular levels of other proteins that are

abnormally stable in vitro, including staphylococcal nuclease (Shortle and Lin 1985), λ Cro (Pakula and Sauer 1989a, b), α-amylase (Declerck et al. 1990; Suzuki et al. 1989; Joyet et al. 1992), kanamycin nucleotidyltransferase (Matsumura et al. 1986; Liao et al. 1986), and T4 lysozyme (Alber and Wozniak 1985; Matsumura et al. 1989).

The diminished growth rate in lactate medium indicates that the thermostable N57I C107A iso-1-cytochrome *c* has a lower specific activity in vivo (Fig. 4), and reveals why this particular protein has not replaced the normal form during evolution. The shift in the redox potential of the protein from 275 mV to 220 mV (Langen et al. 1992; Komar-Panicucci et al. 1992) is undoubtedly responsible for its suboptimal function.

Although the system responsible for degrading normal and mutant forms of iso-1-cytochrome *c* in the mitochondrial intermembrane space has yet to be defined, the mitochondrial proteolytic activities observed in mammalian (Duque-Magalhães and Ferreira 1980; Desautels and Goldberg 1982) and yeast (Zubatov et al. 1984) cells may be involved. The differential susceptibilities of the altered iso-1-cytochromes *c* suggest that the turnover may not be operating by lysosomal degradation of entire mitochondria (Topping and Travis 1974; Allan and Welman 1980; Glaumann et al. 1975). Also, the presumably cytosolic degradation system acting on apo-iso-1-cytochrome *c*, but not apo-iso-2-cytochrome *c*, does not act preferentially on holo forms of cytochrome *c* that differ in thermodynamic stability (Dumont et al. 1990; Liggins et al. 1994). Furthermore, the yeast ATP-dependent protease Pim1, which is located in the mitochondiral matrix (Van Dyck et al. 1994; Suzuki et al. 1994), would not be expected to act on proteins located in the intermembrane space. Furthermore, disruption of *PIM1* did not affect the degradation of a labile form of iso-1-cytochrome *c* (Pearce and Sherman 1995). Although the Yta10 protease, which degrades incompletely synthesized polypeptides in the mitochondrial inner membrane (Pajic et al. 1994), might be suggested to be the protease acting on cytochrome *c*, disruption of *YTA10* also did not affect the degradation of a labile form of iso-1-cytochrome *c*. Furthermore, disruption of the related mitochondrial proteases encoded by *YTA11* and *YTA12* (Schnall et al. 1994), also designated *YME1* (Thorsness et al. 1993) and *RCA1* (Tzagoloff et al. 1994), respectively, did not affect degradation of a labile cytochrome *c* (Pearce and Sherman 1995). Thus the protease responsible for the degradation of cytochrome *c* has yet to be identified.

We also demonstrated that transfer of yeast from aerobic to anaerobic conditions facilitates a rapid fall in the levels of cytochromes $a{\cdot}a_3$, *b* and c_1. Although, not tested by pulse chase labelling of any of these proteins, we suggest that all cytochromes are degraded upon transition from oxidative to fermentative growth as depletion was more rapid than simple dilution of growing cells. As all cytochromes are degraded, it is possible that despite cytochrome *c* being localised in the intermembrane space, and cytochromes $a{\cdot}a_3$, *b* and c_1 in the inner membrane, a coordinated regulation of degradation occurs. It remains to be seen whether any of the above mentioned Yta proteases are involved in this degradation.

The evolutionary constraints determining the formation and maintenance of the normal protein with its specific stability must find a balance between optimal steady-state level and activity. Nevertheless, of all of the eukaryotic cytochromes *c* that have been investigated, iso-1-cytochrome *c* and iso-2-cytochrome *c* are by far the most labile (Polastro et al. 1976). Given the degradation of mitochondrial proteins during repression, we suggest that the lability of the normal iso-1-cytochrome *c* is evolutionarily maintained in order to promote degradation, a condition that may be beneficial to *S. cerevisiae* during transitions between repressed and derepressed conditions.

Acknowledgements We thank Dr. Mark Dumont (University of Rochester Medical School) for useful discussions and suggestions. This investigation was supported by grant RO1 GM12702 from the National Institute of Health.

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