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Thermodynamics of Structural Fluctuations in Lysozyme As Revealed by Hydrogen Exchange Kinetics†

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ABSTRACT: A new method is described that makes use of the empirical enthalpy–entropy compensation behavior of a related series of processes for deriving the activation enthalpy and entropy probability density functions from the corresponding rate constant density function. The method has been applied to data obtained from a study of the temperature dependence of hydrogen–tritium exchange in lysozyme. Analysis of the temperature dependence of t_j, the time required to reach a particular number of hydrogens remaining unexchanged, provides estimates of $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ for the exchange process. The results are consistent with the notion of two mechanisms of exchange characterized by different activation energies. Increases in $\Delta H^{\ddagger}$ are compensated by corresponding increases in $\Delta S^{\ddagger}$. The compensation plot, however, reveals two distinct apparent compensation temperatures, which reflect the operation of two qualitatively different mechanisms of exchange. The faster hydrogens exchange with $\Delta H^{\ddagger}$ values between 8 and 18 kcal·mol^{-1} and are characterized by a high compensation temperature of 470 K. The slower hydrogens exchange with $\Delta H^{\ddagger}$ values that reach 40 kcal·mol^{-1} and display a compensation temperature of $\simeq$360 K. The latter is associated with a thermal unfolding mechanism of exchange.

There is now considerable evidence for a dynamic view of protein structure. Theoretical and experimental evidence [see the review by Gurd & Rothgeb (1979)] points to a broad distribution of closely related conformational states and to a diverse range of internal motions in proteins. Hydrogen isotope exchange is one of a number of techniques that can provide considerable information about protein conformational dynamics by measuring the accessibility of the amide protons and the interior of the protein to solvent. One pathway of exchange appears to be associated with major cooperative unfolding of the protein (Woodward et al., 1975; Ellis et al., 1975, Hilton & Woodward, 1979; Knox & Rosenberg, 1980). However, under conditions that favor the folded form, most amide protons exchange with solvent from the folded conformation without contributions from the major unfolding process.

There is no general agreement as to the types of internal motion that mediate exchange from the native state. A number of models have been proposed that differ both in the amplitude and degree of cooperativity of motion involved and in the medium (i.e., within the protein matrix or in bulk solvent) in which exchange is assumed to take place. The local unfolding model involves relatively large amplitude motions and cooperative hydrogen bond rupture of regions of protein secondary structure that expose the normally protected amide sites to bulk solvent where exchange takes place (Englander, 1975; Hvidt & Nielson, 1966).

By contrast, the penetration model proposes that the exchange catalyst migrates to buried sites within the protein and that exchange occurs within the protein interior. A description of the penetration process has been given by Lumry & Rosenberg (1975) in the "mobile defect" hypothesis. Fluctuations in the bonding network allow a redistribution of internal protein free volume, which provide the pathways for water and water–ion migration to and from the buried exchange sites. These mechanisms have been reviewed in detail elsewhere (Woodward & Hilton, 1979; Gurd & Rothgeb, 1979; Englander, 1980; Barksdale & Rosenberg, 1982).

It has proved very difficult to identify unambiguously the types of internal motion responsible for hydrogen exchange from the native state. Much of this difficulty can be attributed to the problems of analyzing the simultaneous exchange of large numbers of hydrogens (Hamming, 1962; Barksdale & Rosenberg, 1982). On a more general level, the free energy is known not to be a good source of information for cooperative processes (Lumry, 1980a,b; Benzinger, 1969, 1971). These considerations force us to seek mechanistic information in the temperature and pressure derivatives of the free energy. The

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temperature dependence of hydrogen exchange rates has been studied for a number of proteins including ribonuclease A (Woodward & Rosenberg, 1971), trypsin (Woodward et al., 1975), soybean trypsin inhibitor (Ellis et al., 1975), and lysozyme (Wickett et al., 1974). These studies and the studies of single proton exchange by nuclear magnetic resonance (NMR) (Hilton & Woodward 1978, 1979) indicate the existence of two pathways for exchange characterized by low and high activation energies that correspond to an exchange process from the native state and one involving cooperative unfolding, respectively. The previous studies of activation energies, however, were not sufficient to describe the full spectrum of activation energies present as they suffered from inadequacies in the analytical procedures used (Barksdale & Rosenberg, 1982).

In this paper, therefore, the temperature dependence of the complete out-exchange of lysozyme is reexamined in detail. A preliminary paper of these results has been given previously (Gregory et al., 1981). Improved analytical methods are given that provide estimates of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ as functions of the number of hydrogens remaining unexchanged. An experimentally observed relationship between enthalpy and entropy is used together with the rate constant probability density function obtained from a Laplace inversion of the data (Knox & Rosenberg, 1980) to provide probability density functions for the activation enthalpy and entropy.

Experimental Procedures

Materials. Lysozyme (dialyzed and lyophilized grade I) was obtained from Sigma Chemical Co. ^{14}C-Labeled formaldehyde (50 mCi·mmol^{-1}) and tritiated water (100 mCi·mL^{-1}) were obtained from New England Nuclear. Sephadex G-25, medium grade, was obtained from Pharmacia. All other materials were either AnalaR or reagent grade as supplied by Mallickrodt and Fisher Scientific. The scintillation cocktail was as previously described (Carter et al., 1978).

Methods. Lysozyme was labeled with H^{14}CHO by reductive methylation in 0.2 M borate, pH 9.35 (Rice & Means, 1971), to yield a labeled product of 0.5 μCi·(mg of protein)$^{-1}$. Tritium in-exchange was achieved by incubating lysozyme (^{14}C specific activity $\simeq$ 0.01 μCi·mg^{-1}) with tritiated water buffered to pH 8.35 at 40 °C for 18 h, conditions that are known to effect complete in-exchange of tritium (Wickett et al., 1974). The protein concentration and tritium activity of the in-exchange mixture were $\simeq$45 mg·mL^{-1} and 3 mCi·mL^{-1}, respectively. The exchange rates were determined by using the two-column separation technique of Englander (1963) with some modification. An aliquot of the in-exchange was separated from excess tritium by passage through a Sephadex G-25 column equilibrated at the desired experimental pH at 2 °C. Two 5-mL aliquots of the out-exchange were obtained and diluted in an equal volume of buffer to yield a lysozyme concentration of $\simeq$1.5 mg·mL^{-1}. One sample was incubated at the desired experimental temperature (±0.02 °C), while the other was incubated at 25 °C and served as the control. Subsequent separations were performed at a pH 1 unit below the experimental pH at 2 °C. ^{14}C and ^{3}H activities were determined by exhaustive counting on a Beckman LS-230 liquid scintillation counter. Protein concentrations were determined from the ^{14}C activity of protein standards, the protein concentrations of which had been established previously by optical density measurements at 280 nm with a Cary 118 spectrophotometer.

Buffers employed in the out-exchange were adjusted to maintain a constant OH^- ion concentration with changes in temperature. In this way, contributions to the thermodynamic parameters due to the ionization of water are eliminated.

Results

Construction of Complete Out-Exchange Curves. The hydrogen exchange reaction of peptides is catalyzed mainly by hydrogen and hydroxyl ions at low temperatures. The minimum exchange rate in random-coil polypeptides and for single protons in bovine pancreatic trypsin inhibitor occurs in the pH range $2 \leq pH \leq 4.5$ (Englander & Poulsen, 1969; Woodward & Hilton, 1979). The pH range employed in the present experiments ($5 \leq pH \leq 11.5$) assures that only base-catalyzed exchange occurs so that the individual exchange rate k_i of the ith proton may be represented by

$$k_i = k_i'[OH^-]^{\chi} \quad (1)$$

Here, k_i' represents the pH-independent rate constant, which may include contributions from the chemical exchange step as well as from those conformational events that bring catalyst and peptide together. The order of the reaction with respect to the hydroxyl ion concentration is measured by χ which takes a value of 1 for random-coil polypeptides but which deviates from first-order behavior for exchange in proteins (Woodward & Hilton, 1979). Although the isoelectric point of lysozyme is high (p$I \simeq$ 11.0), the net charge on the protein shows little change over the pH range $5 \leq pH \leq 11$. As a consequence, the conformation of lysozyme is essentially unaffected by changes in pH over this range. The effective time range during which exchange can be observed can be extended by taking advantage of this fact and performing exchange at different pH values. Out-exchange curves were constructed by the methods described by Knox & Rosenberg (1980). In the present experiments, hydrogen–tritium exchange was measured at five different temperatures and at pOH values of 9.0, 6.5, and 4.5 and for data at 15 and 5 °C also at pOH 2.5. The out-exchange curves, plotted with log $H(t)$ as a function of log t, were normalized to pOH 6.5 by displacing the curves at pOH 9.0 and 4.5 along the log t axis until an optimal overlap with the data at pOH 6.5 was achieved. Here $H(t)$ is the number of hydrogens remaining unexchanged per protein molecular at time t. The displacement along the log t axis was found to be independent of the pH and temperature of the experimental run and indicated a value of d log k_i/dpOH = Δ log t/ΔpOH = $\chi \simeq$ 0.8–0.9, in agreement with previous findings (Knox & Rosenberg, 1980).

The construction of the out-exchange curves in this way extends the effective time range of the experiments to about 9 orders of magnitude. Plots of log $H(t)$ as a function of log t are shown in Figure 1. No correction for equilibrium isotope effects has been applied.

Determination of Thermodynamic Parameters. Hydrogen exchange of a protein with n sites is given by

$$H(t) = \sum_{i=1}^{n} \exp(-k_i t) \quad (2)$$

Since the rank order of exchange is preserved as the temperature is varied (Woodward & Rosenberg, 1971; Wickett et al., 1974; Barksdale & Rosenberg, 1982), it is possible to evaluate the activation parameters at constant $H(t)$ values. It should be emphasized that the conservation of rank order of exchange in lysozyme as the temperature is varied is essential for this type of analysis. Since the order in which the sites exchange does not vary with temperature, the values of the activation parameters determined at fixed $H(t)$ values will reflect weighted averages derived from approximately the same group of sites at each temperature. The use of the index, $H(t)$,

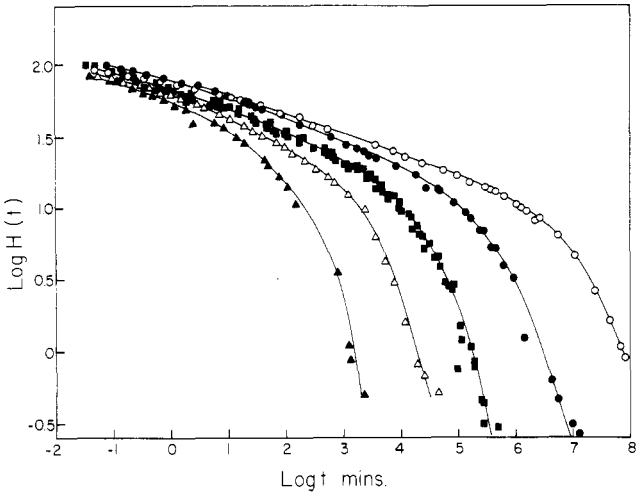

FIGURE 1: Out-exchange of tritium from lysozyme corrected to pH 7.5: (○) 5 °C, (●) 15 °C, (■) 25 °C, (△) 35 °C, and (▲) 45 °C. Time t is expressed in minutes.

does not, of course, imply that each site exchanges completely before the next site but rather that the contributions to the activation parameters of a number of simultaneously exchanging sites remain approximately the same at different temperatures. Differentiation of eq 2 yields an apparent first-order average rate function:

$$\langle k\rangle_j = \frac{-\mathrm{d}\ln H(t)}{\mathrm{d}t} = \frac{\sum_{i=1}^{n} k_i \exp(-k_i t_j)}{\sum_{i=1}^{n} \exp(-k_i t_j)} \qquad (3)$$

where the subscript j serves to emphasize the particular temperature at which $\langle k\rangle$ is evaluated. In general, t_j, the time required to reach a particular $H(t)$ value, is different at different temperatures. Indeed, the derivative $\mathrm{d}t_j/\mathrm{d}T$ itself may be employed to evaluate the hydrogen exchange activation parameters. The use of $\mathrm{d}t_j/\mathrm{d}T$ in this way is a generalization of the procedure first derived by Woodward & Rosenberg (1971) and provides, simply and directly, the weighted average activation energy:

$$\bar{E} = \langle kE\rangle/\langle k\rangle$$

Differentiation of eq 2 with respect to temperature yields

$$\frac{\mathrm{d}H(t)}{\mathrm{d}T} = -t_j\sum_{i=1}^{n}\frac{\mathrm{d}k_i}{\mathrm{d}T}\exp(-k_i t_j) - \frac{\mathrm{d}t_j}{\mathrm{d}T}\sum_{i=1}^{n} k_i \exp(-k_i t_j) \qquad (4)$$

Since the activation parameters are being evaluated at constant $H(t)$ values, we define $\mathrm{d}H(t)/\mathrm{d}T = 0$. Substituting $\mathrm{d}k_i/\mathrm{d}T = k_i E_i/(RT^2)$ in eq 4 yields

$$t_j\sum_{i=1}^{n}\frac{k_i E_i}{RT^2}\exp(-k_i t_j) = -\frac{\mathrm{d}t_j}{\mathrm{d}T}\sum_{i=1}^{n} k_i \exp(-k_i t_j)$$

which may be rearranged to give

$$\mathrm{d}\ln t_j/(R\,\mathrm{d}T^{-1}) = \langle kE\rangle/\langle k\rangle \qquad (5)$$

where the averages $\langle kE\rangle$ and $\langle k\rangle$ are given by

$$\langle kE\rangle = \sum_{i=1}^{n} k_i E_i \exp(-k_i t_j)/\sum_{i=1}^{n}\exp(-k_i t_j)$$

and

$$\langle k\rangle = \sum_{i=1}^{n} k_i \exp(-k_i t_j)/\sum_{i=1}^{n}\exp(-k_i t_j)$$

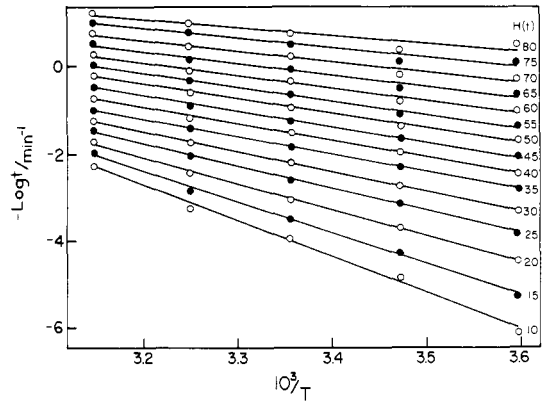

FIGURE 2: Temperature dependence of time, t_j, required to reach a given $H(t)$ value derived from the data shown in Figure 1.

Equation 5 may be compared with the derivative $\mathrm{d}\ln\langle k\rangle/\mathrm{d}T$, which is obtained by differentiating eq 3 with respect to T

$$\frac{\mathrm{d}\ln\langle k_j\rangle}{R\,\mathrm{d}T^{-1}} = \frac{\langle kE\rangle}{\langle k\rangle} + t_j\left(\frac{\langle kE\rangle\langle k^2\rangle}{\langle k^2\rangle} - \frac{\langle k^2E\rangle}{\langle k\rangle}\right) \qquad (6)$$

The additional terms $\langle k^2\rangle$ and $\langle k^2E\rangle$ are defined by analogy with $\langle k\rangle$ and $\langle kE\rangle$. Equation 6 indicates higher order joint moments of k and E that confuse the interpretation of the activation parameters as functions of $H(t)$. For this reason, the use of $\mathrm{d}t_j/\mathrm{d}T$ is preferred and has been employed here. Values of $\log t_j$ were interpolated at constant $H(t)$ values from an enlargement of Figure 1. Plots of $-\log t_j$ against T^{-1} constructed at constant $H(t)$ values are shown in Figure 2. Values of $\bar{E}$ are given by

$$E = 2.303R\ \mathrm{d}\log t_j/\mathrm{d}T^{-1}$$

from which estimates of the average activation parameters $\overline{\Delta H^{\ddagger}}$, $\overline{\Delta G^{\ddagger}}$, and $\overline{\Delta S^{\ddagger}}$ may be derived from the following equations:

$$\overline{\Delta H^{\ddagger}} = \bar{E} - RT$$

$$K^{\ddagger} = h/(k_B T t_j)$$

$$\overline{\Delta G^{\ddagger}} = -RT\ln K^{\ddagger}$$

$$\overline{\Delta S^{\ddagger}} = (\overline{\Delta H^{\ddagger}} - \overline{\Delta G^{\ddagger}})/T$$

Figure 3 shows plots of $\overline{dH^{\ddagger}}$ and $\overline{\Delta S^{\ddagger}}$ calculated at 298 K and pH 7.5. The plot of $\overline{\Delta H^{\ddagger}}$ against $H(t)$ is in reasonable agreement with the apparent activation energies determined previously by Wickett et al. (1974) after account is taken of ΔH°_{w} for the ionization of water. The fastest hydrogens exchange with $\overline{\Delta H^{\ddagger}}$ values between 8 and 18 kcal·mol^{-1} while the slowest hydrogens exchange with an activation enthalpy that rises rapidly to a value of 40 kcal·mol^{-1}. The qualitative features of the plot of $\overline{\Delta S^{\ddagger}}$ as a function of $H(t)$ are similar to those observed for $\overline{\Delta H^{\ddagger}}$. The fastest hydrogens exchange with $\overline{\Delta S^{\ddagger}}$ values that are limited to the range $-38 \leq \overline{\Delta S^{\ddagger}} \leq -15$ cal·deg^{-1}·mol^{-1}. At lower $H(t)$ values $\overline{\Delta S^{\ddagger}}$ rises sharply to a value of 40 cal·deg^{-1}·mol^{-1} at $H(t) = 10$.

Enthalpy–Entropy Compensation. One of the more interesting features of the data is that the increases of $\overline{\Delta H^{\ddagger}}$ with $H(t)$ are compensated by increases in $\overline{\Delta S^{\ddagger}}$. That is, for a related series of processes, in this case hydrogen exchange at different $H(t)$ values, there exists a linear or almost linear relationship between the entropy and enthalpy changes. En-

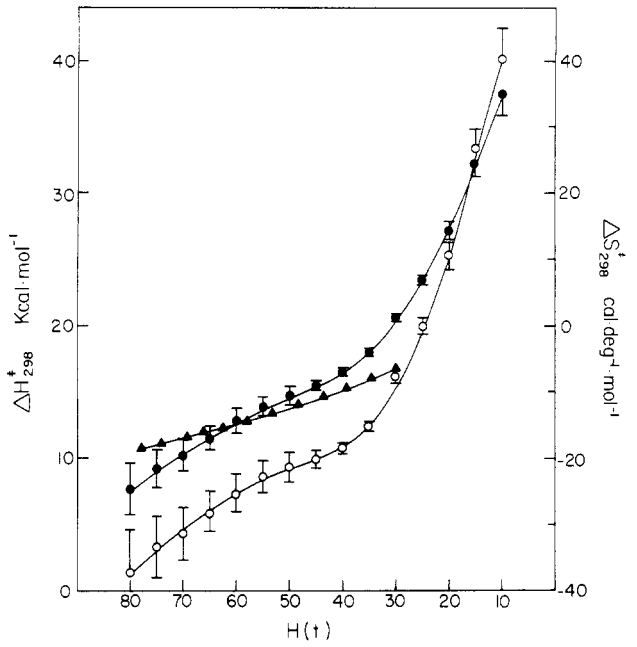

FIGURE 3: Activation enthalpy (●) and entropy (○) calculated at 298 K as functions of $H(t)$ for exchange from lysozyme. (▲) $\langle k\Delta H^{\ddagger}\rangle/\langle k\rangle$ for native exchange derived from the $\Delta H^{\ddagger}$ probability density function as described in the text.

thalpy–entropy compensation is commonly found in a wide variety of reactions in water and in many protein processes (Lumry & Rajender, 1970). The compensation relationship is expressed for a related series of processes by

$$\overline{\Delta H_j^{\ddagger}} = \alpha(T) + T_c\overline{\Delta S_j}^{\ddagger} \quad (7)$$

or

$$\overline{\Delta G_j^{\ddagger}} = \alpha(T) + (T_c - T)\overline{\Delta S_j}^{\ddagger} \quad (8)$$

where T_c is the compensation temperature. Values of $\overline{\Delta G}^{\ddagger}$ and $\overline{\Delta S}^{\ddagger}$ calculated at the harmonic mean temperature = 298 K for different $H(t)$ values are plotted in Figure 4. The compensation plot reveals two distinct apparent compensation temperatures that take values of 470 and 360 K for the fast and slow exchanging hydrogens, respectively.

Determination of Activation Enthalpy and Entropy Distributions. In previous sections of this paper the difficulties of analyzing data for the simultaneous exchange of large numbers of hydrogens have been overcome by invoking the conservation of rank order of exchange and by employing average rate functions. Recently, however, Knox & Rosenberg (1980) have introduced a Laplace inversion technique adapted from a method of Austin et al. (1975) to determine rate constant distribution functions from hydrogen exchange data. The method expresses the probability density function for the exchange rates without invoking exchange classes and, when the inversion is performed numerically, is quite model independent.

The hydrogen exchange of proteins is represented by eq 2:

$$H(t) = \sum_{i=1}^{n} \exp(-k_i t)$$

When n is sufficiently large, as is the case even for small proteins, then the sum in eq 2 may be replaced by an integral:

$$H(t) = \int_0^{\infty} f(k)\exp(-kt)\,dk = \mathcal{L}f(k) \quad (9)$$

Here $f(k)$ is the probability density function for the exchange

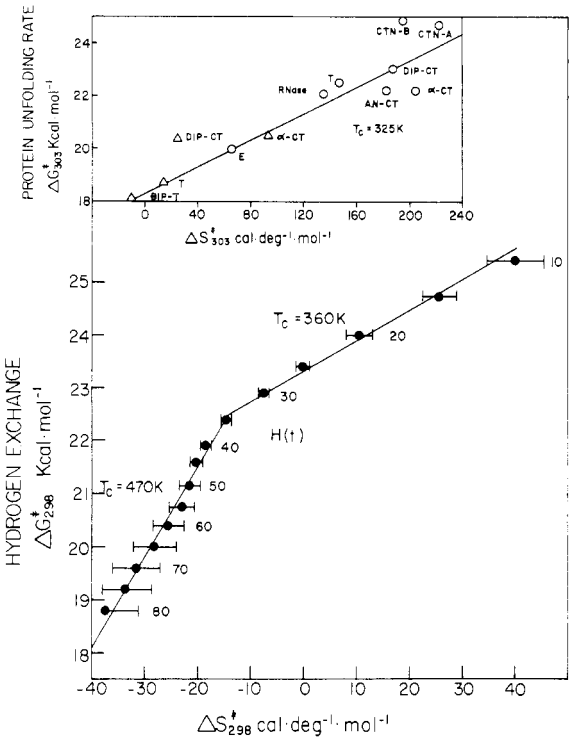

FIGURE 4: Enthalpy–entropy compensation plotted as $\overline{\Delta G}^{\ddagger}$ against $\overline{\Delta S}^{\ddagger}$ at 298 K. (●) Isotope exchange of lysozyme; unfolding rate process for reversible unfolding of proteins and protein derivatives: (○) unfolding in water, pH 2.0, (△) unfolding in 8 M urea, pH 7.2 [data of Pohl (1969)]. Abbreviations: T, trypsin; E, elastase; RNase, ribonuclease; α-CT, α-chymotrypsin; CTN-A and CTN-B, chymotrypsinogen A and B, respectively; DIP, diisopropyl phosphoryl derivative; AN, anhydro derivative.

rate constants, which may be recovered by Laplace inversion of eq 9 (Knox & Rosenberg, 1980):

$$f(k) = \mathcal{L}^{-1}H(t) \quad (10)$$

It is apparent from the relationships

$$k = (k_B T/h)K^{\ddagger}$$

and

$$\Delta G^{\ddagger} = -RT \ln K^{\ddagger}$$

that by suitable transformations of variables, eq 9 may be converted into a distribution of activation free energy. We have

$$H(t) = \frac{k_B T}{h}\int_0^{\infty} f(k)\exp(-kt)\,dK^{\ddagger} = \int_0^{\infty} kf(k)\exp(-kt)\,d\ln K^{\ddagger} = \frac{1}{RT}\int_0^{\infty} kf(k)\exp(-kt)\,d\Delta G^{\ddagger} \quad (11)$$

where $kf(k)d\Delta G^{\ddagger}/(RT)$ represents the number of hydrogens that exchange with activation free energies between $\Delta G^{\ddagger}$ and $\Delta G^{\ddagger} + d\Delta G^{\ddagger}$ at a temperature T. The activation enthalpy and entropy distribution functions can be obtained from eq 11 provided suitable variable transformations can be found for $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$. Austin et al. (1975) have given a method for deriving the distribution of activation energies, $f(E)$, assuming a constant preexponential term, A in the Arrhenius equation $K = A\exp[-E/(RT)]$. The method also requires that A and E be independent of temperature. Instead, we will derive the activation enthalpy and entropy distributions by employing variable transformations suggested by the thermodynamic analysis given in the previous sections.

The most obvious choice of function for a variable transformation of the integral in eq 11 is the Gibbs energy:

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$$

However, the transforms $d\Delta H^{\ddagger} = d\Delta G^{\ddagger}$ and $d\Delta S^{\ddagger} = 1/T\, d\Delta G^{\ddagger}$ require the assumptions that $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$, respectively, remain constant over the ranges of integration employed. The assumption of a constant $\Delta S^{\ddagger}$ is equivalent to that employed by Austin et al. (1975). Examination of Figure 3 shows that $\Delta S^{\ddagger}$ varies by approximately 80 cal·deg^{-1}·mol^{-1} over the range of $H(t)$ values employed here, so that the assumption of a constant $\overline{\Delta S^{\ddagger}}$ is not applicable in the present work. However, the additional information required to perform the transformation is availble through the enthalpy–entropy compensation behavior of the system. In terms of the enthalpy and entropy distribution functions, the compensation equations, eq 7 and 8, indicate that values of $\Delta H^{\ddagger}$ are conditional on the values of $\Delta S^{\ddagger}$. The appropriate variable transforms are therefore

$$d\Delta S^{\ddagger} = \frac{d\Delta G^{\ddagger}}{T_c - T} \qquad \Delta S^{\ddagger} = \frac{\Delta G^{\ddagger} - \alpha(T)}{T_c - T} \tag{12}$$

and

$$d\Delta H^{\ddagger} = T_c\, d\Delta S^{\ddagger} \qquad \Delta H^{\ddagger} = \alpha(T) + T_c\Delta S^{\ddagger} \tag{13}$$

Combining eq 11–13 gives eq 14 and 15.

$$H(t) = \frac{T_c - T}{RT}\int_0^{\infty} kf(k)\exp(-kt)\, d\Delta S^{\ddagger} \tag{14}$$

$$H(t) = \frac{T_c - T}{RTT_c}\int_0^{\infty} kf(k)\exp(-kt)\, d\Delta H^{\ddagger} \tag{15}$$

In practice, the distribution functions are obtained by Laplace inversion of eq 9 followed by appropriate transformations of $f(k)$ and k to give

$$g(\Delta G^{\ddagger}) = \frac{kf(k)}{RT} \qquad \Delta G^{\ddagger} = -RT\ln\frac{kh}{k_B T}$$

$$g(\Delta S^{\ddagger}) = g(\Delta G^{\ddagger})(T_c - T) \qquad \Delta S^{\ddagger} = \frac{\Delta G^{\ddagger} - \alpha(T)}{T_c - T}$$

and

$$g(\Delta H^{\ddagger}) = \frac{g(\Delta S^{\ddagger})}{T_c} \qquad \Delta H^{\ddagger} = \alpha(T) + T_c\Delta S^{\ddagger}$$

The method assumes that the compensation temperature obtained from an analysis of $-\log t_j$ is also applicable to the individual rates in the rate constant density function. That this is true is apparent from an examination of eq 5 since the observation of a common compensation temperature, T_c, for different $H(t)$ values can only occur of the individual $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values themselves compensate with a compensation temperature equal to T_c.

The activation parameter probability densities were constructed from appropriate transformations of the rate constant density function (Knox & Rosenberg, 1980):

$$f(k) = \frac{ba^{-n}}{\Gamma(n)}(k - c)^{n-1}\exp[-(k - c)/a] \qquad k > c$$
$$f(k) = 0 \qquad k < c \tag{16}$$

Equation 16 is the inverse Laplace transform of the power law function:

$$H(t) = b(1 + at)^{-n}\exp(-ct) \tag{17}$$

Data at 25 °C was fitted to eq 17 by using a nonlinear least-squares procedure that provides estimates of all the parameters. The use of eq 16 and 17 and in particular the assignment of the parameter c as the exchange rate constant for the unfolding pathway (Knox & Rosenberg, 1980) have been recently challenged by Halvorsen (1981). However, reanalysis of the data of Knox & Rosenberg (1980) with the numerical Fredholm integral inversion program CONTIN (Provencher, 1979, 1980) indicates that the original assignment of the parameter c in eq 17 is correct (Gregory, 1982).

A detailed study of the temperature dependence of numerically derived distribution functions is currently in progress. However, given the general agreement between the distributions obtained numerically and those derived with eq 16, the power law function will serve here to illustrate the main features of the activation parameter distributions. In addition, eq 16 allows the two compensation temperatures to be directly assigned to appropriate regions of the distribution. The rate constants for exchange from the folded conformation are distributed according to

$$f(k)_n = ba^{-n}k^{n-1}\exp(-k/a) \tag{18}$$

The corresponding density, for, say $\Delta H^{\ddagger}$, is then given by

$$g(\Delta H^{\ddagger})_n = \frac{(T_{c,n} - T)kf(k)_n}{RTT_{c,n}} \tag{19}$$

which has the dimensions mol·kcal^{-1} and is plotted as a function of $\Delta H^{\ddagger}$ given by

$$\Delta H^{\ddagger} = \frac{-RTT_{c,n}}{T_{c,n} - T}\ln\frac{kh}{k_B T} - \frac{T\alpha(T)}{T_{c,n} - T} \tag{20}$$

where $T_{c,n}$ is the compensation temperature for exchange from the folded state. The corresponding term for the unfolding pathway may be obtained by subtracting the exchange contribution due to the folded conformation from the total density. Hence

$$g(\Delta H^{\ddagger})_u = \frac{T_{c,u} - T}{RTT_{c,u}}k[f(k) - f(k)_n] \tag{21}$$

and is plotted as a function of $\Delta H^{\ddagger}$ given by

$$\Delta H^{\ddagger} = \frac{-RTT_{c,u}}{T_{c,u} - T}\ln\frac{kh}{k_B T} - \frac{T\alpha(T)}{T_{c,u} - T} \tag{22}$$

where $T_{c,u}$ is the compensation temperature for exchange by the cooperative unfolding process. The total density is given by

$$g(\Delta H^{\ddagger}) = g(\Delta H^{\ddagger})_n + g(\Delta H^{\ddagger})_u \quad \Delta H^{\ddagger} < \Delta H^{\ddagger}_c$$
$$g(\Delta H^{\ddagger}) = 0 \qquad \Delta H^{\ddagger} > \Delta H^{\ddagger}_c \tag{23}$$

where $\Delta H^{\ddagger}_c$ is the activation enthalpy derived from a transformation of the slowest exchange rate, c, in the original rate constant density function.

Note, that normalization of the density should yield the total number of hydrogens, b, that exchange:

$$\int_{H_1}^{H_2} g(\Delta H^{\ddagger})\, d\Delta H^{\ddagger} = b \tag{24}$$

Estimates of the power law and compensation parameters used to derive the activation parameter probability density functions are given in Table I. The probability densities for the activation terms $\Delta G^{\ddagger}$, $T\Delta S^{\ddagger}$, and $\Delta H^{\ddagger}$ are shown plotted on a common energy scale in Figure 5. The probability density for $\Delta H^{\ddagger}$ is broad and skewed to higher enthalpy values, giving a most probable activation enthalpy, $\Delta H^{\ddagger}_p$ of $\simeq$12.5 kcal·mol^{-1}. The density for $T\Delta S^{\ddagger}$ is sharper and indicates a most probable activation entropy of −27 cal·deg^{-1}·mol^{-1}.

The $\Delta H^{\ddagger}$ density obtained here may be compared with those obtained by Austin et al. (1975) for O_2 and CO rebinding to

Table I: Power Law and Compensation Parameters Used To Derive Activation Parameter Probability Density Functions[a]

$b = 90$	$a = 2.43 \times 10^{-2}$ s^{-1}
$n = 0.21$	$c = 1.87 \times 10^{-7}$ s^{-1}
$\alpha(T)_n = 25.0$ kcal·mol^{-1}	$T_{c,n} = 470$ K
$\alpha(T)_u = 23.4$ kcal·mol^{-1}	$T_{c,u} = 360$ K

[a] Power law parameters were obtained from a fit of the data at 25 °C, pH 7.5, to eq 17. Compensation parameters were calculated at 298 K according to eq 8.

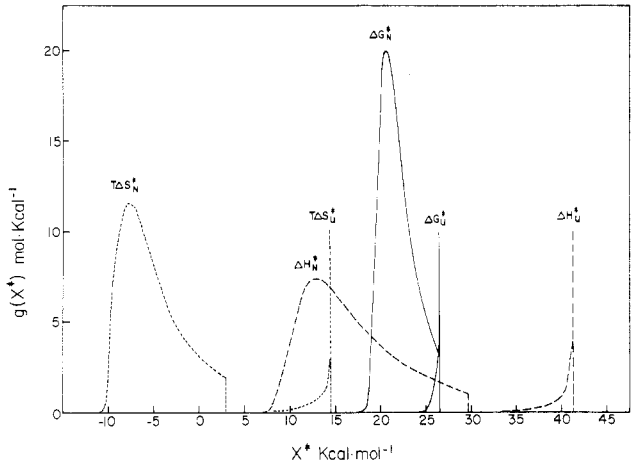

FIGURE 5: Activation parameter probability density functions for isotope exchange from lysozyme calculated at 298 K, pH 7.5: (—) ΔG^*, (– –) ΔH^*, and (- - -) $T\Delta S^*$. n and u represent the density functions for exchange from the native and unfolded protein, respectively.

myoglobin in which $\Delta H^*_p \simeq 2$ kcal·mol^{-1} and 2.5 kcal·mol^{-1}, respectively, and with the data of Alben et al. (1981) for CO rebinding to cytochrome oxidase where $\Delta H^*_p \simeq 10$ kcal·mol^{-1}. The singularity in the probability densities indicates values of $\Delta H^* = 41$ kcal·mol^{-1} and $\Delta S^* = 49$ cal·deg^{-1}·mol^{-1} for the unfolding pathway.

The internal consistancy of the activation enthalpy probability density function and the thermodynamic analysis given in the previous section was checked by calculating $\langle k\Delta H^*\rangle/\langle k\rangle$ as a function of $H(t)$. For this purpose, the activation enthalpy probability density was divided into 20 classes at ΔH^* intervals of 1 kcal·mol^{-1} over the range $8 \leq 27$ kcal·mol^{-1}. The compensation analysis provides values of the rate constant, k_i, which together with estimates of the class size, A_i, and ΔH^*_i values, can be used to calculate values of $\langle k\Delta H^*\rangle/\langle k\rangle$ and $H(t)$:

$$\frac{\langle k\Delta H^*\rangle}{\langle k\rangle} = \frac{\sum_{i=1}^{20} k_i \Delta H^*_i A_i \exp(-k_i t)}{\sum_{i=1}^{20} k_i A_i \exp(-k_i t)} \quad (25)$$

and

$$H(t) = \sum_{i=1}^{20} A_i \exp(-k_i t) \quad (26)$$

Values of $\langle k\Delta H^*\rangle/\langle k\rangle$ calculated in this way for exchange from the folded conformation are shown plotted as a function of $H(t)$ in Figure 3. The calculated and observed values of $\langle k\Delta H^*\rangle/\langle k\rangle$ are in generally good agreement.

Discussion

Magnitude of Activation Barriers. The data displayed in Figures 3 and 4 together with the bimodal probability density functions shown in Figure 5 appear to be consistent with the previous notion of two competing pathways for exchange (Woodward et al., 1975; Ellis et al., 1975; Hilton & Woodward, 1979; Knox & Rosenberg, 1980). Exchange of the slowest exchanging hydrogens is generally assumed to occur by a cooperative unfolding process represented by

$$N^* \underset{k_b}{\overset{k_f}{\rightleftharpoons}} U^* \xrightarrow{k_{OH}[OH^-]} U \quad (27)$$

where k_f and k_b are the forward and backward rate constants for a two-state unfolding process, respectively, and k_{OH} is the base-catalyzed chemical exchange rate. N and U represent the folded and unfolded states, respectively, while an asterisk (*) denotes the tritium label. When the lifetime of the unfolded state is long compared with the rate of the chemical exchange, $k_{OH}[OH^-] \gg k_f + k_b$, then the exchange is determined by k_f, as shown by Segawa et al. (1981) for lysozyme in 4.5 M LiBr over the temperature range of the thermal unfolding transition.

By contrast, the conditions of the present study favor the folded form and are consistent with the assumption of a steady-state concentration of U*:

$$k_b + k_{OH}[OH^-] \gg k_f$$

The apparent first-order rate constant, k_{obsd}, is given by (McDaniel & Smoot, 1956; Hvidt, 1964)

$$k_{obsd} = k_f k_{OH}[OH^-]/(k_b + k_{OH}[OH^-]) \quad (28)$$

With the further assumption that $k_b \gg k_{OH}[OH^-]$, eq 28 simplifies to

$$k_{obsd} = \frac{k_f}{k_b} k_{OH}[OH^-] = K_u k_{OH}[OH^-] \quad (29)$$

where K_u is the equilibrium constant for reversible unfolding. The activation enthalpy and entropy corresponding to k_{obsd} are given simply by

$$\Delta H^*_{obsd} = \Delta H^\circ_u + \Delta H^*_{OH}$$

and

$$\Delta S^*_{obsd} = \Delta S^\circ_u + \Delta S^*_{OH} + R \ln [OH^-] \quad (30)$$

Substitution of the values $\Delta H^*_{OH} = 4$ kcal·mol^{-1} and $\Delta S^*_{OH} = -9$ cal·deg^{-1}·mol^{-1} determined recently for the base-catalyzed exchange reaction in poly(DL-alanine) (R. B. Gregory, L. Crabo, A. J. Percy, and A. Rosenberg, unpublished results) together with appropriate corrections to account for the OH^- ion activity in eq 30 provide the values $\Delta H^\circ_u = 38$ kcal·mol^{-1} and $\Delta S^\circ_u = 88$ cal·deg·mol^{-1}. The calorimetrically determined standard thermodynamic functions for the lysozyme unfolding transition are rather higher with $\Delta H^\circ_u = 56$ kcal·mol^{-1} and $\Delta S^\circ_u = 140$ cal·deg^{-1}·mol^{-1} at 298 K, pH 7.0 (Pfeil & Privalov, 1976). The discrepancy is rather large and may be due to a number of effects. First, it should be realized that the unfolding transition has a melting temperature that is between 30 and 60 °C higher than the experimental temperature. In addition, the hydrogen exchange data for the unfolding process are derived largely from experiments conducted at pH 9.0, which makes direct comparison with the data of Pfeil & Privalov (1976) difficult. Second, it should be noted that agreement between the thermodynamic parameters determined by hydrogen exchange and those determined calorimetrically is only possible when the protein contains a core that becomes solvent accessible only during cooperative thermal unfolding. Competition from a low activation enthalpy exchange process from the native state may reduce the observed value of ΔH^*_{obsd}.

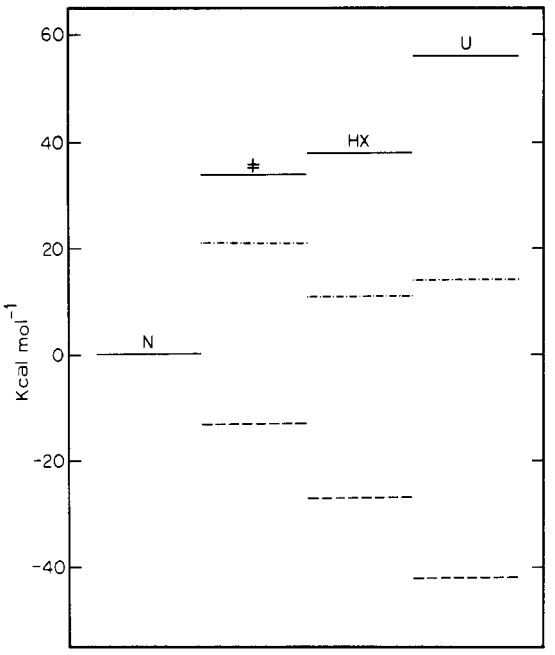

FIGURE 6: A comparison of thermodynamic parameters for the activated complex for unfolding (‡), hydrogen exchange of the slowest protons (HX), and the unfolded state (U) with respect to the native state (N) at 298 K: (-·-) ΔG, (—) ΔH, and (--) $-T\Delta S$.

Finally, the discrepancy may reflect a real difference in the conformations observed by hydrogen exchange and by calorimetry. A comparison of the thermodynamic values determined here with those of the activated complex for unfolding (Segawa et al., 1973) and for unfolded lysozyme (Pfeil & Privalov, 1976), shown in Figure 6, suggests that, in terms of the enthalpy changes, the conformations observed by hydrogen exchange resemble the activated complex more closely than the unfolded form, although the entropy change is much larger than that characterizing the activated complex. An important feature of the unfolding rate process is its negligible heat capacity of activation and positive activation volume (Lumry & Biltonen, 1969; Johnson et al., 1954). Formation of the activated complex for unfolding therefore involves a swelling of the protein with no exposure of hydrophobic groups to bulk water (Lumry & Biltonen, 1969). The results given here suggest that exchange of the slowest protons occurs from a state or range of states along the unfolding reaction coordinate that resemble the activated complex in terms of the degree of H bonding but with increased conformational freedom. A similar observation has been made for the exchange of some single indole protons in lysozyme. At high temperatures the exchange of the Trp-28 NH is characterized by an activation enthalpy of 120 kcal·mol^{-1} in agreement with the calorimetrically determined values for unfolding while the activation enthalpy for the exchange of Trp-108, Trp-111, and Trp-123 is only $\simeq$95 kcal·mol^{-1}, suggesting that exchange of these indole protons occurs from a state that does not resemble the fully unfolded conformation (Wedin et al., 1982).

Low Activation Energy Pathway. It has been argued previously that the relatively low activation enthalpy for exchange of the majority of hydrogens would preclude local unfolding processes as a pathway for exchange (Woodward et al., 1975; Rosenberg & Enberg, 1969) but would be consistent with the small amplitude motions and restricted secondary bond rearrangements proposed in the penetration model. However, as noted by Englander (1980), thermal unfolding processes are characterized by large heat capacity changes (Brandts, 1964; Lumry & Biltonen, 1969) so that at low temperatures relative to the melting temperature, unfolding may occur with small enthalpy changes and a local unfolding pathway of exchange may well be characterized by a low activation energy. These considerations clearly diminish the value of enthalpy changes in distinguishing mechanism. Similar difficulties also attend the use entropy changes for characterizing the nature of accessible states.

Enthalpy–Entropy Compensation. Enthalpy–entropy compensation is commonly observed for rate and equilibrium processes in proteins and aqueous solutions. One source of compensation arises from a simple correlation of errors in the estimates of ΔH and ΔS (Krug et al., 1976a,b). However, in the present work, the values of the compensation temperatures, the linearity when $\Delta G^{\ddagger}$ is plotted as a function of $\Delta S^{\ddagger}$, and the fact that the same experimental technique gives rise to two distinct compensation temperatures indicate that the compensation observed here represents a valid extrathermodynamic relationship and is not due to a correlation of errors in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. Various aspects of compensation behavior have been discussed previously (Lumry & Rajender, 1970; Lumry, 1980a,b). For the present purposes, it is sufficient to note that many reactions of proteins and aqueous solutions display compensation behavior with characteristic compensation temperatures that indicate the existence within the measured process of a linkage to a common subprocess among the related series of processes. For complex systems such as proteins, it is often difficult to identify exactly which subprocess is being revealed in the compensation behavior by the variation of a particular variable. However, we should emphasize that the apparent linear relationship between the enthalpy and entropy of activation employed here to construct probability density functions for the activation parameters requires no mechanistic assumptions as to the source of compensation. In fact any function may be employed provided it expresses the relationship between the enthalpy and entropy and explicit terms.

Enthalpy–entropy compensation has been observed for the low activation energy process for the exchange of tryptophan NH in lysozyme (Wedin et al., 1982) and for the exchange of some protons in BPTI (C. K. Woodward, I. Simon, and E. Tüchsen, unpublished results), where the authors noted an increase in activation enthalpy as the exchange rate decreased.

In the present work two different compensation temperatures are revealed. The value of T_c = 360 K is similar in magnitude to the melting temperature for unfolding expected under the conditions of the present work and to the value (T_c = 325 K) obtained by Pohl (1969) for protein unfolding rates and supports the conclusion that exchange of the slowest hydrogens is associated with a thermal unfolding mechanism.

The T_c values of 470 K observed for the exchange of the faster hydrogens are more difficult to interpret, and their use in characterizing the accessible state is subject to some uncertainty. Here we shall consider just two aspects of the accessible state. The first concerns the degree of exposure of hydrophobic groups to bulk solvent. The hydration of hydrophobic side chains is a very expensive process in terms of free energy and is associated with large heat capacity changes that, through the heat capacity integral $\int_0^T \Delta C_p \, dT'$, contribute both to $\Delta H°$ and $\Delta S°$, which display a compensation temperature of 280 K (Lumry & Rajender, 1970; Lumry, 1980a). The second concerns the volume – entropy relationship. As Lumry & Biltonen (1969) have noted, the entropy of the folded protein is strongly dependent on the amount of internal free volume. A good example is provided by the activation parameters for the unfolding rate process described previously

in which an increase in entropy is associated with a positive activation volume of unfolding. The activation volumes for isotope exchange from the folded conformation are small and positive (Carter et al., 1978) and would be associated with only a small increase in entropy. The high T_c value that is observed, indicates a process that is dominated by enthalpy changes and would be consistent with a mechanism of bond rearrangements with considerably restricted conformational freedom. However, such a description would be appropriate for both the penetration and local unfolding models so that a distinction in mechanism is not possible. Indeed, if exposure of hydrophobes to bulk water is restricted in the accessible state, then the two models, to a great extent, do become indistinguishable. We suspect in common with Englander (1980) that the lability of hydrogen bonds is a major factor in determining exchange rates not only because strong bonding reduces the probability of exchange during a catalyst–peptide encounter but also because of its more general effects on the patterns of internal bond rearrangements.

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