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Protein Hydrogen Exchange in Denaturant: Quantitative Analysis by a Two-Process Model†

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Recent measurements have shown that hydrogen exchange (HX) rates of the amides of ribonuclease A (RNase A) are a function of denaturant concentration, even at concentrations well below the denaturation transition (Mayo & Baldwin, 1993). This behavior was interpreted as being due to a combination of two mechanisms, termed limited structural fluctuation and local unfolding. The authors also concluded that the local unfolding reactions entailed a novel structural transition, termed global unlocking.

Two issues must be addressed in considering the HX behavior of proteins in denaturant: the mechanism of exchange from the native protein and the significance of exchange from the unfolded state. This paper addresses the latter of these issues, using a quantitative formalism derived from existing HX models (Englander & Kallenbach, 1983; Wagner & Wüthrich, 1979; Woodward & Rosenberg, 1971; Woodward & Hilton, 1980). In particular, the two routes of HX via native-like and globally unfolded states are integrated to allow a quantitative analysis of the transition between the two routes. Exchange from native and native-like states typically dominates the observed rates in the absence of denaturant, but becomes less significant relative to the unfolded state in more strongly denaturing conditions, including conditions below the global unfolding transition. It is concluded that the available data are insufficient to discriminate between the various models for HX from native-like states.

THE MODEL

Proton exchange from protein amides is generally described using a kinetic mechanism developed in Linderstrom-Lang's laboratory (Hvidt & Nielsen, 1966). Under typical experimental conditions ("EX2 regime") this kinetic mechanism attributes the slow HX rates of native proteins to a fast equilibration between exchange-incompetent and exchange-competent conformations, prior to a relatively slow chemical exchange step (eq 1):

$$\text{HX incompetent} \overset{K_{op}}{\rightleftharpoons} \text{HX competent} \overset{k_x}{\longrightarrow} \text{exchanged} \quad (1)$$

The observed exchange rate constant for the EX2 regime is given by (Hvidt & Nielsen, 1966)

$$k_{obs} = \frac{k_{op}}{k_{op} + k_{cl}} k_x = \frac{K_{op}}{1 + K_{op}} k_x \quad (2)$$

where k_{op} and k_{cl} are the rate constants for the pre-equilibration between competent ("open") and incompetent ("closed") forms; the equilibrium constant for the pre-equilibration step is given by $K_{op} = k_{op}/k_{cl}$. Values of k_x, the intrinsic rate constant of exchange for the competent amide, depend on pH and local sequence and have been directly determined using short, unstructured peptides (Bai et al., 1993).

Experimentally determined rate constants are often normalized relative to the intrinsic exchange rate constant k_x to obtain a phenomenological protection factor. The Carlsberg formulation allows one to assign a structural interpretation to the protection factor. It makes no assumptions, however, about the structural nature of the global events leading to exchange competence. Clearly, global unfolding of the protein

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can lead to exchange competence of the protein chain (Robertson & Baldwin, 1991). Conformational fluctuations in the native state of the protein can also allow local regions of the chain to become competent. The two-process model described by Woodward and Hilton (1980) formalizes the observation that both global unfolding of proteins and some type of local fluctuation from the native state can lead to exchange. We now present a further development wherein we explicitly include the denaturant dependences of the two processes in a quantitative fashion. To interpret measurements of HX rates in terms of equilibria between conformations having different exchange competence, we construct an equilibrium model which includes competent conformations arising from both global unfolding and local fluctuations. The denaturant dependence of these equilibria are then used to predict the total amounts of exchange competent species (and their observed exchange behavior) under different conditions.

The global unfolding process has been relatively well characterized in many proteins, including RNase. In particular, the denaturant dependence of global unfolding may be modeled relatively simply, using either the linear free energy or stoichiometric binding models (Pace, 1975). We will use the former in our analysis below.

The structural nature of local fluctuations which can lead to exchange competence is not well characterized. The degree to which local fluctuations involve local unfolding of the chain is expected to be related to the denaturant dependence of the free energy difference between the native and locally fluctuated conformations (Schellman, 1978; Mayo & Baldwin, 1993). In order to assess whether the denaturant dependence of the observed HX measurements can be explained solely via the effects of global unfolding, we make the assumption that the local fluctuations have no denaturant dependence and hence are as folded as the native state. With these assumptions, we can show that all the observed denaturant dependence of Mayo and Baldwin's measurements can be accounted for by the denaturant dependence of the global unfolding reaction alone. We make no further assumptions concerning the extent or nature of the fluctuations which lead to local exchange competence, nor whether the individual fluctuations are correlated with one another.

Note that conformations arising by local fluctuations from the native state are defined only *locally*, with reference to an individual amide. These different locally defined species are not mutually disjoint and thus cannot be included in a single equilibrium. Instead, we define a separate equilibrium for each amide i, consisting of three species: a native conformation, N_i, with the ith amide in a nonexchanging environment; a locally fluctuated conformation, I_i, with the ith amide in an exchange-competent environment; and an unfolded conformation, U, with the ith amide (and, in fact, every amide) in an exchange-competent environment.

Since we assume that state I_i is indistinguishable from N_i in any global unfolding measurement, all the individual three-state equilibria are related via the global unfolding reaction. Thus all the conformations explored by the protein in solution may be classified as either folded or unfolded on the basis of global properties such as CD or viscosity. Those conformations which are folded may then be further divided on the basis of local properties into those which are competent or incompetent for exchange at a particular amide. Only this second division depends on which amide is under consideration. Note that this implies that the sum $[N_i] + [I_i]$ is independent of i. These points are illustrated in Figure 1.

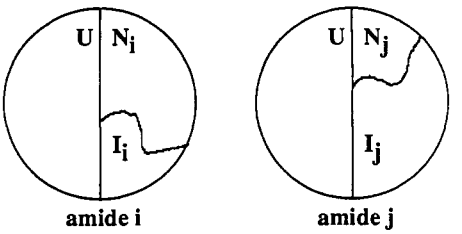

FIGURE 1: Conformational phase space of a protein may be divided into three regions corresponding to unfolded, native, and intermediate species. The distinction between regions U and N + I is made according to global unfolding criteria such as CD and is independent of the particular amide under consideration. The distinction between N and I regions is made based on local properties of the polypeptide chain. Some conformations may belong to both intermediate I_i and I_j: the various intermediate forms are not necessarily mutually disjoint.

With reference to a particular amide, we define the following equilibrium:

$$I_i \overset{K_{1,i}}{\rightleftharpoons} N_i \overset{K_{2,i}}{\rightleftharpoons} U \tag{3}$$

with intrinsic equilibrium constants

$$\frac{[I_i]}{[N_i]} = K_{1,i} \qquad \frac{[U]}{[N_i]} = K_{2,i} \tag{4}$$

The equilibria between N_i and I_i and between N_i and U correspond to the two parallel processes by which the ith amide can become competent for exchange. Unfortunately, the individual equilibria are not directly measurable. One can measure, however, the equilibrium between all globally folded and globally denatured forms. We define this equilibrium as K_g. It is independent of the choice of any particular amide. One can also measure the equilibrium between all exchange-competent and exchange-incompetent forms, which we define as K_{hx}. Unlike K_g, this requires reference to a specific amide. The corresponding free energies for global unfolding and acquisition of exchange competence can be expressed in terms of the intrinsic equilibrium constants:

$$\Delta G_g = -RT \ln K_g = -RT \ln \frac{[U]}{[N_i] + [I_i]} = -RT \ln \frac{K_{2,i}}{1 + K_{1,i}} \tag{5}$$

$$\Delta G_{hx} = -RT \ln K_{hx} = -RT \ln \frac{[I_i] + [U]}{[N_i]} =$$

$$-RT \ln (K_{1,i} + K_{2,i}) = -RT \ln [K_{1,i} + (1 + K_{1,i})K_g] \tag{6}$$

Equation 6 indicates that the observed HX behavior depends on both the global and local conformational equilibria. Thus, if the global unfolding free energy is known, then the observed HX behavior, ΔG_{hx}, can be determined from eq 6 with $K_{1,i}$ as a parameter.

RESULTS

Denaturant Dependence of ΔG_g and ΔG_{hx}. We first show that the denaturant dependence of the $N_i \rightleftharpoons U$ transition ($K_{2,i}$) is the same as the denaturant dependence of the global unfolding transition (K_g). The standard treatment of the denaturant dependence of the global unfolding transition is the linear free energy model (Pace, 1975):

$$\Delta G_g = \Delta G_g^0 - m_g[D] = -RT \ln (K_g^0 e^{m_g[D]/RT}) \tag{7}$$

In this model, m_g is related to the differential interaction of the denaturant with the folded and unfolded forms of the protein. The superscript 0 indicates values in the absence of denaturant.

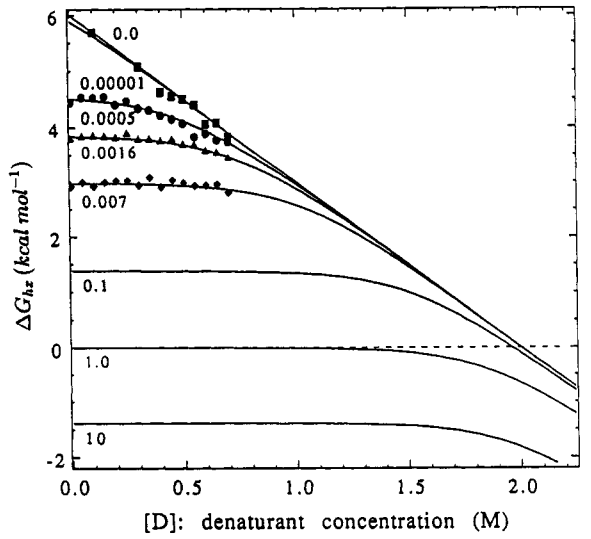

FIGURE 2: Model predictions for single proton HX rates as functions of denaturant concentration as in eq 9. The plot was made using ΔG_g^0 = 6 kcal/mol; m_g = 3 kcal/(mol·M). Values of $K_{1,i}$ are indicated by each curve. The symbols are data from Mayo and Baldwin's Figure 1 after subtracting 3.6 kcal/mol as suggested by the original authors (C58, H12, E49, and N44 from top to bottom). Note that there are two types of behavior: for large and small $K_{1,i}$, the data are basically linear (C58, N44); for intermediate range $K_{1,i}$, the curves are nonlinear (H12, E49). The transition point, where $K_{2,i} = K_{1,i}$, is at [D] = $-\infty$, –0.30, 0.48, 0.71, 1.01, 1.52, 1.86, and 1.98 for each of the respective curves. For $K_{1,i}$ ranging between 0 and 0.1, all curves converge at the global unfolding midpoint: [D] = 2 M.

Combining eqs 5 and 7, and assuming that the transition represented by $K_{1,i}$ is insensitive to denaturant, we have

$$-RT \ln \frac{K_{2,i}}{1 + K_{1,i}} = -RT \ln \frac{K_{2,i}^0 e^{m_g[D]/RT}}{1 + K_{1,i}} \quad (8)$$

Thus the global unfolding dependence on [D] is the same as the dependence of $K_{2,i}$ on [D]. Both are invariant with respect to the intermediate state I_i, as expected.

The denaturant dependence of ΔG_{hx} is more complex. When eqs 6–8 are combined, the observed ΔG_{hx} as a function of denaturant concentration [D] is given as

$$\Delta G_{hx} = -RT \ln (K_{1,i} + K_{2,i}^0 e^{m_g[D]/RT}) = -RT \ln [K_{1,i} + (1 + K_{1,i}) K_g^0 e^{m_g[D]/RT}] \quad (9)$$

This is not a linear function of [D]. This arises because ΔG_{hx} is derived from the population ratio of N_i relative to both U and I_i. Under conditions where $K_{1,i} \gg K_{2,i}$, then $[I_i] \gg [U]$, and HX will be dominated by local fluctations. Where $K_{2,i} \gg K_{1,i}$, then $[U] \gg [I_i]$, and HX will be dominated by global unfolding. The limiting slopes of the plot of ΔG_{hx} vs [D] will be 0 and m_g in the two respective cases.

Values of ΔG_g(D) and m_g are obtainable using optical probes of global structure such as peptide CD (Mayo & Baldwin, 1993). Given these values, the model predicts a family of curves which depend only on $K_{1,i}$ (eq 9). In Figure 2 we show the results of fitting eq 9 to the data of Mayo and Baldwin. The only adjustable parameter for each curve is $K_{1,i}$. The curves are extended to higher denaturant concentrations to clearly illustrate their biphasic nature. The current two-process model fits the observed HX data well, without invoking specific structural models for denaturant dependence of exchange from the native state.

Relation between ΔG_{hx}^0 and m_{hx}. Mayo and Baldwin demonstrated a correlation between ΔG_{hx}^0 and m_{hx}. The present model also predicts a relation between ΔG_{hx}^0 and m_{hx}, since both depend on $K_{1,i}$. Combining eqs 7 and 9 leads to

$$\Delta G_{hx}^0 = +RT \ln \left[\frac{m_{hx}}{m_g} (1 + 1/K_g^0) - 1 \right] \quad (10)$$

This relation is illustrated by the curve in Figure 3. For those amides with low values of m_{hx}^0, there is a very little constraint on possible values of ΔG_{hx}^0, and there is no expectation that ΔG_{hx}^0 should reach a particular value when m_{hx}^0 is zero. Mayo and Baldwin interpreted the apparent linear correlation between ΔG_{hx}^0 and m_{hx}^0 as supporting their model of a common exchange mechanism for their previously identified class 1 and 2a amides, involving denaturant dependent local unfolding of the protein. The value of the apparent intercept led them to postulate a "global unlocking" step in all local unfolding reactions. Figure 3 demonstrates that this correlation is related to the transition between two exchange mechanisms, one of which is exchange via global unfolding. Because the cor-

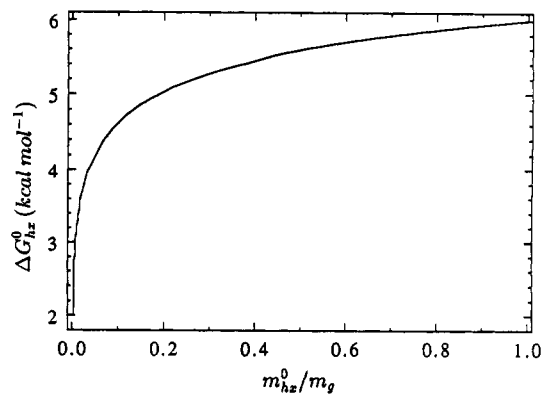

FIGURE 3: Relation between ΔG_{hx}^0 and m_{hx}^0. Note that our model allows no ΔG_{hx} greater than ΔG_g and that m_{hx} cannot be greater than m_g nor less than zero.

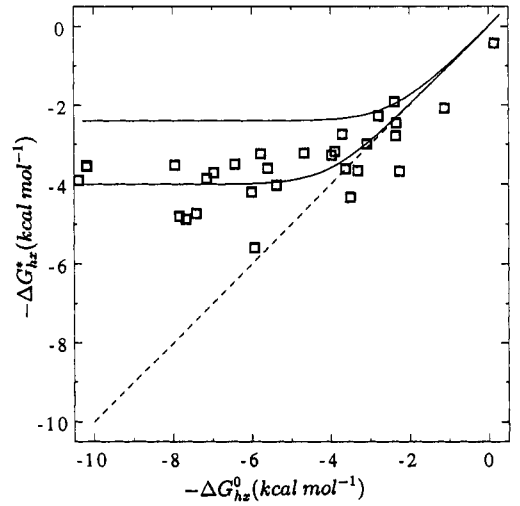

FIGURE 4: Comparison between quantitative model prediction and data from Kim and Woodward's Table 1 (Figure 4). The HX rate constants, k_{obs}'s, are converted to free energies. For 0 M urea: $\Delta G_{hx}^0 = -RT \ln [k_{obs}/(k_x - k_{obs})]$ where k_x are calculated according to Bai et al. (1993). For all data in the figure, $\Delta G_{hx}^0 \approx -RT \ln (k_{obs}/k_x)$. For 8 M urea an additional correction is applied to all the amides due to the urea effect on k_x (Loftus et al. 1986), as suggested by Kim and Woodward: $\Delta G_{hx}^{8M} = -RT \ln [k_{obs}/(k_x/2.5 - k_{obs})] \approx -RT \ln (2.5\, k_{obs}/k_x)$. The upper solid line is calculated for ΔG_g^0 = 9 kcal/mol and m_g = 0.825 kcal/(mol·M), as suggested by Kim et al. (1993) and Kim and Woodward (1993). The lower solid line is the least-squares fit to the data with variable m_g but fixed ΔG_g^0 = 9 kcal/mol, and the best fit is m_g = 0.625 kcal/(mol·M).

relation is not expected to be linear, there is no need to postulate a global unlocking step.

Correlation between ΔG_{hx} *at* $[D] = 0$ *and* $[D]^*$. We can also apply the quantitative analysis to the data of Kim and Woodward (1993) on BPTI. They have measured values of k_{obs} in the presence and absence of 8 M urea. BPTI is predominantly folded under both of these conditions. Again, from eq 6 we have

$$\Delta G^*_{hx} = -RT \ln \left(\frac{e^{-\Delta G^0_{hx}/RT}(1 + K^*_g) + K^*_g - K^0_g}{1 + K^0_g} \right) \quad (11)$$

where the asterisk stands for conditions at any urea concentration: $\Delta G^*_g = \Delta G^0_g - m_g[D]^*$. Equation 11 is compared to the normalized data of Kim and Woodward on HX in BPTI (Figure 4).

DISCUSSION

It is believed that protein HX rates which are slow relative to rates in short, unstructured oligopeptides reflect a dynamic structural process. A clear example of such a process leading to exchange competence is the global unfolding of a protein. Globally unfolded proteins have HX rates comparable to those of unstructured peptides (Robertson & Baldwin, 1991). A variety of local fluctuations can also lead to exchange competence. Either global or local processes can dominate exchange, depending on their respective values of K_{op} (Hvidt & Nielsen, 1966).

This realization has been expressed by Woodward and coworkers as the two-process model (Woodward & Hilton, 1980). Excellent descriptions of the model are given by Kim and Woodward (1993) and by Englander and Kallenbach (1983). We have specifically addressed the denaturant dependences of the two processes and show that the observed exchange in RNase A and BPTI can be described quantitatively using only a simple model for the denaturant dependence of global unfolding. Whatever their structural nature, a denaturant dependence of local fluctuations need not be invoked to explain the observed data. For each amide, the crossover between the two regimes is determined by the relative values of $K_{1,i}$ and $K_{2,i}$, corresponding to the relative stability of U and I_i (regardless of the stability of each relative to N_i).

The physical meaning of the experimentally measurable quantities ΔG_{hx} and m_{hx} becomes apparent in light of the present model. The former reports the equilibrium between all exchange competent and incompetent species, while the latter reports the equilibrium between the two exchange-competent states, I_i and U:

$$e^{-\Delta G_{hx}/RT} = \frac{[I_i] + [U]}{[N_i]}, \qquad \frac{m_{hx}}{m_g - m_{hx}} = \frac{[U]}{[I_i]}$$

Thus the existence and stability of equilibrium intermediates under native conditions can be quantitatively assessed. They differ significantly from the unfolded state in their dependences on denaturant.

A global unlocking step was postulated by Mayo and Baldwin to explain why amides which have little or no dependence on denaturant (i.e., $m_{hx} = 0$) do not have $k_{obs} = k_x$. The present model obviates the need for such a step: at very low m_{hx}, the dependence of ΔG_{hx} on m_{hx} becomes infinitely steep (Figure 3). The reason Mayo and Baldwin did not observe amides with low values of ΔG_{hx} (i.e, where k_{obs} approaches k_x) is that they were experimentally unable to measure rate constants for faster amides. Could these faster rates be measured, the present model predicts a nonlinear correlation in the plot of ΔG^0_{hx} vs m_{hx}. This is a testable prediction. The rates of exchange from these faster amides can be measured by modifying the experimental protocol (work in progress).

The stated purpose of the Mayo and Baldwin experiment was to determine the denaturant dependence of exchange due to local fluctuations of the native state, on the assumption that this dependence would reveal something of the structural nature of these fluctuations. This remains an important issue. Any analysis, however, must take into account the contribution of the unfolded state to the apparent denaturant dependence, and this requires that exchange be measured under conditions where global unfolding does not dominate the observed exchange. We have presented a quantitative formalism for doing this.

The EX2 kinetic scheme, described above, allows one to determine equilibria simply by measuring the rate constant for an irreversible labeling process (eq 2). The ability to distinguish the two species is determined not by their relative concentrations but by their relative concentrations scaled by their intrinsic rate constants, which can differ by many orders of magnitude. This ability is not restricted to HX experiments; an excellent discussion of another use is given by Vas and Boross (1974). Results from such analyses should be considered with caution, however: K_{op} may not be measured under the same conditions as K_g, and the value of k_x determined from model systems may not be the same as the actual rate constant for the exchanging form under experimental conditions.

Mayo and Baldwin noted a discrepancy of 3.6 kcal/mol between the values of ΔG^0_g and ΔG^0_{hx} of Cys 58. Because Cys 58 exchanges primarily through global unfolding, even in the absence of denaturant, these two values might be expected to be equal. Such discrepancies are seen in systems other than RNase A. A discrepancy of 1.4 kcal/mol exists between the global unfolding free energies of BPTI determined by calorimetry and HX (Kim & Woodward, 1993; Kim et al., 1993). Bai et al. (1994) have also observed an offset of 2 kcal/mol for cytochrome *c*. Mayo and Baldwin attributed the RNase A discrepancy to a difference in the intrinsic rate constant for exchange from the unfolded states. A possible explanation for such a difference could be the presence of residual structure in the unfolded protein at low concentrations of denaturant, although residual structure in heat-denatured RNase A does not cause such an effect (Robertson & Baldwin, 1991). More direct explanations include differences in the solution conditions (e.g., D_2O vs H_2O). The present model offers no independent explanation of the discrepancy. We have followed Mayo and Baldwin and implicitly assumed a uniform protection factor of ~400 in our treatment of the observed data.

The contribution to observed HX rates from the globally unfolded state is unlikely to be as simple as suggested. Nonetheless, this simple model does explain the available data. It demonstrates the role of the globally unfolded state in determining HX behavior, even at concentrations of denaturant well below the global unfolding transition. It also indicates the conditions that must be met to assess the effects of perturbants on HX from conformations other than the globally unfolded form.

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