Modulation of Buried Ionizable Groups in Proteins with Engineered Surface Charge

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Most ionizable residues in proteins are found at the surface, involved in energetically favorable interactions with water. Many proteins can actually accommodate a small number of ionizable residues at buried positions, in particular when these internal residues are required for important roles in catalysis,[1,2] ion transport and homeostasis,[3−5] light-activated processes,[6] and, in general, energy transduction processes.[7] The ability to use rational design to introduce buried ionizable groups with tailored properties in suitable protein scaffolds should, therefore, be an important addition to the protein engineering toolbox. Recent work by Garcia-Moreno and co-workers has shown that proteins can indeed tolerate hydrophobic-to-ionizable-residue mutations.[7,8] In most cases the buried residues undergo a large pK_a shift (downward for buried lysines and upward for buried carboxylic acids); the ionizable residues are actually neutral at physiological pH values, thus minimizing the unfavorable interactions with the hydrophobic environment. Clearly, the next goal should be the development of approaches to modulate the pK_a and the ionization state of the buried residue. We provide experimental evidence here that this goal can be achieved through re-engineering of the surface charge distribution.

Charges at exposed surface positions are expected to be hydrated and exert only comparatively weak Coulomb effects. Still, the sum of many such small effects may have important consequences, and in fact, there is growing evidence[9−11] that the design of surface charge distribution is a very efficient method to modulate and enhance relevant protein properties, such as stability and folding−unfolding barriers. Furthermore, since charge−charge interactions are long-range, we may further expect surface charges to affect buried ionizable residues[12] and modify their pK_a values, as in this case Coulomb interactions may occur to a significant extent through the protein proper, which has a lower dielectric constant than that of water. To examine the magnitude of Coulomb interactions between internal and surface groups, we took advantage of the availability of two suitable variants of *E. coli* thioredoxin, which shall be referred to as 3x and 6x. These proteins were characterized previously.[13] 3x is a consensus-stabilized variant with three mutations that do not involve ionizable residues (A22P, I23V, P68A). 6x includes three additional charge-reversal mutations at exposed positions (K3E, K18E, K69E) (Figure 1a); this variant has altered surface electrostatics with respect to the wild type and 3x forms.

The leucine residue at position 58 in *E. coli* thioredoxin is fully buried in the hydrophobic interior (ASA value strictly equal to zero). In the wild-type protein, the closest-atom separation distances

between this residue and surface residues 3, 18, and 69 (charge-reversed in 6x) are, respectively, 11.5, 13.1, and 8.3 Å. We have introduced the L58K mutation in both the 3x and 6x backgrounds. The L58K substitution does not seem to have any detectable effects on the structure of the protein, as evidenced by the almost identical circular dichroism spectra and by the fact that the four variants (3x, 6x, $3x_{L58K}$, and $6x_{L58K}$) retain similar levels of enzymatic activity (in both the insulin reduction and thioredoxin reductase assays[14]) which are also similar to that of wild-type thioredoxin (Figure S1 and Table S1 in the Supporting Information).
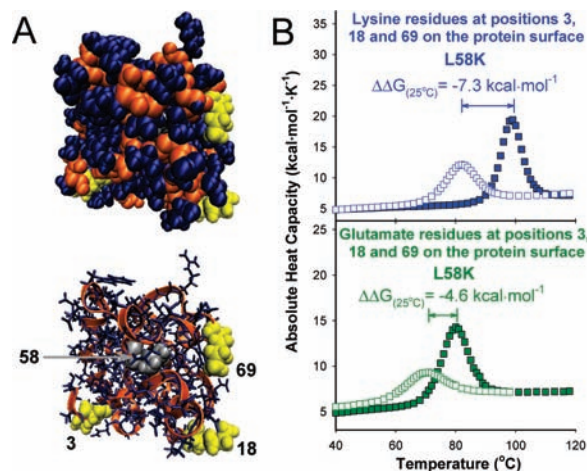

Figure 1. (A) Structure of *E. coli* thioredoxin showing the buried and surface residues at positions 58 (in gray) and 3, 18, and 69 (in yellow) mutated in this study, protein backbone (in orange) and side chains (in blue). The structure in the upper panel employs van der Waals radii for all atoms to highlight the buried character of Leu58. (B) Absolute heat capacity versus temperature profile for the four variants studied in this work (upper panel, 3x background; lower panel, 6x background). Associated standard errors are smaller than the size of the data points. The effect of the L58K mutation on the protein free energy (taking the unfolded state observed at high temperature as reference) is indicated for both 3x and 6x backgrounds. These $\Delta\Delta G$ values have been derived from the calorimetric profiles using a model-free Gibbs−Helmholtz integration. The symbol code for this panel refers to the different protein variants: closed blue (3x); open blue ($3x_{L58K}$), closed green (6x); open green ($6x_{L58K}$).

On the other hand, the L58K substitution has a clear destabilizing effect, which is smaller in the 6x background than in the 3x background). This is evident even from a simple visual inspection of the scanning calorimetry profiles of Figure 1B. A model-free, Gibbs−Helmholtz integration of the calorimetric data (Figure S3) indicates that, in terms of free energy, the destabilizing effect of the L58K mutation is ∼3 kcal/mol smaller in the case of the 6x

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background (Figure 1B). This very large discrepancy is suggestive of stabilizing Coulomb interaction between the surface charges and a buried charge through a low dielectric constant medium. That is, while in the $3x_{L58K}$ variant the lysine residue at position 58 is to a significant extent deprotonated at pH 7, it is likely to be mostly charged in the $6x_{L58K}$ variant and, therefore, involved in stabilizing charge−charge interactions with some (or all) of the negative charges created by the surface charge-reversal mutations in the 6x background.

To gain further support for the above interpretation, we determined the titration profile of the lysine residue at position 58 by difference potentiometry[15] (i.e., using the fact that the titration of a single group in a protein can be assessed from the difference between the titration profiles of two suitable chosen variants: see Supporting Information for details). The potentiometric difference $3x_{L58K}$-3x (Figure 2A) indicates a depressed pK_a value of ∼6.4 for the Lys-58 in the 3x background (indicating significant deprotonation at pH 7). On the other hand, the potentiometric difference $6x_{L58K}$-6x is close to zero within the measured pH range (Figure 2A), a result which is consistent with a $pK_a > 9.5$ for the lysine residue in the 6x background and, consequently, with a charged lysine at pH 7. That this situation is associated to Coulombic interactions is further supported by experimentally detected salt effects (Figure S5 in Supporting Information).
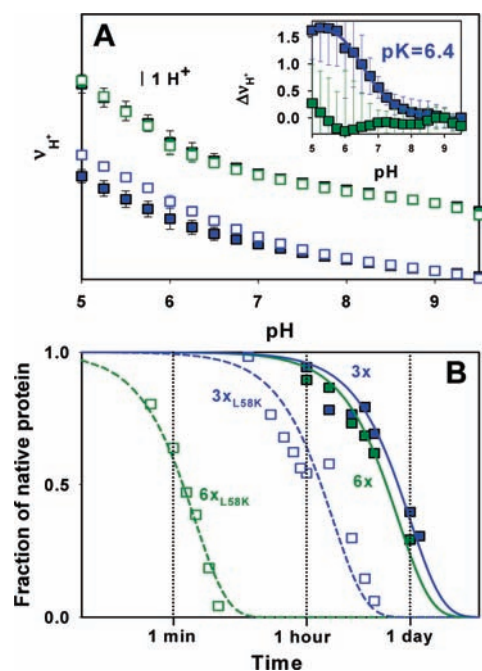

Figure 2. (A) Potentiometric titration curves (i.e., plots of number of protons bounds per molecule of protein vs pH) for the variants 3x, 6x, $3x_{L58K}$, $6x_{L58K}$. Curves for the 3x and 6x backgrounds have been shifted for the sake of clarity. The inset shows the L58K difference titration profiles for the 3x and 6x backgrounds. The continuous line is the best fit of the model described in the Supporting Information to the 3x-background data. From this fit, a pK_a value of 6.4 is obtained. (B) Resistance of the variants 3x, 6x, $3x_{L58K}$, $6x_{L58K}$ to degradation by thermolysin at 25 °C, pH 7. Lines are fits of a single-exponential decay. Note the logarithmic time scale.

Protonation of the buried lysine has a clear effect on protein dynamics, as indicated by two widely different types of experimental measurements: (A) heat capacity values provide a thermo-dynamic measure of energy fluctuations in proteins.[16,17] The value of the low-temperature heat capacity for the $6x_{L58K}$ variant is significantly higher than the values for the other three variants (Figures 1 and S2). This indicates larger energy fluctuations in $6x_{L58K}$, likely reflecting enhanced native-state structural fluctuations and transient unfolding processes. (B) recent work[18] has shown that native-state flexibility can be reliably probed by the susceptibility to proteolysis, and indeed, we find a much higher susceptibility to proteolysis by thermolysin in the $6x_{L58K}$ variant (Figures 2B and S4). It ought to be emphasized that, based on these two types of measurements, the 3x and 6x background variants display similar dynamics. The picture emerges, therefore, of increased conformational flexibility linked to the buried charged lysine in the 6x background (perhaps allowing some water penetration and stabilization of the buried positive charge). A similar situation has been described[19] for the K38 residue in staphylococcal nuclease, which titrates with a normal pK_a despite being buried.

Overall, our results support the notion that the essential properties (pK_a value, protonation state, local dynamics) of buried ionizable groups in proteins can be efficiently modulated through the rational design of the surface charge distribution, thus paving the way for the protein engineering exploitation of charge burial.

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