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COMMUNICATIONS

Published on Web 09/18/2004

An Experimental Strategy to Evaluate the Thermodynamic Stability of Highly Dynamic Binding Sites in Proteins Using Hydrogen Exchange

Josephine C. Ferreon, James B. Hamburger, and Vincent J. Hilser*

Department of Human Biological Chemistry and Genetics, and Sealy Center for Structural Biology, University of Texas Medical Branch, Galveston, Texas 77555-1068

Received June 24, 2004; E-mail: vjhilser.utmb.edu

Proteins are conformationally heterogeneous, and native state hydrogen−deuterium exchange (HX) has emerged as a powerful tool for quantitatively characterizing the thermodynamics associated with this heterogeneity.[1,2] The importance of HX is that the conformational excursions can be quantified in energetic terms, and the results can be resolved at the level of individual residues. Unfortunately, experimental constraints render an energetic characterization of the most conformationally dynamic regions of proteins inaccessible by classic HX techniques. Specifically, complete exchange at most dynamic regions occurs during the dead-time of the experiment, making an assessment of the stability impossible. It is therefore of significant value to develop an experimental strategy that can provide a quantitative, residue-specific description of the energetics of highly dynamic regions.

In this communication we report an experimental strategy that provides unique insight into the energetics of the conformationally dynamic RT loop that comprises a part of the binding site of the C-terminal SH3 domain of SEM5. There is a large body of experimental work that shows that the RT loops of various SH3 domains are highly dynamic[3−5] and show no protection as monitored by HX,[4,5] even though several amides appear to be occluded from solvent in the high-resolution structure.[4] It has also been shown that the observed dynamics decrease upon binding ligand.[5] This means that the native-state ensemble in the absence of ligand exists as an equilibrium between binding-competent (BC) and binding-incompetent (BI) states (Figure 1) and that, upon binding, the equilibrium is shifted toward BC. The goal is to provide a quantitative thermodynamic characterization of this equilibrium.

The strategy is to target a surface-exposed residue in the RT loop (i.e., Ser 170) for mutation to glycine (Gly). As the side chain of residue 170 projects into solution, it makes neither intramolecular contacts nor contacts with its peptide ligand (Sos). It is well-known that Gly substitutions at solvent-exposed sites effect a change in the folding/unfolding equilibrium of proteins by virtue of the increased conformational space accessible to the Gly-containing protein in the denatured state[6] (i.e. $K_u(G170) = \Omega_u K_u(S170)$ where Ω_u is the ratio of conformational space in the denatured state that is available to Gly relative to Ser). This effect is observed even when no intramolecular contacts are changed by the Gly substitution.

The approach described here is similar to previous Gly substitution studies,[6,7] except that in this case the coupling between conformational and binding equilibrium is harnessed to deduce the energetics of the dynamic region. Specifically, the mutation is targeted to a site that is known to be dynamic, but HX is monitored at sites that exchange only as a result of global unfolding. For those global unfolding sites that are in rapid equilibrium (i.e. they exchange through the EX2 mechanism),[1] HX provides direct access to the folding/unfolding free energy difference between the Ser and the Gly variant through the ratio of the exchange rates, $k_{ex,G}$, at the global unfolding sites, [i.e. $\Delta\Delta G_{HX}$ (S170G) = $RT \ln(k_{ex,G}(G170)/k_{ex,G}(S170))$].

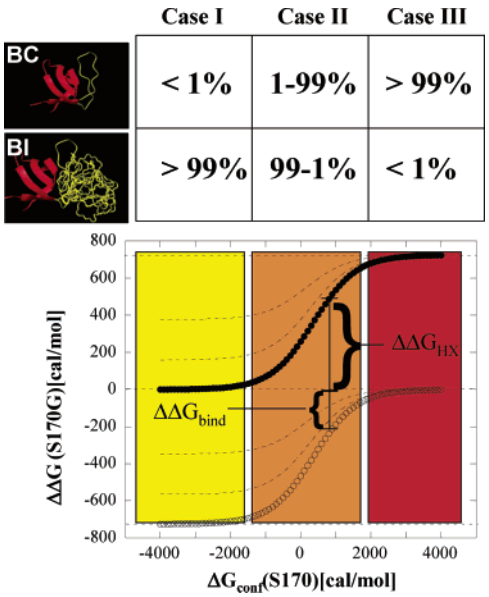

Figure 1. Graphical representation of the expected difference in binding affinity obtained from ITC (open symbols) and stability obtained from HX of globally unfolding residues (closed symbols) plotted for different values of the equilibrium between binding competent (BC) and incompetent (BI) states [i.e. $\Delta G_{conf}(S170) = -RT \ln K_{conf}(S170) = -RT \ln([BI]/[BC])$]. Three cases are highlighted. In Case I, the equilibrium favors the BI states, and the $\Delta\Delta G_{bind}$ is maximal, whereas $\Delta\Delta G_{HX}$ is minimal. Conversely, in Case III, the equilibrium favors the BC states, and $\Delta\Delta G_{bind}$ is minimal, whereas $\Delta\Delta G_{HX}$ is maximal. In Case II, the equilibrium is poised between the two extremes. For this simulation, Ω_u and Ω_{conf} (see eqs 1 and 2) are modeled as being equal. Dashed curves represent different simulation parameters wherein $\Omega_{conf} < \Omega_u$, which is the likely scenario.

If the conformational excursions are dramatic enough, such that under native conditions the loop is significantly disordered, the Gly-containing protein will sample more available conformational space, and the equilibrium constant for conformational fluctuations will be affected (i.e. $K_{conf}(G170) = \Omega_{conf} K_{conf}(S170)$). A consequence of this preexisting conformational equilibrium, under otherwise native conditions, is that the effects of the mutation on the global stability will be decreased relative to the case with no fluctuations and will adhere to the following relationship:

$$\Delta\Delta G_{HX}(S170G) = RT \ln\left(\frac{k_{ex,G}(G170)}{k_{ex,G}(S170)}\right) = RT \ln\left(\frac{(1 + K_{conf}(S170))\cdot\Omega_u}{(1 + \Omega_{conf}\cdot K_{conf}(S170))}\right) \quad (1)$$

Furthermore, as discussed below, such dramatic excursions will necessarily affect the binding affinity for the peptide (Figure 1).

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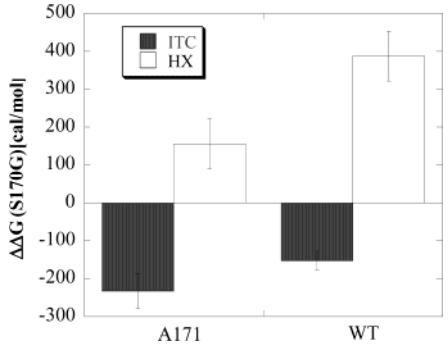

Figure 2. $\Delta\Delta G_{HX}$ (white) and $\Delta\Delta G_{bind}$ (dark) for the WT and the A171 variant of SEM5. As predicted by the model in Figure 1, large $\Delta\Delta G_{bind}$ values are associated with low $\Delta\Delta G_{HX}$ values, and vice versa. The HX results were obtained from the average ratios of exchange for 17 globally unfolding amides (eq 1). Error bars in HX correspond to the standard deviation of the mean. The mean and error bars in ITC are derived from the binding of SEM5 to two peptide variants (P3 and P3A -Sos).[8]

Thus, the thermodynamic insight afforded by this mutation strategy is facilitated by monitoring the effects of the mutation on two experimental observables: (1) the difference in binding free energy, $\Delta\Delta G_{bind}$, obtained from isothermal titration calorimetry (ITC) and (2) the difference in stability of the molecule as determined from the $\Delta\Delta G_{HX}$ at globally unfolding sites.[1]

The significance of the behavior shown in Figure 1 is two-fold. First, the magnitudes of $\Delta\Delta G_{bind}$ and $\Delta\Delta G_{HX}$ are inversely correlated. Second, regardless of the values of Ω_u and Ω_{conf}, the distance between the two curves yields the conformational free energy difference between the denatured states of the Ser- and Gly-containing protein (i.e. $-RT \ln \Omega_u$). This is evident when the function for $\Delta\Delta G_{bind}$,

$$\Delta\Delta G_{bind}(\text{S170G}) = -RT \ln\left(\frac{(1 + \Omega_{conf}\cdot K_{conf}(\text{S170}))}{(1 + K_{conf}(\text{S170}))}\right) \quad (2)$$

is subtracted from the function for $\Delta\Delta G_{HX}$ (eq 1). The value of Ω_u, which can only be determined if both the $\Delta\Delta G_{bind}$ and $\Delta\Delta G_{HX}$ are known, is important because it provides a means of determining a lower limit for the conformational equilibrium constant between binding-competent and incompetent states [i.e. setting $\Omega_{conf} = \Omega_u$ and solving for K_{conf} (S170); see below].

To test the approach, the effect of the Ser-to-Gly mutation at position 170 was monitored in the context of the wild type (WT) and the A171 variant of SEM5. Inspection of the HX and ITC results (Figure 2) reveal that the global stability and the binding affinity of the molecule are clearly affected to different extents in the two SEM5 variants.

Of note is that the results agree with the behavior predicted by Figure 1. Namely, for a particular site in the protein (in this case residue 170), a relatively high value for $\Delta\Delta G_{HX}$ should be accompanied by a relatively low value for $\Delta\Delta G_{bind}$, and vice versa. The determining factor as to the relative effect of the Ser-to-Gly mutation on the HX and ITC results is the equilibrium between binding-competent and -incompetent states. If the equilibrium favors the binding-competent states (Case III in Figure 1), the effect of the mutation will be seen primarily in $\Delta\Delta G_{HX}$. If the equilibrium favors the binding-incompetent (Case I in Figure 1), the effect will be manifested primarily in the $\Delta\Delta G_{bind.}$ Thus, Figure 2 indicates that the conformational equilibrium more heavily favors the binding-incompetent states in the A171 variant relative to the WT protein. In fact, from the relationships shown in Figure 1 and eq 2, the lower limit for the population of binding-incompetent states, P_{conf}(S170),

$$P_{conf}(\text{S170}) = \frac{K_{conf}(\text{S170})}{(1 + K_{conf}(\text{S170}))} \quad (3)$$

can be shown to be 0.25 and 0.50 for the WT and the A171 variant, respectively. These values indicate that the energetic consequences of fluctuations in the RT loop of SEM5 are indeed significant.

The importance of being able to resolve the energetics of fluctuations in proteins cannot be overstated. It is clear that differences in the structure of a binding interface between two variants of a protein will result in differences in binding affinity. However, as suggested by the results presented here, interpretation of the mechanism of such differences may not be straightforward. For regions that are highly dynamic, mutations not only affect the topology of the interaction surface, they also perturb the conformational equilibrium between the ensemble of binding-competent and -incompetent states. Such a situation is exemplified by the surfaced-exposed Gly substitution described in this study, demonstrating that even in the absence of measurable changes in the binding interface a change in affinity can nonetheless be effected. Provided conformational fluctuations in other proteins display the same type of dramatic excursions reported for SEM5, the method described here should prove valuable for obtaining a quantitative energetic characterization.

In conclusion, we have presented a strategy for evaluating the stability of highly dynamic regions of proteins, which takes advantage of the coupling between binding and stability. The ability to link thermodynamic quantities with dynamic behavior in proteins is the cornerstone to a molecular level understanding of such processes as catalysis, allosterism, and signal propagation.

Acknowledgment. This work was supported by the National Institutes of Health (R01-GM13747) and the Welch Foundation (H-1461).

Supporting Information Available: Derivations of eqs 1 and 2 and experimental details. This material is available free of charge via the Internet at http:// pubs.acs.org.

References

(1) Bai, Y.; Sosnick, T. R.; Mayne, L.; Englander, S. W. *Science*, **1995**, *269*, 192−197.
(2) (a) Swint-Kruse, L.; Robertson, A. D. *Biochemistry* **1996**, *35*, 171−180. (b) Hvidt, A.; Nielsen, S. O. *Adv. Protein Chem.* **1966**, *21*, 287−386. (c) Kim, K.-S.; Fuchs, J. A.; Woodward, C. K. *Biochemistry* **1993**, *32*, 9600−9608. (d) Chamberlain, A. K.; Handel. T.; Marqusee, S. *Nat. Struct. Biol.* **1996**, *3*, 782−778. (e) Radford, S. E.; Buck, M.; Topping, K. D.; Dobson, C. M.; Evans, P. A. *Proteins* **1992**, *14*, 237−248. (f) Fuentes, E. J.; Wand, A. J. *Biochemistry* **1998**, *37*, 3687−3698. (g) Fuentes, E. J.; Wand, A. J. *Biochemistry* **1998**, *37*, 9877−9883. (h) Itzhaki, L. S.; Neira, J. L.; Fersht, A. R. *J. Mol. Biol.* **1997**, *270*, 89−98. (i) Feng, H.; Takei, J.; Lipsitz, R.; Tjandra, N.; Bai, Y. *Biochemistry* **2003**, *42*, 12461−12465.
(3) (a) Arold, S.; O'Brien, R.; Franken, P.; Strub, M.; Hoh, F.; Dumas, C.; Ladbury, J. E. *Biochemistry* **1998**, *37*, 14683−1469. (b) Wang, C.; Pawley, N. H.; Nicholson, L. K. *J. Mol. Biol.* **2001**, *313*, 873−887. (c) Wittekind, M.; Mapelli, C.; Lee, V.; Goldfarb, V.; Friedrichs, M. S.; Meyers, C. A.; Mueller, L. *J. Mol. Biol*. **1997**, *267*, 933−9521. (d) Mittermaier, A.; Kay, L. E. *Protein Sci*. **2004**, *13*, 1088−1099.
(4) Ferreon, J. C.; Volk, D. E.; Luxon, B. A.; Gorenstein, D. G.; Hilser, V. J. *Biochemistry* **2003**, *42*, 5582−5591.
(5) Ferreon, J. C.; Hilser, V. J. *Protein Sci*. **2003**, *12*, 982−996.
(6) (a) D'Aquino, J. A.; Gómez, J.; Hilser, V. J.; Lee, K. H.; Amzel, L. M.; Freire, E. *Proteins* **1996**, *25*, 143−156. (b) Blaber, M.; Zhang, X. J.; Lindstrom, J. L.; Pepiot, S. D.; Baase, W. A.; Matthews, B. W. *J. Mol. Biol*. **1994**, *235*, 600−624. (c) Hernandez, E. L.; Serrano, L. *Protein Struct., Funct., Genet.* **1995**, *22*, 340−349.
(7) (a) Maity, H.; Lim, W. K.; Rumbley, J. N.; Englander, S. W. *Protein Sci.* **2003**, *12*, 153−160. (b) Huyghues-Despointes, B. M.; Langhorst, U.; Steyaert, J.; Pace, C. N.; Scholtz, J. M. *Biochemistry* **1999**, *38*, 16481−16490.
(8) Ferreon, J. C.; Hilser, V. J. *Protein Sci.* **2003**, *12*, 447−457.

JA046255K