LETTERS

Lack of Evolutionary Conservation at Positions Important for Thermal Stability in the Yeast ODCase Protein

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Mutations destabilizing the spatial structure of proteins can persist in populations if they are fixed by drift or compensated by other mutations. The prevalence and dynamics of these processes remain largely unrecognized. A suitable target to screen for both deleterious and compensatory mutations is the *URA3* gene in yeast. We identified 13 positions in which a single missense substitution causes substantially strong thermal sensitivity. We then applied mild mutagenesis resulting in roughly one base substitution per gene and found that only reversions to an original amino acid can compensate for the thermal instability. However, the 13 positions are not visibly conserved across 53 species of Ascomycota, despite that the gene product is an enzyme of stable function and high efficiency. This shows how much fitness penalties for amino acid substitutions are background dependent, underscoring the role of complex intragenic interactions in the evolution of proteins.

A large fraction of amino acid substitutions are deleterious to fitness because they result in a substantial decrease or increase of protein stability (Pakula and Sauer 1989; Chiti et al. 2003; Pál et al. 2006; Drummond and Wilke 2008). However, a simple model assuming effective purging of all mutations altering optimal stability is probably insufficient to explain the observed variation in coding regions of DNA. Extant proteins appear to contain large numbers of mutations that have been subject to positive selection, even if their primary function was to compensate for damage instead of contributing new adaptations (Fay et al. 2002; Bazykin et al. 2004; Eyre-Walker 2006). Moreover, adaptations to one environment may initiate compensatory adjustments to other environments (MacLean et al. 2004). It has been proposed that evolution of proteins can be, to a large extent, governed by specific cycles in which a substitution leading to an excessive increase or decrease of stability is followed by a number of substitutions with an opposite, and thus compensatory, effect (DePristo et al. 2005; Poon et al. 2005; Camps et al. 2007). Fixation of the initially destabilizing mutation is facilitated by strong drift or functional advantage associated with the change of structure. The latter is best exemplified by mutations providing resistance to antibiotics (Björkman et al. 2000; Maisnier-Patin et al. 2002). The question arises whether substantial amounts of within-protein compensatory interactions can accumulate in proteins in which functional shifts were rare or absent.

We studied the yeast *URA3* gene coding for orotidine 5′-phosphate decarboxylase (ODCase). This enzyme is responsible for the synthesis of uridine 5′-phosphate, an essential precursor of RNA and DNA. Not only its catalytic function but also turnover, pH optimum, and susceptibility to inhibitors are similar in organisms that are as phylogenetically distant as *Escherichia coli* and *Saccharomyces cerevisiae* (Harris et al. 2000). It was found that active yeast ODCase converts 5-fluoro orotic acid (5-FOA) into a toxic compound. This is helpful in screening for loss of function mutations in *URA3* because only mutants can grow on media containing 5-FOA (Boeke et al. 1984). We focused on mutations leading to thermal sensitivity (ts) because this marks a decrease in the stability of the protein's 3D structure (Chakshusmathi et al. 2004).

We used the BY 4742 *ura3Δ* strain with *URA3* residing on a plasmid. Our experiments consisted of two phases (see Materials and Methods, Supplementary Material online, for full description). First, we screened for ts mutations in the *URA3* locus, *ura3(ts)*. We did it by looking for mutants able to grow on medium supplied with 5-FOA (and uracil) at 37 °C but not at 23 °C. This required that the Ura3 protein was inactivated only at the higher temperature. Among mutants meeting this criterion, 14 contained a single amino acid substitution in one of 13 different positions (table 1 and fig. 1). These mutants were used in the second phase of the experiment, that is, a screen for mutations compensating the ts effect. This meant that an ability to grow at 37 °C on media lacking uracil was restored. Several such mutants were found for every *ura3(ts)* allele. Analysis of the DNA sequence showed that in each of them the amino acid substitution causing thermal sensitivity was absent, that is, replaced by the original amino acid (table 1). We conclude that only reversion of the ts mutations could provide efficient compensation for thermal sensitivity. We regard the screen as saturated because the compensatory mutations were found always and exactly at the ts position although they could have happened at every other one.

We also looked for imperfect or partial compensation. We used the same 14 *ura3(ts)* mutants in a similarly intense mutagenesis and screen for compensation at 30 °C. The phenotypes were usually nonreproducible, indicating that the difference in stability of mutant proteins between 23 and 30 °C was too small. When loss of thermal sensitivity at 30 °C was reproducible, it was always associated with reversion to the original amino acids. These results show that genetic screens tend to reveal mutants with rather strong phenotypic effects. It cannot be excluded that if we were able to find less damaging mutations then the spectrum of compensatory substitutions would be richer than simple reversions.

Thermal sensitivity prevents growth at high temperatures and often slows it down even at permissive ones. This poses fitness costs and therefore we asked whether the 13

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Table 1
Thermally Sensitive (ts) Mutations in the Budding Yeast's ODCase

Ts Mutation[a]	Secondary Structure[b]	ConSurf Score[c]	Substitutions in Other Ascomycota[d]
H13**R**	Other	6	A(21), E(3), H(9), K(3), L(2), P(2), Q(1), **R**(1), S(5), T(5)
M25**K**	α Helix	5	A(1), E(4), F(15), I(1), **K**(1), L(6), M(9), P(1), R(3), S(1), V(3), Y(5)
T30**A**	Other	5	**A**(6), C(1), E(10), K(9), L(4), M(6), N(2), Q(1), R(1), S(3), T(8)
L32**S**	β Strand	7	A(3), C(2), D(1), K(14), L(14), N(7), Q(2), R(2), **S**(2), T(2)
L45**P**	α Helix	6	A(1), D(1), E(5), F(6), I(2), K(14), L(12), N(1), R(1), S(1), T(7)
V74**D**	α Helix	5	**D**(6), E(12), F(2), G(1), I(3), L(3), P(2), S(3), T(11), V(8), Y(1)
S81**F**	α Helix	6	A(16), E(3), G(2), H(1), K(6), L(8), P(1), S(7), T(6), V(1), Y(1)
Y105**D**	α Helix	8	**D**(1), G(1), I(5), K(19), N(2), Q(8), S(1), T(3), V(1), Y(10)
I118**N**	β Strand	9	A(23), D(7), I(9), K(1), **N**(2), Q(1), S(1), T(3)
I118**S**	β Strand	9	A(23), D(7), I(9), K(1), N(2), Q(1), **S**(1), T(3)
R145**G**	Other	3	A(6), C(1), E(3), **G**(4), K(1), L(2), N(1), P(12), R(11), S(2), T(2), Y(1)
A172**V**	α Helix	5	A(16), D(5), E(4), K(3), L(3), R(2), S(5), T(4), **V**(1), Y(1)
R242**G**	Other	4	D(3), F(7), **G**(11), I(10), (l), P(2), Q(1), R(10), T(1), V(2)
Y251**S**	α Helix	4	A(11), D(2), E(4), G(4), L(2), P(2), Q(1), R(11), **S**(1), T(1), Y(11)

[a] Mutation description: original amino acid (underlined), position within the protein, substitution leading to thermal sensitivity (bold).
[b] The budding yeast ODCase is composed of α helix (42%), β strand (15%), turns (11%), and other structures (32%).
[c] ConSurf rank 1 refers to least conserved, 9 to most conserved positions (Landau et al. 2005).
[d] Numbers in parentheses indicate the number of species with the specified amino acid.

positions are evolutionarily conserved. We compared the amino acid sequences of ODCase available in the SwissProt database for 53 species of Saccharomycotina and Pezizomycotina, the two best studied and closely related subphyla of Ascomycota (Fitzpatrick et al. 2006). The 3D structure of the yeast ODCase has been resolved (Miller et al. 2000); therefore, we applied the ConSurf program, which calculates individual conservation scores for all

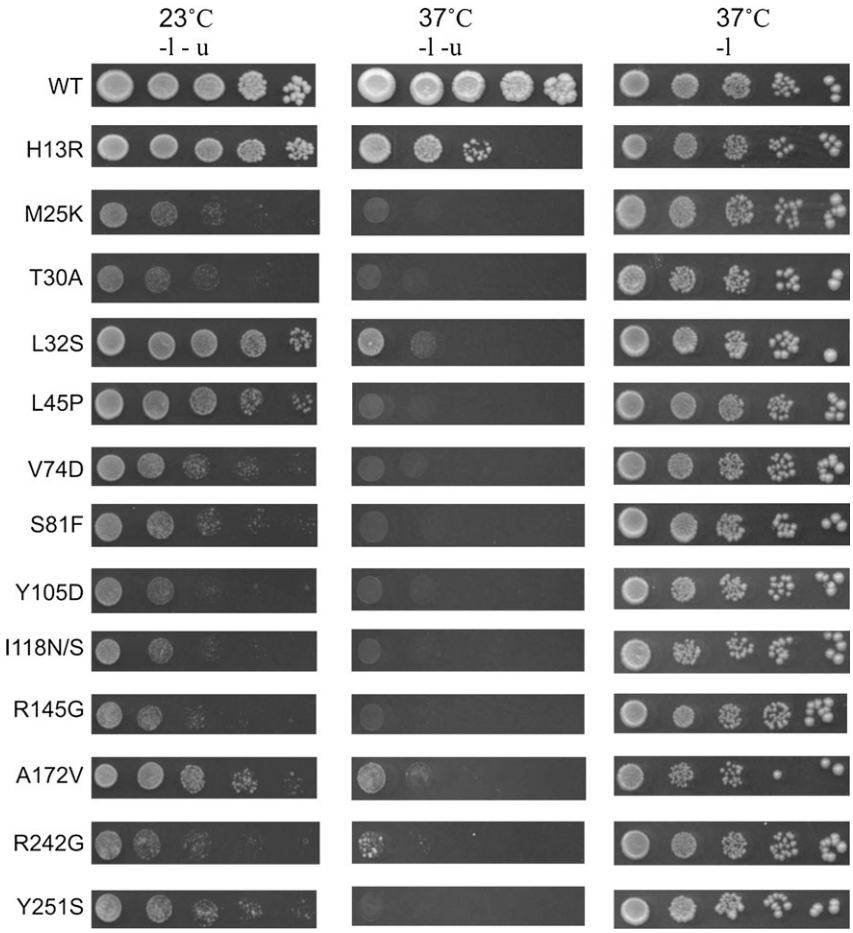

FIG. 1.—Thermally sensitive mutations at *URA3* caused by single base substitutions. Mutation designations include original amino acid, position within the protein, and substitution leading to thermal sensitivity. Series of 10-fold dilutions were prepared and overlaid on synthetic agar media in which leucine or uracil was dropped out, −l and −u, respectively. Absence of leucine stabilized the plasmid. Absence of uracil tested for the ODCase activity. The two columns from the left show that mutants grew better at 23 °C than at 37 °C suggesting thermal sensitivity of Ura3. Indeed, growth at 37 °C was restored when uracil was added (right column).

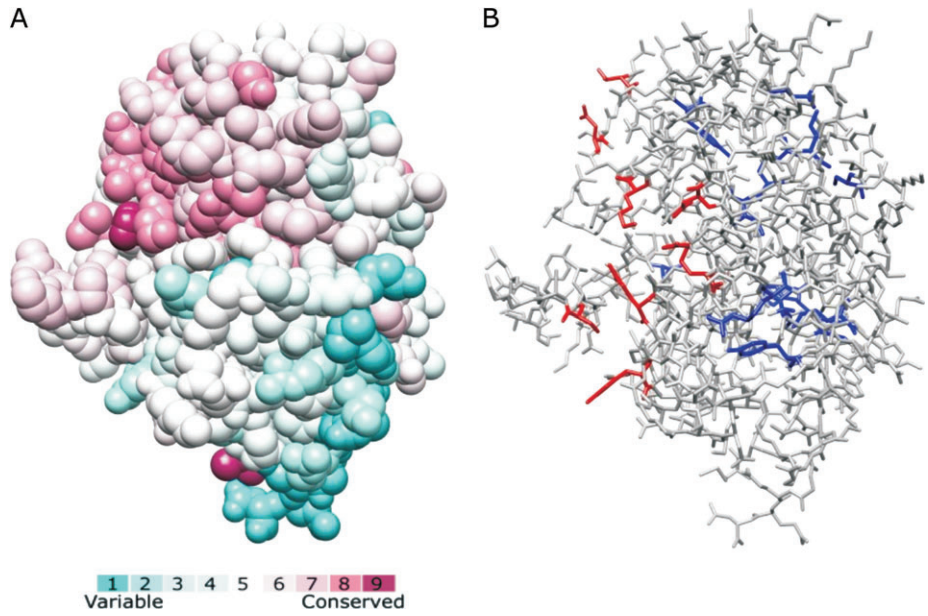

FIG. 2.—Evolutionary conservation across ODCase. A single subunit is shown, the enzyme works typically as a dimer with two active sites (Miller et al. 2000). (*A*) Colors quantify variation among 53 species of Saccharomycotina and Pezizomycotina. Conservation scores are converted into ranks and presented on a color scale: The least variable residues are dark blue (rank 1), the most variable are dark purple (rank 9). Evolutionary conservation was estimated using the ConSurf program, which calculates conservation for every amino acid using phylogenetic methods (Landau et al. 2005). (*B*) Positions of the mutant sites studied here (blue) and the residues critical for enzymatic function of the ODCase (red).

residues and overlays them on the spatial model of the query protein (Landau et al. 2005). Figure 2 shows that the yeast ODCase exhibits both conserved and variable domains; however, the more conserved residues tend to surround its catalytic center (Miller et al. 2000). Using the 53 sequences, we found that the mean conservation score of the 13 mutant positions was −0.276. The negative score indicates a tendency toward increased conservation, but it is weak and not significantly different from zero, that is, the mean normalized conservation score of the whole protein ($n_1 = 13$, $n_2 = 276$, $t = 0.982$, $P = 0.327$). Not only the mean value but also the frequency distribution of conservation scores of the 13 positions was not different from that of the whole protein (fig. 3). When the analysis was restricted to 28 species of Saccharomycotina, the mean score for the studied mutant positions was lower, −0.462, but again not statistically different from the proteinwide average ($n_1 = 13$, $n_2 = 276$, $t = 1.643$, $P = 0.101$).

We calculated the ratio of nonsynonymous-to-synonymous substitutions, K_a/K_s, for each codon of 53 ODCases using the Selecton program (Stern et al. 2007). The mean K_a/K_s was 1.073. Scores higher than one, suggesting positive selection, were found for 52% of the codons; for 12% of the codons, K_a/K_s was sufficiently high to suggest statistically significant positive selection. Among the ts positions, 7 of 13 had K_a/K_s higher than one, but in all cases, the bias was rather small and nonsignificant. Thus, the ts sites do not show definite signs of positive selection, although this type of selection is likely responsible for a nontrivial share of all base substitutions in *URA3*. This result is not surprising as positive selection for compensatory mutations is believed to operate at sites with moderate rather than large (de)stabilizing effects (Ferrer-Costa et al. 2007).

Table 1 shows 37 cases when a residue causing a strong ts effect in *S. cerevisiae* is present in other Ascomycota, both in Saccharomycotina (15) and Pezizomycotina (22). There is no straightforward explanation for this variation. Fungal populations are reportedly large, which favors purifying selection over drift-mediated fixation. Beneficial shifts of

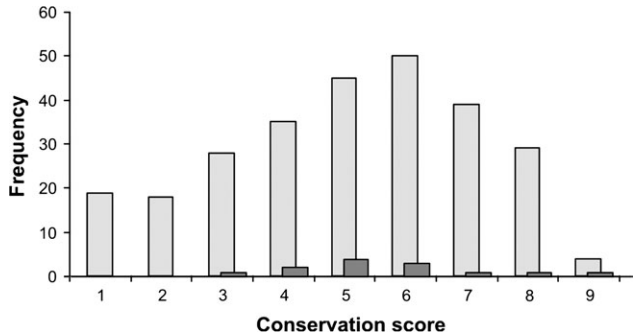

FIG. 3.—Lack of evolutionary conservation of ts mutants. Frequency distribution of conservation ranks at the 13 mutant sites (dark bars) and the whole protein (light bars).

function, counterbalancing the negative effects of destabilization, do not appear likely in an enzyme that has a single substrate and an extreme efficiency (Harris et al. 2000). A more plausible explanation for the observed lack of conservation is that missense mutations were fixed earlier in other codons and facilitated substitutions at the ts positions by decreasing fitness penalties. These would be constraint-relieving mutations. Their relieving effect would be only potential as long as the disturbing mutations were not yet present. On the other hand, there are signs of positive selection in this gene, suggesting that there were also mutations whose beneficial effect, and thus accelerated fixation, depended on earlier destabilizations. Thus, perhaps both constraint-relieving mutations and typical compensatory mutations contributed to relaxation of stabilizing selection at positions important for the structural stability of ODCase.

Supplementary Material

Materials and Methods is available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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