

HIV-1 Protease Catalytic Efficiency Effects Caused by Random Single Amino Acid Substitutions

Mariona Parera, Guerau Fernández, Bonaventura Clotet, and Miguel Angel Martínez

Fundació irsiCaixa, Universitat Autònoma de Barcelona, Barcelona, Spain

Protein evolution has occurred by successive fixation of individual mutations. The probability of fixation depends on the fitness of the mutation, and the arising variant can be deleterious, neutral, or beneficial. Despite its relevance, only few studies have estimated the distribution of fitness effects caused by random single mutations on protein function. The human immunodeficiency virus type 1 (HIV-1) protease was chosen as a model protein to quantify protein's tolerability to random single mutations. After determining the enzymatic activity of 107 single random mutants, we found that 86% of single mutations were deleterious for the enzyme catalytic efficiency and 54% lethal. Only 2% of the mutations significantly increased the catalytic efficiency of the enzyme. These data demonstrate the vulnerability of HIV-1 protease to single random mutations. When a second random mutagenesis library was constructed from an HIV-1 protease carrying a highly deleterious single mutation (D30N), a higher proportion of mutations with neutral or beneficial effect were found, 26% and 9%, respectively. Importantly, antagonist epistasis was observed between deleterious mutations. In particular, the mutation N88D, lethal for the wild-type protease, restored the wild-type catalytic efficiency when combined with the highly deleterious mutation D30N. The low tolerability to single random substitutions shown here for the wild-type HIV-1 protease contrasts with its *in vivo* ability to generate an adaptive variation. Thus, the antagonist epistasis between deleterious or lethal mutations may be responsible for increasing the protein mutational robustness and evolvability.

Introduction

Robustness defined as a protein's tolerance to substitutions is of fundamental importance to understand natural or artificial protein evolution. Recently, it has been shown that mutational robustness can enhance proteins' capacity to evolve (Aharoni et al. 2005; Bloom, Labthavikul, et al. 2006). Protein thermodynamic stability seemed to be responsible for conferring protein robustness (Bloom et al. 2005; Wilke et al. 2005; Bloom, Drummond, et al. 2006; Bloom, Labthavikul, et al. 2006). It has also been claimed that selection might favor proteins that are robust to genetic change (Taverna and Goldstein 2002; Wilke and Adami 2003; Wagner 2005; Pal et al. 2006), which may lead to an increase in selectively neutral variants.

Proteins are to a certain extent tolerant to single mutations. Examples are bacteriophage T4 lysozyme and *Escherichia coli lac* repressor in which 84% and 65%, respectively, of single mutants were functional (Rennell et al. 1991; Markiewicz et al. 1994). We and others have also demonstrated the protein robustness to multiple substitutions (Martinez et al. 1996; Guo et al. 2004; Bloom et al. 2005). Although the former studies have provided important information about protein tolerance to amino acid changes, they may not reflect the protein tolerance to single substitutions because when several mutations are present simultaneously in the same genotype, they interact to determine the overall fitness, a phenomenon also termed epistasis (Phillips et al. 2000). Because the evolution of natural proteins is thought to have occurred by successive fixation of individual mutations (Smith 1970), it may be relevant to explore protein tolerance to single substitutions in order to understand the evolution of natural proteins.

To characterize protein tolerance to single random substitutions, a randomly mutated library of the human immunodeficiency virus type 1 (HIV-1) protease was con-

structed. We then determined the catalytic efficiency of 107 mutants carrying unique single amino acid substitutions. It has been suggested that genotypes of reduced fitness generally experience a higher fraction of mutations with beneficial effect than genotypes of high fitness (Wilke et al. 2003). Thus, a second randomly mutated library from a HIV-1 protease carrying a highly deleterious single mutation (D30N) was generated. Similarly, we analyzed the catalytic efficiency of single random variants. We decided to study the HIV-1 protease for 2 reasons. First, numerous studies have described the HIV-1 protease variability and polymorphisms found in naive or protease inhibitor-treated infected individuals (Wu et al. 2003; Ceccherini-Silberstein et al. 2004), which suggested the high evolvability of this protein. Second, HIV-1 proteins are subjected to a higher mutational burden than cellular proteins due to the error-prone nature of HIV-1 replication. It has been hypothesized that genomes experiencing high mutational burden may face selective pressure to evolve proteins that are more tolerant to change (Guo et al. 2004).

Materials and Methods

Construction of Random HIV-1 Protease Mutation Libraries in Lambda Phage

Two separate error-prone polymerase chain reaction (PCR) mutagenesis protocols that generate complementary mutational spectra were performed. In the first protocol, PCR was carried out in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.5 mM MnCl₂, 1 μM of each oligonucleotide, 5 units *Taq* polymerase (Promega, Madison, WI), and biased deoxynucleoside triphosphate concentrations (30 μM deoxycytidine triphosphate/1 mM deoxythymidine triphosphate/30 μM deoxyadenosine triphosphate/1 mM deoxy-guanosine triphosphate, Promega), as previously described (Vartanian et al. 1996). Cycling parameters were as follows: 50× (95°C, 30 s; 55°C, 30 s; 72°C, 10 min). Input DNA was 1 ng of a pBluescript SK plasmid containing a wild-type HIV-1 protease DNA (HXB2 strain) or a mutated (D30N) HIV-1 protease DNA per 100 μl of reaction. The second PCR mutagenesis protocol was performed with Mutazyme in the GeneMorph kit (Stratagene, La Jolla,

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E-mail: mmartinez@irsicaixa.es.

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Table 1
Mutational Spectra and Mean Mutation Frequencies

	Wild-type HIV-1 Protease ^a		Mutated (D30N) HIV-1 Protease ^b
	<i>Taq</i> (mutagenic buffer and biased dNTPs)	Mutazyme	<i>Taq</i> (mutagenic buffer and biased dNTPs)
A → N, T → N	88.7%	49.2%	92.2%
G → N, C → N	9.5%	42.7%	7.1%
Deletions	1.5%	6.9%	0.7%
Insertions	0.3%	1.2%	0%
Mutation frequency ^c	1.3×10^{-2}	0.83×10^{-2}	0.95×10^{-2}

^a Of the 973 individual protease clones sequenced, 663 were obtained from the *Taq* mutagenic buffer and biased dNTPs protocol and 310 were obtained from the Mutazyme protocol.

^b With mutated (D30N) HIV-1 protease (382 clones sequenced), only one error-prone PCR mutagenesis protocol was employed.

^c Average mutation frequency is the number of mutations in a set of n clones divided by $n \times$ length of target clone (i.e., 297 bp).

NOTE.—dNTP, deoxynucleoside triphosphate.

CA). Mutational spectra are described in table 1. In both protocols the following PCR oligonucleotides were employed: HIVPRLSFNF (sense, 5'-GGG GAATTC TTCCTTAACTTCCCTCAG-3', HXB2 residues 2240–2258; underline indicates an *EcoRI* restriction site) and Xho8R (antisense, 5'-GGGAGGGGCTCGAGTCA AAGGCCATCCATTCTGGC-3', HXB2 residues 2588–2604; underline indicates an *XhoI* restriction site; stop codons are indicated in bold face). The resulting PCR products were digested with *EcoRI* and *XhoI*, isolated, and ligated to lambda DNA (Uni-ZAP XR Vector Kit, Stratagene). The ligations were packaged (Uni-ZAP XR Giagapack Cloning Kit, Stratagene), tittered, and amplified according to standard procedures. The composition of the library was determined by nucleotide sequencing of the encoded HIV-1 protease gene included in individual phage colonies. Phage DNA from individual colonies was PCR amplified and sequenced with the flanking oligonucleotides T3 (5'-AATTAACCCTCACTAAAGGG-3') and T7 (5'-TCGAGGTCGACGGTATC-3') using the ABI PRISM dRhodamine terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequence alignment and editing was performed with the Sequencer version 4.1 (GeneCodes, Ann Arbor, MI) software program.

Determination of Protease Enzymatic Activities

The catalytic efficiencies of the different HIV-1 proteases were calculated using a phage lambda-based genetic screening, as previously described (Sices and Kristie 1998; Martinez et al. 2000; Cabana et al. 2002; Martinez and Clotet 2003; Parera et al. 2004). Briefly, *E. coli* JM109 cells containing the plasmid p2X-cI.HIV were transformed with plasmid pC.I.HIV-cro. The resulting cells were grown in the presence of 0.2% maltose, harvested by centrifugation, and suspended to 2.0 optical density at 600 nm (OD₆₀₀)/ml in 10 mM MgSO₄. Cells (200 μl) were infected with 5×10^7 pfu of phages containing the different HIV-1 proteases. After 15 min at 37°C, the cells were washed with 1 ml of 10 mM MgSO₄, harvested by centrifugation, and suspended in 1 ml of Luria broth (LB) medium containing 12.5 μg

of tetracycline, 0.2% maltose, 10 mM MgSO₄, and 0.1 mM isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The cell cultures were incubated at 37°C for 3 h and harvested by centrifugation. An additional cycle of selective growth was done by suspending the infected cells with a fresh aliquot (200 μl) of JM109 pC.I.HIV-cro cells. After 2 selective growth cycles, the titer of the resulting phage was determined by coplating the cultures with 200 μl of *E. coli* XL-1 Blue cells (OD₆₀₀ = 2.0/ml in 10 mM MgSO₄) on LB plates using 3 ml top agar containing 12.5 μg of tetracycline/ml, 0.2% maltose, and 0.1 mM IPTG. After incubation at 37°C for 6 h, the plaques were counted for growth scores. The catalytic efficiency of every mutant was calculated as the mean ± standard error of at least 3 independent replicates.

Statistical Analysis

The classification of mutations as deleterious, neutral, or beneficial was based on a paired *t*-test included in the GraphPad Prism version 4.00 for Windows, San Diego, CA (<http://www.graphpad.com/prism/Prism.htm>).

Results

HIV-1 Protease Vulnerability to Single Random Amino Acid Substitutions

The HIV-1 protease is an aspartic protease consisting of 2 identical 99-amino acid monomers. The viral protease is the enzyme required for processing the gag and gag-pol polyproteins to yield mature, infectious virions. We used PCR mutagenesis to generate a randomly mutated library of the HIV-1 protease (HXB2 strain) (table 1) averaging 2.2 amino acid substitutions per mutant. Sequencing of 973 individual protease clones identified 107 mutants carrying unique single amino acid substitutions (fig. 1). The 107 single substitutions were distributed through the protein and affected 68 different residues (fig. 1). To determine the enzymatic activity of the different identified single-mutant proteases, a phage lambda-based genetic screen was used. This genetic screen is based on the phage lambda regulatory circuit in which the viral repressor cI is specifically cleaved to initiate the lysogenic to lytic switch (Sices and Kristie 1998). Introduction of an HIV-1 protease in a wild-type phage will cleave a mutant cI repressor containing a specific HIV-1 protease cleavage site, allowing the phage to go into the lytic replication cycle. As we have previously demonstrated, the cI repressor cleavage is directly proportional to the protease catalytic efficiency (Martinez et al. 2000; Cabana et al. 2002; Martinez and Clotet 2003; Parera et al. 2004). Likewise, we have also shown that the enzyme activities determined with this genetic screen were affecting to a similar extent the ex vivo replication capacity of the virus (Cabana et al. 2002; Fernandez G, Clotet B, Martinez MA, submitted). The introduction of different protease variants in the HIV-1 infectious clone pNL4.3luc⁺R⁻E⁻A confirmed that HIV-1 growth was proportional to the enzyme activity observed with the genetic screen used here (Fernandez G, Clotet B, Martinez MA, submitted) (data not shown). This HIV-1 protease genetic screen model has to be seen as a complement to the classical biochemical approach for monitoring protease proteolytic activity. The enzymatic activities of the different single-variant proteases analyzed in this study were related to

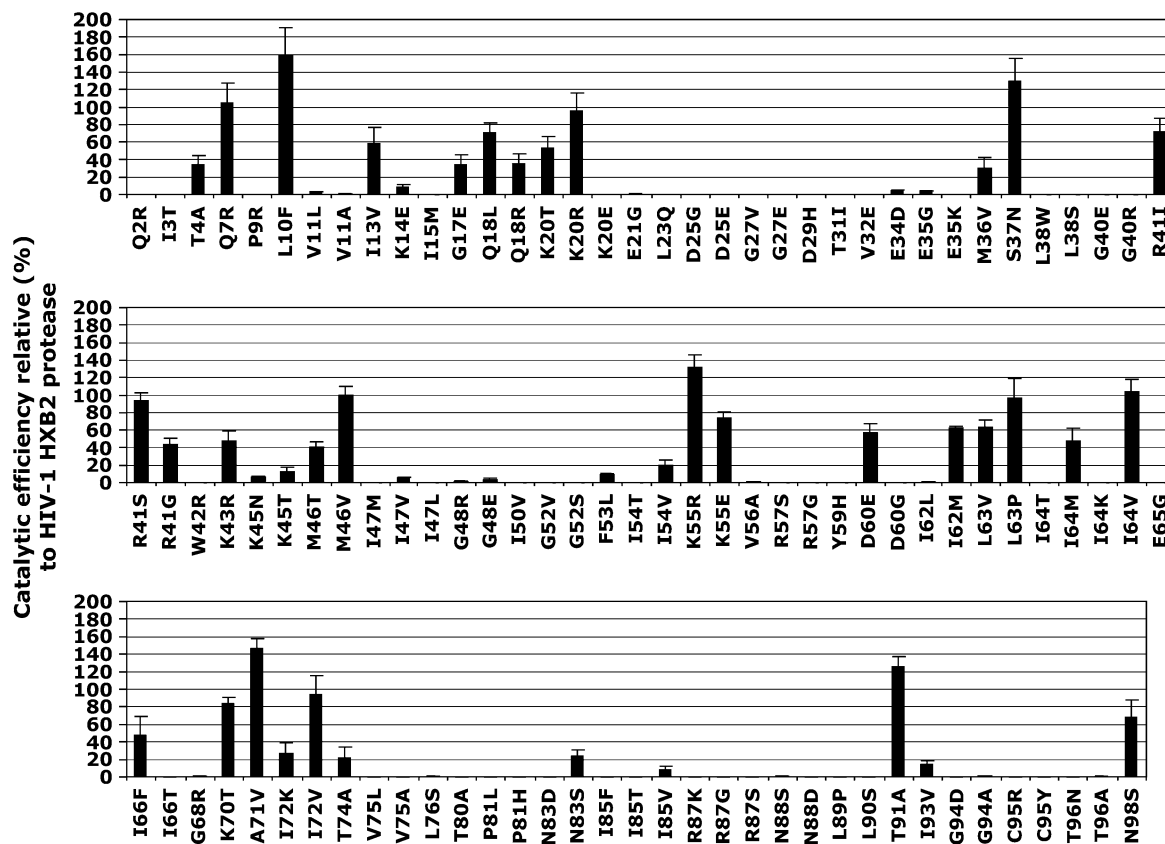


FIG. 1.—Comparative catalytic efficiency of 107 HIV-1 protease mutants carrying unique single amino acid substitutions. The catalytic efficiency of the single protease variants was compared with HXB2 protease (100%). Three independent replicates were performed for each sample. Error bars correspond to standard errors.

the activity of the wild-type HXB2 protease (100%) (fig. 1). Fifty-eight (54.2%) mutations were lethal (t -test, $P < 0.05$), 34 (31.7%) were deleterious (t -test, $P < 0.05$), 13 (12.1%) had no significant effect (t -test, $P < 0.05$), and 2 (1.9%) were significantly beneficial (t -test, $P < 0.05$) (fig. 1). The average effect on the catalytic efficiency of the 34 deleterious mutations was -71.6% . For the 13 neutral and 2 beneficial mutations, the average effect was 1.0% and 52.5% , respectively. As expected, several lethal or highly deleterious mutants were located in conserved residues critical to the structure and function of the protease (Loeb et al. 1989; Rao et al. 1991; Ishima et al. 2001) as the active site (amino acids 22–34), the flap (amino acids 47–52), and amino acids 84–94 which encompass a single α -helix that interacts with the substrate. Nevertheless, the former preserved coding regions were not the only residues in which lethal or deleterious mutations were found (fig. 1). Residues located outside the above 3 critical regions in which we found lethal or highly deleterious mutants (V11, E21, L38, G40, V56, Y59, G68, V75, L76, T80, P81, G94, C95, and T96) were absent or rarely mutated in drug-naïve infected individuals (Wu et al. 2003; Ceccherini-Silberstein et al. 2004), confirming the possible in vivo lethal or deleterious effect caused by the former mutations. In contrast, neutral or beneficial mutations (L10, S37, L63, I64, and A71) were located in very well known polymorphic positions in the virus isolated from infected individuals (Wu et al. 2003; Ceccherini-Silberstein et al. 2004). Interestingly, 2 mutations at positions L10

and A71 could be strongly beneficial. It may be argued that our assay does not fully reflect the requirements for functionality of HIV-1 protease in vivo. Nevertheless, site-directed mutagenesis of residues L10 and A71 has demonstrated that viruses carrying these substitutions could be as infectious as the wild-type virus (Mammano et al. 2000). A 3-dimensional image representing the crystallographic HIV-1 protease structure showed that most of the neutral or beneficial mutations were located at the peripheral areas of the enzyme and almost entirely positioned in surface loops far from the active-site and substrate-binding regions (not shown). In short, these results demonstrate the low tolerability of the HIV-1 protease to single random mutations.

Tolerability to Single Random Amino Acid Substitutions of a Low-Fitness HIV-1 Protease

To explore the tolerance to single random amino acid substitutions of a low-fitness HIV-1 protease, we decided to generate a second randomly mutated protease library using, as starting template, a mutated HXB2 protease carrying the single substitution D30N. It has been demonstrated that proteases carrying the substitution D30N, a primary mutation associated to the protease inhibitor nelfinavir resistance, had a very low catalytic efficiency (Martinez-Picado et al. 1999; Cabana et al. 2002). Similarly, the D30N substitution reduced the viral replication capacity (Martinez-Picado et al. 1999). In this study, we estimated that the mutant

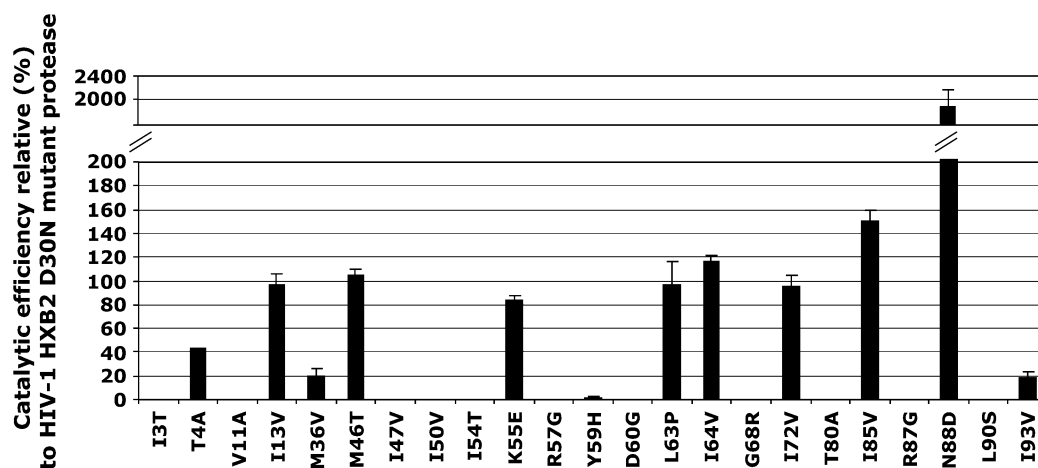


FIG. 2.—Comparative catalytic efficiency of 23 HIV-1 protease mutants carrying the D30N substitution together with unique single amino acid substitutions. The catalytic efficiency of the single protease variants was compared with the D30N mutant protease (100%). Three independent replicates were performed for each sample. Error bars correspond to standard errors.

D30N HXB2 protease had a $2.6\% \pm 0.4\%$ of the wild-type HXB2 proteolytic activity. Sequencing of 382 individual clones of this mutant library showed an average of 1.7 amino acid substitutions per clone (table 1).

Fifty-nine unique single mutants were identified, of which 23 were identical to single variants found in the first mutant library. Assessment of the catalytic efficiency of these 23 mutants was performed (fig. 2), and the results were compared with those obtained by the homologous 23 mutants obtained from the first library. In this second mutant library, the enzymatic activities of the different single mutants were compared with the activity of the mutant D30N HXB2 protease (100%) (fig. 2). Now, 12 (52.2%) mutations were lethal (t -test, $P < 0.05$), 3 (13.0%) were deleterious (t -test, $P < 0.05$), 6 (26.1%) had no significant effect (t -test, $P < 0.05$), and 2 (8.7%) were significantly beneficial (t -test, $P < 0.05$) (fig. 2). The average effect on the catalytic efficiency of the 3 deleterious mutations was -73% ; these percentages were 4.0% and 950% for the 6 neutral and the 2 beneficial mutations, respectively. When the same analysis was performed with the homologous 23 variants obtained from the wild-type HXB2 protease, a lower proportion of mutations with neutral or beneficial effect were observed. These values were 57%, 26%, 17%, and 0% for lethal, deleterious, neutral, and beneficial mutations, respectively. These results suggested that the same mutations, when expressed together with the highly deleterious mutation D30N, may have a different effect on the protease catalytic efficiency. Of note, the mutation N88D that was lethal for the wild-type HXB2 protease (fig. 1) improved the catalytic efficiency of the enzyme to numbers obtained with the wild-type HXB2 protease (fig. 2). Mutations I64V and I85V also improved the catalytic efficiency of the mutant D30N HXB2 protease, but their effect was modest. Similar to the N88D mutation, the I85V mutation was highly deleterious for the wild-type HXB2 protease (fig. 1). Both mutations, N88D and I85V, were rarely found in isolates from drug-naïve infected individuals. Nevertheless, they do not confer drug resistance but can be detected in isolates from protease inhibitor-treated individuals (Wu

et al. 2003). This result showed that the former mutations may act as compensatory or stabilizing substitutions in the presence of accompanying deleterious mutations, suggesting that antagonistic epistasis may buffer the effects of deleterious mutations.

Discussion

There are many examples of proteins highly robust to mutations. They include several enzymes that can tolerate many amino acid changes (Martinez et al. 1996; Guo et al. 2004). Protein robustness seems to be a selectable trait because neutral mutations can be a key to future evolutionary innovations (Wagner 2005). A recent evolution experiment demonstrated the evolutionary advantages of neutral mutations by showing that human or bacterial enzymes can acquire new functions without losing their original functions (Aharoni et al. 2005). This apparent selective advantage of robustness contrasts with the low tolerability to single substitutions demonstrated in this study for the HIV-1 protease, even with the high mutational burden faced by this protein. Here, we show that up to 54% of random single amino acid substitutions were lethal. Moreover, the average catalytic efficiency effect for the 32% of deleterious but nonlethal mutations was -72% . This high vulnerability to single mutations contrasts with the high HIV-1 protease genetic variability found within infected individuals (Wu et al. 2003; Ceccherini-Silberstein et al. 2004). Especially intriguing is the extremely rapid evolvability displayed by this protein after protease inhibitor treatment (Condra et al. 1995). HIV-1 protease can rapidly acquire mutations that lead to drug resistance but that barely affect its catalytic efficiency (Martinez-Picado et al. 1999; Nijhuis et al. 1999). Moreover, up to 22 (22%) different residues have been associated with drug resistance (Johnson et al. 2005). Although the high mutation rates and large population size of HIV-1 are obviously favoring the rapid evolvability of the HIV-1 protease (Domingo et al. 1997), protein evolution also depends on a reduced lethality of mutations (Aharoni et al. 2005; Wagner 2005; Bloom, Labthavikul, et al. 2006).

After showing the low tolerability to single random substitutions of a high-fitness protein, we decided to explore the tolerance to single random amino acid substitutions of a low-fitness HIV-1 protease. The chosen low-fitness protein was the HXB2 protease carrying the single mutation D30N. This substitution is primarily associated to resistance to the HIV-1 protease inhibitor nelfinavir (Johnson et al. 2005). It is very well known that this mutation impairs the enzyme's catalytic efficiency and the virus replication capacity (Martinez-Picado et al. 1999; Cabana et al. 2002). Interestingly, we found a higher proportion of mutations with a neutral or beneficial effect with the low-fitness protease. When epistatic relationships were searched between the highly deleterious mutation D30N and the different single random mutations, strong antagonistic epistasis was found with some mutations. In particular, the substitutions N88D and D30N turned out to be strongly epistatic. Both mutations reduced the catalytic efficiency of the wild-type protease (N88D was lethal and D30N strongly deleterious), but together, their effect was better. Because the mutation D30N confers some resistance to the drug and emerges first during *in vivo* protease inhibitor resistance development (Martinez-Picado et al. 1999; Cabana et al. 2002), the appearance of the mutation N88D would restore the catalytic defect originated by the D30N substitution. Indeed, the D30N and N88D mutations are specifically associated with resistance to nelfinavir in HIV-1-infected individuals (Patick et al. 1998).

In a number of recent studies carried out with RNA viruses, including HIV-1, a tendency toward antagonistic epistasis has been observed (Bonhoeffer et al. 2004; Burch and Chao 2004; Sanjuan et al. 2004). The existence of antagonistic epistasis is not restricted to RNA viruses, and it has also been found in bacteria, eukaryotic proteins, and digital organisms (Maisnier-Patin et al. 2002; Lenski et al. 2003; Bridgham et al. 2006). Especially interesting is the recent work performed by Bridgham et al. (2006). They demonstrate the role of antagonistic epistasis in the evolution of eukaryotic proteins such as the hormone receptors. In this study the antagonist epistatic interaction of 2 mutations introduced into an ancestral sequence recapitulates the evolution of present-day receptor specificity. Similar to our findings, an occasional highly deleterious mutation was rescued by a partner mutation that conferred a beneficial trait.

It has been hypothesized that antagonistic epistasis is characteristic of hypersensitive genomes (Elena et al. 2006; Sanjuan and Elena 2006); our findings may explain how antagonist epistasis can increase the mutational robustness and evolvability of a protein highly vulnerable to single random mutations.

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