Vol 459|4 June 2009|doi:10.1038/nature08009

ARTICLES

Chaperonin overexpression promotes genetic variation and enzyme evolution

Nobuhiko Tokuriki[1] & Dan S. Tawfik[1]

Most protein mutations, and mutations that alter protein functions in particular, undermine stability and are therefore deleterious. Chaperones, or heat-shock proteins, are often implicated in buffering mutations, and could thus facilitate the acquisition of neutral genetic diversity and the rate of adaptation. We examined the ability of the *Escherichia coli* GroEL/GroES chaperonins to buffer destabilizing and adaptive mutations. Here we show that mutational drifts performed *in vitro* with four different enzymes indicated that GroEL/GroES overexpression doubled the number of accumulating mutations, and promoted the folding of enzyme variants carrying mutations in the protein core and/or mutations with higher destabilizing effects (destabilization energies of >3.5 kcal mol^{-1}, on average, versus ~1 kcal mol^{-1} in the absence of GroEL/GroES). The divergence of modified enzymatic specificity occurred much faster under GroEL/GroES overexpression, in terms of the number of adapted variants ($\geq$2-fold) and their improved specificity and activity ($\geq$10-fold). These results indicate that protein stability is a major constraint in protein evolution, and buffering mechanisms such as chaperonins are key in alleviating this constraint.

Proteins are thought to evolve under strong stability constraints. Their relatively small margin of stability prevents the acquisition of a large fraction of mutations, and thus severely limits the rate of evolution[1–3]. Mutations that alter protein functions are particularly destabilizing[4,5]. Thus, protein variants that carry mutations with the potential to endow new, or improved, functions are likely to be purged despite, or even because of, their innovative potential[1]. Mechanisms that buffer the destabilizing effects of mutations therefore have a critical role in maintaining higher genetic diversity and expediting the rate of adaptation[1,6].

Chaperones, or heat-shock proteins (HSPs), are essential to eukaryotic and prokaryotic cells as they assist the folding, disaggregation, transport and function of proteins. They buffer the effects of environmental changes, as indicated by the marked increase in their levels under various stress conditions[7,8]. Chaperones also comprise a buffering system to genetic perturbations, and thus promote genetic diversity[9,10]. In eukaryotes, partial reduction of HSP90 results in unmasking of heritable phenotypic variation[11–13]. Eukaryotic chaperones may also mediate adaptive evolution by buffering the deleterious effects of mutated genes that mediate new functions[10]. The *Escherichia coli* GroEL/GroES chaperonins were shown to rescue heat-sensitive protein mutants[14], and bacteria facing high mutational loads are fitter under GroEL/GroES overexpression[15,16]. However, chaperone functions are very complex, in not only assisting protein folding but also affecting the development and signal transduction networks of the cell and alleviating the toxic effects of aggregated proteins. Their buffering mechanisms also vary from one organism, and chaperone, to another[9,16–19], and some cases of chaperone-related adaptations do not involve a direct rescue of mutated proteins[17,19].

We explored GroEL, which, with its cofactor GroES, is the most intensely studied and only chaperone in *E. coli* essential under all growth conditions[8]. Indeed, GroEL/GroES interacts with about 250 different *E. coli* proteins, which constitute $\sim$10% of all soluble *E. coli* proteins[20]. We aimed to establish an *in vitro* experimental system for a systematic and quantitative assessment of the capability of this chaperonin to promote the acquisition of genetic diversity by assisting the folding of destabilized enzyme mutants. We were particularly interested in the potential role of GroEL/GroES in rescuing mutants carrying altered or improved enzymatic functions.

The experimental system

We performed a random mutational drift of several enzymes in the presence of GroEL/GroES overexpression (Fig. 1). Mutagenesis of the target enzyme gene was performed *in vitro* by error-prone polymerase chain reaction (PCR) optimized to give $\sim$1.5 random mutations per gene per generation (Supplementary Table 1). The mutated genes were transformed to *E. coli* for overexpression, in parallel with GroEL/GroES overexpression or in its absence. Colonies carrying individual enzyme variants were grown, the bacteria were lysed and the levels of enzymatic activity were assayed. The enzyme-coding genes were re-cloned after each round of mutagenesis and screening, and subsequently transformed into a fresh sample of *E. coli* (Fig. 1). This procedure eliminated the role of factors related to bacterial fitness and other intergenic effects, and restricted the study to specific mutations within the coding region of the target enzymes and to the effect of GroEL/GroES overexpression on these mutations.

We tested four enzymes: glyceraldehydophosphate dehydrogenase from *E. coli* (GAPDH) and human carbonic anhydrase 2 (CA2), which are known clients of GroEL (proteins bound and refolded by GroEL)[20,21]; a variant of *Pseudomonas* sp. phosphotriesterase (PTE), named PTE-vr (see Methods), which was confirmed to be a GroEL/GroES client by our experiments; and *E. coli* triose phosphate isomerase (TIM), which is not a GroEL/GroES client[20,22]. All four enzymes were overexpressed in a soluble and functional form in *E. coli* in a chaperonin-independent manner: protein levels, indicated by both SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and enzyme activity, were similar under GroEL/GroES overexpression (growth with arabinose) compared to no chaperonin overexpression (growth with glucose).

As expected, mutagenesis led to a substantial decrease in enzymatic activity, and 20–40% of the 360 randomly picked variants in the initial screen showed no measurable activity. Enzyme variants that retained

[1]Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel.

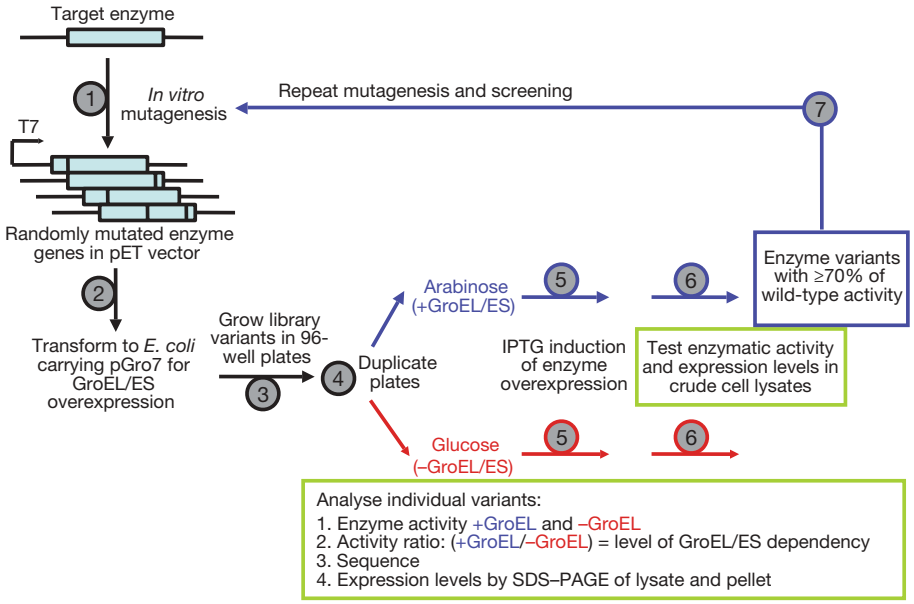

Figure 1 | The experimental setup. The target enzymes were subjected to random mutagenesis (step 1). The mutated genes were cloned into a pET expression vector and transformed to *E. coli* carrying the pGro7 plasmid, which conditionally overexpressed GroEL/GroES (step 2). In each round, 360 randomly picked colonies were individually re-grown in liquid media within 96-well plates (step 3). The plates were duplicated. To one set of plates, arabinose was added to induce GroEL/GroES overexpression. Glucose was added to the parallel set to suppress GroEL/GroES expression (step 4). Overexpression of the mutated enzyme variants was subsequently induced by IPTG addition (step 5). The cells were lysed, enzyme activity with and without GroEL/GroES overexpression was recorded (step 6), and representative variants were analysed (green box). To continue the drift, clones showing activity above the chosen threshold under arabinose induction were taken for the next round of mutagenesis and screening (step 7).

sufficient activity (150 individual clones, on average) were taken for the next round of mutagenesis and screening (Fig. 1, step 7). For GAPDH, PTE and TIM, we defined the threshold as ≥70% of the wild-type activity under GroEL/GroES overexpression. The threshold in the case of CA2, which was less tolerant to mutagenesis, was defined as ≥30%. The mutational drift, or 'mutation-accumulation' experiment, therefore occurred under purifying selection to purge deleterious mutations, and the soluble expression and enzymatic function of the drifting enzyme variants was maintained throughout. The overexpression of GroEL/GroES, the high mutation rates, small population sizes and relatively stringent selections used here exaggerate the effects of both GroEL/GroES and mutations. However, these conditions were necessary to enable the monitoring of GroEL/GroES effects for hundreds of different mutations, and thereby provide a clear and quantitative picture of how buffering the destabilizing effects of mutations accelerates neutral and adaptive evolution. Potential implications regarding more realistic evolutionary scenarios are mentioned in the Discussion.

Divergence of GroEL/GroES-dependent variants

In the first round, 93% of the GADPH variants were active above the defined activity threshold (Fig. 2, upper panel; the dashed vertical line separates all variants above the threshold of ≥70% of wild-type activity). However, when the drifting variants were tested in parallel with no GroEL/GroES overexpression, only 85% were sufficiently active. By the third generation, 60% of variants were sufficiently active with GroEL/GroES, but only 43% in its absence. Overall, ~30% of the drifting variants had chaperonin-dependent activity, whereby the activity observed with and without GroEL/GroES overexpression varied by a factor of between 2 and 30. In the fourth generation, the percentage of active variants actually increased, and the population did not become more GroEL/GroES-dependent. This tendency was observed when the GADPH drift was repeated (Supplementary Fig. 1a), and is probably due to the drift reaching a stage where the stability of the enzyme is compromised to the level that non-synonymous mutations do not readily accumulate (Supplementary Table 1)[23]. The enzymes PTE and CA2 had a similar behaviour. By the third generation, the fraction of variants for which levels of activity are chaperonin-dependent became 44% and 34%, respectively (Supplementary Table 2 and Supplementary Fig. 1b, c).

A control GADPH drift in the absence of GroEL/GroES overexpression indicated that none of the drifting variants that retained sufficient activity (Fig. 2, lower panel, left of the dashed line) was GroEL/GroES-dependent (ratio of +GroEL/−GroEL of ≤2). Chaperonin-dependent variants were observed among low-activity variants (right of the dashed line), but they gradually disappeared as the drift progressed. Drifts without GroEL/GroES overexpression were not performed with the other enzymes owing to the laborious nature of these experiments. However, the comparison of mutations found in GroEL/GroES-dependent and -independent variants validated the role of GroEL/GroES in rescuing stability-compromised variants in both GADPH and PTE (next section). An additional control experiment was performed with TIM—an enzyme that does not seem to interact with GroEL/GroES[20,22]. The drifting TIM populations showed a relatively small fraction of GroEL/GroES-dependent variants (4–10%; Supplementary Fig. 1d). The results of all drift experiments are summarized in Supplementary Table 2.

Because both the target enzymes and GroEL/GroES were overexpressed (≥10,000 molecules per cell), the effect of the endogenous chaperones was negligible. To support further the direct role of GroEL/GroES in rescuing the drifted enzyme variants, we compared the active enzyme levels for a representative sample of variants in the presence of overexpression of GroEL on its own (with no overexpression of GroES) and of two additional *E. coli* chaperones: DnaJ/DnaK/GrpE and trigger factor. These led to almost no increases in activity under the conditions in which GroEL/GroES overexpression led to 2–17-fold increases (Supplementary Table 3 and Supplementary Fig. 2). Pool-down assays also indicated the interaction of the target enzymes and their mutants with GroEL/GroES (Supplementary Fig. 3).

Characteristics of GroEL/GroES-dependent variants

Analysis of the type, location and stability effects of mutations revealed notable differences between neutral variants that drifted with GroEL/GroES assistance (≥2-fold difference in activity with and without GroEL/GroES overexpression) and those that did not (a ratio of

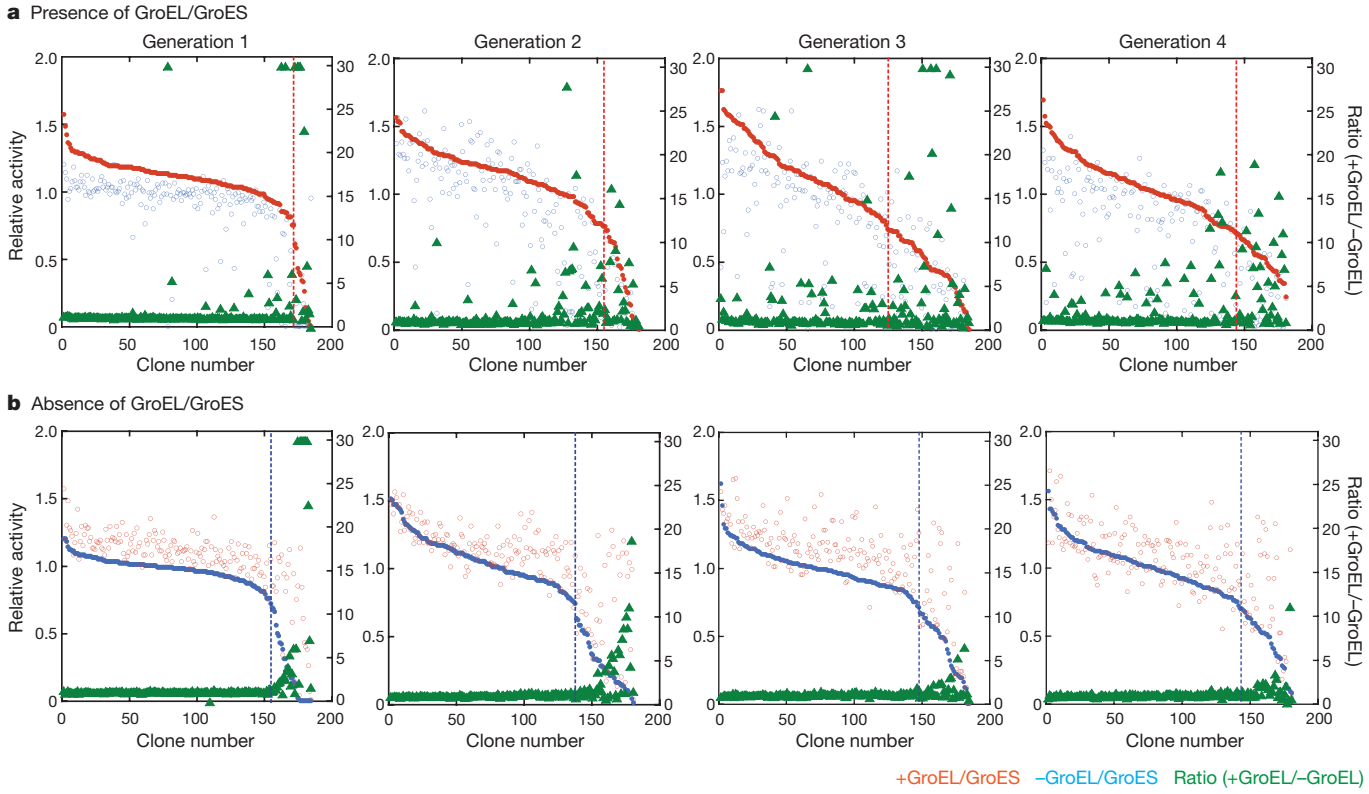

Figure 2 | GAPDH mutation-accumulation experiments. a, GADPH was drifted while overexpressing GroEL/GroES. Enzymatic activity was assayed for 180 randomly picked clones, and for each clone activity with GroEL/GroES overexpression relative to wild-type GADPH is presented (red circles; left *y* axis). Variants exhibiting ≥70% of wild-type activity (left of the dashed vertical line) were taken for the next round of mutagenesis and selection. Each clone was expressed in parallel without GroEL/GroES overexpression (blue open circles; left *y* axis), and the ratio of activities (+GroEL/−GroEL) is also presented (green triangles; right *y* axis). **b**, GAPDH drifted under the same conditions, yet without GroEL/GroES overexpression. Presented are the activities with and without GroEL/GroES overexpression (red open circles and blue circles, respectively; left *y* axis) and the GroEL/GroES-dependency (green triangles) for each clone. Variants exhibiting ≥70% of wild-type activity (left of the dashed vertical line) were taken for the next round.

<2). In total, 139 variants and 559 mutations for GAPDH from both the third and fourth generations of the drifts were analysed. The GroEL/GroES-independent variants included variants from the drift in the absence of GroEL/GroES, and variants from the drift under GroEL/GroES overexpression that show no chaperonin dependency (both groups showed similar features). For a start, the GroEL/GroES-independent variants were found to carry less than half the number of amino acid exchanges, or non-synonymous mutations, per gene (N_a): an average N_a value of 1.6, on average, versus 3.5 in the GroEL/GroES-dependent variants (all values, and analysis of their statistical significance, are provided in Supplementary Table 4). The fraction of non-synonymous mutations was accordingly lower: $N_a/(N_a + N_s) = 0.46$ versus 0.69 (where N_s is the average number of synonymous mutations, and $N_a + N_s$ is the total number of mutations).

The GroEL/GroES-dependent variants also tolerated a higher fraction of mutations in core residues. Mutations in buried residues are more likely to yield aggregated folding intermediates, and are more destabilizing than exposed surface mutations (average *Escherichia* destabilization energies, $\Delta\Delta G$ values, of $+1.4\,\mathrm{kcal\,mol^{-1}}$, versus $+0.6$)[24]. Consequently, evolutionary rates of core residues are much slower[25]. Indeed, 48% of the mutated residues in GroEL/GroES-dependent variants exhibited relatively low exposed surface areas: ASA values (relative accessible surface area, whereby 0 and 1 correspond to totally buried and exposed residues, respectively) of ≤0.25, compared to 41% in the GroEL/GroES-independent variants. The fraction of highly exposed residues mutated in the absence of GroEL/GroES (ASA values ≥0.6) was concomitantly higher (30% versus 21% with GroEL/GroES overexpression; Supplementary Fig. 4). In agreement with the core–surface distributions, the mutations found in the GroEL/GroES-dependent variants are predicted to be more destabilizing: the average $\Delta\Delta G$ per mutation is $+1.0\,\mathrm{kcal\,mol^{-1}}$, compared to $+0.6$ for the GroEL/GroES-independent variants. The GroEL/GroES-independent variants also carry threefold more stabilizing mutations: 9% of mutations with $\Delta\Delta G$ of $<-1\,\mathrm{kcal\,mol^{-1}}$, compared with 3% in the GroEL/GroES-dependent variants. Altogether, the GroEL/GroES-dependent variants carried more than twice the number of core mutations, and had a predicted average stability loss per variant of $+3.5\,\mathrm{kcal\,mol^{-1}}$, compared to $+0.9$ for GroEL/GroES-independent variants. The PTE variants from the third generation of the drift (in total 131 variants and 315 mutations) showed a very similar trend (Supplementary Table 4 and Supplementary Fig. 4). There are obviously additional chaperonin effects that relate to folding intermediates, but the FoldX stability-prediction algorithm can only address the native state.

The increase in activity in the presence of GroEL/GroES overexpression is largely due to the ability of the chaperonin to assist the folding of mutants with compromised stability and problematic folding intermediates, thus giving rise to a larger fraction of soluble, active enzyme. This hypothesis was supported by the observed correlation between enzyme activity and the relative occurrence in the soluble fraction versus that in the aggregated fraction (Supplementary Table 5 and Supplementary Fig. 5). As exemplified (Fig. 3a), wild-type GADPH had similar activity with or without GroEL/GroES overexpression, and in both cases >90% of the enzyme was in the soluble fraction. In contrast, drifting GADPH variants that exhibited GroEL/GroES dependency showed a low soluble fraction in the absence of chaperonin overexpression, and a much higher fraction in its presence.

Divergence of new function

The early steps in the divergence of new enzymes are presumed to involve mutations that increase a latent, weak promiscuous activity. Such activities may provide an initial selective advantage under changing

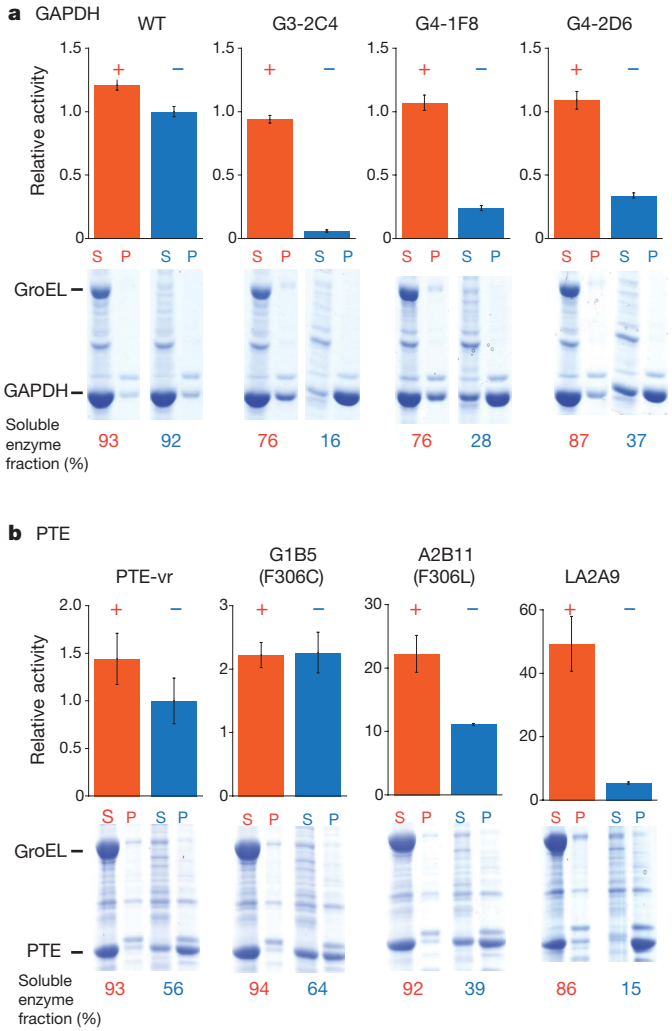

Figure 3 | Changes in activity and protein levels of enzyme variants expressed with and without GroEL/GroES overexpression. **a**, Wild-type GAPDH and its representative neutrally drifting variants. **b**, The starting point for the PTE drift (PTE-vr) and its variants with improved esterase activity. The bars represent relative enzymatic activities in crude lysates with GroEL/GroES overexpression (red) and without it (blue). Error bars represent standard deviations of six independent repeats. The supernatant and insoluble pellet fractions of the above lysates (S and P, respectively) were analysed by SDS–PAGE, and the percentage of enzyme in the soluble fraction was determined by the relative intensities of the supernatant and pellet bands.

environments, and can be subsequently improved by adaptive mutations and become the primary activity of the evolving enzyme[26,27]. However, mutations that endow enzymes with new, or improved, substrate and reaction specificities are, on average, destabilizing and tend to occur in more buried residues than neutral, non-adaptive mutations[4,5]. To examine whether GroEL/GroES may alleviate these stability constraints, we choose PTE—an enzyme that hydrolyses the pesticide paraoxon with high catalytic efficiency (k_{cat}/K_M of $>10^7\,M^{-1}s^{-1}$). We examined the adaptive evolution of PTE to increase its weak, promiscuous esterase activity (k_{cat}/K_M for the target ester substrate 2-naphtylhexanoate is $\sim10^2\,M^{-1}s^{-1}$). PTE-vr was subjected to random mutagenesis as before, and the resulting library ($\sim$10,000 transformants) was subjected to a chromogenic screen whereby colonies exhibiting esterase activity turn red.

The number of red colonies in the library expressed with GroEL/GroES (arabinose induction) was far greater than in its absence (agar with glucose; data not shown). The red-coloured colonies were subsequently grown, lysed and tested for enzymatic activity with 2-naphtylhexanoate in 96-well plates. This screen also indicated a significant difference in favour of the selection with chaperonins, in terms of the number and the fold-improvements in esterase activity (Fig. 4a). Differences in the selectivity of the evolved variants (esterase/paraoxonase rates; Supplementary Table 6) indicated changes in the active site of the mutants, irrespective of changes in their stability and expression.

Almost all the variants showing improved esterase activity carried a mutation of Phe 306 into Cys, Ser or Leu (Supplementary Table 7). Phe306Cys was previously identified in PTE variants evolved towards a similar ester substrate[28]. However, variants selected under GroEL/GroES overexpression, and especially those showing the highest improvements in esterase activity, carried the Phe306Leu mutation that has not been seen in previous attempts to evolve PTE. Two variants carrying only these point mutations were identified: A2B11 (Phe306Leu) from the library with GroEL/GroES overexpression, and G1B5 (Phe306Cys) from the library selected without GroEL/GroES. The Phe306Leu mutation showed higher improvement in esterase activity than Phe306Cys (11.8-fold in k_{cat}/K_M, versus 2.6-fold for Phe306Cys), but also had deleterious effects on the folding of PTE that inhibited its identification in the absence of GroEL/GroES overexpression. Indeed, on GroEL/GroES overexpression, the Phe306Leu mutant exhibited a 2.4-fold increase in the levels of soluble protein and a concomitant increase in enzymatic activity. In contrast, the Phe306Cys mutation was barely affected by GroEL/GroES overexpression (Fig. 3b).

The single-mutation variants were subjected to another round of directed evolution with and without GroEL/GroES overexpression, as described previously. The same trends were observed with a larger number of improved variants under GroEL/GroES overexpression, and with higher improvements in both activity (Fig. 4b) and selectivity (Supplementary Table 6). Overall, the evolutionary route under GroEL/GroES produced variants exhibiting up to 44-fold higher esterase activity, and 54-fold higher selectivity, compared to the starting point (PTE-vr). The same route in the absence of GroEL/GroES yielded only 3.4-fold improvements in activity, and 4.2-fold higher selectivity. The kinetic parameters (k_{cat}, K_M) determined with purified enzymes confirmed the improvements observed in cell lysates (Fig. 4c and Supplementary Table 7).

As observed with neutrally drifting variants, enzymatic activities and expression levels in the soluble versus the aggregated fraction were correlated, indicating that GroEL/GroES overexpression is indeed rescuing the variants evolved in its presence (Supplementary Fig. 5b). Variants selected under GroEL/GroES overexpression showed higher activity under GroEL/GroES overexpression ($\leq$9.1 versus $\leq$2.4 for variants selected without GroEL/GroES) and a concomitant increase in the soluble fraction ($\leq$5.8-fold versus $\leq$2.2-fold, respectively; Supplementary Table 7). Notably, many variants selected with GroEL/GroES overexpression show little, or no, improvement in activity without GroEL/GroES. For example, in crude lysates variant LA2A9 (diverged from the Phe306Leu mutant) showed twofold improvement in esterase activity with GroEL/GroES overexpression, but twofold decrease without it (Fig. 3b).

As shown with neutral GAPDH and CA2 variants, overexpression of GroEL on its own and of other *E. coli* chaperones (DnaJ/DnaK/GrpE and trigger factor) gave almost no increase in activity with the evolved GroEL/GroES-dependent PTE mutants (Supplementary Table 3). *In vitro* refolding experiments showed that significant refolding was obtained in the presence of GroEL/GroES (10–100%; with wild-type PTE, and with several evolved mutants tested), but no refolding was observed in buffer, or with GroEL on its own (Supplementary Table 7).

Discussion

Theoretical and experimental studies indicate that seemingly neutral, or hidden, variation has a key role in adaptation towards new environments, at the level of organisms and individual genes[6,26,29,30]. However, the destabilizing effects of mutations can severely limit the acquisition of such neutral variations[2,31]. Over 80% of mutations are deleterious due to their destabilizing effects, which in turn lead to reduced protein

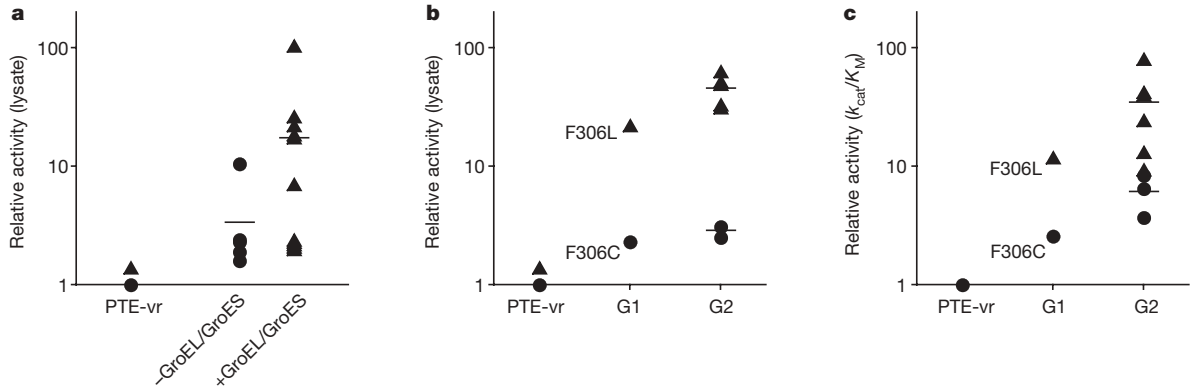

Figure 4 | Adaptive evolution of *P. diminuta* PTE towards higher esterase activity. Triangles represent variants evolved and assayed under GroEL/GroES overexpression, and circles represent variants without GroEL/GroES overexpression. Shown are activities relative to the starting point PTE-vr. **a**, Activity levels in crude lysates of variants identified in the first round of mutation and selection. **b**, Activities of second-round variants. Annotated are two single-mutant variants: Phe306Leu (A2B11), which comprised the starting point for the next round under GroEL/GroES overexpression, and Phe306Cys (G1B5) for the selection without it. **c**, k_{cat}/K_M values measured with the purified enzyme variants isolated from these selections (data are provided in Supplementary Table 7).

levels[1,32]. Similarly, most mutations that improve enzymatic activities undermine stability[4,5]. For example, the development of antibiotic resistance in TEM-1 β-lactamase is dependent on the accumulation of a compensatory mutation that makes up for the stability losses associated with mutations that alter the enzyme's substrate specificity[4]. Owing to decreases in the level of soluble, functional enzyme, such mutants may show only small improvement in the cellular rates of enzymatic activity. Decreased stability can also result in insoluble aggregates that reduce fitness[33]. Our results show that rescuing stability-impaired mutants by increasing the fraction of soluble, functional enzyme has marked effects on evolutionary rates.

The experiment also provides a quantitative assessment of the buffering capability of GroEL/GroES. On average, in the absence of GroEL/GroES, the stability of neutral variants seem to be compromised by $\sim$1 kcal mol^{-1} (predicted values, assuming additivity of stability effects, are 0.9 and 1.2 kcal mol^{-1} for PTE and GADPH, respectively). The overexpression of GroEL/GroES shifted this threshold by about 2.5 kcal mol^{-1} to an average level of $\sim$3.6 kcal mol^{-1}. A significant enrichment of stabilizing compensatory mutations also occurred in GroEL/GroES-independent variants (Supplementary Fig. 4). Stabilizing mutations are scarce[3,24]. Therefore, evolution, whether neutral or adaptive, that depends on such mutations is far slower. This is indeed reflected in >2-fold lower total number of non-synonymous mutations found in variants drifting without GroEL/GroES (Supplementary Table 4).

GroEL/GroES clients seem to be characterized by low folding propensities and high translation rates (as is the case in our *in vitro* system)[34]. The chaperonin buffered mutants are therefore likely to suffer not only from low stability, but also from lower folding propensities and/or slower folding rates. The buffering capacity of GroEL/GroES is, however, not unlimited. The FoldX predictions indicate that highly destabilizing mutations (>5 kcal mol^{-1}) were purged whether GroEL/GroES was overexpressed or not (Supplementary Table 4 and Supplementary Fig. 4). Indeed, many variants that drifted for two, let alone four, generations under GroEL/GroES overexpression appear to be very close to the 5 kcal mol^{-1} threshold (Supplementary Table 8), and variants evolved for new function were also severely compromised (for example, LA2A9 described above). Thus, once the extended margin provided by the GroEL/GroES is consumed, the dependency on stabilizing compensatory mutations returns[1,4,23]. The limited buffering capacity is also reflected in the fact that the fraction of mutants that are GroEL/GroES-dependent did not exceed a certain value (for example, ≤35% for the GAPDH drift). This implies that a significant fraction of destabilizing mutations cannot be rescued by GroEL/GroES. The plateau may also be the result of balancing forces such as the appearance of stabilizing compensatory mutations. Indeed, when the GroEL/GroES-dependent PTE variant A2B11 (Phe306Leu) was subjected to evolution in the absence of GroEL/GroES overexpression, a second mutation (Ile274Ser) appeared (variant LG2B6; Supplementary Table 7). Ile274Ser hardly improves catalytic activity (1.1-fold), but it increases stability and lowers GroEL/GroES dependency. This stabilizing mutation may mimic natural processes of reducing chaperone dependency and thus providing 'chaperone space' for other mutants.

The buffering capacity is also protein-dependent, because only a subset of *E. coli* proteins interacts with GroEL/GroES[20,22,34]. Also, drifts we conducted with two known GroEL/GroES clients (green fluorescent protein and TEM-1 β-lactamase data not shown) did not consistently indicate GroEL/GroES-dependency. However, although the wild-type protein does not interact with chaperonins (for example, TIM tested here), its mutants may interact. A number of TIM mutants became GroEL/GroES-dependent, although, relative to the other enzymes tested here, TIM mutants clearly showed a lower tendency to become GroEL/GroES-assisted. The experimental system developed here could be applied to test this correlation in a larger number of proteins.

Our *in vitro* findings have potential implications on natural evolution. Mutation rates in bacteria increase under stress, and so do chaperone levels[35,36]. The combination of increased mutation rates and chaperone overexpression can markedly accelerate the rate of acquisition of new functions, as demonstrated here. However, the balance within living *E. coli* cells, between the capacity to buffer mutations and the rate by which mutations accumulate, is obviously different than the one applied here. Indeed, under stress conditions the available chaperone capacity can also decrease, as shown for HSP90, and the phenotype of previously buffered mutations unravelled[11–13].

Finally, the experimental system described here provides a way of performing directed evolution *in vitro*, whereby, for enzymes that comprise effective chaperonin clients, overexpression of GroEL/GroES can significantly increase the number and fold-improvement of enzyme variants with a new, or improved function—not only at crude cell lysates, but also of the actual kinetic parameters of the evolving enzyme variants (Fig. 4c).

METHODS SUMMARY

Wild-type CA2, GAPDH, TIM and engineered PTE-vr were used as the starting points. Gene libraries were created by random mutagenesis using error-prone PCR with the 'wobble' base analogue dPTP, and gene libraries for the evolution of esterase activity of PTE-vr were created with dPTP and 8-oxo-dGTP. The mutated enzyme variants were cloned into pET vector, and transformed to *E. coli*

BL21 (DE3). Agar colonies were individually picked and transferred for growth in 96-well plates. Following arabinose, or glucose, addition, enzyme expression was induced with IPTG. The bacteria were lysed, and the levels of enzymatic activity were assayed in 96-well plates. GAPDH activity was measured by a coupled enzyme assay based on the decrease of absorbance at 340 nm due to NADH oxidation. Paraoxonase activity of PTE was measured by following the increase of absorbance at 405 nm. PTE libraries were screened for esterase activity on agar plates using FAST Red dye that creates a red precipitate with the 2-naphtol product, followed by a spectrophotometeric screen in 96-well plates at 500 nm. The hydrolase activity of CA2 was measured with *p*-nitrophenyl acetate by following the increase of absorbance at 405 nm. TIM activity was determined by a coupled enzyme assay based on the decrease of absorbance at 340 nm due to NADH oxidation. GAPDH and PTE variants were purified over a Strep-tactin Sepharose column (IBA).

Computations. The stability effect of mutations ($\Delta\Delta G$ values) found in GAPDH and PTE variants was predicted based on crystal structures (PDB accession codes 1S7C and 1HZY, respectively) using the FoldX algorithm. Surface accessibility (ASA) values of residues were calculated by the web-based program ASA-view (http://gibk26.bse.kyutech.ac.jp/~shandar/netasa/asaview/). A complete list of all mutations and their predicted $\Delta\Delta G$ and ASA values is provided in Supplementary Table 8.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 4 June 2008; accepted 20 March 2009.

1. DePristo, M. A., Weinreich, D. M. & Hartl, D. L. Missense meanderings in sequence space: a biophysical view of protein evolution. *Nature Rev. Genet.* **6,** 678–687 (2005).
2. Wilke, C. O., Bloom, J. D., Drummond, D. A. & Raval, A. Predicting the tolerance of proteins to random amino acid substitution. *Biophys. J.* **89,** 3714–3720 (2005).
3. Zeldovich, K. B., Chen, P. & Shakhnovich, E. I. Protein stability imposes limits on organism complexity and speed of molecular evolution. *Proc. Natl Acad. Sci. USA* **104,** 16152–16157 (2007).
4. Wang, X., Minasov, G. & Shoichet, B. K. Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. *J. Mol. Biol.* **320,** 85–95 (2002).
5. Tokuriki, N., Stricher, F., Serrano, L. & Tawfik, D. S. How protein stability and new functions trade off. *PLOS Comput. Biol.* **4,** e1000002 (2008).
6. Wagner, A. *Robustness and Evolvability in Living Systems.* (Princeton Univ. Press, 2005).
7. Martin, J. & Hartl, F. U. Chaperone-assisted protein folding. *Curr. Opin. Struct. Biol.* **7,** 41–52 (1997).
8. Thirumalai, D. & Lorimer, G. H. Chaperonin-mediated protein folding. *Annu. Rev. Biophys. Biomol. Struct.* **30,** 245–269 (2001).
9. Fares, M. A., Moya, A. & Barrio, E. GroEL and the maintenance of bacterial endosymbiosis. *Trends Genet.* **20,** 413–416 (2004).
10. Rutherford, S. L. Between genotype and phenotype: protein chaperones and evolvability. *Nature Rev. Genet.* **4,** 263–274 (2003).
11. Rutherford, S. L. & Lindquist, S. Hsp90 as a capacitor for morphological evolution. *Nature* **396,** 336–342 (1998).
12. Queitsch, C., Sangster, T. A. & Lindquist, S. Hsp90 as a capacitor of phenotypic variation. *Nature* **417,** 618–624 (2002).
13. Sangster, T. A., Lindquist, S. & Queitsch, C. Under cover: causes, effects and implications of Hsp90-mediated genetic capacitance. *Bioessays* **26,** 348–362 (2004).
14. Van Dyk, T. K., Gatenby, A. A. & LaRossa, R. A. Demonstration by genetic suppression of interaction of GroE products with many proteins. *Nature* **342,** 451–453 (1989).
15. Fares, M. A., Ruiz-González, M. X., Moya, A., Elena, S. F. & Barrio, E. Endosymbiotic bacteria: GroEL buffers against deleterious mutations. *Nature* **417,** 398 (2002).
16. Maisnier-Patin, S. *et al.* Genomic buffering mitigates the effects of deleterious mutations in bacteria. *Nature Genet.* **37,** 1376–1379 (2005).
17. Cowen, L. E. & Lindquist, S. Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. *Science* **309,** 2185–2189 (2005).
18. Sangster, T. A. & Queitsch, C. The HSP90 chaperone complex, an emerging force in plant development and phenotypic plasticity. *Curr. Opin. Plant Biol.* **8,** 86–92 (2005).
19. Bobula, J. *et al.* Why molecular chaperones buffer mutational damage: a case study with a yeast Hsp40/70 system. *Genetics* **174,** 937–944 (2006).
20. Kerner, M. J. *et al.* Proteome-wide analysis of chaperonin-dependent protein folding in *Escherichia coli. Cell* **122,** 209–220 (2005).
21. Persson, M., Carlsson, U. & Bergenhem, N. C. GroEL reversibly binds to, and causes rapid inactivation of, human carbonic anhydrase II at high temperatures. *Biochim. Biophys. Acta* **1298,** 191–198 (1996).
22. Chapman, E. *et al.* Global aggregation of newly translated proteins in an *Escherichia coli* strain deficient of the chaperonin GroEL. *Proc. Natl Acad. Sci. USA* **103,** 15800–15805 (2006).
23. Bershtein, S., Golding, K. & Tawfik, D. Intense drifts yield robust and evolvable consensus proteins. *J. Mol. Biol.* **379,** 1029–1044 (2008).
24. Tokuriki, N., Stricher, F., Schymkowitz, J., Serrano, L. & Tawfik, D. S. The stability effects of protein mutations appear to be universally distributed. *J. Mol. Biol.* **369,** 1318–1332 (2007).
25. Sasidharan, R. & Chothia, C. The selection of acceptable protein mutations. *Proc. Natl Acad. Sci. USA* **104,** 10080–10085 (2007).
26. Amitai, G., Gupta, R. A. & Tawfik, D. S. Latent evolutionary potentials under the neutral mutational drift of an enzyme. *HFSP Journal* **1,** 67–78 (2007).
27. McLoughlin, S. Y. & Copley, S. D. A compromise required by gene sharing enables survival: implications for evolution of new enzyme activities. *Proc. Natl Acad. Sci. USA* **105,** 13497–13502 (2008).
28. Roodveldt, C. & Tawfik, D. S. Shared promiscuous activities and evolutionary features in various members of the amidohydrolase superfamily. *Biochemistry* **44,** 12728–12736 (2005).
29. Le Rouzic, A. & Carlborg, O. Evolutionary potential of hidden genetic variation. *Trends Ecol. Evol.* **23,** 33–37 (2007).
30. Fontana, W. & Schuster, P. Continuity in evolution: on the nature of transitions. *Science* **280,** 1451–1455 (1998).
31. Bershtein, S., Segal, M., Bekerman, R., Tokuriki, N. & Tawfik, D. S. Robustness-epistasis link shapes the fitness landscape of a randomly drifting protein. *Nature* **444,** 929–932 (2006).
32. Yue, P., Li, Z. & Moult, J. Loss of protein structure stability as a major causative factor in monogenic disease. *J. Mol. Biol.* **353,** 459–473 (2005).
33. Lindner, A. B., Madden, R., Demarez, A., Stewart, E. J. & Taddei, F. Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. *Proc. Natl Acad. Sci. USA* **105,** 3076–3081 (2008).
34. Noivirt-Brik, O., Unger, R. & Horovitz, A. Low folding propensity and high translation efficiency distinguish *in vivo* substrates of GroEL from other *Escherichia coli* proteins. *Bioinformatics* **23,** 3276–3279 (2007).
35. Radman, M., Matic, I. & Taddei, F. Evolution of evolvability. *Ann. NY Acad. Sci.* **870,** 146–155 (1999).
36. Taddei, F. *et al.* Role of mutator alleles in adaptive evolution. *Nature* **387,** 700–702 (1997).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements Financial support by the EU via the MiFEM consortium, NIH grant number W81XWH-07-2-0020, and Israel Science Foundation are gratefully acknowledged. We are thankful to P. Goloubinoff for his advice, V. Kolotonouv for technical assistance, L. Serrano for the use of FoldX, and A. Horovitz and S. Rutherford for their help in refining this manuscript.

Author Contributions N.T. and D.S.T. designed the study, analysed the data and wrote the manuscript. N.T. performed the experiments.

Author Information Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to D.S.T. (tawfik@weizmann.ac.il).

METHODS

Gene cloning and library construction. To identify candidates for the drift experiment, we screened the published lists of chaperone targets[37] for enzymes with chromogenic substrates that can be assayed in crude bacterial lysates. GADPH and CAII met these criteria. Two other candidates (green fluorescent protein and TEM-1 β-lactamase) were explored but the preliminary results were inconclusive. TIM also fulfilled the screening requirement, and served as a control for an enzyme that has been shown not to interact with GroEL/GroES. Finally, PTE (phosphotriesterase from *Pseudomonas diminuta*), for which an array of chromogenic substrates is available[38], was tested and found to be a GroEL/GroES target. Wild-type CA2, GAPDH, TIM and engineered PTE-vr were used as the starting points for the neutral drift. PTE-vr is related to the previously described PTE-S5-1.1, which was evolved for higher *E. coli* expression and 2-naphtyl acetate hydrolase activity[38,39]. The PTE-vr was cloned with NcoI and HindIII sites into a modified pET32-trx[40] for the neutral drift, and into pET-strep for the adaptive evolution experiments. The genes encoding GAPDH and TIM were amplified from the ASKA collection[41] and re-cloned into pET32(b) (Novagen) with NdeI and HindIII sites. For purification and further characterization, GAPDH and its drifted variants were cloned into pET-strep using NcoI and HindIII sites. The CA2-pET construct was described previously[42]. The gene encoding GroEL was amplified from pGro7 plasmid[43] and re-cloned into pBAD33 with NdeI and HindIII sites (pBAD33-GroEL).

Libraries were generated by random mutagenesis with error-prone PCR using the wobble base analogues dPTP and 8-oxo-dGTP (TriLink). The first 20 PCR cycles with primers flanking the cloned genes were performed with BioTaq polymerase (Bioline) in 50-μl reactions using 1 ng of plasmid template, 0.5 μM dPTP for GAPDH, PTE and TIM neutral drifts, 1 μM dPTP for CA2, and 1 μM dPTP and 20 μM 8-oxo-dGTP for PTE's adaptive evolution. The PCR products were treated with DpnI to destroy the template plasmid, purified, and then used as a template (20 ng) for another 15 PCR cycles, this time performed with nesting primers and BioTaq polymerase. The product of this final PCR was restricted with restriction enzymes described above (NEB, 3 h, 37 °C) and ligated into the modified pET as described above. This protocol created with high reproducibility libraries carrying an average of ~1.5 mutations per gene (Supplementary Table 1). This rate provided approximately one amino acid exchange per generation. Lower mutation rate rates would have resulted in screening a large fraction of variants that carry no mutations, and higher rates would result in having multiple mutations appearing simultaneously, and a very low fraction of mutants being viable.

Activity assays. Phosphotriesterase activity was assayed by measuring the increase in absorbance at 405 nm with paraoxon (0.5 mM) in 50 mM Tris-HCl, pH 7.5, 0.005% Triton X-100. 2-Naphtylhexanoate (2NH) hydrolysis was assayed by a coupled reaction assay based on the increase of absorbance at 500 nm due to the conjugation of the released 2-naphtol and FAST Red (Sigma) with 0.2 mM 2NH and 0.4 mM FAST Red in 50 mM Tris-HCl, pH 7.5, 0.005% Triton X-100.

GAPDH activity was measured by a coupled enzyme assay based on the decrease of absorbance at 340 nm due to NADH oxidation provided by Sigma. The GAPDH reaction mixture (50 mM Tris-HCl, pH 7.5, 6.7 mM 3-phosphoglyceric acid, 1.1 mM adenosine 5′ triphosphate, 0.12 mM β-nicotinamide adenine dinucleotide, reduced form (β-NADH, Sigma), 3.3 mM L-cysteine, and 8 units per ml 3-phosphoglyceric phosphokinase (Sigma)) was pre-incubated at 30 °C for 1 h, and the reaction was initiated by the addition of the GADPH sample.

TIM activity was determined by a coupled enzyme assay based on the decrease of absorbance at 340 nm due to NADH oxidation[44]. The substrate, glyceraldehyde-3-phosphate, was prepared from DL-glyceraldehyde-3-phosphate diethyl acetal monobarium salt as described by the supplier (Sigma). The TIM reaction mixture contained 1 mM D-glyceraldehyde-3-phosphate, 0.24 mM NADH, and 20 mg ml^{-1} glycerol-3-phosphate dehydrogenase, in 50 mM Tris-HCl (pH 7.5).

The esterase activity of CA2 was assayed by measuring absorbance at 405 nm with 4-nitrophenyl acetate (0.5 mM) in 50 mM Tris-HCl, pH 7.5.

Coexpression chaperones, target enzymes and activity assays. *E. coli* BL21 (DE3) cells containing the pGro7 (GroEL/GroES), pBAD33-GroEL (GroEL), pKJE7 (DnaK/DnaJ/GrpE) or pTf16 (trigger factor) plasmid[43] were transformed with the libraries or a variant of GAPDH, PTE, CA2 or TIM, and plated on Luria Broth (LB) agar with 100 μg ml^{-1} ampicillin (amp) and 34 μg ml^{-1} chloramphenicol (cam). Single colonies were picked and grown overnight in 96-deep-well plates at 30 °C with shaking, in LB medium with 100 μg ml^{-1} amp and 34 μg ml^{-1} cam. These overnight cultures were used to inoculate (at 1:20 dilution) within 96-deep-well plates cultures in a fresh LB amp, cam, medium. The medium contained 0.2% (w/v) arabinose or glucose to induce or suppress GroEL/GroES expression, respectively. Cells were grown at 37 °C in the case of GAPDH, CA2 and TIM, or at 30 °C in the case of PTE, with shaking for about two hours, to D_{600} of 0.6–1.0, and then pelleted and resuspended in LB media pre-incubated at 37 °C or 30 °C with amp and 1 mM IPTG to induce overexpression of the target enzyme. After 1 h incubation at 37 °C in the case of GAPDH, CA2 and TIM, or 30 °C in the case of PTE, the D_{600} of these cultures were measured using a plate reader. Cells were then pelleted and stored at −20 °C overnight. Cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 μg ml^{-1} lysozyme, 0.5 units per ml benzonase, and 0.1% TritonX-100). The lysates were clarified by centrifugation and assayed for enzymatic activity in 200-μl reactions (20 μl crude lysate was used for assaying CA2; 20 μl of 20-times diluted lysate for GAPDH, or 20 μl of 400-times diluted lysate for PTE; and 20 μl of 400-times diluted lysate for TIM).

Co-fractionation of GAPDH and PTE with GroEL/GroES. The enzymes GAPDH or PTE, and mutants thereof, were overexpressed in *E. coli* together with GroEL and His-tagged GroES (or with non-tagged GroES). The cells were lysed with 50 mM Tris-HCl, pH 8.0, 0.01% TritonX-100, 10 mM $MgCl_2$, 25 units per ml benzonase, 10 mM glucose and 20 units per ml hexokinase. ADP was added immediately after lysis, and the clarified lysate was purified by IMAC affinity chromatography. After 4 washes with 10-times column volume of 20 mM imidazole in Tris pH 8, 500 mM NaCl, 10 mM $MgCl_2$, 10 mM KCl and 1 mM ADP, the complex was eluted by 500 mM imidazole in Tris, pH 8, and analysed by SDS–PAGE.

Random mutational drift. Libraries were transformed into *E. coli* BL21 (DE3) cells carrying the pGro7 plasmid. About 360 transformants were picked into liquid medium in 96-deep-well plates, and GroEL/GroES overexpression was induced with arabinose as described above. At this library size the chances of haphazard fixation are rather low ($1/360 < 1\%$) so the library could maintain high polymorphism, and the sampling is therefore statistically significant as indicated, for example, in the analysis of the mutations. The enzymatic activities were assayed in the crude lysates, and 180 clones showing the detectable activity (≥2-fold above background rates) with GroEL/GroES induction were randomly picked. These 180 variants were then screened in parallel with, and without, overexpression of GroEL/GroES, and the levels of enzymatic activity were determined. The wild type was used as reference in two replica wells. For the subsequent round of neutral drift, variants with the threshold enzymatic activity defined as neutral were picked (≥70% of the wild-type activity of GAPDH, PTE-vr and TIM, and ≥30% of CA2). On average, 150 neutral variants were taken for the next round. Their overnight cultures were combined, and the plasmid DNA purified and used as the template for the next round of mutagenesis by error-prone PCR.

Directed evolution. Gene libraries derived from PTE-vr were transformed into *E. coli* BL21 (DE3) cells carry the pGro7 plasmids, and plated on LB-amp-cam agar plates containing 0.2% arabinose (for overexpression of GroEL/GroES) or 0.2% glucose (for suppression of GroEL/GroES). About 10^4 colonies were screened for each set (arabinose or glucose) using FAST Red[38,42]. From each set, about 280 colonies showing the fastest development of red colour were picked from replica plates, grown in 96-well plates, and assayed for 2NH hydrolysis. Thirty variants that showed the highest 2NH hydrolase activity were picked, and the plasmids were purified and retransformed into fresh *E. coli* BL21 (DE3)-pGro7 competent cells, and then their activities with 2NH and paraoxon were tested in triplicate.

Enzyme purification and kinetics. The evolved PTE and GAPDH variants were purified using Strep-tactin affinity columns (IBA) according to the manufacturer's protocol. Initial rates were determined as described above using paraoxon (0.5 to 1,000 μM) and 2NH (2 to 2,000 μM). The kinetics parameters K_M and K_{cat} were determined by fitting the initial rates to the Michaelis–Menten model using KaleidaGraph (Synergy Software). GroEL and GroES were purified as described previously[45].

Protein refolding of PTE variants. 10 μM of purified PTE variants was denatured in 30 mM HCl for 1 h at 25 °C and diluted 100-fold (final 0.1 μM PTE) into 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM $MgCl_2$ and 1 mM ATP in the absence or presence of 0.2 μM GroEL or GroEL/GroES overnight at 25 °C. The enzymatic activity was measured to determine the fraction of refolded active enzyme.

Computation of stability effects (ΔΔ*G*) by FoldX[46,47]. We applied the published four-step procedure: first, the structures of GAPDH (PDB 1S7C) and PTE (PDB 1HZY) were optimized; second, mutant three-dimensional structures were created by introducing one mutation at a time, and locally optimizing the structure; third, the energy functions of the mutants were computed and compared with the wild-type function; fourth, the actual free energy values were calculated based on the FoldX correlation function between experimental and calculated data ($\Delta\Delta G^{Experimental} = (\Delta\Delta G^{FoldX} + 0.078)/1.14$ (ref. 48).

37. Kerner, M. J. *et al.* Proteome-wide analysis of chaperonin-dependent protein folding in *Escherichia coli*. *Cell* **122**, 209–220 (2005).
38. Roodveldt, C. & Tawfik, D. S. Shared promiscuous activities and evolutionary features in various members of the amidohydrolase superfamily. *Biochemistry* **44**, 12728–12736 (2005).

39. Roodveldt, C. & Tawfik, D. S. Directed evolution of phosphotriesterase from *Pseudomonas diminuta* for heterologous expression in *Escherichia coli* results in stabilization of the metal-free state. *Protein Eng. Des. Sel.* **18,** 51–58 (2005).
40. Aharoni, A. *et al.* Directed evolution of mammalian paraoxonases PON1 and PON3 for bacterial expression and catalytic specialization. *Proc. Natl Acad. Sci. USA* **101,** 482–487 (2004).
41. Kitagawa, M. *et al.* Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. *DNA Res.* **12,** 291–299 (2005).
42. Gould, S. M. & Tawfik, D. S. Directed evolution of the promiscuous esterase activity of carbonic anhydrase II. *Biochemistry* **44,** 5444–5452 (2005).
43. Nishihara, K., Kanemori, M., Kitagawa, M., Yanagi, H. & Yura, T. Chaperone coexpression plasmids: differential and synergistic roles of DnaK–DnaJ–GrpE and GroEL–GroES in assisting folding of an allergen of Japanese cedar pollen, Cryj2, in *Escherichia coli*. *Appl. Environ. Microbiol.* **64,** 1694–1699 (1998).
44. Misset, O. & Opperdoes, F. R. Simultaneous purification of hexokinase, class-I fructose-bisphosphate aldolase, triosephosphate isomerase and phosphoglycerate kinase from *Trypanosoma brucei*. *Eur. J. Biochem.* **144,** 475–483 (1984).
45. Yifrach, O. & Horovitz, A. Transient kinetic analysis of adenosine 5′-triphosphate binding-induced conformational changes in the allosteric chaperonin GroEL. *Biochemistry* **37,** 7083–7088 (1998).
46. Guerois, R., Nielsen, J. E. & Serrano, L. Predicting changes in the stability of proteins and protein complexes: a study of more than 1000 mutations. *J. Mol. Biol.* **320,** 369–387 (2002).
47. Schymkowitz, J. *et al.* The FoldX web server: an online force field. *Nucleic Acids Res.* **33,** W382–W388 (2005).
48. Tokuriki, N., Stricher, F., Schymkowitz, J., Serrano, L. & Tawfik, D. S. The stability effects of protein mutations appear to be universally distributed. *J. Mol. Biol.* **369,** 1318–1332 (2007).