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Comment on Enhancement of the Catalytic Activity of a 27 kDa Subtilisin-like Enzyme from *Bacillus amyloliquefaciens* CH51 by in Vitro Mutagenesis

Thrombosis is a condition characterized by blood coagulating within blood vessels. The blood clots are referred to as thrombus and contain protein called fibrin. A recent study suggests that thrombosis can be treated by drugs containing fibrinolytic enzymes.[1] It has been shown that a specific bacterial species, *Bacillus amyloliquefaciens*, secretes the enzyme AprE51, which is proven to have fibrinolytic activity. AprE51 was mutated into several variants, in which the mutant form, AprE51-6 (S101W, G169A, V192A), was found to have the most efficient fibrinolytic activity.[1] The authors observed an increase in catalytic efficiency of AprE51-6 on the basis of the kinetic parameters K_M and k_{cat} in comparison with the catalytic efficiency of the wild type, AprE51. They also measured the thermal stability of the two enzymes, AprE51 and AprE51-6, by interpreting the half-life of denaturation at different temperatures.

Whereas the authors (Kim et al.) of the original paper[1] discovered the mutant form of the enzyme, AprE51-6, to have the highest rate of fibrinolytic activity, we found that further analysis of the structural and kinetic information could lead to a structure−function relationship of the enzyme. This information is expected to be valuable in the development of more effective variants of the enzyme that can be used as drugs. In this paper, we will show our thermodynamic analysis of AprE51 and its mutant form AprE51-6.

To derive the thermodynamic variables, enthalpy ($\Delta H_a°$), entropy ($\Delta S_a°$), and Gibbs free energy of activation ($\Delta G_a°$) of protein thermal stability, we first determined the rate constant (k) of protein thermal denaturation. The rate constant is determined using eq 1 and the half-life values ($t_{1/2}$) of the enzyme's thermal stability at various temperatures as listed in the Kim paper.[1] Because the thermal denaturation was shown to follow first-order kinetics, the rate constant is represented by eq 1.

$$k = \frac{\ln 2}{t_{1/2}} \quad (1)$$

Next, we use the Eyring equation, eq 2, to obtain the standard molar Gibbs free energy of activation ($\Delta G_a°$) values. In eq 2, R is the gas constant (8.3145 J/K mol), T is the temperature in Kelvin scale, h represents Planck's constant (6.6261×10^{-34} J s), and k_B is the Boltzmann constant (1.3807×10^{-23} J/K).[2]

$$\Delta G_a^o = -RT \ln \frac{kh}{k_B T} \quad (2)$$

The resulting values of $\Delta G_a°$ as a function of temperature are illustrated in Figure 1A. The linear relationship between $\Delta G_a°$ and temperature allows us to obtain the variables $\Delta H_a°$ and $\Delta S_a°$, on the basis of eq 3 as the slope and the y-axis intercept represent $-\Delta S_a°$ and $\Delta H_a°$, respectively.

$$\Delta G_a^o = \Delta H_a^o - T\Delta S_a^o \quad (3)$$

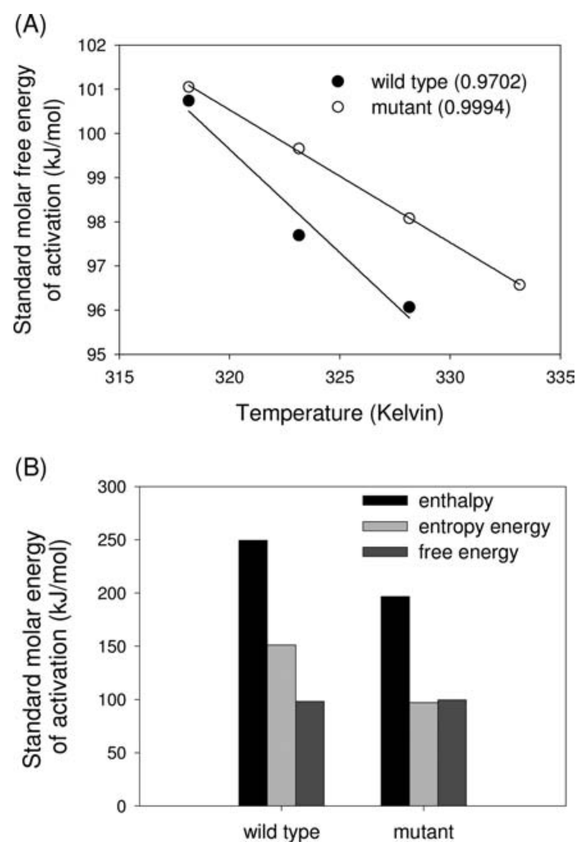

Figure 1. (A) Temperature-dependent standard molar free energy of activation of thermal stability for wild-type AprE51 and the AprE51-6 mutant. Standard molar enthalpy and entropy of activation were obtained from fitting a linear equation ($y = ax + b$) into the data. R^2 of the fitting is shown in the legend. (B) Standard enthalpy, entropy energy, and free energy of activation of protein denaturation. Standard entropy energy and free energy values correspond to the values at 50 °C. Graph preparation and fitting were performed using SigmaPlot (version 11, Systat Software Inc., San Jose, CA, USA).

Figure 1B shows the relationship of the three thermodynamic variables between the wild type and mutant forms of the enzyme.

Structural characteristics of the enzymes can be inferred from the thermodynamic variables. First, the thermal denaturation kinetics is unfavorable in terms of enthalpy but favorable in terms of entropy for both forms of the enzyme. Because $\Delta H_a°$ > 0, it is anticipated that breaking of noncovalent interactions is required for the enzyme to reach the transition state in thermal denaturation. $\Delta S_a°$ > 0 means the process might require release of small particles such as ions or water molecules from the

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protein molecule. Second, whereas both thermodynamic parameters (enthalpy and entropy) play a role in the denaturation process for both types of the enzyme, the degree of their influence depends on the type of enzyme. For example, the enthalpy value is 250% of the free energy in the case of the wild type, but it is 200% for the mutant, suggesting the structural stability is different for the two enzymes in terms of thermodynamics. Third, although there are significant variations in enthalpy and entropy between the two forms of the enzyme, the free energy of activation for denaturation shows little variation. Whereas $\Delta\Delta H_a^\circ$, defined as ΔH_a° (mutant) − ΔH_a° (wild type), is −52.7 kJ/mol and $T\Delta\Delta S_a^\circ$ at 50 °C is −54.1 kJ/mol, $\Delta\Delta G_a^\circ$ is just 1.4 kJ/mol at that temperature. This means that $\Delta\Delta H_a^\circ$ is compensated by $T\Delta\Delta S_a^\circ$. This phenomenon is called enthalpy−entropy compensation, which is often observed in weakly coupled systems[3] including proteins[4−7] and small chemicals.[8−13]

One possible mechanism for this compensatory behavior is that the three mutations (S101W, G169A, V192A) cause the enzyme to be more flexible, leading to more conformational states, whereas the noncovalent interactions responsible for the native structure of the enzyme are becoming weaker, as suggested by its ΔS_a° and ΔH_a° values, respectively. This proposed interpretation is in agreement with the functional characteristic of the enzyme, catalytic efficiency (k_{cat}/K_m).[2] Typically binding and catalytic transformation of a substrate require flexibility of the enzyme.[3] The mutant form has a lower value of K_m and a higher value of k_{cat}, indicating a higher affinity to the substrate and a higher turnover number than the wild type. According to our theoretical analysis, the mutant form might have higher flexibility than the wild type. Therefore, we can propose that the higher catalytic efficiency exhibited by the mutant enzyme is a consequence of structural flexibility induced by the mutations at the three residues. This is particularly interesting because we are able to establish a link between the functional property of the enzyme (catalysis) and its structural characteristics (flexibility), demonstrating the usefulness of thermodynamic analysis.

The conformational heterogeneity of binding sites was proposed to be the main contributor for binding and catalytic promiscuity. Thus, by using structural adaptations or conformational heterogeneity, enzymes and antibodies were demonstrated to catalyze and/or bind to structurally diverse target molecules.[14−16] With respect to the present study these facts imply that although the mutant enzyme has a higher catalytic efficiency, one concern arises on the basis of our analysis. If the mutant adopts more conformations, as we propose, then it will have an elevated potential to bind and possibly cleave proteins other than its intended substrate, fibrin. The potential promiscuous catalysis would cause a problem in using this mutant form of the enzyme as a therapeutic agent. To be used as a therapeutic agent, specificity is also an important criterion to consider in addition to effectiveness. Although the authors examined the specificity of the wild and mutant form and found no significant difference, the specificity evaluation was conducted using only one type of substrate. We suggest that specificity assessments be conducted with more diverse forms of substrates, thus evaluating promiscuity of the enzyme.

In our analysis we assumed that the wild type and the mutant forms shared the same transition state in the thermal denaturation. To consolidate our conclusions derived from this assumption, we suggest an equilibrium thermodynamic study of protein denaturation (for example, see refs17 and 18) using two forms of the enzyme because both types will have an essentially identical thermodynamic state. If our assumption is correct, then the wild type will have larger values of both ΔS° (= $S^\circ_{denatured} - S^\circ_{native}$) and ΔH° (= $H^\circ_{denatured} - H^\circ_{native}$), whereas the overall effect will be small as a result of miniscule $\Delta\Delta G^\circ$.

In conclusion, our thermodynamic analysis establishes a possible linkage between the structure and function of the enzyme on the basis of two independent experiments, kinetics of thermal denaturation and enzyme kinetic data. Suggestions for future studies include verification of our hypothesis using the experiment proposed here and, importantly, experiments to examine the specificity of the wild type and mutant enzymes. This is crucial for the effective application of the enzyme in a therapeutic setting as indicated by the original paper.

Beenish Viqar,[†]
Adarsh Gopinath,[†]
Jordan D. Dimitrov,[§,#,⊗]
Jonghoon Kang,*[,†]
[†]Department of Biology, Valdosta State University, Valdosta, Georgia 31698, United States
[§]Centre de Recherche des Cordeliers, Université Pierre et Marie Curie, Unité Mixte de Recherche S 872, Paris, France
[#]Université Paris Descartes, Unité Mixte de Recherche S 872, Paris, France
[⊗]INSERM, U872, Paris, France

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 1 (229) 333-7140. Fax: 1 (229) 245-6585. E-mail: jkang@valdosta.edu.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Kim, J.; Kim, J. H.; Choi, K. H.; Kim, J. H.; Song, Y. S.; Cha, J. Enhancement of the catalytic activity of a 27 kDa subtilisin-like enzyme from *Bacillus amyloliquefaciens* CH51 by in vitro mutagenesis. *J. Agric. Food Chem.* **2011**, *59*, 8675−8682.
(2) Chang, R. *Physical Chemistry for the Chemical and Biological Sciences*; University Science Books: Sausalito, CA, 2000.
(3) Haynie, D. T. *Biological Thermodynamics*; Cambridge University Press: Cambridge, U.K., 2001.
(4) Lumry, R.; Rajender, S. Enthalpy−entropy compensation phenomena in water solutions of proteins and small molecules: a ubiquitous property of water. *Biopolymers* **1970**, *9*, 1125−1227.
(5) Kang, J.; Warren, A. S. Enthalpy-entropy compensation in the transition of a monospecific antibody towards antigen-binding promiscuity. *Mol. Immunol.* **2007**, *44*, 3623−3624.
(6) Thielges, M. C.; Zimmermann, J.; Yu, W.; Oda, M.; Romesberg, F. E. Exploring the energy landscape of antibody-antigen complexes: protein dynamics, flexibility, and molecular recognition. *Biochemistry* **2008**, *47*, 7237−7247.
(7) Kang, J.; Auerbach, J. D. Thermodynamic characterization of dissociation rate variations of human leukocyte antigen and peptide complexes. *Mol. Immunol.* **2009**, *46*, 2873−2875.
(8) Leffler, J. E. The enthalpy−entropy relationship and its implications for organic chemistry. *J. Org. Chem.* **1955**, *20*, 1202−1231.
(9) Labuza, T. P. Temperature/entropy/enthalpy compensation in food reactions. *Food Technol.* **1980**, *34*, 67−77.

(10) Rudra, S. G.; Singh, H.; Basu, S.; Shivhare, U. S. Enthalpy entropy compensation during thermal degradation of chlorophyll in mint and coriander puree. *J. Food Eng.* **2008**, *86*, 379−387.
(11) Fisher, N. R; Purnell, C. B.; Kang, J. Comment on effect of heating on the stability of quinolones in milk. *J. Agric. Food Chem.* **2010**, *58*, 13020−13021.
(12) Brock, J. R; Rosa, B. Q; Kang, J. Comment on color loss in ethanolic solutions of chlorophyll *a*. *J. Agric. Food Chem.* **2011**, *59*, 9045−9047.
(13) Kechinski, C. P.; Guimarães, P. V.; Noreña, C. P.; Tessaro, I. C.; Marczak, L. D. Degradation kinetics of anthocyanin in blueberry juice during thermal treatment. *J Food Sci.* **2010**, *75*, C173−C176.
(14) Khersonsky, O.; Tawfik, D. S. Enzyme promiscuity: a mechanistic and evolutionary perspective. *Annu. Rev. Biochem.* **2010**, *79*, 471−505.
(15) James, L. C.; Roversi, P.; Tawfik, D. S. Antibody multispecificity mediated by conformational diversity. *Science* **2003**, *299*, 1362−1367.
(16) James, L. C.; Tawfik, D. S. Conformational diversity and protein evolution − a 60-year-old hypothesis revisited. *Trends Biochem. Sci.* **2003**, *28*, 361−368.
(17) Solá, R. J.; Griebenow, K. Chemical glycosylation: new insights on the interrelation between protein structural mobility, thermodynamic stability, and catalysis. *FEBS Lett.* **2006**, *580*, 1685−1690.
(18) Dalal, K.; Pio, F. Thermodynamics and stability of the PAAD/DAPIN/PYRIN domain of IFI-16. *FEBS Lett.* **2006**, *580*, 3083−3090.