V. THE CORRELATION OF RIBONUCLEASE EXCHANGE KINETICS WITH THE TEMPERATURE-INDUCED TRANSITION*

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SUMMARY

RNase hydrogen exchange kinetics are similar to the characteristically complex hydrogen exchange of proteins in general. We have analyzed these kinetic data in terms of distributions of first order rates.

Two classes of exchanging sites in tritiated RNase can be identified and isolated by their temperature dependence. The pH and cosolvent effects on each class have been measured separately. Each class contains a distribution of first order rates evenly distributed over the range. The rank order of exchange is pH and temperature independent.

One class exchanges with an apparent activation energy ($E_{app}^*$) similar to that of random conformation polypeptides and substituted amides, $\approx 22$ kcal. The second class exchanges with an $E_{app}^*$ of $\approx 60$ kcal. The number of sites exchanging in each class is pH dependent. At pH 3.15 about 50% of the measured sites exchange with low $E_{app}^*$, while at pH 6 about 90% exchange with low $E_{app}^*$.

To account for the range and temperature dependence of the high $E_{app}^*$ distribution of exchange rates we propose a model involving an equilibrium between the folded and thermally unfolded state. This model is supported by (a) the agreement between observed and calculated rates estimated from the literature values of the thermal unfolding equilibrium constant, (b) the pH dependence of the high $E_{app}^*$ exchange, and (c) the urea and ethanol dependence of the high $E_{app}^*$ exchange.

It is proposed that the low $E_{app}^*$ exchange is from the folded protein, and as such offers a uniquely sensitive probe of the conformational dynamics of the folded state.

The pH dependence of the low $E_{app}^*$ rates is less than one-third that observed in model random conformational polypeptides.

The elucidation of molecular motion and structure is the primary goal of hydrogen exchange studies. However, the usefulness of this approach with proteins has been limited because of difficulties arising from the interpretation of kinetic data in relationship to protein structure. The prevalent model for hydrogen exchange is that proposed by the Carlsberg group (1); this model has been expanded (2) and clarified (3) but not appreciably altered. In the model, exchange from a single site is represented as $N \xrightarrow{k_1} I \xrightarrow{k_2} J$ exchange, where $N$ represents the nonexchanging form and $J$ the exchanging form of the site.

The model is simple and thus appealing, yet its general nature implies only that the attenuation of a first order rate is formally accounted for by an equilibrium between a reacting and nonreacting species. Under certain limiting conditions the apparent rate constant, $\beta$, can be expressed as a function of $k_1$, $k_2$, and $k_3$, the relative magnitudes of which determine the specific form for $\beta(1-3)$. It is assumed that $k_1$ is the conformation-independent first order exchange constant, the models for which are simple peptides and synthetic homopolymers. The $k_1$ and $k_2$ represent the conformational influence and thus contain the pertinent information about protein structure. The choice of a particular equation for $\beta$ rests on a priori assumptions about the relative magnitudes of $k_1$ and $k_2$. However, since these constants have not been associated with definite conformational processes little physicochemical meaning can be derived because the values of $k_1$ and $k_3$ from hydrogen exchange cannot be compared with analogous constants obtained by independent methods.

Efforts to assign $k_1$ and $k_3$ to reversible cooperative unfolding, a well defined kinetic process, have been frustrated by the fact that the observed exchange is not first order. Attempts have been made to fit exchange data with the minimum number of linear independent terms, each of which are thought to correspond to a definite structural unit of the protein. The assumption made in this kind of analysis is that the conformation-independent rate constant, $k_3$, is identical for all peptide amide sites; that is, that a group of sites exchanging by the same mechanism will have a common rate. This assumption, however, is not valid since oxidized RNase, an unfolded, random conformation heteropolymer, does not exchange with simple first order kinetics (4). Since we assume that each single site in oxidized RNase exchanges with a first order rate constant, $k_{ex}$, the observed rate for oxidized RNase represents a distribution of $k_{ex}$ values.1

1 The notation $k_{ex}$ is not intended to replace $k_3$ which denotes the rates of substituted amides, small peptides, or synthetic homopolymers (1-3). The $k_{ex}$ is an experimentally measured rate for...
Fig. 1. The temperature dependence of out-exchange from fully tritiated RNase, pH 3.15. $H_{\text{molecule}}$, hydrogen per molecule.

The distribution of first order rates, coupled with a rather large experimental error, makes the kinetic analyses of the observed exchange hazardous. However, conditions are optimized with RNase for which the thermodynamics and kinetics of the cooperative thermal and urea transitions, as well as the exchange properties of the unfolded protein, are well known.

RNase has been tritiated in three ways. (a) All sites are labeled; (b) only sites exchanging with $E^*_{\text{app}}$ of $\approx 22$ kcal are labeled; and (c) only sites exchanging with $E^*_{\text{app}}$ of $\approx 60$ kcal are labeled. The temperature, pH, and solvent perturbation of exchange kinetics in each case has been measured, and the data have been analyzed in terms of distributions of first order rate constants. The temperature and pH data are reported in this paper, the solvent data in the following paper (5).

METHODS

The methods and materials used in this study are the same as those reported previously (6) except for the following. RNase A was obtained from Sigma (bovine RNase, type IIIA, batch 27B-8600) and used without further purification. The temperature was maintained within $0.01^\circ$ by a water bath; in the experiments below room temperature the filtration columns were jacketed with water circulating from the bath. In experiments above room temperature the filtration columns were kept at room temperature. The calculations of hydrogens unexchanged per molecule ($H_{\text{rem}}$) were performed as described (6, 7) using a molar extinction of $9.80 \times 10^4$ for RNase. The pH 3.15 experiments were buffered with $0.04 \text{ M glycine-HCl}$ and the pH 6 experiments with $0.05 \text{ M acetate}$. The pH of all buffers was determined at room temperature and no correction was made for heat of ionization.

**Fully Tritated RNase**—The conditions are essentially those described by Slobodian and Fleischer (8). The RNase is dissolved in glass-distilled water (20 mg per ml) and the pH brought to pH 6.0 with dilute NaOH (1 to 2 drops). Then 1 or 2 ml are mixed with an equal volume of tritiated water (5 mCi per ml) and the pH again checked. This solution is maintained at 62° for 15 min or 45° for 12 hours. In both cases no increase of in-exchange is obtained by extending the time, and the subsequent out-exchange experiments are identical for both methods of in-exchange. For this reason the treatment at 62° for 15 min is used routinely. After heating at 62° the protein solution is equilibrated at the temperature and pH of the out-exchange experiment for 1 hour before the zero time filtration is done.

**Partially Tritiating RNase in Folded State**—RNase (20 mg per ml) is dissolved in two times concentrated buffer and the pH checked. The protein solution (2 to 3 ml) is then put into a glass vial and placed in a water bath at the indicated temperature (10° at pH 3.15; 15°, or 28° at pH 6). In a separate vial an equal volume of tritiated water (5 mCi per ml) also is placed in the bath. After 6 to 8 hours of preliminary incubation the contents of the vials are mixed (in a cold room) and the solution returned to the bath. The in-exchanging solution is kept at the preliminary incubation temperature for 18 to 21 hours after which there is no increase in the number of $H_{\text{rem}}$.

**Out-exchange from Fully Tritated Protein**—After the fully in-

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Experimental conditions} & \textbf{Temperature interval} & \textbf{$k_{\text{col/molc}}$} & \textbf{$E^*_{\text{app}}$} \\
\hline
Out-exchange from fully tritiated RNase, pH 3.15 (data in Fig. 1) & 10-15° & 2.0 ± 0.2 & 22 ± 5 \\
& 15-20 & 2.0 ± 0.2 & 23 ± 3 \\
& 20-28 & 2.6 ± 0.3 & 21 ± 2 \\
& 28-31 & 2.5 ± 0.3 & 55 ± 6 \\
& 31-34 & 2.3 ± 0.1 & 50 ± 3 \\
& 34-37 & 3.5 ± 0.6 & 78 ± 10 \\
& 37-40 & 2.3 ± 0.3 & 53 ± 8 \\
& 40-40 & 7.5 ± 0.5 & 64 ± 3 \\
Out-exchange from fully tritiated RNase, pH 6 (data in Fig. 6) & 10-15 & 2.0 ± 0.2 & 22 ± 3 \\
& 15-20 & 2.1 ± 0.2 & 25 ± 3 \\
& 20-28 & 2.3 ± 0.2 & 18 ± 3 \\
& 28-35 & 2.4 ± 0.2 & 23 ± 3 \\
& 35-40 & 3.9 ± 0.3 & 52 ± 3 \\
Out-exchange from RNase partially tritiated at 10°, pH 3.15 (data in Fig. 3) & 10-15 & 2.2 ± 0.5 & 25 ± 7 \\
& 15-20 & 1.7 ± 0.3 & 18 ± 6 \\
& 20-31 & 3.5 ± 0.5 & 20 ± 3 \\
& 31-34 & 5.8 ± 0.2 & 19 ± 3 \\
Out-exchange from RNase, pH 5.19, 10°, plateau (data in Fig. 5) & 28-31 & 2.9 ± 0.2 & 64 ± 5 \\
& 31-34 & 2.8 ± 0.2 & 64 ± 4 \\
& 34-37 & 6.5 ± 0.5 & 57 ± 3 \\
\hline
\end{tabular}
\caption{Activation energies of RNase hydrogen exchange}
\end{table}
Fig. 2. Out-exchange from RNase, pH 3.15, 10°; from the fully tritiated protein, X-X; from the protein partially tritiated in the pH 3.15, 10° folded state, O-O. H/molecule, hydrogen per molecule.

Fig. 3. The temperature dependence at pH 3.15 of out-exchange from RNase partially tritiated in the pH 3.15, 10° folded state. H/molecule, hydrogen per molecule.

Fig. 4. Diagram explaining the conditions of the temperature change experiments. —, out-exchange from the fully tritiated protein at temperatures, T₁ and T₂. Points A and B are at identical values of hydrogen per molecule unexchanged on these curves. For the temperature change experiment, the out-exchange at T₁ from the fully tritiated protein is carried to the plateau value of hydrogen per molecule unexchanged (Point A), then the temperature is raised to T₂. The ensuing out-exchange rate is shown by ——, which is superimposable on the T₂ solid curve after Point B if A and B are both taken as zero time. See text for discussion.

The exchangeable hydrogens measured in these experiments are considered to be predominantly peptide amide hydrogens. Model compound studies show that all side chain-labile hydrogens exchange with half-lives <2 to 3 min (1), and since the zero time filtration requires 4 min, we assume that labile hydrogens of side chains are not measured. Also, the number of Hseq after the zero time filtration is consistently within 3 to 5 hydrogens of the number of peptide amide hydrogens, albeit the equilibrium isotope effect, which is a source of some disagreement (9, 10), must be
taken into account before conclusions are drawn for absolute numbers. However, since the absolute number of sites involved does not alter the conclusions of this investigation, the question was not further probed.

RESULTS

Out-exchange at pH 3.15

Out-exchange from Fully Tritiated RNase—The temperature dependence of out-exchange at pH 3.15 from fully tritiated RNase is shown in Fig. 1. Although the kinetics are not first order, we have presented evidence that the observed exchange kinetics of proteins represent the simultaneous measurement of many first order processes (4) and that therefore the curves in Fig. 1 show the temperature dependence of distributions of first order rates. The apparent activation energies ($E_{app}^*$) of such distributions can be estimated between each temperature interval.

Fig. 5. Temperature change experiment of out-exchange from the pH 3.15, 10º plateau, Point A in Fig. 4. O——O. Out-exchange from the fully tritiated protein with zero time set at the hydrogen per molecule (H/molecule) unexchanged equal to the 10º plateau value, Point B in Fig. 4. ■——■. The temperature is changed from 10º to that indicated in the figure.

Fig. 6. The temperature dependence of out-exchange from fully tritiated RNase, pH 6.0. H/molecule, hydrogen per molecule.

Fig. 7. Temperature change experiments with RNase, pH 6.0. The closed symbols are exchange from Point A, Fig. 4. The open symbols are exchange from Point B, Fig. 4. H/molecule, hydrogen per molecule.
by the method described in "Appendix I." The confidence limits of this way are compiled in Table I. In the temperature intervals from 10-28°C the exchange has an $E_{\text{app}}$ of 21 to 23 kcal, whereas in the intervals from 28-40°C $E_{\text{app}}$ is between 50 and 80 kcal. The curves for the low $E_{\text{app}}$ exchange level off to a very slow, but measurable rate around 18 hours; the curves for the high $E_{\text{app}}$ do not level off (Fig. 1).

Experimental Separation of High and Low $E_{\text{app}}$ Groups—The leveling off of the low $E_{\text{app}}$ exchange rate in Fig. 1 facilitates partial tritiation of RNase in two reproducible ways: (a) in-exchange under conditions of low $E_{\text{app}}$ exchange, namely 10°C, pH 3.15, 18 hours; (b) fully tritiated RNase, then out-exchange at 10°C, pH 3.15, 18 hours, leaving the unexchanged sites labeled. RNase partially labeled in these ways was studied separately.

As pointed out by Englander and Poulsen (9), the in- and out-exchange of trace label is governed by the out-exchange rate constant; therefore the in- and out-exchange at constant temperature and pH have identical kinetics. Then at constant pH the out-exchange kinetics at 10°C from RNase partially tritiated at 10°C will be the same as that at 10°C from fully tritiated RNase. This is what we observe, as shown in Fig. 2.

When the out-exchange from RNase tritiated at 10°C, pH 3.15, is measured at higher temperatures, the curves in Fig. 3 are obtained. Activation energies of 18 to 25 kcal have been estimated for temperature intervals from 10-31°C, Table I.

The temperature dependence of RNase partially labeled in the second (above) way can also be measured separately. In this case the fully tritiated protein is out-exchanged at 10°C until the plateau is reached (about 18 hours), then the temperature is raised; subsequent exchange is followed in the usual way. This type of experiment is diagrammed in Fig. 4. When the temperature is raised from 10°C (Point A, Fig. 4) the kinetic data shown in Fig. 5, ○, are obtained, zero time being the time of the temperature change. The closed symbols (■) in Fig. 5 show the kinetics of exchange from fully tritiated RNase at the same temperature (data in Fig. 1) with zero time moved to the $H_{\text{ren}}$ value of the 10°C plateau (Point B, Fig. 4). The apparent activation energies in the intervals from 28-34°C range from 57 to 64 kcal, Table I.

Out-exchange at pH 6.0

The out-exchange from fully tritiated RNase, pH 6.0, as a function of temperature is shown in Fig. 6. The $E_{\text{app}}$ for the pH 6 exchange in all intervals from 10-35°C is ~22 kcal, while that in the interval 35-40°C is ~33 kcal. Comparison of Fig. 1 and Fig. 6 shows that the number of sites exchanging with low $E_{\text{app}}$ is larger for pH 6.0 than for pH 3.15. However, at pH 6.0, as at pH 3.15, the exchange with low $E_{\text{app}}$ is characterized by leveling off of the first order plots. Thus, temperature change experiments analogous to those at pH 3.15 may be done from any of these plateaus. The 10°C and 28°C plateaus were chosen in order to cover the widest possible range. In all cases the pH 6 temperature change experiments have the same results as those at pH 3.15, i.e. the exchange from each plateau initiated by a rise in temperature coincides with exchange directly from the fully tritiated protein at the higher temperature, Fig. 7.

pH Dependence of Partially Labeled RNase—The pH dependence in the range pH 3.15 to pH 5 for the low $E_{\text{app}}$ exchange is shown in Fig. 8. There is a small increase in rate, but the general shape of the curves is retained.

The pH dependence of the pH 3.15 high $E_{\text{app}}$ exchange is shown in Fig. 9. In this experiment we selectively label the pH 3.15 high $E_{\text{app}}$ class by letting the fully tritiated protein out-exchange at pH 3.15, 10°C, until the plateau is reached, and then raising the pH. These pH change experiments are directly analogous in design to the temperature change in Fig. 4. The results are also similar in that, when temperature is held constant, the kinetics of exchange after the pH is raised are identical with the...
exchange kinetics at the single, higher pH beginning zero time at equal \( H_{\text{rem}} \). This is shown in Fig. 10 in which the pH 6 curve from Fig. 9 is redrawn along with the exchange curves from the fully tritiated protein at pH 3.15 and pH 6.0.

**Discussion**

In general the exchange kinetics of a heteropolymer represents the net effect of a great number of parallel independent reactions. The amount of label retained, \( H_{\text{rem}} \), at any time, \( t \), can be expressed

\[
H_{\text{rem}} = \sum_{i=1}^{N} H_i e^{-\beta_i t}
\]

where \( \beta_i \) is the apparent first order rate constant for the \( i \)th site. Equation 1 is subject only to the restriction that \( \sum H_i = N \) where \( N \) is the total number of sites exchanging. The experimental data (\( H_{\text{rem}}, t \)) can be described mathematically by a sum of exponentials, but such exponential terms are unlikely to correspond to a class of sites exhibiting common properties of exchange. However, a division of sites into classes with common properties is implicit in all physical interpretation of exchange kinetics. It is our intention to choose rate groups in a way that will facilitate our proving that under restricted conditions a rate group does correspond to a class of sites exchanging by a common mechanism characterized by the structural behavior of the molecule.

We have presented evidence that the curves in Fig. 1 represent distributions of first order rates (4). Activation energies for such distributions can be estimated for a given temperature interval only if the temperature coefficients of the apparent rate constants are identical. When the ratio of times necessary to obtain the same degree of exchange at two temperatures, \( t_1/t_2 \), remains constant during the exchange, the activation energy can be estimated (11), as shown in “Appendix I.” The ratio \( t_1/t_2 \) for pairs of curves is constant within the rather large experimental errors, Table I. Since we are not trying to characterize the temperature dependence of a single site but rather to estimate the average apparent \( E^* \) as a function of temperature, the sharp break in the \( E^*_{\text{app}} \) values in Table I is well outside the limits of experimental error. It should be noted that the limits of the time resolution of the method is such that there is not a single or even a few values of \( H_{\text{rem}} \) at which the \( t_1/t_2 \) of all temperature intervals can be obtained. Therefore calculated \( E^* \) values of different temperature intervals are for different rate constants.

There is, however, evidence for a common temperature coefficient for rates in a given temperature interval in the results in Figs. 5 and 7 of the experiments diagrammed in Fig. 4. Referring to Fig. 4, if there is a common \( E^* \) in the interval \( T_1 \) to \( T_2 \) for the sites exchanging before \( A \) and \( B \), then the contribution of each first order term to the total exchange kinetics is in the same rank order and there are no sites exchanging before \( A \) that do not contribute to exchange before \( B \). The exchange kinetics from \( A \) are superimposable on the kinetics from \( B \) at all temperatures and pH values measured, Figs. 5 and 7. This would follow from a temperature independent rank order, i.e. if there were a common \( E^* \) for all the rates measured. A second type of supporting evidence comes from the internal consistency of the pH and solvent effects on the temperature dependence.

Bearing qualifications of the calculated \( E^*_{\text{app}} \) in mind, we will attempt to establish that (a) there are two rate groups at pH 3 which represent specific classes of sites having two corresponding mechanisms for exchange, one a low \( E^* \) process and one a high \( E^* \) process, and (b) the physical basis for the difference in mechanism is accounted for by the model below. Our arguments are based on the combined results of the temperature, pH, and solvent perturbation of RNase hydrogen exchange.

Although the data do not exclude a third process with an intermediate value of \( E^* \), our postulation of two exchange processes is the simplest way to account sensibly for the temperature dependence. The conditions of most of our experiments were designed to avoid the intermediate region so that one of the mechanisms would clearly predominate.

**Model for Temperature Dependence of RNase Hydrogen Exchange**—The model is as follows. The class of sites exchanging with low \( E^*_{\text{app}} \) is composed of sites which exchange from the folded state of the protein. The exchange of such a tritium (T)-labeled site may be expressed

\[
\text{Folded T} \xrightarrow{k_{\text{app}}} \text{unfolded T} \text{H + TOH}
\]

The apparent rate constant for this exchange, \( k_{\text{app}} \) (low \( E^* \)), is a function of \( k_{\text{eq}} \), the chemical exchange constant, for that site and of all solvent and protein influences different for the site in folded RNase versus the same site in random conformation RNase.

The model further proposes that the class of sites exchanging with high \( E^*_{\text{app}} \) consists of sites whose exchange is preceded by the temperature-induced, reversible unfolding of the protein. The mechanism for exchange from these sites in the folded protein may be represented by the scheme

\[
\text{Folded T} \xrightarrow{k_f} \text{unfolded T} \xrightarrow{k_u} \text{unfolded H + TOH}
\]

The rate constants, \( k_f \) and \( k_u \), are the rates of reversible unfolding and refolding of RNase as measured by Pohl (12) and \( k_f/k_u = K_{\text{eq}} \) as measured by Brandts and Hunt (13). The \( k_u \) is the exchange rate from the thermally unfolded RNase.

The solution for the apparent over-all rate of Equation 3 is the same as given by Hvidt (14) for a formally identical case, and exchange of a site in the high \( E^*_{\text{app}} \) class is

\[
k_{\text{app}}(\text{high } E^*) = \frac{k_f}{k_f + k_u + k_n}
\]

and when \( k_f \ll k_u \) and \( k_u \ll k_n \)

\[
k_{\text{app}}(\text{high } E^*) = K_{\text{eq}} \cdot k_u
\]

Ideally, we could calculate the value and temperature dependence of \( k_{\text{app}} \) (high \( E^* \)) predicted by this model and compare it with that observed. The hitch is \( k_{\text{eq}} \), the exchange rate constant for the thermally unfolded state. We assume that \( k_u \) is a function of \( k_{\text{eq}} \), the first order chemical exchange constant. As documented previously (4), random conformation oxidized RNase exchanges with a distribution of \( k_{\text{eq}} \) and therefore we expect a distribution of first order rates for the exchange of the high \( E^* \) class. This makes calculation of expected half-lives somewhat less straightforward, but since we are not trying to calculate individual rate constants but rather to estimate relative distributions.

The cases are not equivalent. We use the model in Equation 3 as a specific one for part of the exchanging sites under limited conditions of pH and temperature, while Hvidt and Nielsen use it generally for all exchanging hydrogens. Further, we use different models to evaluate \( k_{\text{eq}}, k_f, \) and \( k_u \), which are definite, pH dependent, molecular parameters.
tions, we treat the constants for the exponential terms as average rate constants ("Appendix II").

A second point concerning $k_a$ is the choice of the correct model compound for the exchange from the thermally unfolded protein. There are two limiting possibilities: (a) The exchange rate distribution of the thermally unfolded state is similar to that of the random conformation and therefore $k_a \approx k_{\text{uu}}$ in value and distribution, or (b) the exchange rate distribution of thermally unfolded RNase is similar to that of the folded state and therefore $k_a \approx k_{\text{app}}$ (low $E^*$) in value and distribution. We have estimated both $k_{\text{app}}$ (low $E^*$) and $k_{\text{uu}}$ ("Appendix III") and have calculated the expected distribution of $k_{\text{app}}$ (high $E^*$) for both cases in a variety of temperature and urea concentrations.

In Fig. 11 the calculated distributions are compared to those observed for the high $E^*_\text{app}$ exchange at pH 3.15. Values calculated using $k_a = k_{\text{uu}}$ are clearly different from those observed. On the other hand, when $k_a = k_{\text{app}}$ (low $E^*$) is used there is good agreement between the calculated and observed rate distributions at the pH 3.15 high $E^*_\text{app}$ class at all conditions of temperature and urea measured. Further support for the validity of our model in Equation 3 comes from the experimental activation energy for this class. The value for the high $E^*_\text{app}$, Table I, fits reasonably well with a $\Delta H^0 = 50$ kcal for $K_{eq}$ (13) and low $E^*_\text{app} = 22$ kcal.

While the agreement illustrated in Fig. 11 does not by itself constitute definitive proof of the veracity of our model, when considered together with the pH and solvent dependence of exchange, these data lend a strong argument in favor of the model. These points are expanded in the following paper (5).

**pH Dependence of Low $E^*_\text{app}$ Hydrogen Exchange**—The most striking difference between the pH 6 and the pH 3 exchange kinetics is that the high $E^*_\text{app}$ exchange represents only the last 10 to 15 hydrogens at pH 6 compared with ~866 sites at pH 3. Clearly, the high and low $E^*_\text{app}$ rate groups are composed of different sites at different pH. To focus on this point we have measured the pH dependence of exchange from RNase partially labeled so that all sites exchange at pH 3.15 with low $E^*_{\text{app}}$. Fig. 8 shows the out-exchange at several pH values, 28°C, of RNase partially labeled in this way. Since we assume that $k_{\text{app}}$ (low $E^*$) for each site is a function of $k_a$ for that site, we would expect that a nearly 1000-fold increase of OH− ion should lead to a corresponding increase of $k_{\text{uu}}$ and consequently of $k_{\text{app}}$ (low $E^*$).

This is the case for oxidized RNase where the rates measured at pH 3 become too fast to measure at pH 6 by this method. In native RNase, however, the rate increase with increasing OH− concentration is considerably less (Fig. 8), the observed rate increase from pH 3.8 to pH 5.0 being 2 to 2.5 fold. From model studies (1, 3), the observed rate increase for the same pH interval is a factor of 15; when corrected for electrostatic effects (15), the expected factor is 9. This indicates that there are pH dependent factors other than $k_{\text{uu}}$ which contribute to $k_{\text{app}}$ (low $E^*$).

**Distribution of Low $E^*_\text{app}$ Exchange Rates**—In our model all sites in each class exchange in a temperature-independent rank order over a range of rates. One may then ask how the rates are distributed over the range. It is possible that there are two (or more) fairly discrete clusters of rates within the complete range. In the more extreme cases, this would show up as a discontinuity in the exchange kinetics. Experimentally, however, this would be difficult to observe since at any one temperature at a given pH only a small part of the rate distribution is detected by our method, many rates being either too fast or too slow. By working at pH 6, where almost all of the sites exchange with low $E^*_\text{app}$, it is possible to calculate a large part of the distribution by combining the 10°C, 28°C, and 35°C measurements. With the assumption that the rank order of low $E^*_\text{app}$ exchange is temperature independent we can calculate the rates of each increment of $H_{\text{rem}}$ which becomes measurable with the respective temperature increment. Using $E^* = 22$ kcal we can then calculate what the distribution at 10°C would be for the $H_{\text{rem}}$ measured at 28°C and 35°C. The results of these calculations, Fig. 12, are consistent with our interpretation that exchange from the folded protein by a low $E^*_\text{app}$ occurs by an apparently continuous and evenly spread distribution of rates.

The range of the low $E^*$ rate distribution is also noteworthy.

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**Fig. 11.** Comparison of the observed exchange rate distribution for sites exchanging with high $E^*_\text{app}$ with that calculated as in "Appendix II." The observed distribution of average $k_{\text{rem}}$ ($k_{\text{app}}$) for out-exchange at pH 3.15 from the pH 3.15 10°C plateau (the high $E^*$ class), . The calculated distribution of average $k_{\text{app}}$ from Equation 5 with $k_a = k_{\text{app}}$ (low $E^*$), . The calculated distribution of average $k_{\text{app}}$ from Equation 5 with $k_a = k_{\text{uu}}$, .

**Fig. 12.** The distribution of average $k_{\text{app}}$ ($k_{\text{app}}$) for RNase, pH 6.0, 10°C. See text for details.
If our model is correct, the range of rates for exchange from folded RNase is 75-fold larger than that of random conformation oxidized RNase.

Summary of pH and Temperature Dependence of RNase Hydrogen Exchange. The main observations of this study are these.

1. The width of the exchange rate distribution of folded RNase is about 75-fold wider than that of random conformation RNase at the same conditions.

2. At pH 3.15 and at pH 6.0, part of the sites exchange with an apparent low activation energy, while the rest exchange with an apparent high activation energy.

3. The rates in each class are apparently evenly distributed over the range.

4. The high \( E^* \) exchange rate distributions at pH 3.15 are accounted for in value and in temperature and urea dependence by the model in Equation 3, when \( k_a \) is taken as approximately equal to the distribution for the latter (low \( E^* \)). This model involves an equilibrium between the folded and the thermally unfolded state, and the site in question exchanges from the thermally unfolded state. The calculated rates using \( k_a = k_{app} \) (low \( E^* \)), in good agreement with those observed, indicate that the exchange rate for the thermally unfolded state is more similar to the exchange rate from the folded state than to that from the random conformation.

5. The number of sites exchanging in each class is pH dependent. At pH 3.15 about 50% of the measured sites exchange with low \( E^* \), while at pH 6.0 about 90% exchange with low \( E^* \). If, as we propose in Equation 2, the low activation energy mechanism occurs only for exchange from the folded protein and not from the thermally unfolded protein, and if, as seems evident (16), the unfolding is two state, then we must account for the fact that all (or nearly all) RNase-labile protons may exchange while the protein is in the folded state.

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**APPENDIX I**

Calculation of \( E^* \) for Distribution of First Order Rates—If we consider the exchange at two temperatures, \( T_1 \) and \( T_2 \), from one site, \( H_a \), for any point with equal fraction of label retained, \( H_{rem} = e^{-E^*/RT_1} = e^{-E^*/RT_2} \) and \( -b_{T_1} = -b_{T_2} \). Thus \( t_1/t_2 \) is constant at any given \( H_{rem} \) value in the temperature interval. Now consider the simultaneous exchange of all sites as in Equation 1. If the ratio of times for an equal degree of total exchange at two temperatures for a single \( H_{rem} \) along the first order plots, \( t_1/t_2 \), is constant, then the rates whose measured have a common activation energy (6, 11). Then \( t_1/t_2 = \beta_{T_1}/\beta_{T_2} = e^{-E^*/RT_1}/e^{-E^*/RT_2} \) where \( \beta \) is the apparent overall exchange rate, and
$E^* = (T_1 \times T_3 \times R \times 2.303 \log t_3/i_3)/(T_1 - T_3)$ (6)

**APPENDIX II**

Expression of Distributions of First Order Rates in Plots of $H_{rem}$ versus Average $k_{app}$ (high $E^*$)—The observed $k_{app}$ (high $E^*$) is estimated by graphically fitting (17, 18) first order plots of the observed kinetics in Fig. 5 with three linear terms, each of which is the average value of a distribution of first order rates for $n$ $H_{rem}$, the extrapolated number of hydrogens per molecule unexchanged on the ordinate.

The calculated values of the average $k_{app}$ (high $E^*$) are computed from Equation 5 using $K_{app}$ at the appropriate temperatures and urea concentrations from Brandts and Hunt (13), and using for $k_a$ the distributions of $k_{app}$ (low $E^*$) and of $k_{cz}$ estimated as in “Appendix III.”

Equation 5 is valid only if $k_f \ll k_b$ and $k_u \ll k_b$ (14). The values of $k_f$ and $k_b$ for RNase, estimated at pH 3.15 from the data of Pohl (12), meet these requirements.

Neither the values of the average $k_{app}$ (high $E^*$) nor the number of $H_{rem}$ with that average $k_{app}$ are considered to have any structural significance. The distribution of rates is considered to be continuous over the whole range, but the experimental error does not warrant fitting the curves with more than three terms.

**APPENDIX III**

Estimation of $k_{app}$ (low $E^*$) and of $k_{cz}$—The distribution of $k_{app}$ (low $E^*$) for the 60 sites measurable at pH 3.15, $10^9$, is obtained by fitting the $10^9$ data in Fig. 3 with three linear terms. The values obtained are hydrogen per molecule unexchanged $= 15e^{-2.4 \times 10^{-2}t} + 30e^{-2.4 \times 10^{-2}t} + 15e^{-1.8 \times 10^{-2}t}$ with $t$ in seconds. Distributions at other temperatures were computed using an activation energy of 22 kcal.

Since one cannot label the high $E^*_{app}$ class unless the disulfide bonds are intact, measuring directly the $k_{cz}$ of the high $E^*_{app}$ class requires breaking the disulfide bonds simultaneously and instantaneously after the separation of partially labeled protein from the excess tritium. Because the $k_{cz}$ of the high $E^*_{app}$ class cannot be directly measured, we must extract its distribution from the observed exchange kinetics of the fully tritated oxidized RNase. This cannot be done by simply using the rates of the slowest 60 exchanging protons from oxidized RNase because to do so one must assume that the sites in the folded protein exchange in the same rank order as the sites in oxidized RNase. That this assumption is not valid is evidenced by the fact that the rates of the exchange of the pH 3.15 low $E^*_{app}$ class in 4 M urea overlap completely the rates of the pH 3.15 high $E^*_{app}$ class in 4 M urea, that is, the exchange rates in 4 M urea of the sites in both classes extend over the entire range of the distribution. This can be documented in the following way. The $k_{app}$ of both the high and low $E^*_{app}$ classes in 4 M urea is computed, as shown in Table II. Then the sites are put in order of ascending $k_{app}$, Table II, and plotted in that order against $H_{rem}$. This plot can be compared to that obtained from the observed exchange of the fully tritiated protein in 4 M urea. These plots are shown in Fig. 13 and it can be seen that the agreement is very good. Therefore we have taken as the $k_{cz}$ of the high $E^*$ class 60 sites with rate values extending evenly over the entire rate distribution of fully tritiated oxidized RNase.

The $k_{cz}$ distribution for the high $E^*_{app}$ class at $2^\circ$ pH 3.15, computed for 60 sites evenly distributed over the range for the fully tritiated oxidized RNase (4) is hydrogen per molecule unexchanged $= 10e^{-5.8 \times 10^{-4}t} + 30e^{-2.6 \times 10^{-4}t} + 15e^{0.8 \times 10^{-4}t}$ with $t$ in seconds.

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