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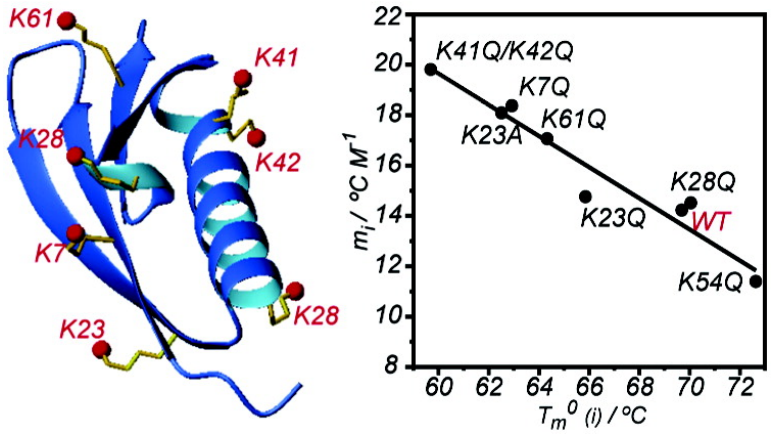
J. Am. Chem. Soc., **2005**, 127 (27), 9690-9691• DOI: 10.1021/ja051352e • Publication Date (Web): 15 June 2005



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Published on Web 06/15/2005

On the Origin of the Thermostabilization of Proteins Induced by Sodium Phosphate

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Thermal stability is an important requirement for biotechnological applications of enzymes.[1] There are different ways to modulate protein stability, including site-directed mutagenesis and the addition of cosolutes to the media.[2] The effect of different cations and anions in protein stability was first empirically described a long time ago in the Hofmeister series,[3] and it has been the object of intense research since then.[4] Protein−cosolute interactions and changes in the solvent-excluded volume have been identified as key players,[5] but, in general, the effect of cosolute ions in protein stability cannot be predicted yet. The influence of charged residues in the thermal stability of proteins has been demonstrated,[6] but, again, no definitive model is available. To gain insight into the molecular mechanism of anion-induced thermal stability, we have studied the effect of phosphate concentration on the thermal stability of the IGg binding domain of protein L from *Peptostreptococcus magnus* (Prot L) and on a series of surface mutants in which different lysine residues had been replaced by noncharged residues.[7] Thermal unfolding has been monitored by far UV circular dichroism (CD) at increasing concentrations of sodium phosphate at pH 6.0.

Figure 1 shows that increasing phosphate concentrations causes a linear increase at the midpoint in the thermal denaturation of wild type (WT) Prot L (T_m(WT)), going from 70.4 °C at 20 mM to 84.0 °C at 1000 mM sodium phosphate. The slope (m_{WT}) provides a measure of the dependence of protein thermal stability with phosphate concentration, whereas the intercept (T_m^0(WT)) corresponds to the melting temperature of the protein in the absence of cosolute.[8] In contrast, equivalent amounts of sodium chloride added to the solution do not result in any protein stabilization (open circles in Figure 1).

To explore the interaction between charged residues and phosphate, lysine residues on the protein surface were replaced by noncharged ones using site-directed mutagenesis; six single mutants and one double mutant were studied (K7Q, K23Q, K23A, K28Q, K54Q, K61Q, K41Q/K42Q). A hydrophobic core double mutant (I6L/L58V) was included as a control. All mutants and the wild type protein show very similar CD spectra, indicating that protein structures are conserved. Figure 2 shows that changes in melting temperature induced by sodium phosphate are linear for all mutant proteins but with different slopes m_i. We found an m_{WT} value of 14.3 °C·M^{-1}, whereas mutant proteins range in values between 11.4 (m_{K54Q}) and 19.8 °C·M^{-1} ($m_{K41Q/K42Q}$). Characteristic T_m^0 values for each mutant ($T_m^0(i)$) were obtained from the ordinate intercepts, showing a significant correlation with the solvent accessible area of the mutated lysine (Figure S1), in full agreement with previously published results about the effects of ionizable residue mutations on staphylococcal nuclease stability.[9]

Figure 3 shows a plot of the values of m_i (parameter that accounts for the change on $T_m(i)$ induced by phosphate) versus $T_m^0(i)$ (mutant

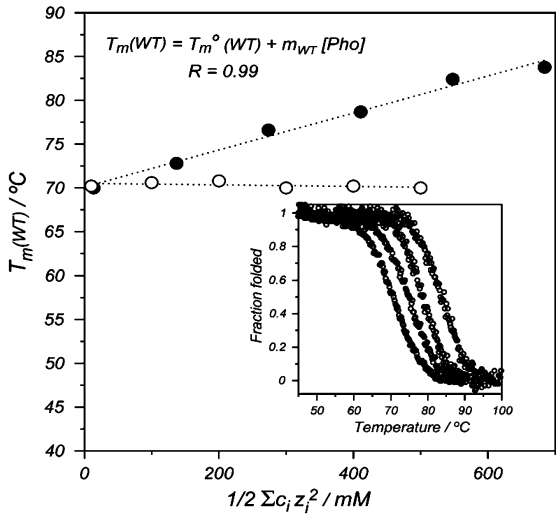

Figure 1. Main plot: dependence of T_m with the ionic strength of sodium phosphate (●) and sodium chloride (○) for wild type protein L. Circles represent experimental points, and the dotted line corresponds to the best linear fit. Inset: fraction of folded protein versus temperature for WT Prot L at increasing concentrations (200, 400, 600, and 1000 mM) of sodium phosphate.

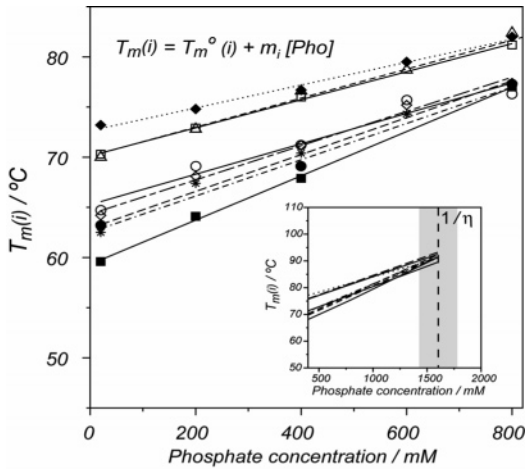

Figure 2. Phosphate-induced stabilization of Prot L mutants: K54Q (♦), K28Q (□), WT (△), K23Q (○), K61Q (◊), K7Q (∗), K23A (●), K41Q/K42Q (■). Inset: extrapolations of the lines up to the saturating phosphate concentration ($1/\eta$ = 1.61 M). The shadowed area reflects the standard deviation of the saturation concentration.

i thermal stability in the absence of phosphate) for each of the mutants. The two parameters show a remarkable linear correlation with $R = 0.97$. Dashed lines locate the position of the wild type in the plot. $T_m^0(i)$ is an intrinsic property of each protein and a reporter of the molecular stability in the absence of cosolute. The observed

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10.1021/ja051352e CCC: $30.25

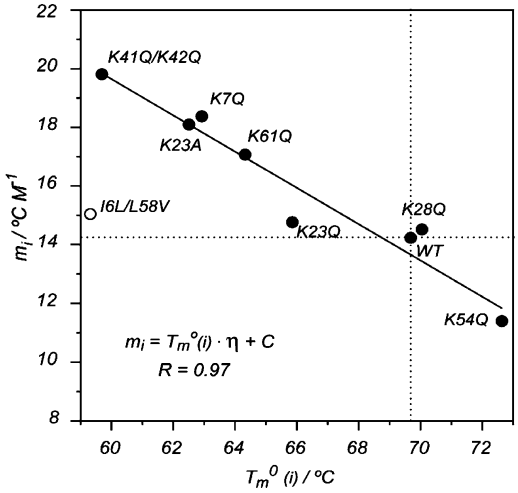

Figure 3. Correlation between $T_{mi}{}^0$ and m_i (the slopes obtained in Figure 2) for protein L mutants. The dashed line marks the values observed for wild type protein L. The open circle corresponds to the hydrophobic core double mutation I6L/L58V.

linear dependency shows that phosphate-induced stabilization is sensitive to perturbations on the surface of the protein and is *affected by the same principles that account for the contribution of charged residues to thermal stability.*

Taking as a reference WT Prot L, surface mutations of charged residues that decrease the stability of the protein in the absence of phosphate will always enhance the stabilizing effect produced by phosphate (upper-left sector in Figure 3). On the other hand, an increase in the stability of the protein upon mutation reduces the stabilizing effect of the cosolute (bottom-right sector in Figure 3).

The linear dependency of m_i with $T_m{}^0(i)$ establishes that the different plots of $T_m(i)$ versus phosphate should cross at a phosphate concentration given by the reciprocal of the slope of the linear correlation of Figure 3 ($\eta^{-1} = 1.61$ M). This value falls outside the temperature range of the spectropolarimeter, but extrapolations to high phosphate concentration are shown in the inset of Figure 2.

The above-mentioned linear correlation is not restricted to mutations that preserve the net charge since it applies as well to the double mutant K41Q/K42Q and to WT (see Figure 3). It is also followed by different mutations at the same position (K23A and K23Q), which indirectly corresponds to a charge-conserving mutation from glutamine to alanine at the protein surface. Thus, the correlation is conserved for mutations affecting the protein surface, even if they do not alter the charge state at a particular position. On the contrary, the hydrophobic core double mutant I6L/L58V does not follow the trend, indicating that the observed correlation is only reporting on the contribution of surface residues to protein stability.

Phosphate ions increase the stability of all Prot L mutants included in this study. This effect cannot be accounted for by shielding of unfavorable electrostatic interactions at higher ionic strength since NaCl does not provide an equivalent stabilization. In addition, the observed correlation between $T_m{}^0(i)$ and m_i is maintained when glutamine is replaced by alanine, a perturbation that conserves the number and location of charged residues.

According to our results, the changes in stability introduced with the mutation and the phosphate-induced thermostabilization have equivalent origins. Thermostability has been related to the optimization of electrostatic interactions on protein surfaces,[10] but stabilizing interactions between charges of opposite sign are, in general, not very effective since the enthalpic gain is balanced with a loss of entropy due to mobility restrictions in the side chains.[11] The correlation between surface accessibility and $T_m{}^0(i)$ (Figure S1) suggests that solvation contributes to the observed effects. The better correlation obtained when accessibility is calculated using an ensemble of NMR structures instead of a crystal structure also suggests a possible role for side chain flexibility.

Wild type proteins, shaped and selected by evolution, are expected to show a quasi optimal distribution of charged residues. This assumption provides an explanation for the observation that most surface mutations lead to a decrease in $T_m{}^0$. The surface charge network, once perturbed by the mutation, would hold a reduced number of accessible conformations for the remaining charged residues (both positive and negative). Phosphate interactions with charged side chains (directly or through the solvating water) might increase the conformational entropy of the folded state by partially quenching the mutual electrostatic repulsion between like charges, by offering alternative configurations of the surface electrostatic interaction network or by affecting the inner solvation layer. A saturating phosphate concentration would equalize the contribution of different surface networks, explaining the common melting temperature for all mutants with the same hydrophobic core and providing an explanation for the observed dependency between $T_m{}^0(i)$ and m_i.

We have used site-directed mutagenesis of surface residues to demonstrate the relationship between the intrinsic thermal stability of the different mutants and their sensitivity to phosphate-induced thermostabilization. This suggests that both parameters are mechanistically related. Our results offer an easy tool to experimentalists to characterize protein surfaces and highlight the role of ionic cosolutes in the modulation of protein thermal stability. The clear difference between the effect of phosphate and chloride ions demonstrates that the observed stabilization is not simply a result of changes in ionic strength. Preliminary results from our group show that thermal stabilization by different anions follows the Hofmeister series. We are actively pursuing this line of research.

Acknowledgment. We thank Dr. Karin Kloiber and Dr. Robert Konrat for helpful discussions. This work was supported by funds from MCyT (BIO2004-5436) to M.P. and from the Ramon y Cajal program to O.M.

Supporting Information Available: Materials and methods and plots of the correlation between $T_m{}^0(i)$ and solvent-exposed area. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Krishna, S. H. *Biotechnol. Adv.* **2002**, *20*, 239−267.
(2) Collins, K. D. *Methods* **2004**, *34*, 300−311.
(3) Hofmeister, F. *Arch. Exp. Pathol. Pharmakol.* **1888**, *24*, 247−260.
(4) Kuntz, W.; Lo Nostro, P.; Ninham, B. W. *Curr. Opin. Colloid Interface Sci.* **2004**, *9*, 1−18.
(5) (a) Saunders, A. J.; Davis-Searles, P. R.; Allen, D. L.; Pielak, G. J.; Erie, D. A. *Biopolymers* **2000**, *53*, 293−307. (b) Nishimura, C.; Uversky, V. N.; Fink, A. L. *Biochemistry* **2001**, *40*, 2113−2128.
(6) Perl, D.; Mueller, U.; Heinemann, U.; Schmid, F. X. *Nat. Struct. Biol.* **2000**, *7*, 380−383.
(7) We have used as wild type a mutated version of protein L (Y47W) widely used as a model system for studies in protein folding and stability: Kim, D. E.; Fisher, C.; Baker, D. *J. Mol. Biol.* **2000**, *298*, 971−984.
(8) A thermal melt of protein L in 10 mM MES pH 6.0 gave a $T_{mi}{}^0$ value identical to the one extrapolated from Figure 1.
(9) Meeker, A. K.; Garcia-Moreno, B.; Shortle, D. *Biochemistry* **1996**, *35*, 6443−6449.
(10) Loladze, V. V.; Ibarra-Molero, B.; Sanchez-Ruiz, J. M.; Makhatadze, G. I. *Biochemistry* **1999**, *38*, 16419−16423.
(11) Perl, D.; Schmid, F. X. *Chembiochem* **2002**, *3*, 39−44.

JA051352E