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SHORT COMMUNICATION

How many ionizable groups can sit on a protein hydrophobic core?

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ABSTRACT

Full or partial burial of ionizable groups in the hydrophobic interior of proteins underlies the large modulation in group properties (modified pK value, high nucleophilicity, enhanced capability of interaction with chemical moieties of the substrate, etc.) linked to biological function. Indeed, the few internal ionizable residues found in proteins are known to play important functional roles in catalysis and, in general, in energy transduction processes. However, ionizable-group burial is expected to be seriously disruptive and, it is important to note, most functional sites contain not just one, but several ionizable residues. Hence, the adaptations involved in the development of function in proteins (through *in vitro* engineering or during the course of natural evolution) are not fully understood. Here, we explore experimentally how proteins respond to the accumulation of hydrophobic-to-ionizable residue substitutions. For this purpose, we have constructed a combinatorial library targeting a hydrophobic cluster in a consensus-engineered, stabilized form of a small model protein. Contrary to naïve expectation, half of the variants randomly selected from the library are soluble, folded, and active, despite including up to four mutations. Furthermore, for these variants, the dependence of stability with the number of mutations is not synergistic and catastrophic, but smooth and approximately linear. Clearly, stabilized protein scaffolds may be robust enough to withstand many disruptive hydrophobic-to-ionizable residue mutations, even when they are introduced in the same region of the structure. These results should be relevant for protein engineering and may have implications for the understanding of the early evolution of enzymes.

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Key words: protein stability; mutation effects; disruptive mutations; protein robustness; polar group burial; enzyme catalysis.

INTRODUCTION

Ionizable residues in proteins tend to be located at the surface, where they can establish favorable interactions with the aqueous solvent. Still, a small number of ionizable groups are often found in the hydrophobic interior of many proteins. This is perhaps not too surprising, since burial (full or partial) may place ionizable groups in local environments leading to the large modulation in properties (large pK shift, for instance) required for catalysis.[1,2] Indeed, internal ionizable residues are known to play important functional roles, not only in catalysis but also in ion transport, homeostasis, light-activated processes and, in general, energy transduction.[1–5] Certainly, ionizable-group burial is expected to be disruptive. Nevertheless, recent pioneering work by Garcia-Moreno and coworkers has demonstrated that many proteins can tolerate one hydrophobic-to-ionizable substitution.[3,4] That is, mutating a hydrophobic residue to, for instance, glutamate or lysine is clearly destabilizing, but the protein may remain folded and functional after mutation, provided that its stability prior to mutation was sufficiently high. Following this lead, we have shown that the pK value of an internal residue thus created can be modulated using rational protein engineering.[6] These results are highly relevant for our understanding of molecular evolution, as they support that, likely, special structural

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adaptations are not required to make possible the presence of functionally important internal ionizable residues and, therefore, the development of function may be determined to a significant extent by protein stability,[3,4] an scenario which is fully consistent with the known proposal that stability promotes evolvability.[7] Furthermore, they are also relevant from the point of view of protein engineering, as clearly demonstrated by the recent design of a switchable Kemp eliminase on the basis of a single hydrophobic-to-ionizable mutation at the bottom of an hydrophobic cavity.[8]

However, there is one essential issue to consider in this context. The studies referred to above have demonstrated that proteins can tolerate ONE hydrophobic-to-ionizable mutation, while many functional sites contain SEVERAL ionizable and/or charged residues. Examples abound:[1] several carboxylic acids are considered essential catalytic groups in glycosidases, ribonucleases, aspartic proteases and hen-egg white lysozyme; two lysine residues are thought to be involved in the catalytic mechanism of acetoacetate decarboxylase; the catalytic triad in serine proteases includes two ionizable residues (histidine and aspartate); two arginines interact with the carboxylate groups of the substrate in the active site of aspartate aminotransferase; glutamate and histidine residues play essential roles in the catalytic mechanism of triosephosphate isomerases; the pK values of the Schiff's base with retinal and several aspartic acid residues change during the photocycle in bacteriorhodopsin, driving the functionally relevant proton transfers[9]; internal arginine and histidine residues have been proposed to play an essential role in preventing proton permeation through the water channel aquaporin.[10]

In view of the above, the two key questions would actually seem to be: "are special adaptations (other than high stability) required to allow the introduction of SEVERAL functionally important internal ionizable residues?" and "can a protein tolerate SEVERAL hydrophobic-to-ionizable residue substitutions?". It would appear that the answers to these related questions should be "yes" and "no", respectively. First of all, it is relevant here that the recent directed-evolution studies of Tokuriki and Tawfik,[11] aimed at increasing protein evolvability through buffering of destabilizing mutation effects, did not lead to a significant number of hydrophobic-to ionizable residue substitutions. These authors performed random drifts *in vivo* for proteins under chaperonin overexpression. They certainly found an increase in the number of mutations in core residues (as compared with the results of random drifts carried out without chaperonin overexpression). However, for glyceraldehydophosphate dehydrogenase and phosphotriesterase none of the core mutations reported as arising from drifts with chaperonin overexpression involved the replacement of hydrophobic residues with ionizable or charged residues (see the ~400 mutations reported in Supporting Information Table 8 of Tokuriki and Tawfik[11]). From a more general viewpoint, it is well-known that apolar residues in protein structures cluster together, reflecting the fact that hydrophobicity (loosely defined as the tendency of apolar moieties to avoid aqueous environments) is one of the main thermodynamic forces that drive folding. Introducing one hydrophobic-to-ionizable residue substitution will perturb one structurally essential hydrophobic cluster. However, introducing several such mutations at neighboring positions could be expected to essentially destroy the cluster and prevent the protein from folding properly.

Overall, the results and reasoning summarized above would seem to argue against the feasibility of introducing several hydrophobic-to-ionizable residue substitutions in the same region of a protein. Despite this expectation, we deemed of interest to perform an experimental test of the possibility of going beyond the single-mutation level in this context. For this purpose, we constructed a combinatorial library targeting a hydrophobic cluster in a consensus-engineered, stabilized form of *E. coli* thioredoxin[12] and found that, contrary to the naïve expectation, many of the variants randomly selected from the library are soluble, folded and active, despite including a comparatively large number of hydrophobic-to-ionizable residue substitutions.

MATERIALS AND METHODS

The combinatorial library of thioredoxin variants was constructed using gene—assembly mutagenesis,[13] as previously described.[12] For ease of purification, the variants had a His_6 tag attached to the amino-terminal (i.e., roughly opposite to the targeted hydrophobic cluster). The purification of these His_6-tagged variants was carried out as previously described.[12] All the 40 variants randomly selected for purification were soluble in the high-salt buffer used in the last step of the purification. However, significant precipitation was observed in many of them upon dialysis against the low-salt buffer (50 m*M* Hepes, pH 7) used in the calorimetric and spectroscopic experiments, with 50% of the variants precipitating completely. These insoluble variants, therefore, were not subjected to biophysical characterization. Reductase activity was measured using a turbidimetric assay of the thioredoxin catalyzed rate of reduction of insulin,[14] as previously described.[6]

Differential scanning calorimetry experiments were carried out in a capillary VP-DSC microcalorimeter (MicroCal, General Electric) as previously described.[12] Far-UV circular dichroism spectra were acquired using a Jasco (Tokyo, Japan) J-715 spectropolarimeter, as previously described.[6]

Native PAGE was performed as described by the manufacturer (Instruction Manual Mini-PROTEAN ® Tetra Cell, BIO-RAD), using a continuous buffer system. Pro-

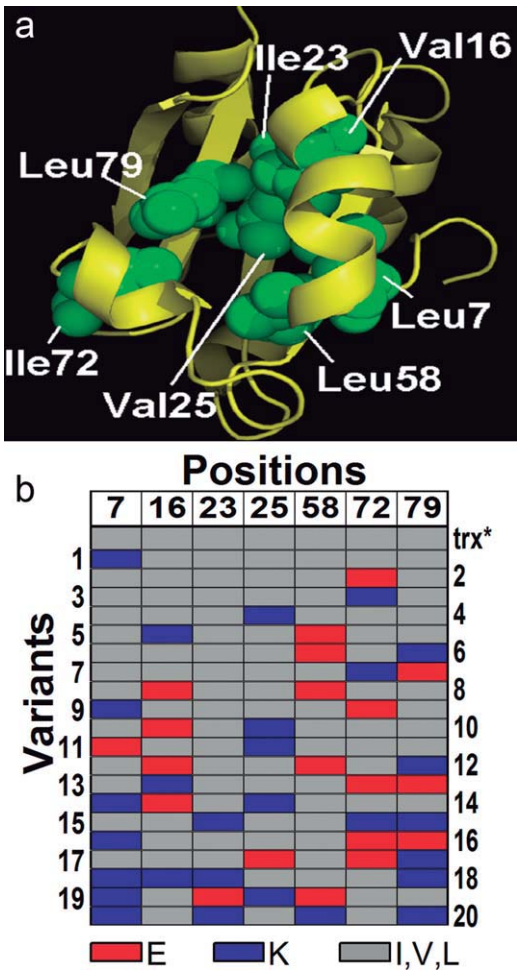

Figure 1

(**a**) Structure of thioredoxin (2trx) showing (green) the seven buried hydrophobic residues targeted for mutation. The side-chain accessible surface areas (in Å^2) for these residues are 0 (Leu7), 0 (Val16), 5.0 (Ile23), 0 (Val25), 0 (Leu58), 6.2 (Ile72), and 4.6 (Leu79). [Note that Ile75, also belonging to the same cluster, was not targeted because of its proximity to the active-site disulfide bridge]. A combinatorial library including all combinations of three possibilities (no mutation, mutation to Lys and mutation to Glu) over the seven positions was constructed. (**b**) Sequences of the library variants selected and analyzed in terms of structure, function and stability. Residues at the seven positions targeted are color-coded as gray (the hydrophobic side-chain present in the background trx* variant) red (Lys) and blue (Glu).

tein separation was carried out on 6% polyacrylamide gels. Totally 43 m*M* imidazole and 35 m*M* HEPES, pH 7.4 was used as continuous buffer according to McLellan.[15] Coomassie Blue staining was done following standard protocols. Final protein concentration was around 0.6 mg/mL. In order to assess the protonation state of our Lys/Glu trx* variants, a new set of protein variants was prepared to be used as charge electrophoretic markers. Thus, using trx* as background, a number of variants from a library targeting surface positions (manuscript in preparation) were selected for charge mutations. Since only solvent-exposed positions were mutated in these marker variants, there is little uncertainty regarding their total charge with respect to the trx* background. In particular, five different protein markers were obtained and purified as described previously.[12] Details regarding their sequence and extra charge taking trx* as reference are as follows: T14K/T54K/A105K (+3), H6E/T14K/T54K/Q62K/Q85K/Q98E/A105K (+3), T14K/T54K/N83D/Q85K/Q98E (+1), A68E/N83D/A105K (–1) and A50E/A68E/N83D/Q85K (−2). All 20 library variants [Fig. 1(b)] were studied by native PAGE (see Fig. S1 in Supporting Information for a representative example). To a good degree of approximation, integer values for the net charge (with respect to trx*) could be assigned to all the library variants through comparison with the markers (see Table I).

RESULTS AND DISCUSSION

In anticipation of the destabilizing character of the intended hydrophobic-to-ionizable residue mutations, we used as background a highly-stable (and fully active) variant of *E. coli* thioredoxin (trx*) previously obtained through consensus engineering.[12] We targeted 7 fully buried (accessible surface area ∼ 0) positions in the hydrophobic cluster involving the central β-sheet and helices 11–17 and 59–70 [Fig. 1(a)]. For each position we

Table I
Net Charges of the Thioredoxin Variants Described in Figure 1 and Possible Interpretations in Terms of Integer Charges at the Ionizable Residues

Variant	Net charge	Interpretations
Trx*	0	
1	1	K7+
2	−1	E72−
3	1	K72+
4	0	K25n
5	1	K16+ E58n
6	1	E58n K79+
7	0	K72+ E79− K72n E79n
8	−1	E16− E58n E16n E58−
9	0	K7+ E72− K7n E72n
10	−1	E16− K25n
11	0	E7− K25+ E7n K25n
12	0	E16− E58n K79+ E16n E58− K79+ E16n E58n K79n
13	−1	K16+ E72− E79− K16n E72− E79n K16n E72n E79−
14	1	K7+ E16− K25+ K7n E16n K25+ K7+ E16n K25n
15	3	K23+ K72+ K79+
16	−1	K7+ E72− E79− K7n E72− E79n K7n E72n E79−
17	−1	E25− E72− K79+ E25n E72− K79n E25− E72n K79n
18	3	K7+ K16+ K23+ K79n K7+ K16+ K23n K79+ K7+ K16n K23+ K79+ K7n K16+ K23+ K79+
19	1	K7+ E23n K25n E58n K7+ E23− K25+ E58n K7+ E23n K25+ E58− K7n E23n K25+ E58n
20	3	K7+ K23+ K58+ K79n K7+ K23+ K58n K79+ K7+ K23n K58+ K79+ K7n K23+ K58+ K79+

Note that values of the net charges are given with referente to trx* (i.e., net charge of variant minus net charge of trx*).

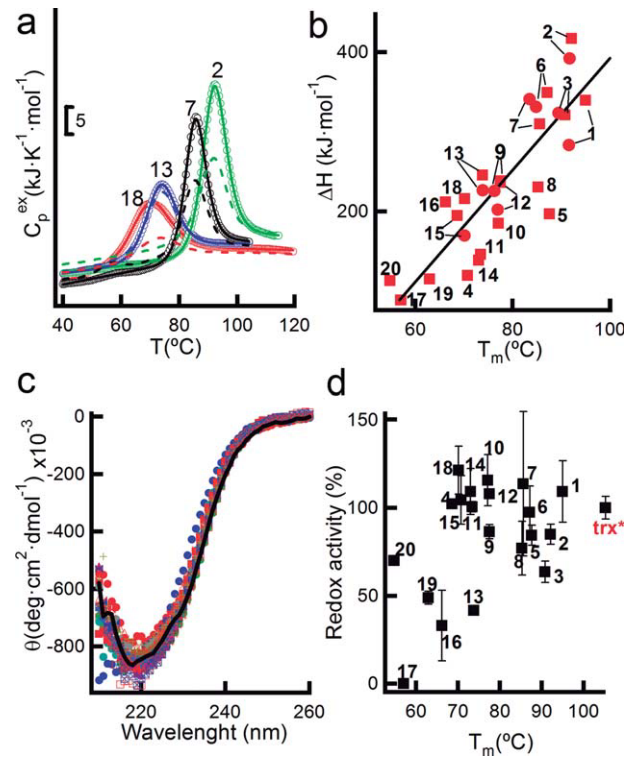

Figure 2

(**a**) Representative scanning calorimetry thermograms for the thermal denaturation of the thioredoxin variants studied in this work [sequences given in Fig. 1(b)]. Reheating runs are shown with dashed lines. Continuous lines are the best fits of a pseudo-two-state model to the first-heating data. (**b**) Plot of denaturation enthalpy versus denaturation temperature including all the studied thioredoxin variants. (**c**) Far-UV circular dichroism spectra for all the 20 studied thioredoxin variants. The spectra of the background trx* variant is shown with a continuous black line. (**d**) Plot of reductase activity (as percentage of the activity of the trx* background variant) versus denaturation temperature for all the 20 studied thioredoxin variants. Note that all variants (except variant number 17) display significant redox activity. The numbers in all the panels refer to the variants listed in Figure 1(b). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

considered three possibilities (no mutation, mutation to glutamate, mutation to lysine) and constructed a combinatorial library including all combinations of the 3 possibilities over the 7 positions. The library comprised $3^7 = 2187$ variants of which 40 were randomly selected for purification. Of these, 20 variants [Fig. 1(b)] were soluble in low-salt buffer at the tenth of mg/mL level at least, and were amenable to characterization in terms of stability [Figs. 2(a,b) and 3(a)], structure [Fig. 2(c)] and function [Figs. 2(d) and 3(b)]. Examination of the sequences of the soluble [Fig. 1(b)] and low-solubility [Fig. 4(a)] variants reveals that a large number of mutations and the presence of a lysine at position 58 may contribute to the insolubility (see Fig. 4 for details). Circular dichroism spectra did not support drastic structural alterations in the soluble variants [Fig. 2(c)], which, in fact, retained in most cases significant reductase activity [Figs. 2(d) and 3(b)]. Scanning calorimetry showed well-defined thermal-denaturation transitions which displayed significant reversibility in most cases [Fig. 2(a)]. Calorimetric transitions were well described by pseudo-two-state model including a van't Hoff enthalpy to describe the temperature dependence of the unfolding equilibrium constant [Fig. 2(a)]. Van't Hoff enthalpy values were found in some cases to be higher than the corresponding calorimetric enthalpies. Van't Hoff to calorimetric enthalpy ratios larger than unity have been previously reported for *E. coli* thioredoxin and attributed to partial dimerization in both, the native and the unfolded state.[16] For a significant number of variants (13), the quality of the pretransition and post-transition baselines allowed reliable estimates of the unfolding heat capacity change to be

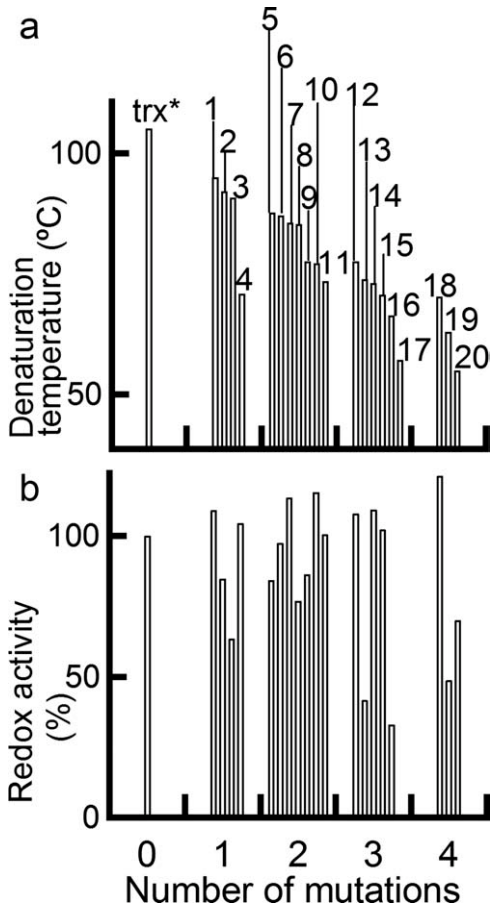

Figure 3

Denaturation temperature (**a**) and redox activity (**b**) of the thioredoxin variants specified in Figure 1(b) plotted versus the number of hydrophobic-to-ionizable residue mutations. Note in (a) the approximately linear dependence of the denaturation temperature value with the number of mutations. Redox activity values in (b) are given as percentages of the activity of trx* background variant. Note that all variants (except 17) display significant redox activity.

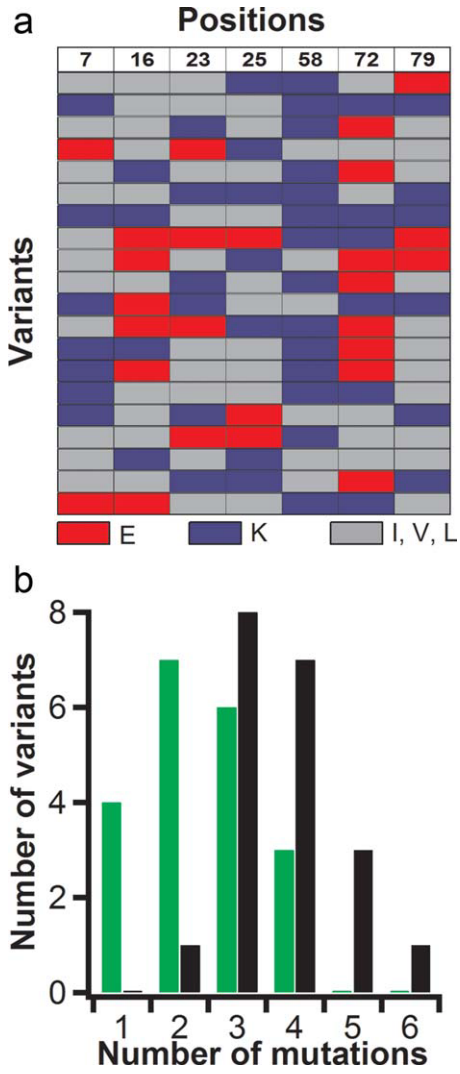

Figure 4

(**a**) Sequences of 20 thioredoxin variants that precipitated in the low-salt buffer used in the biophysical characterization studies. The color code is the same as that used in Figure 1(b) for the 20 variants that showed significant solubility in the low-salt buffer and were characterized in terms of structure, function and stability (Figs. 2 and 3). Note the presence of L58K in most of the insoluble variants. (**b**) Number of variants versus number of mutations distributions for the soluble variants [green, sequences given in Fig. 1(b)] and the insoluble variants (gray, sequences given in panel a of this figure). Note that insoluble variants tend to have a larger number of mutations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

obtained from the experimental thermograms. These ΔC_P ranged between 4 and 7 kJ K^{-1} mol^{-1} with an average value of 5.1 $\pm$ 0.8 kJ K^{-1} mol^{-1}, in agreement with the experimental value for wild type thioredoxin unfolding: 5.8 $\pm$ 0.8 kJ K^{-1} mol^{-1}.[17] A plot of calorimetric enthalpy versus denaturation temperature [Fig. 2(b)] including all studied variants is roughly linear with a slope of 7.0 $\pm$ 0.8 kJ K^{-1} mol^{-1}. The fact that this slope is somewhat higher that the average ΔC_P value derived from the experimental calorimetric baselines, together with the dispersion observed in the plot of Figure 2(b), may be reflecting local structural reorganizations concomitant with the introduction of the ionizable groups at internal positions (see further below). Note, nevertheless, that the enthalpy scatter seen in Figure 2(b) has little influence on stability due to enthalpy-entropy compensation (see Fig. 5 and Fig. S2 and S3 in Supporting Information).

The most striking results were revealed by the values of the denaturation temperature, a distinct proxy of protein stability (see Fig. 5 for details on the relation between T_m and the unfolding free energy). As was to be expected, there is a stability price to be paid for the progressive destruction of a protein hydrophobic cluster. However, the dependence of stability on the number of hydrophobic-to-ionizable mutations [Fig. 3(a)] is not synergistic and catastrophic, but smooth and approximately linear, with a slope of about -10° per mutation, which, in terms of free energy is roughly equivalent to a decrease of about 7–8 kJ mol^{-1} per mutation (see Fig. 5). It is interesting to note in Figure 5 that the unfolding free energy at 37°C is only slightly above zero for the lowest stability variants. This suggests that the number of hydrophobic-to-ionizable substitutions that can be accepted is determined to a significant extent by the exhaustion of the stability at physiological temperature of the trx* variant used as background.

Finally, native PAGE was used to assess the total charge of the 20 variants studied. Table I collects the net charge values obtained as well as the possible interpretations in terms of integer charges on the mutated residues. While unique interpretations are not possible for most variants, it is clear that a significant number of the introduced ionizable residues must be charged in our library variants. To properly discuss this result, it is useful to review two different scenarios that emerge from published studies on buried ionizable groups in proteins. Statistical analyses of protein structures support that "wild-type" ionizable groups can sometimes be fully buried, provided that they form stabilizing hydrogen bonds and or salt bridges with other groups.[18,19] Introduction of hydrophobic-to-ionizable substitutions, on the other hand, likely leads to a different scenario, in particular when the ionizable groups become charged. For instance, recent molecular dynamics simulations on 18 variants of staphylococcal nuclease in which internal positions have been replaced by ionizable residues one at a time[20] suggest that backbone reorganization, localized partial unfolding, water penetration and increase in hydration occur concomitantly with residue charging. It is to be noted that this partial exposure of internal groups upon charging may actually be convenient from an enzyme engineering viewpoint, as the charged residue will be accessible to the substrate

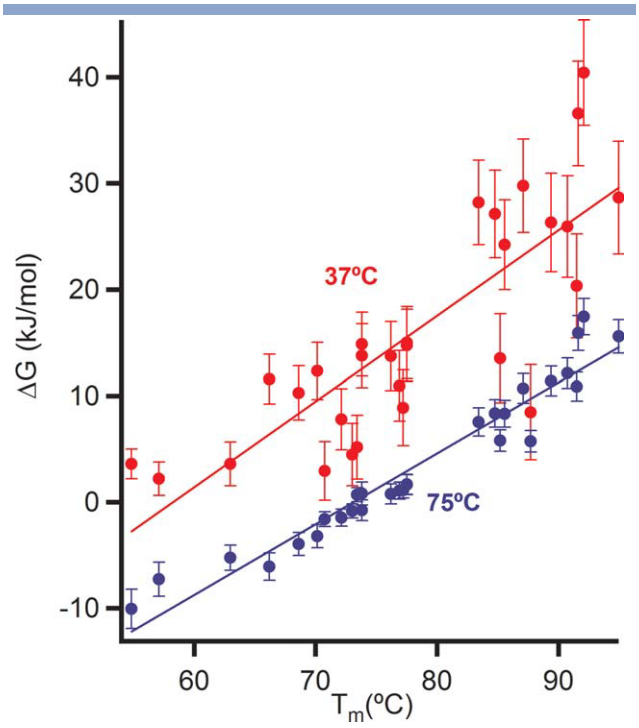

Figure 5

Correlation between unfolding free energies and denaturation temperature values for the variants characterized in this work (Figs. 1 and 2). ΔG values were calculated using the integrated Gibbs-Helmholtz equation:

$$\Delta G = \Delta H_m \cdot \left(1 - \frac{T}{T_m}\right) + \Delta C_P \cdot \left[T - T_m - T \cdot \ln\left(\frac{T}{T_m}\right)\right],$$

with the denaturation temperatures (T_m) and unfolding enthalpies at the T_m's determined from each calorimetric experiment [see Fig. 2(b) in the main text], together with an average value for the unfolding heat capacity change of 5.1 kJ K^{-1} mol^{-1} (see Fig. S2 in Supporting Information for a similar calculation using the actual ΔC_P values derived from the individual calorimetric experiments). This calculation assumes that the T_m value provides a good estimate of the temperature at which the free energies of the native and unfolded states are equal, even if there are deviations from two-state behavior (for details see Sanchez-Ruiz[24]). The ΔG values are calculated for 75 and 37°C, the noise being larger for the latter temperature reflecting the very long temperature-extrapolation. The estimated errors shown were calculated by the Monte Carlo method assuming Gaussian distributions for T_m, ΔH, and ΔC_P with standard deviations of 1.5°, 24.6 kJ mol^{-1} (from T_m and ΔH reproducibility in replicated experiments) and 0.76 kJ K^{-1} mol^{-1}, respectively. Straight lines are the best linear least squares fits and are meant to describe the general trends (actually, a small curvature may be seen in the ΔG vs. T_m plots). The slopes of these lines are 0.67 ± 0.03 kJ K^{-1} mol^{-1} (75°C) and 0.85 ± 0.11 kJ K^{-1} mol^{-1} (37°C). Accordingly, a decrease of 10° in denaturation temperature translates into a decrease of 7–8 kJ mol^{-1} in unfolding free energy. The ΔG values given in this figure should be considered as estimates, as they have not been corrected for potential kinetic distortions associated to partial irreversibility in the denaturation processes. Nevertheless, the ΔG vs. T_m correlations shown clearly support the use of the denaturation temperature as a proxy of thermodynamic stability in this case. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

but will still be in a microenvironment that leads to the modulation in properties useful in catalysis. For instance, NMR structure determination suggests that the accessibility to solvent of the catalytic Glu92 is roughly 10% in the Kemp eliminase[8] recently designed by DeGrado and coworkers (Ivan Korendovych and William DeGrado, personal communication); still, the presence of Glu92 in a hydrophobic cavity could enhance its basicity and facilitate the dehydration associated with substrate binding.

Overall, the existence of the two scenarios we have described above (fully buried charged "wild-type" groups stabilized by hydrogen bonds and/or salt bridges versus partial exposure to water upon charging of the residues resulting from a hydrophobic-to-ionizable substitution at an internal position) is consistent with the notion that the protein moeity, being rigid, and not polarizable, can stabilize internal charges in particular locations but is not likely to stabilize randomly introduced internal charges.[21] The relevant questions here are which scenario applies to the stabilization of the charges newly introduced (Table I) and how that stabilization relates to the unexpected success in preparing variants with a significant number of hydrophobic to-ionizable residue substitutions. Simple structural modeling (see Fig. S4 and S5 in Supporting Information) suggests that the thioredoxin scaffold used could accommodate a significant number of the fully buried ionizable groups in their neutral forms, at least in single-mutant variants. However, no specific design for stabilization of buried charges is involved in our multiple-mutant variants. Therefore, it appears likely that the charges created (Table I) are stabilized by increased-conformational-flexibility/partial-exposure mechanism. Certainly, we cannot rule out that favorable interactions between the polar groups of the glutamate and lysine residues (hydrogen-bonding, opposite charge interactions) may also contribute to avoid a precipitous drop in stability with the number of mutations. Note, however, that variants 18 and 20 [Fig. 1(b)] with 4 mutations to lysine, have denaturation temperature values [Fig. 3(a)] similar to that of variant 19, which has two mutations to glutamate and two mutations to lysine. Finally, it is also plausible that the methylene moieties of the lysine and glutamate side-chains could still participate in some kind of stabilizing hydrophobic interaction and thus contribute to the stabilization of our multiple mutant variants.

CONCLUSIONS

Although it must be recognized that the results reported here are to some extent open for molecular interpretation (see last paragraph in the preceding section), it is highly relevant that the stability/number-of-mutations dependence is nearly linear and, consequently, that variants with a large number of hydrophobic-to-ionizable residue substitutions can be readily prepared. In fact, variants with four mutations can be prepared,

although significantly less stable than the trx* background, show denaturation temperatures similar to those of many proteins from mesophilic organisms. It is likely that variants with even larger number of mutations could be obtained by using a further stabilized thioredoxin scaffold as background. Our results, therefore, have obvious implications for protein engineering and evolution that we briefly summarize below.

Replacing an internal hydrophobic residue with an ionizable one may lead to a polar group with the anomalous properties useful in enzyme catalysis (modified pK value, high nucleophilicity, enhanced capability of interaction with chemical moieties of the substrate, etc.) and several such internal ionizable residues may be required for biological function. We have shown that proteins may be robust enough to withstand many disruptive hydrophobic-to-ionizable residue mutations, even in the same region of the structure. Furthermore, we have provided a first experimental estimate of the associated pattern of stability penalties, which may be useful when selecting stabilized scaffolds for protein engineering tasks, such as the design of novel activities. Finally, we may speculate that the capability of high-stability proteins to accumulate many disruptive mutations may have played a role in the development of early enzyme catalysts. It is relevant in this context that ancestral reconstruction has revealed large stability enhancements for the oldest proteins.[22,23]

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REFERENCES

1. Harris TK, Turner GJ. Structural basis of perturbed pK_a values of catalytic groups in enzyme active sites. IUBMB Life 2002;53:85–98.
2. Warshel A, Sharma PK, Kato M, Parson WW. Modeling electrostatic effects in proteins. Biochim Biophys Acta 2006;1764:1647–1676.
3. Isom DG, Cannon BR, Castañeda CA, Robinson A, Garcia-Moreno BE. High tolerante for ionizable residues in the hydrophobic interior of proteins. Proc Natl Acad Sci USA 2008;105:17784–17788.
4. Isom DG, Castañeda CA, Cannon BR, Velu PD, Garcia-Moreno BE. Charges in the hydrophobic interior of proteins. Proc Natl Acad Sci USA 2010;107:16096–16100.
5. Karp DA, Stahley MR, Garcia-Moreno BE. Conformational consequences of ionization of Lys, Asp, and Glu buried at position 66 in staphylococcal nuclease. Biochemistry 2010;49:4138–4146.
6. Pey AL, Rodríguez-Larrea D, Gavira JA, Garcia-Moreno B, Sanchez-Ruiz JM. Modulation of buried ionizable groups in proteins with engineered surface charge. J Am Chem Soc 2010;132:1218–1219.
7. Bloom JD, Labthavikul ST, Otey CR, Arnold FH. Protein stability promotes evolvability. Proc Natl Acad Sci USA 2006;103:5869–5874.
8. Korendovych IV, Kulp DW, Wu Y, Cheng H, Roder H, DeGrado WF. Design of a switchable eliminase. Proc Natl Acad Sci USA 2011;108:6823–6827.
9. Luecke H, Richter H-T, Lanyi JK. Proton transfer in bacteriorhodopsin at 2.3 ansgstrom resolution. Science 1998;280:1934–1937.
10. Agre P. The aquaporin water channels. Proc Am Thorac Soc 2006;3:5–13.
11. Tokuriki N, Tawfik DS. Chaperonin overexpression promotes genetic variation and enzyme evolution. Nature 2009;459:668–673.
12. Rodríguez-Larrea D, Perez-Jimenez R, Sanchez-Romero I, Delgado-Delgado A, Fernandez JM, Sanchez-Ruiz JM. Role of conservative mutations in protein multi-property adaptation. Biochem J 2010;429:243–249.
13. Bessette PH, Mena MA, Nguyen AW, Daugherty PS. Construction of designed protein libraries using gene assembly mutagenesis. Methods Mol Biol 2003;231:29–37.
14. Holmgren A. Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. J Biol Chem 1979;254:9627–9632.
15. McLellan T. Electrophoresis buffers for polyacrylamide gels at various pH. Anal Biochem 1982;126:94–99.
16. Ladbury JE, Wynn R, Hellinga HW, Sturtevant JM. Stability of oxidized *Escherichia coli* thioredoxin and its dependence on the protonation of the aspartic acid residue in the 26 position. Biochemistry 1993;32:7526–7530.
17. Georgescu RE, Garcia-Mira MM, Tasayco ML, Sanchez-Ruiz JM. Heat capacity análisis of oxidized Escherichia coli thloredoxin fragments (1–73, 74–108) and their noncovalent complex. Evidence for the burial of apolar surface in protein unfolded states. Eur J Biochem 2001;268:1477–1485.
18. Rashin AA, Honig B. On the environment of ionizable groups in globular proteins. J Mol Biol 1984;173:515–531.
19. Bush J, Makhatadze GI. Statistical analysis of protein structures suggests that buried ionizable residues in proteins are hydrogen bonded or form salt bridges. Proteins 2011;79:2027–2032.
20. Damjauovic A, Brooks BR, Garcia-Moreno B. Conformational relaxation and water penetration coupled to ionization of internal groups in proteins. J Phys Chem A 2011;115:4042–4053.
21. Kim J, Mao J, Gunner MR. Are acidic and basic groups in buried proteins predicted to be ionized? J Mol Biol 2005;348:1283–1298.
22. Gaucher EA, Govindarajan S, Ganesh OK. Paleotemperature trend for Precambrian life inferred from resurrected proteins. Nature 2008;451:704–707.
23. Perez-Jimenez R, Ingles-Prieto A, Zhao ZM, Sanchez-Romero I, Alegre-Cebollada J, Kosuri P, Garcia-Manyes S, Kappock TJ, Tanokura M, Holmgren A, Sanchez-Ruiz JM, Gaucher EA, Fernandez JM. Single-molecule paleoenzymology probes the chemistry of resurrected enzymes. Nat Struct Mol Biol 2011;18:592–596.
24. Sanchez-Ruiz JM. Ligand effects on protein thermodynamic stability. Biophys Chem 2007;126:43–49.
25. Sharp K. Entropy-enthalpy compensation: fact or artifact? Protein Sci 2001;10:661–667.