Mechanisms of Hydrogen Exchange in Proteins from Nuclear Magnetic Resonance Studies of Individual Tryptophan Indole NH Hydrogens in Lysozyme†

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ABSTRACT: The individual rates of solvent exchange of the six tryptophan indole NH hydrogens of lysozyme in ^2H_2O have been measured over a wide range of temperatures by using ^1H NMR. Two distinct mechanisms for exchange have been identified, one characterized by a high activation energy and the other by a much lower activation energy. The high-energy process has been shown to be associated directly with the cooperative thermal unfolding of the protein and is the dominant mechanism for exchange of the most slowly exchanging hydrogen even 15 °C below the denaturation temperature. Rate constants and activation energies for the folding and unfolding reactions were obtained from the experimental exchange rates. At low temperatures, a lower activation energy mechanism is dominant for all hydrogens, and this can be associated with local fluctuations in the protein structure which allow access of solvent. The relative exchange rates and activation energies can only qualitatively be related to the different environments of the residues in the crystal structure. There is provisional evidence that a mechanism intermediate between these two extremes may be significant for some hydrogens under restricted conditions.

Proteins in solution undergo a wide range of molecular motions as revealed by a variety of experimental and theoretical techniques (Karplus & McCammon, 1981). Some of the first evidence for protein motions came from the observation of hydrogen exchange in globular proteins (Hvidt & Linderstrøm-Lang, 1954). In hydrogen exchange, labile hydrogens (e.g., amide hydrogens from the protein backbone or indole NH hydrogens from tryptophan side chains) are replaced by hydrogens from the solvent. If deuterium- or tritium-labeled solvent is used, the rates of exchange can be measured directly. The time required for exchange can range from seconds to years depending upon conditions such as pH and temperature (Hvidt & Nielsen, 1966).

Over the last 2 decades, much research has been done relating hydrogen exchange rates with protein motions (Hvidt & Nielsen, 1966; Englander et al., 1972; Woodward & Hilton, 1979). A two-step reaction scheme has generally been used to explain the phenomenon of hydrogen exchange in native proteins. The first step in this scheme represents the reactions bringing the exchangeable groups and solvent into contact, the second step being the chemical exchange step. Several models have been proposed to explain the physical nature of the first step in this reaction scheme. The question of which of the various models best describes the mechanism of hydrogen exchange under given conditions is a matter of current controversy. In one model, the solvent molecules penetrate into the interior of the protein matrix through cavities opened by small molecular fluctuations, and exchange takes place in the interior of the protein. This model has recently been used to explain exchange results from myoglobin (Richards, 1979), the bovine pancreatic trypsin inhibitor (BPTI) (Woodward & Hilton, 1980), and lysozyme (Knox & Rosenberg, 1980). An alternative model, involving local unfolding and the breaking of several adjacent hydrogen bonds, has recently been used to explain results from lysozyme (Nakanishi et al., 1973), ribonuclease (Schreier & Baldwin, 1977), and hemoglobin (Englander et al., 1980). A model which combines aspects of the above has been used to explain exchange results from BPTI (Wüthrich et al., 1980). In this multistate model, hydrophobic clusters fluctuate with respect to each other while each cluster preserves its basic structure; solvent molecules reach the labile hydrogens along pathways opened by the fluctuations of the hydrophobic clusters.

Most of the studies of hydrogen exchange have used ^3H-labeling methods, but UV, IR, and Raman spectroscopic methods have also been used. These methods give data on the bulk exchange of all labile hydrogens or the bulk exchange of selected groups of labile hydrogens. The use of bulk exchange data to predict the exchange behavior of individual hydrogens is not universally accepted [discussion after Englander et al. (1980)]. Recently, nuclear magnetic resonance (NMR) spectroscopy has been used to follow the exchange rates of individual hydrogens (Glickson et al., 1971; Campbell et al., 1975; Hilton & Woodward, 1979; Wagner & Wüthrich, 1979).

In this study, proton NMR has been used to measure the individual rates of exchange of the indole NH hydrogens from the tryptophan residues in hen egg white lysozyme. Lysozyme contains six tryptophan residues which are found in widely different environments in the protein structure (Blake et al., 1978). The resonances of the indole NH hydrogens are well resolved and have previously been assigned in the spectrum (Cassels et al., 1978; Lenkinski et al., 1979). Of particular interest here is the dependence of the exchange rates on temperature and the insight that this gives into the mechanisms of the exchange reactions.

Experimental Procedures

Lysozyme from hen egg white (EC 3.2.1.17) was obtained from Sigma and was further purified by dialysis at pH 3.0. Fourier-transform ^1H NMR spectra were recorded at 270 and

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300 MHz with Bruker spectrometers and at 498 MHz with a home-built spectrometer at the Francis Bitter National Magnet Laboratory. The spectrometer probe temperature was calibrated for each experiment (Van Geet, 1968) with a precision of ±0.3 °C. Hydrogen exchange rates were determined by following the decrease in peak height of the NMR signals of individual hydrogens after lyophilized lysozyme was dissolved in a deuterated acetate/^2H_2O buffer, pH 3.8. The samples were 5.5 ± 0.5 mM in lysozyme, and the buffer was 150 mM in acetate. The reported pH values are the meter readings, with no adjustments for isotope effects. For exchange rates in the range 10^{-2}–10^{-5} s^{-1}, the entire experiment took place in the NMR spectrometer probe; 15–20 spectra were collected in sequence for each experiment. For exchange rates outside this range, aliquots were removed at timed intervals from lysozyme solutions maintained in a constant-temperature water bath. The exchange process was quenched by quickly freezing the aliquots to 195 K. The NMR spectrum of each aliquot was later recorded under conditions where exchange would be minimal. The two experimental methods are mathematically equivalent (Glickson et al., 1971). When the two methods were compared at a standard temperature, the experimental results were the same within experimental error. The exchange rates for tryptophan itself (50 mM, pH 3.8, in acetate/H_2O buffer) and for the tryptophan residues in thermally denatured lysozyme were determined by using saturation recovery and saturation transfer experiments with a Redfield 2–1–4 pulse sequence (Waelder & Redfield, 1977; Redfield & Gupta, 1971).

The denaturation equilibrium of lysozyme was studied in ^2H_2O and H_2O in the temperature range 70–78 °C. A fresh aliquot of lysozyme solution (5.5 mM, pH 3.8, in acetate buffer) was used at each temperature to minimize the effect of irreversible denaturation. After 10–15 min was allowed in the spectrometer probe for the establishment of thermal and chemical equilibrium, a series of three NMR spectra was obtained. The peak areas of eight well-resolved and previously assigned resonances were measured in each spectrum. For the $H^{\epsilon 1}$ of His-15, both the native and denatured resonances were measured. For the other resonances, only the native peak areas were measured. The calculation of thermodynamic values from peak area data has been previously described (McDonald et al., 1971).

Results and Discussion

Experimental Measurements. The kinetics of hydrogen exchange under all conditions were observed to follow, within experimental error, a first-order rate law, and the rate constants were determined by least-squares fitting procedures. The rates of exchange of the amino acid tryptophan itself (Figure 1) are in good agreement with values reported previously (Waelder & Redfield, 1977; Campbell et al., 1977; Nakanishi et al., 1978). The Arrhenius plot (Figure 1) is linear over the entire temperature range studied (37–77 °C) and shows an activation energy of 11.6 ± 0.6 kcal mol^{-1}. Individual resonances of the native and denatured species can be observed separately in the spectra, because the rate of transition between the two species is slow on the NMR time scale (McDonald et al., 1971). It was therefore possible to measure independently exchange rates from the two species. At pH 3.8 and at temperatures above 77 °C, lysozyme exists predominantly as the fully denatured species, and the bulk exchange rates from the tryptophans of the denatured species are closely comparable to those for the amino acid (Figure 1). Below 72 °C, lysozyme exists predominantly in its native conformation, and the exchange rates for the six individual tryptophan residues

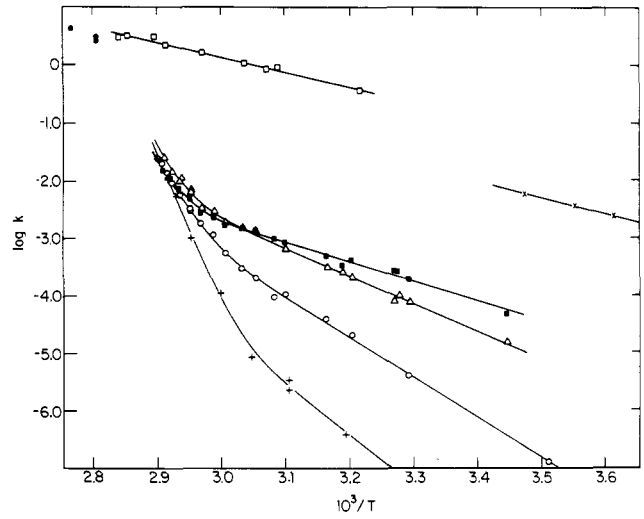

FIGURE 1: Temperature dependence of the first-order rate constants (in s^{-1}) for exchange of indole NH hydrogens in ^2H_2O for tryptophan (□), unfolded lysozyme (●), and Trp-28 (+), Trp-63 (×), Trp-108 (■), Trp-111 (○), and Trp-123 (△) of native lysozyme. The solid lines are least-squares fits of the data as described in the text. Each data point represents the mean of two to four trials and has an uncertainty of ±0.04 log unit (or 10%). The values for Trp-63 have an uncertainty of ±0.10 log unit (or 25%).

Table I: Activation Energies for Indole NH Hydrogen Exchange[a]

residue	high-temp process[b]	low-temp process[b]
Trp-28	120 ± 8	40.2 ± 5.7
Trp-62		<13.0
Trp-63		13.2 ± 2.5
Trp-108	93 ± 12	15.5 ± 0.6
Trp-111	92 ± 6	31.6 ± 0.7
Trp-123	97 ± 10	22.0 ± 0.6

[a] In kcal mol^{-1}. For tryptophan itself, the value is 11.6 ± 0.6 kcal mol^{-1}. [b] Values obtained from the least-squares fitting procedure described in the text, using the data shown in Figure 1. For Trp-62, only an average upper limit could be obtained, and for Trp-63, only the low-temperature process could be observed.

of native lysozyme are all slower than for the amino acid itself. The exchange process for Trp-62 could not be measured; the rates of exchange over the entire temperature range of 4–72 °C were too fast to be measured by following changes in peak heights but too slow to be measured by the saturation recovery method. The rates therefore must be in the range 1–10^{-2} s^{-1} over the whole temperature range, and this enables the maximum average activation energy to be estimated to be 13 kcal mol^{-1}. Exchange rates for Trp-63 were too fast to be measured at temperatures above 17 °C (Figure 1). For the four other tryptophan residues, the exchange was slow enough for the rates to be measured accurately over a wide temperature range. The shapes of the Arrhenius plots show, however, for each of these four residues that a single activation energy does not describe the kinetics over the entire temperature range. At least two processes could be identified, with a process of higher activation energy becoming dominant in each case at temperatures above 60 °C. For the initial analysis, the data were fitted to a two-process model, using a least-squares program, and the fits are shown in Figure 1. The activation energies for each process are given in Table I, and the high quality of the fits indicates that the model is a reasonable one for all four residues.

The equilibrium constant for the denaturation of lysozyme was determined over the range 70–78 °C. The linear plot (Figure 2) shows that the denaturation equilibrium is ade-

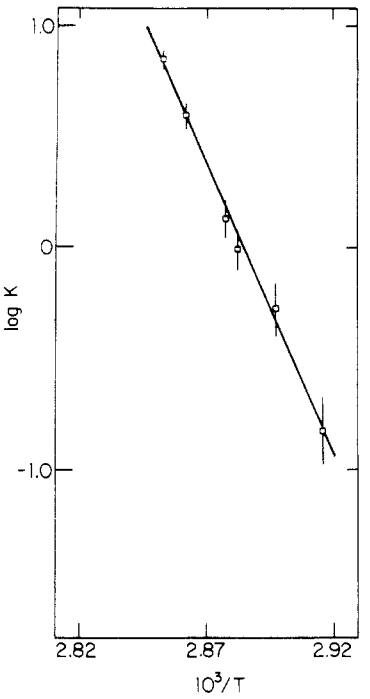

FIGURE 2: Temperature dependence of the equilibrium constant for denaturation. The data represent the mean and standard deviation of the values individually determined by study of the following resonances: His-15 H^{ϵ_1}; Leu-17 H^{δ_2}; Trp-28 H^{ζ_3}; Trp-108 H^{ζ_3}; and four H^{α}. The solid line is the best straight line fit of the data and has a slope of 119 kcal mol^{-1}.

quately described by a two-state model. For denaturation in ^2H_2O, the denaturation temperature is 73.6 °C, and the thermodynamic values are $\Delta H = 119 \pm 5$ kcal mol^{-1} and $\Delta S = 343 \pm 13$ cal mol^{-1} deg^{-1} (Figure 2). These values were calculated from the best fit of the data with the assumption that ΔH is temperature independent. This assumption is only approximately correct, because there is a slight increase of ΔH of denaturation with temperature for most proteins.

General Description of Exchange Kinetics. The reaction scheme that has been used most widely to describe hydrogen exchange in proteins assumes that exchange requires two separate steps (Hvidt & Nielsen, 1966). In the first step, the native protein (E) in which the hydrogen is not accessible to solvent is in equilibrium with a form X. X may be the unfolded state of the protein, but in general, it is any conformation in which the hydrogen is accessible to solvent. In the second step, the actual chemical exchange of the labile hydrogen takes place. For study of a specific hydrogen in the protein, we can write for exchange with ^2H_2O solvent

$$EH \underset{k_2}{\overset{k_1}{\rightleftharpoons}} XH \xrightarrow{k_3} P$$

where EH and XH are species in which the hydrogen has not been exchanged and P is the protein in which the hydrogen has been replaced by deuterium. The general solution of the coupled differential equations

$$\frac{d[EH]}{dt} = -k_1[EH] + k_2[XH] \quad (1)$$

$$\frac{d[XH]}{dt} = k_1[EH] - (k_2 + k_3)[XH] \quad (2)$$

indicates that the kinetics will be complex in general and will involve two rate constants (Hvidt, 1964, 1973).

Hvidt & Nielsen (1966) have pointed out, however, that there are two important limiting cases for hydrogen exchange in proteins, EX1 kinetics where $k_1 + k_2 \ll k_3$ and EX2 kinetics where $k_1 + k_3 \ll k_2$. In either case, apparent first-order kinetics, such as found experimentally in this work, can be observed. This will be the case if sufficient time has elapsed between dissolution of the protein in ^2H_2O and observation of the exchange reaction to allow the more rapidly decaying term in the general kinetic solution to become insignificant. Then the observed rate constant will be

$$k_{obsd} = \frac{k_1 + k_2 + k_3 - \sqrt{(k_1 + k_2 + k_3)^2 - 4k_1k_3}}{2} \quad (3)$$

For EX1 kinetics, the observed rate constant can then be simplified to k_1 and

$$[EH] = [EH]_0 \exp(-k_1t) \quad (4)$$

$$[XH] = \frac{k_1(k_1 + k_2)}{k_2k_3}[EH]_0 \exp(-k_1t) \quad (5)$$

In the EX2 case

$$[EH] = [EH]_0 \exp\left(-\frac{k_1k_3}{k_2}t\right) \quad (6)$$

$$[XH] = \frac{k_1}{k_2}[EH]_0 \exp\left(-\frac{k_1k_3}{k_2}t\right) \quad (7)$$

where $[EH]_0$ is the initial concentration of EH.

In the NMR experiment, the chemical shifts of the labile hydrogen resonances will in general be different for the EH and XH species, because the protein conformation is assumed to be different in the E and X states. If the interconversion of the two states is slow compared with the chemical shift difference, the hydrogen exchange of EH and XH can be observed separately. This would be the case, for example, for the indole NH hydrogens of lysozyme if E is the native state and X the fully denatured one. Otherwise, an average resonance will be observed for EH and XH. This is the likely situation where X represents a state of the protein which is not dramatically different from the native state. Whether or not EH and XH are observed separately, the observed first-order rate constant will be the same. In addition, examination of eq 4–7 shows that for EX2, and often for EX1 also, [EH] $\gg$ [XH].

For exchange in H_2O, measured by the saturation recovery method, the kinetic scheme discussed above is not applicable. For the cases studied in this work, exchange from tryptophan itself or from the denatured protein, the observed rate constants are essentially equivalent to k_3 in the above scheme.

Exchange at High Temperatures. At the highest temperature at which exchange from the native form of lysozyme could be measured (72 °C), the rates of exchange of the four most slowly exchanging indole NH hydrogens are essentially identical. This suggests that the exchange proceeds through a common mechanism, and as the midpoint of the thermal denaturation of lysozyme under these conditions is 73.6 °C, it is reasonable to examine the hypothesis that the state from which exchange takes place (XH) is the completely unfolded state.

The resonances of the indole NH hydrogens in the native state are well separated from those in the unfolded state, and thus the exchange of EH can be measured directly. At 72 °C, the rate for all four indole NH hydrogens is $(2.7 \pm 0.6) \times 10^{-2}$ s^{-1}. At this temperature, the equilibrium constant for the unfolding process is $K = k_1/k_2 = 0.42$ (Figure 2), a value close to that reported under similar conditions from calorimetric studies (Pfeil & Privalov, 1976). The rate of exchange from tryptophan itself under these conditions is 2.8 ± 0.3 s^{-1}, and

Table II: Parameters Describing Unfolding Process[a] at 72 °C

parameter	value
ΔH^b	119 ± 5 kcal mol^{-1}
ΔS^b	343 ± 13 cal mol^{-1} deg^{-1}
K^b	0.42 ± 0.10
k_1	(2.8 ± 0.8) × 10^{-2} s^{-1}
k_2	(6.6 ± 1.7) × 10^{-2} s^{-1}
k_3^c	2.8 ± 0.3 s^{-1}

[a] For indole NH hydrogens of Trp-28, -108, -111, and -123 by using the two-state model described in text. The observed average rate of exchange, k_{obsd}, was (2.7 ± 0.6) × 10^{-2} s^{-1}. [b] Average value measured from eight resolved resonances in the lysozyme spectrum; see Figure 2. [c] Value for tryptophan itself at this temperature.

as the rate of exchange from the fully unfolded lysozyme is, at higher temperatures, found to compare closely to that of tryptophan (Figure 1), this value may be adopted as k_3. By substitution of the values in eq 3, the calculated rates are determined to be $k_1 = (2.8 \pm 0.8) \times 10^{-2}$ s^{-1} and $k_2 = (6.6 \pm 1.7) \times 10^{-2}$ s^{-1} (Table II). As $k_3 = 30(k_1 + k_2)$ and $k_{obsd} = 0.96k_1$, this corresponds closely to EX1 kinetics and is far from the requirements for EX2 kinetics. Further, from the general solution of eq 1 and 2 (Hvidt, 1964, 1973), first-order kinetics would be a good approximation after approximately $5/k_3$, or 2 s. At least 20 s elapsed between dissolution of the samples in ^2H_2O and the accumulation of the first spectrum; hence, the observation of first-order kinetics is fully expected.

The value of k_1 may be compared with the limited data for the rate of unfolding of lysozyme at the midpoint of denaturation. In 2.5 M guanidinium chloride (25 °C), the unfolding rate = 2.3 × 10^{-3} s^{-1} (Tanford, 1970; Tanford et al., 1973), and in 4.5 M LiBr (40 °C), the unfolding rate = 3.8 × 10^{-2} s^{-1} (Segawa et al., 1973). The present value of k_1 is of the same order as these values, although the different conditions make further comparison of little value, and it is consistent with the conclusion that a mechanism involving total denaturation describes the observed exchange rates.

For the most slowly exchanging indole NH hydrogen, that of Trp-28, the Arrhenius plot is linear for about 15 °C below the denaturation temperature. As the temperature is reduced, k_3 decreases sufficiently slowly that exchange by way of the fully unfolded form will continue to satisfy approximately the conditions of EX1 kinetics, at least over this temperature range. The activation energy of 120 ± 8 kcal mol^{-1} thus corresponds to the activation energy of the unfolding reaction with rate constant k_1. This value is essentially identical with the value for the enthalpy of denaturation (ΔH) of lysozyme, 119 ± 5 kcal mol^{-1}, over the temperature range 70–78 °C (Figure 2). Calorimetric studies give comparable values (128 kcal mol^{-1} at 74 °C and 111 kcal mol^{-1} at 57 °C) (Pfeil & Privalov, 1976) as do UV studies (127 kcal mol^{-1} at 75 °C) (Nakanishi et al., 1973). This result is fully consistent with the unfolding model, as it has been observed previously that the activation energies for the unfolding of proteins [d(ln k_1)/d(1/T)] are similar or slightly less than the thermodynamic enthalpies of denaturation [d(ln K)/d(1/T)] (Pohl, 1969; Segawa et al., 1973). The data show that the activation energy for the refolding of lysozyme under these conditions is small, less than ±10 kcal mol^{-1}, indicating that k_2 is little changed between 67 and 77 °C.

Thus, both kinetic and thermodynamic results are fully consistent with the argument that Trp-28 exchanges most efficiently by way of complete unfolding at temperatures above about 60 °C. For the three other slowly exchanging indole NH hydrogens (Trp-108, -111, and -123), it is apparent from Figure 1 that only at the very highest temperatures could a similar conclusion be valid, because at lower temperatures they exchange more rapidly than Trp-28. Fitting the Arrhenius plots to the two-process model does, however, show that for these hydrogens the high-temperature process has in each case a high activation energy, above 90 kcal mol^{-1}. The values are, however, significantly lower than that of Trp-28, and this point is discussed below. For the remaining two tryptophans (Trp-62 and -63), the rates of exchange are faster than the unfolding rate even at the highest temperatures at which measurements were made (72 °C).

This analysis has assumed that the unfolding equilibrium can be adequately described by a two-state model. Such a model is sufficient to explain fully thermodynamic studies of lysozyme (Privalov, 1979), but from a number of kinetic studies, a three-state model has been proposed for the refolding of lysozyme (Tanford et al., 1973; Kato et al., 1981). The present data cannot be used to determine the rates of the two steps in a three-state model. The exchange rates discussed here would, however, be expected to reflect the rate of the first unfolding step, the major cooperative unfolding.

Exchange at Lower Temperatures. The exchange rates of the tryptophan residues become progressively more different as the temperature is reduced. Below 55 °C, the rates of exchange are much faster than expected for the total unfolding mechanism. Linear Arrhenius plots are observed for each residue (Figure 1), but even the highest activation energy (40 kcal mol^{-1} for Trp-28) is much lower than the enthalpy of denaturation for lysozyme (91 kcal mol^{-1} at 47 °C). There is a clear relationship between these low-temperature activation energies and the low-temperature exchange rates (high activation energies are associated with slow exchange rates). In addition, all the activation energies measured are greater than that for tryptophan itself, by between 2 and 28 kcal mol^{-1}, and all the measured rates are slower, by factors of between 10 and 10^6.

The temperature dependence of the exchange of the tryptophan indole NH hydrogens below 55 °C resembles that reported for the amide hydrogens of lysozyme and other proteins under conditions where exchange does not occur from fully unfolded protein (Woodward & Hilton, 1979). A two-step reaction scheme could be used to analyze the present data, but such an analysis has limitations. Unlike the situation for the high-temperature exchange, the mechanism leading the protein structure to the XH form, the structure of the XH form, and the values of k_1, k_2, and k_3 cannot be established independently. Nevertheless, the present data do provide some information about the factors contributing to the exchange processes.

In the lysozyme structure, four of the six tryptophan indole NH hydrogens (Trp-62, -63, -108, and -123) are partly exposed to solvent (Glickson et al., 1971). It is therefore conceivable that exchange could take place directly without the need for a conformational alteration to a state with the hydrogens fully exposed. Indeed, the activation energies for Trp-62 and -63 are no more than 2 kcal mol^{-1} greater than that of tryptophan itself. There is, however, no direct correlation between calculated exposures and exchange rates (Cassels et al., 1978), and it is likely that fluctuations in the protein structure are important for the exchange kinetics. For the other two tryptophan residues (Trp-28 and -111), the exchange rates are slower and activation energies higher. For these two resonances, the indole NH hydrogens in the crystal structure are fully buried, and this indicates that fluctuations of the structure are essential here to permit exchange to take place.

The much smaller values obtained for the low-temperature activation energies (Table I), compared with those for complete unfolding, imply that the fluctuations are of limited scale (Woodward & Hilton, 1979). Whether these are more accurately described as giving rise to penetration of solvent or to local unfolding cannot be established at present. One structural property expected in either case to be important in the exchange kinetics is the state of hydrogen bonding (Englander et al., 1980). Three of the six tryptophan indole NH hydrogens (Trp-28, -108, and -111) are defined to be involved in internal hydrogen bonds (Grace, 1980). No absolute correlation between hydrogen bonding and the exchange parameters is, however, obvious. On the contrary, comparison of the data for Trp-108 and Trp-123 shows that the existence of such hydrogen bonding has no unique retarding effect on the exchange rates or activation energies. The lack of absolute correlation of either exposures or hydrogen bonding with the exchange kinetics is perhaps not surprising. The structural parameters are defined for the static crystal structure of the protein, but hydrogen exchange is a result of the dynamic features of the molecule.

It is interesting that the exchange reaction for each hydrogen has a characteristic activation energy, which is observed to be constant over a wide temperature range. That such behavior is consistent with a model involving specific local conformational fluctuations may be seen by comparison with the results of the kinetics of 180° flips of aromatic rings of tyrosine and phenylalanine about the C^{β}–C^{γ} bonds. This behavior has been observed now in many proteins and is clearly associated with molecular fluctuations (Karplus & McCammon, 1981). For a given protein, characteristic rates and activation energies exist for each residue. The activation energies, which may be as high as 40 kcal mol^{-1} (Karplus & McCammon, 1981), have been observed to be unchanged over temperature ranges of even 70 °C (Campbell et al., 1976).

Exchange at Intermediate Temperatures. At temperatures below 57 °C, the fastest mechanism for hydrogen exchange for all the tryptophan residues of lysozyme involves solvent penetration or local fluctuations which lead to exposure. For the residues of Trp-62 and -63, which are close to the protein surface, this mechanism is dominant even at the highest temperatures at which exchange was measured. For the other four tryptophan residues, however, a mechanism involving total unfolding of the protein becomes increasingly important as the temperature is raised above 57 °C. At 72 °C, this appears to be the dominant mechanism for these residues. Fitting the exchange rates to two processes over the entire temperature range, however, suggests that the apparent activation energies derived for the complete unfolding process are not identical for the four residues. Those for Trp-108, -111, and -123 are 90–95 kcal mol^{-1}, while for Trp-28 the value is nearly 120 kcal mol^{-1}. It is apparent from Figure 1 that it is only for Trp-28 that the experimental data define this value well. This is emphasized in Figure 3 where the predicted rates for the low-energy process are subtracted from the observed rates. The possibility cannot, however, be eliminated that over the approximate temperature range 57–67 °C for Trp-108, -111, and -123 a slightly different mechanism applies to the exchange. For example, fluctuations of a larger scale than those responsible for the low-temperature exchange but smaller than those leading to complete denaturation could result in more rapid exchange for Trp-108, -111, and -123 than for the deeply buried Trp-28. Further studies are needed to test this possibility, but it seems reasonable that such large fluctuations could occur close to the denaturation temperature.

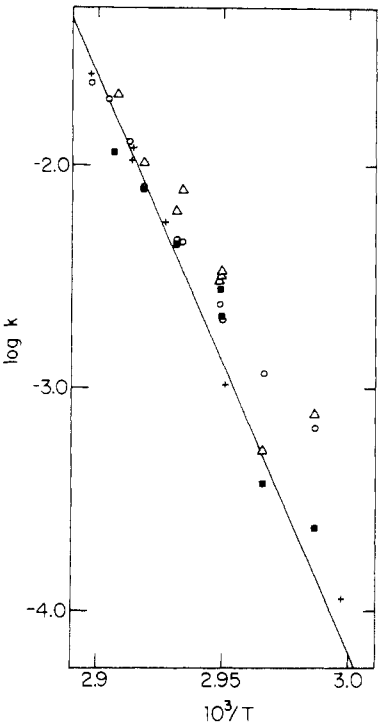

FIGURE 3: Plot of the high-temperature data of Figure 1 after subtraction of the predicted contribution of the low activation energy process for Trp-28 (+), Trp-108 (■), Trp-111 (○), and Trp-123 (△). The solid line is a least-squares fit to the data for Trp-28 and has a slope of 120 kcal mol^{-1}.

Conclusions

The results presented in this paper for the rates of solvent exchange of the indole NH hydrogens of the six tryptophan residues of lysozyme reveal that at least two distinct processes, one of high activation energy and one of much lower activation energy, can contribute to the observed exchange rates. Preliminary studies of amide NH exchange in lysozyme reveal that this is also true for these hydrogens. The high-energy process is associated directly with the cooperative thermal unfolding of lysozyme and in the case of the most slowly exchanging indole NH hydrogen is the dominant mechanism even 15 °C below the denaturation temperature. Only at low temperature is a lower activation energy process dominant for all hydrogens. Although residues close to the surface may be directly accessible to solvent in the native state of the protein, this low-energy process can in general be associated with fluctuations in the protein structure which permit either local unfolding or solvent penetration. There is provisional evidence for a third process, observed close to the denaturation temperature for some hydrogens, which is attributed to large-scale fluctuations which do not lead to total unfolding.

For the denaturation process, it has been possible to determine the rates of unfolding and folding of the protein at high temperatures. For the low activation energy process, it is not possible at this stage to define the mechanism in detail. Although some correlation of exchange rates with the degree of exposure and the presence of internal hydrogen bonds as calculated from the crystal structure is found, this is not sufficient to explain fully the relative rates of exchange for different residues. The exchange rates do, however, correlate well with the measured activation energies.

The results given in this paper may be compared with those for the exchange of the hydrogen-bonded amide protons of the β-sheet backbone of BPTI. The individual exchange rates have been correlated with the thermal stability of this protein and a variety of derivatives. This led to the proposal that fluctu-

ations leading to exchange are global in nature (Wüthrich et al., 1980). In other work, however, two processes were identified (Hilton & Woodward, 1979; Woodward & Hilton, 1980). The higher energy process was attributed directly to thermal unfolding, but the low-energy process, observed under less extreme conditions of temperature and pH, was related to local internal motions in the protein which are not correlated with thermal stability (Hilton et al., 1981). The existence of the two processes in lysozyme resembles the latter results. Preliminary studies in this laboratory on a lysozyme homologue of higher stability suggest that for lysozyme, too, the low-energy process is not generally correlated with thermal stability. It is possible, however, that the tentative third process observed over a limited temperature range in lysozyme is analogous to larger scale global fluctuations of the type proposed for BPTI (Wüthrich et al., 1980).

There are many possible mechanisms for hydrogen exchange, and the mechanism which results in the most rapid exchange for a given case will depend on the nature and environment of the hydrogen and on other variables such as temperature and pH. Variation of the conditions, therefore, enables different aspects of the dynamics of protein structure to be investigated.

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