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Viscosity and transient solvent accessibility of Trp-63 in the native conformation of lysozyme

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We have measured the rates of isotope exchange at the nitrogen of the indole ring of Trp-63 of lysozyme and of L-tryptophan as a function of solution viscosity. We have used two cosolvents, glycerol and ethylene glycol, to modify the relative viscosity. We have derived the appropriate kinetic equations for the alternative possibilities that the exchange takes place either in solution or in the intact protein matrix. Because we chose to study the proton-catalyzed exchange reaction, the rate of it is not expected to be diffusion-limited. We confirmed this by measuring the exchange from tryptophan. These results and the known effects of glycerol and ethylene glycol on the solvation of indole allow us to predict that if the exchange reaction takes place in a protein matrix the effects of the two cosolvents when compared under isoviscous conditions should be identical. This is what we find for Trp-63 in lysozyme at 15, 20 and 26 °C. The slope of the linear plot of log k vs. log relative viscosity is 0.6. This strongly supports a model for conformational fluctuations where transient solvation takes place without major changes in protein folding. The most interesting feature of our findings is the fact that a slow reaction admittedly not diffusion-limited shows, when taking place in a protein matrix, a linear dependence on solution viscosity. We suggest that what we observe is the effect of damping of movement of the side chain expressed as a change in the friction along the reaction coordinate in the corresponding phase space. The presence of such effects stresses the validity and usefulness of Kramers model of rate processes for reactions taking place in a protein matrix. Such behavior is predicted by several of the recently proposed general mechanisms of enzyme catalysis.

1. Introduction

Proteins are large molecules and their biological activity is critically dependent on achieving and retaining an appropriate three-dimensional structure. Through work carried out in many laboratories including ours, it has become accepted that the three-dimensional structure fluctuates between many conformational states around the most probable form, a structure representing the free energy minimum [1,2].

Strong indirect evidence links these fluctuations to biological processes such as enzyme activity [3,4] and antigen recognition. The structural nature of such a linkage is less well understood, but for a series of kinases it has been shown crystallographically [5] that upon ligand binding, the two lobes of the protein structure on either side of the cleft harboring the active site close around the ligand. The structure around the site in the cleft is believed to be intrinsically flexible and thus prone to fluctuations [6]. We have recently shown [7] that inhibitor binding to the corresponding cleft in the structure of lysozyme exerts its main effect in the form of strengthening the bonds in two well-defined units of secondary structure located on either side but not directly in the cleft. At this point, it appears to be necessary that we should be

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able to write a formal free energy linkage scheme [8] between ligand binding or enzyme rate and an appropriate thermodynamic expression for structural fluctuations. It is the latter that is generally absent because our knowledge about the thermodynamic character of fluctuations is clearly inadequate.

In this investigation, we focus attention on the class of fluctuations involving transient hydration of previously buried residues. We have chosen the tryptophan residues of lysozyme. Out of six tryptophan residues, five are buried and only transiently accessible to solvent. Previous works [9,10] show large variation in the frequency of access. Simulation of short time span motion of these residues [11] shows that five of the residues behave very similarly and show no discernible motion of the magnitude that would be expected for hydration of the side chain. It is thus reasonable to assume that fluctuations leading to transient access to solvent and hydrogen exchange belong to a subgroup of fluctuations of low probability.

We have chosen to study the time-dependent exposure of Trp-63 by studying the exchange kinetics of indole hydrogen in the presence of cosolvents, utilizing ultraviolet difference spectroscopy [12]. The spread of the exchange rate constants of the six tryptophans [9] allows us to choose conditions where only contributions from Trp-63 are involved in the observed changes and the choice of ultraviolet difference spectroscopy instead of NMR avoids complications due to viscosity effects on the measurements per se.

It is our intention to establish the mechanism by which transient hydration is accomplished. For this purpose, we present models for the expected exchange kinetics, assuming two different and mutually exclusive pathways for hydration, and predict the different effects of the two cosolvents, glycerol and ethylene glycol, on these two models. We find that in both water/glycerol and water/ethylene glycol between 15 and 26 °C, the viscosity of the solvent plays a dominant role. The magnitude and form of the viscosity function agree well with the proposed pathway of exchange involving preferential hydration of the residue in the native protein matrix. The cosolvent effects on the slower hydrogens, judging from data available on tritium exchange, cannot be accounted for by such a mechanism alone and we must also invoke the pathway characterized by transient solvation of the indole side chain.

The most interesting observation, however, is a demonstration of the validity of Kramers model for rate processes in describing a reaction that takes place in a highly viscous protein matrix. Previous experiments in this field have as a rule utilized ligand-binding reactions with very low activation barriers where it is difficult to separate viscosity effects on the transport of reactants in real space from friction effects in reaction space.

2. Materials and methods

Egg white lysozyme (Sigma grade 1), L-tryptophan (Sigma grade), deuterium oxide (99.8 atom%) and glycerol (Sigma grade, lot-25F-0032) were all obtained from Sigma. Ethylene glycol (99% spectrophotometric grade) was from Aldrich. Glycine (ammonia-free) was from Matheson-Coleman and acetic and hydrochloric acids (A.R.) were from Mallinckrodt.

The exchange of a deuterium atom at the nitrogen of the indole ring with a hydrogen or vice versa results in a small change in the ultraviolet spectrum of indole observable between 290 and 294 nm. This change has been used previously to follow the hydrogen exchange kinetics of indole side chains in proteins [12]. For our purpose, the exchange of fully deuterated compounds with water/cosolvent mixtures provided advantages in definition of both H^+ activity and the viscosity of the cosolvent/water mixture. To accomplish the initial exchange of hydrogen for deuterium we dissolved dry lysozyme or tryptophan in deuterium oxide. The solution was stored overnight at room temperature and centrifuged at $15\,000 \times g$ for 5 min before use. The experiments were carried out with a Cary model 219 spectrophotometer coupled to a Compaq 'Deskpro' computer. An experiment was started by mixing 60–80 μl of the deuterium oxide solution containing protein or amino acid with 2 ml of buffer or buffer/cosolvent mixtures. The mixing device at low cosolvent concentration was a previously described plunger sys-

tem [13]. At higher viscosities more complete mixing was accomplished by use of a normal 1 cm cell and a vibrating teflon device (NSG Precision Cells, Farmingdale, NY). Mixing times were 6–12 s for the amino acid and 15–35 s for the protein. The optimal wavelength to follow the change was 291 nm for tryptophan and 292.7 nm for lysozyme. The sample compartment of the spectrophotometer was thermostatted and kept within $\pm 0.02\,^{\circ}C$ by means of a large circulating bath from Precision Scientific. The buffer solutions were degassed and filtered before introduction into the spectrophotometer. The experiments were extremely sensitive to the presence of air bubbles or particles of dust. We accepted only experiments that resulted in a final stable baseline corresponding to complete in-exchange. The experiments took place at the limit of precision and reproducibility for the instruments. The total absorbance change for the reaction observed was around 0.008 A units. With the instrument operating at a full-scale deflection of 0.02 A units the noise was ± 0.005 A units. Besides computer averaging it was necessary to carry out repetitive runs (5–10) for each of the experimental points. The choice of the pH used was determined by our desire to optimize conditions which would enable us to calculate a reliable rate constant. We show in fig. 1 the pH dependence of exchange for L-tryptophan plotted according to previously published data [12] and for Trp-63 as measured by us. Our choice of pH 2.5 for Trp-63 and 3.8 for tryptophan was a compromise to ensure a measurable rate in a pH region where the protein was still thermally stable and where the expression for the apparent first-order rate constant for the exchange in solution $k_{app} = k_0 + k_H[H^+] + k_{OH}[OH^-]$ can be truncated to $k_{app} = k_H[H^+]$. The activity of H^+ was adjusted and determined after addition of the cosolvent and again measured in each solution at the conclusion of the experiment. H^+ activity is a function of the cosolvent concentration. The reading of a glass electrode calibrated with standard buffers has to be corrected among other factors for changes in junction potential. Corrections for glycerol and ethylene glycol systems are available in the literature [14]. The correction factor d is a well-behaved linear function of cosolvent content up to 45–50% (w/v) of cosolvent. After that point, the magnitude of the factor changes precipitately making experiments at higher cosolvent concentration questionable. The data in the form of absorbance changes as a function of time were smoothed by a standard averaging routine and then fitted to an equation of the form $A - B\exp(-ct)$. All curves were also plotted using a manual curve peeling procedure. The results obtained from both methods were in good agreement. The relative viscosities of the cosolvent mixtures were calculated from the data for $20\,^{\circ}C$ in the Handbook of Chemistry and Physics, 67th edn, CRC., and converted to data for 15 and $26\,^{\circ}C$ by use of the relations previously published for the temperature dependences [15,16].

3. Results

The first series of experiments presented in table 1 compare the out-exchange rates of deuterium from indole obtained in this investigation with the in-exchange rate previously reported [9,12]. Although we compare experiments made at the same nominal pH meter reading, it should be borne in mind that deuterium ion activity in the deuterium oxide solutions used for in-exchange studies represents activities corresponding to a pH meter reading that is 0.4 units higher [17]. We see from the pH dependence of exchange in fig. 1 that we are on the acidic side of the exchange, which allows us to calculate the pH-independent second-order rate constants for in- and out-exchange of deuterium. The ratio of these rate constants,

Table 1

The experimental, apparent first-order rate constant (k, in s^{-1}) and the pH-independent second-order H^+-catalyzed rate constant (k^H, in $s^{-1}\ mol^{-1}$) for L-tryptophan

Reference	k_{exp}	pH	pD	k (15 °C)	k^H (15 °C)
Nakanishi et al. [12]	0.127 (22 °C)	3.8	4.2	0.064 [a]	1.01×10^3
				0.074 [b]	1.17×10^3
Wedin et al. [9]		3.8	4.2	0.078	1.24×10^3
This work		3.8	–	0.084	6.03×10^2

[a] $\Delta H^{\ddagger}$ 66.5 kJ [12].
[b] $\Delta H^{\ddagger}$ 55.2 kJ [9].

when comparing our data to the in-exchange results of Wedin et al. [9], is equal to 2.05. This value is quite close to 2.5 – the only other ratio of this kind reported for the peptide group [18].

Our next step was to consider the relative rates of the 6 exchangeable indole hydrogens of lysozyme in order to estimate the possibilities of observing a single-exponential decay for the tryptophan of our choice. We choose Trp-63 because it is the fastest of the solvent-inaccessible tryptophans (R.D. Gregory, unpublished observations). Published data covering the temperature and pH range of interest [9] show that when observing exchange from Trp-63 the only effective source of interference comes from Trp-62. The next slowest tryptophan, Trp-108, is nearly 100-fold slower in its exchange and its contributions to observed exchange can safely be neglected. Although no direct determinations of the exchange rate of Trp-62 have been reported, we have very good estimates of the slowest possible rate under our conditions [9]. From this we see that Trp-62 is at least 5–6-fold faster than Trp-63. This means that for our study of Trp-63 we have the necessary flat final baseline and the last 75% of exchange is represented by single-exponential decay. This prediction is borne out by experiments. Due to the necessity for very thorough mixing, we as a rule never observe the first 20% of exchange. The first-order rate constant for Trp-63 that we observe at pH 3.8 (table 2) compares well with the value estimated from the curves published by Wedin et al. [9]. The agreement is better than one might expect mostly because the difference due to ion activities as detected by the glass electrode (+0.4 log units) is compensated by the kinetic isotope effect of 0.31 log units.

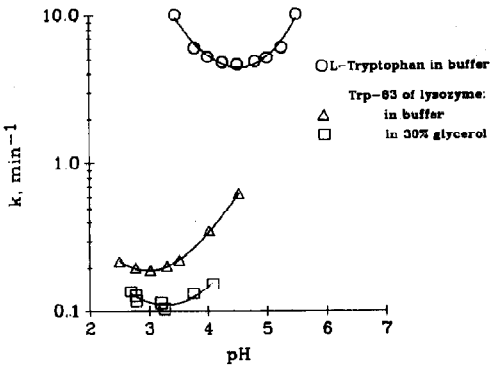

Fig. 1. Apparent first-order rate constant (min^{-1}) for Trp-63 of lysozyme at 15 °C plotted as a function of pH. (△———△) Determined in the absence of cosolvent; (□———□) in the presence of 30% (v/v) glycerol; (○———○) apparent first-order rate constant for exchange from L-tryptophan at 22 °C (from ref. 12).

Table 2

The apparent experimental first-order exchange rate constants (k) for the six tryptophans of lysozozyme at pH 3.8 (pD = 4.2) and 15 °C (in s^{-1} and m^{-1})

Data, unless otherwise indicated, are from ref. 9.

	k (s^{-1})	k (min^{-1})
Trp-62	> 0.026 [c]	1.58
Trp-63	5.76×10^{-3}	0.345
Trp-63 [b]	5.16×10^{-3}	0.310
Trp-108	3.80×10^{-5}	2.28×10^{-3}
Trp-123	1.00×10^{-5}	0.6×10^{-3}
Trp-111	2.1×10^{-7}	1.2×10^{-5}
Trp-28	2.0×10^{-9} [a]	1.7×10^{-7}

[a] An estimate of the lower limit.
[b] This work.
[c] An estimate of magnitude.

The determination of the exchange rate of Trp-63 as a function of pH as seen in fig. 1 shows a minimum around pH 3.2. When comparing this to the pH profile of L-tryptophan in fig. 1, we see a major shift in pH minimum and in the rate at the minimum. The magnitude of the shift is larger than expected for nearest-neighbor interactions but quite within the range observed for buried groups in proteins [19]. We note also that a pH of 2.5 is far enough from minimum to reduce contributions from OH catalysis to 10% or less. We have also determined and plotted in fig. 1 a part of the pH dependence of the exchange rate of Trp-63 in the presence of 30% (v/v) glycerol. The rates, besides showing attenuation, form a curve very similar to that observed for glycerol-free solution. The shift of the minimum towards higher pH is too small to be reliable.

Fig. 2 depicts the effects of glycerol and ethylene glycol on the second-order rate constant k_H from the general equation $k_{app} = k_H a_H$, where k_{app} is the observed first-order rate constant of exchange and a_H the H^+ activity as determined

by the glass electrode and corrected for cosolvent effects on the glass electrode/calomel electrode response. We see that up to a relative viscosity of 5 we can observe no effects of the presence of cosolvent. This does not mean that a small dependence $d \ln k / d \ln \eta < 0.12$ could not be present and remain unnoticed within the error of our measurements. In fact, such a small effect on a reaction between an ion and a dipole is quite plausible resulting from change in the dielectric constant [20].

The somewhat larger solvent effects reported for the exchange rate of the peptide group [21] refer to values that are uncorrected for electrode error and used in a relative fashion. We have previously observed the absence of measurable cosolvent effects on the acid-catalyzed exchange from poly(DL-alanine) (R.D. Gregory, unpublished observations).

The rather complex appearance of fig. 3 represents a compilation of the exchange rates of Trp-63 in the presence of either glycerol or ethylene glycol. The data are gathered at pH 2.5 at three different temperatures and plotted vs. viscosity. The points represent averages and error bars represent the standard deviation for a series of repeat runs. However, in our opinion the largest error in this type of measurement stems from the uncertainty in pH determination for buffer/cosolvent mixtures.

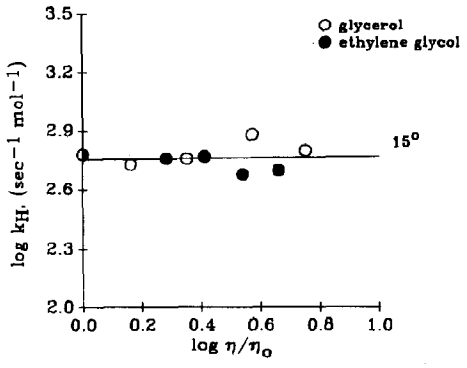

Fig. 2. Second-order rate constant for H^+-catalyzed exchange (k_H, in s^{-1} mol^{-1}) for L-tryptophan at 15°C plotted as a function of relative viscosity (η/η_0) of solutions containing different amounts of cosolvent: (○———○) in the presence of glycerol; (●———●) in the presence of ethylene glycol.

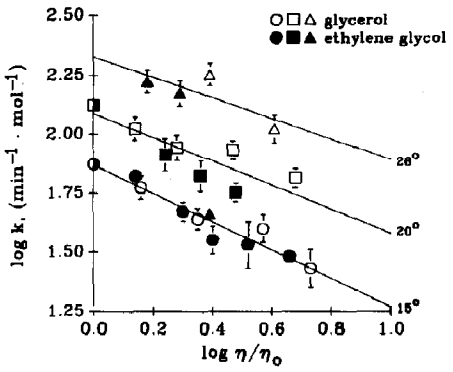

Fig. 3. Second-order rate constant for H^+-catalyzed exchange rate (k_H, in min^{-1} mol^{-1}) for Trp-63 of lysozyme plotted vs. relative viscosity (η/η_0) of solutions containing different amounts of cosolvents; (●———●) at 15°C in the presence of ethylene glycol; (○———○) at 15°C in the presence of glycerol; (■———■) at 20°C in the presence of ethylene glycol; (□———□) at 20°C in the presence of glycerol; (▲———▲) at 26°C in the presence of ethylene glycol; (△———△) at 26°C in the presence of glycerol.

We have here, in addition to a normal uncertainty of 0.02, an error in the correction factor depending on the individual electrodes. This results in our estimate having a systematic error of 0.03 pH units. The averages in fig. 3 arise from a total database of 200 independent determinations for lysozyme and 80 for L-tryptophan.

We would like to draw attention to the behavior of the rate constant at 26°C in the presence of high concentrations of ethylene glycol. Judging from studies of thermal stability in acidic pH regions [22], the T_m in cosolvent-free solutions is around 50°C. In the presence of 30% (v/v) ethylene glycol the rate increases rapidly and at 35% of cosolvent we observe only exchange from another much slower tryptophan. The effect of thermal instability appears thus quite abruptly. This is in good agreement with the cooperative nature of thermal transitions.

The exchange in the presence of glycerol does not show the appearance of such discontinuity.

4. Discussion

If protein structure in solution, at all times, corresponded to the X-ray diffraction image, a large fraction of the peptide bonds and side chains

would remain permanently inaccessible to the solvent. Consequently, the ionizable hydrogens of the peptide bonds or side chains such as indole would not be able to come to isotope equilibrium with hydrogen isotopes introduced into the bulk solvent. We know, however, that all groups, given time, will exchange their protons with those of the solution without help from the thermal unfolding process [23–26]. The transient exposure of all the exchangeable groups in the native state is thus well established. These findings of hydrogen exchange studies are supported by evidence obtained through using other independent methods [2,27–29]. The question we are addressing in this investigation concerns the nature of structural changes associated with these transients.

In reviewing the models that have been proposed for the hydrogen exchange process, we see that in one specific aspect they can all be classified as belonging to one or the other of two classes of mechanisms. In the first type, the actual exchange reaction involving ionized intermediates takes place in the bulk solution where the exchanging group finds itself during the transient structural change. In the second, the reaction takes place in a protein matrix, which although in equilibrium with the bulk solution should, from a thermodynamic aspect, be considered as a separate phase. The logic of our investigation is to develop two quantitative kinetic models based on these two conceptual alternatives. We will then predict the effects of cosolvents and viscosity on the exchange kinetics of hydrogen exchange at a single tryptophan residue in lysozyme. We shall base our predictions on the known effects of the cosolvents and viscosity on the stability of proteins and on the solubility of side chains. Once the validity of the kinetic model has been established, we can proceed to discuss what limits it imposes on our picture of the underlying physical process of structural fluctuations.

4.1. Derivation of formal kinetic equations

The simplest general scheme for exchange at the indole nitrogen of Trp-63 has to consist of two consecutive steps: first, hydration of the previously inaccessible residue followed by the exchange reaction per se, catalyzed by H^+ or OH^- [24,26]. Whereas the exchange reaction can be modeled by a similar reaction at the indole ring in small molecules, the transient hydration step is unique for protein molecules. However, a large number of alternative steps of different probabilities can account for the hydration. The different pathways of hydration for any exchanging site are all, we assume, characterized by rapid equilibria between different substates or conformations for the chain segment and side chains involved. The decisive point is that the exchange reaction itself is slow when compared to these conformational process. This is the reason why the exchange rates for single sites will remain first order; however, the rate constant is now a complicated function of different equilibrium and rate constants. It is interesting to note that under similar circumstances in the study of fluorescence decay, which is very much faster than the conformational processes, equilibrium is not reached and we observe, instead of an apparent first-order rate constant, a distribution of rate constants [30]. The important point is that we would expect for such complex apparent first-order rate constants a nonlinear temperature dependence, reflecting changes in the relative proportions of the different species involved. The temperature dependence of the exchange of all tryptophans in lysozyme is linear in the low-temperature region we are exploring [9]. This is gratifying because our choice of Trp-63 as the first indole to be studied was based on the small distance of the residue from protein-solvent interphase [31] which we expect to limit the number of alterantive mechanisms of hydration.

The kinetic equation describing exchange from a single site can be expressed in terms of the simplest possible reaction mechanism by

$$\underset{\text{inaccessible}}{\overset{\diagdown}{\text{N}}\overset{\diagup}{\text{H}}} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{\text{accessible}}{\overset{\diagdown}{\text{N}}\overset{\diagup}{\text{H}}} \xrightarrow{(OH^-)(H^+)/k_3} \text{exchange} \qquad (1)$$

The above expression corresponds to the classical Carlsberg scheme for hydrogen exchange [32]. Whatever models are proposed for the step or steps corresponding to k_1 and k_2, all of them can be classified by making two possible basic assumptions about the nature of k_3, the exchange rate. Either the exchange during the transient ex-

posure takes place in the bulk solution or, alternatively, the accessible state is not identical but separate from the state in free solution. This distinction is important for interpretation of these and other results. Of course, a choice does not endorse any of the hotly contested exchange mechanisms, partial unfolding or penetration; these models are concerned with the nature of k_1 and k_2.

The Carlsberg mechanism is based on the rigorous assumption that the chemical exchange process characterized by rate constant k_3 is exactly the same as one observes for a similar group in a small peptide in the free solution [33]. The differential equation system corresponding to eq. 1 can easily be solved [34].

The general solution can be truncated if we assume that $k_2 \gg k_3$, k_1 [34], which leads to the following expression for k_{app}, the apparent exchange rate constant

$$k_{app} = K_{eq} k_3$$
$$K_{eq} = k_1/k_2 \qquad (2)$$

It is this mechanism, the EX_2 mechanism, that has been used exclusively to describe the class of mechanisms where K_{eq} represents thermal or partial thermal unfolding equilibrium [25]. Given conditions for the opposite extreme to be valid $k_3 \gg k_1$, k_2 the truncated solution reduces to

$$k_{app} = k_1$$

the so-called EX_1 mechanism. There has never been presented a strong case for the EX_1 type of exchange to have been observed.

The simplest kinetic scheme corresponding to the alternative assumption, namely, that the exchange reaction characterized by k_3 in eq. 3 does not take place in bulk solution but in the protein phase, can be written as

$$(H^+)(H_2O)_4^{out} \underset{k_2}{\overset{k_1'}{\rightleftharpoons}} (H^+)(H_2O)_4^{in}$$
$$+ \text{–ND–} \underset{k_{-x}}{\overset{k_x}{\rightleftharpoons}} \text{–NH–} + (D^+)(H_2O)_4^{in} \qquad (4)$$
$$\downarrow k_3'$$
$$(D^+)(H_2O)_4^{out}$$

$$K_{eq} = \frac{k_1'}{k_2'}$$

This represents a minimum mechanism. A more detailed mechanism might contain additional sequential steps instead of k_x and k_{-x}. However, the kinetic expression we derive in the appendix will remain of the same form. Our type of experiments cannot resolve the apparent rate constants further and thus the introduction of a more complex mechanism, although reasonable and correct, is not justified.

In these equations $[H^+]$ and $[D^+]$ with superscripts in and out refer to nominal concentrations in the protein matrix and in the bulk solution, respectively. We assume that the transient hydration is accomplished by H^+ or OH^- with their accompanying hydration shell. We use four waters for $[H^+]$ based on the observed fourth-order dependence of hydrogen exchange on water [35]. The derivation is of course not dependent on this number. As shown in appendix A, the rate constant corresponding to the above mechanism can be written as

$$k_{app} = k_x K_{eq} [H^+]^{out} \qquad (5)$$

For constant pH, k_x can be replaced by an apparent first-order constant k_y, which allows us to write

$$k_{app} = K_{eq} k_y \qquad (6)$$

This equation is similar to eq. 2 derived for the EX_2 mechanism. Henceforth, this is designated the reverse-EX_2 mechanism. The difference is that K_{eq} in this case refers to distribution of water and catalyzing ion between the bulk solution and the protein phase. Further, k_y, although a rate constant for the actual exchange step, is not necessarily similar to k_3, the rate constant observed for the exchanging group in solution.

The rate expression corresponding to the Carlsberg EX_1 mechanism can also be derived. It is somewhat more complicated, notably being pH dependent. Details of these expressions will be published in another context.

4.2. Application of rate equations to actual data

The validity of EX_2 and reverse-EX_2 as representative kinetic mechanisms depends on the ap-

plicability of the truncations used in our specific case. In other words, are the equilibria representing the transient hydration of Trp-63 established more rapidly than the chemical exchange and are the probabilities for a transient to occur low compared to the probability for the solvent nonaccessible states? We know the apparent first-order rate constant for exchange from L-tryptophan at pH and T values close enough to those of our experimental conditions. NMR studies report k_{app} of 1 s^{-1} [9]. In both cases it is the in-exchange of deuterium. The out-exchange of deuterium we observe is slower by a factor of 2 (see table 2). These values represent a good approximation of k_3 in the EX_2 mechanism.

Studies of the rates of lysozyme unfolding and the position of thermal equilibrium [32] clearly reveal that k_2, the thermal refolding rate, is under our conditions much larger than k_3. This holds true for most protein-unfolding reactions [36]. Thus, the conditions for EX_2 would be fulfilled if exchange took place by thermal unfolding. This is seldom the case [37,38]. Partial unfolding reactions, however, have lower activation barriers if they are more probable than the complete unfolding ergo the rates are probably still faster. Still the most decisive argument for the validity of EX_2 comes from pH dependence in fig. 1 which shows the classical minimum and linear rise expected for EX_2 and not for the EX_1 mechanism.

The case for the alternative exchange model of eq. 6 is more difficult to make. The pH dependence is here not a valid argument because both reverse-EX_2 and reverse-EX_1 are expected to show a dependence on the concentration of catalyst. The rate constant k_x in eq. 5 and k_y in eq. 6 cannot be determined independently. We can only argue with good confidence that k_y describing the exchange process in a more restricted environment than k_3 does for the same process in solution would lead to conditions where $k_y < k_3$. However, it is the relationship of k_x to k_3' in eq. 4 that corresponds to the relationship between k_2 and k_3 in the EX_2 mechanism. Thus, besides estimating k_y as being equal to or less than k_3, we have to obtain an estimate of $k_3' \simeq k_2'$. Fortunately, residue Trp-63 in lysozyme is one of the few residues in proteins for which studies of local motion have been carried out [39]. Studies of this residue after local changes in accessibility are introduced by nearly instantaneous binding of a series of hydrated small inhibitors reveal that the rate of structural relaxation between two structures, one accommodating sugar and water and the other not, is quite fast and that the rate constant at 22°C is greater than 10^3 s^{-1}. It becomes measurably slow only for large multisite inhibitors such as NAG_3. Because hydrated H^+ or OH^- is smaller than glucose we expect the transient hydration to be, if not faster than, then similar to the reaction observed by Dobson and Williams [39]. We can thus say with confidence that under the experimental conditions we use (see tables 1 and 2), $k_3' \gg k_y$ and it is the EX_2 or reverse-EX_2 mechanisms that are applicable to our data.

Before proceeding to consider the cosolvent effects we have observed, we have to examine further the constants and variables in our expression for k_{app}, the apparent first-order rate constant. In the expression $k_{app} = K_{eq}k_3$ (eq. 2), k_3 represents the exchange rate constant for the indole site in solution, $k_3 = k_0 + k_H[H^+] + k_{OH}[OH^-]$, we can ignore k_0 at moderate temperatures [13]. Because we operate by choice to the left of the pH minimum (fig. 1), we can also neglect the term $k_{OH}[OH^-]$ which leaves us with the final expression of $k_3 = k_H[H^+]$. We obtain good estimates for k_3 in our determination of exchange from the indole of tryptophan (fig. 2). The rate constant k_y, although also an apparent first-order rate constant of the form $A[H^+]$, is more complicated because the reaction takes place in a protein matrix. We cannot treat the rate constant k_y as representing the customary low-viscosity limit. The more general rate expression for the high-viscosity case [40,41] can be written as

$$k_y = A/\eta^k \exp(-e/RT) \qquad (7)$$

$$A/\eta^k \exp(-e/RT)$$

where A is a constant now containing $[H^+]$ as well, η the solution viscosity and k the coupling constant between solution viscosity and the friction experienced by the indole group [41]. These

considerations all lead us to the final expressions for the apparent first-order rate constant of exchange for the two exchange pathways

$$EX_2 \quad k_{app} = K_{eq} k_3 \tag{8}$$

$$\text{reverse-}EX_2 \quad k_{app} = K'_{eq} A/\eta_k \exp(-e/RT) \tag{9}$$

Of course k_3 can be expanded according to Eyring-Laidler for the low-viscosity limit as $k_3 = B\exp(-G/RT)$ but, because B is by definition independent of cosolvent and G experimentally shown to be cosolvent-independent, such expansion serves no purpose.

4.3. Cosolvent effects

We can now address the question as to what cosolvent effects we observe and if these can be rationalized in terms of a choice between eqs. 8 and 9 or whether a combination of the two is perhaps most appropriate.

The experimental findings presented in figs. 1–3 can be summarized as follows:

(1) The observed k_{app} for out-exchange of deuterium from Trp-63 of lysozyme at pH 2.5 and at temperatures of 15, 20 and 26 °C varies linearly with viscosity.

(2) The results are independent of the nature of the cosolvent used to modify the viscosity.

(3) The exchange rate constant for indole in L-tryptophan when determined in the same cosolvent system shows the absence of measurable effects of viscosity in the presence of cosolvents.

(4) At 26 °C, whereas the glycerol effects behave normally up to 40% (v/v) cosolvent, the ethylene glycol curve shows a sharp break at 32%, the rate of Trp-63 becoming very fast. The exchange we observe is now from a previously unobserved slowly exchanging tryptophan residue.

We shall now turn our attention first to the classical EX_2 mechanism. The effects of viscosity and ethylene glycol and glycerol as cosolvents on k_3 of eq. 8 are, as pointed out in section 4.2, adequately modelled by the observed effects on exchange from L-tryptophan (fig. 2). The reaction is slow enough not to be viscosity limited; however, we would at first glance expect effects emanating from glycerol and ethylene glycol as cosolvents. Their dielectric constants (42.5 and 37, respectively), although similar, deviate markedly from that of water. However, if we consider the magnitudes of effects on similar acid-catalyzed reactions that have been reported [20], the expected effects are small and within the error of our measurements. The absence of measurable cosolvent effects and specifically of differential effects between the two cosolvents is thus not in disagreement with the EX_2 mechanism.

The case is quite different for the effects of cosolvents on K_{eq} in eq. 8, the equilibrium constant for some type of unfolding. First we have to make it quite clear that it is not the thermal unfolding. It is easy to calculate from calorimetric measurements [42] that the enthalpy associated with K_{eq} in this case would be at least 105 kJ, a far cry from the observed 13 kJ [9]. It is also easy to calculate the expected effect of stabilization for 30% glycerol [42]. In such a case, d log k_{app}/d logη would be approx. 4 and not in the range below unity, which we observe. These arguments, besides excluding unfolding, mitigate against partial thermal unfolding of a substantial nature. The stabilization and thermal effects are too small. Small heat effects and viscosity dependence similar to those in our case have been observed for many rapidly changing hydrogens in lysozyme [13]. We have plotted in fig. 4 the observed viscosity dependence of the average rate constant for tritium exchange from fully labelled lysozyme (R.D. Gregory, unpublished observations). We have in this case replaced the average rate constant with time, the time it takes to observe a similar degree of exchange at different cosolvent concentrations [24,26]. We see again that exchange from Trp-63 falls in line with a whole complement of rapidly exchanging hydrogens.

What remains is the argument that the unfolding could be very local, being mainly characterized by solvation of the tryptophan side chain. Direct measurements of such an unfolding are not easy to accomplish; however, we have a considerable amount of information for such a process in these two cosolvents. Table 3 represents a compilation of data on the free energy of transfer of the indole side chain to ethylene glycol and glycerol solutions [43,44]. We see that the two cosolvents show radi-

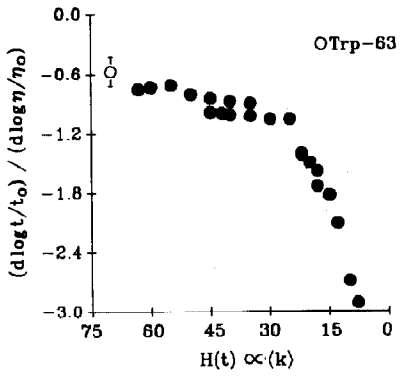

Fig. 4. Viscosity dependence of the average exchange rate constant in lysozyme, expressed as viscosity dependence of the ratio of times necessary to reach the same degree of exchange. The dependence in the form of a derivative is then plotted as a function of the average number of sites remaining unexchanged at the time point for rate determination (from unpublished data of Gregory et al.). The single point for Trp-63 is from this work.

cally different behavior. Calculations are for concentrations giving the same solution viscosity. The difference for the solubilization equilibria is 2500 J. We can of course combine it with the transfer of five peptide bonds, a group giving the highest possible contribution in the opposite direction. The difference is still 1670 J. This translates into differences of the effect on observed rates by a factor of 0.5 log units which is 5-fold larger than the standard deviation for our data.

One could of course argue for an accidental compensation combining both bond breaking and transfer free energies. However, bearing in mind the large heat capacities for transfer quantities

Table 3

Free energies of transfer at 25 °C from water to water/cosolvent systems of indole side chain and the peptide group

Cosolvent	Transfer free energy (J)		
	Indole	Five peptide groups [c]	Sum
42% (w/v) ethylene glycol [a]	−3486	1779	−1707
37% (w/v) glycerol [b]	−1071	1193	125

[a] Data from Nozaki and Tanford [43].
[b] Data from Gekko [44].
[c] For the peptide of triglycine.

[45], the probability of accidental compensation at 15, 20 and 26 °C is quite remote.

The cosolvent effects on the rate constants of the alternative pathway (eq. 9) can also be predicted with good confidence. First, cosolvent affects K'_{eq}, the distribution constant for solvent and catalyst ions between the solution and the protein phase; but because we adjust our solutions always to constant a_{H^+}, H^+ activity, the only effect we expect to see would be changes in the protein phase per se, i.e., cosolvent-induced conformational changes. There is no direct evidence for such changes at the moderate glycerol and ethylene glycol concentrations we use. Viscosity, of course, appears directly as a variable in eq. 10. Our concern here is with the magnitude of the empirical coupling constant k. The question of whether internal structural movement is dampened by external viscosity is addressed by the values of k, the values reported ranging from about 0.8 [41] to much smaller values of ~ 0.5 [46]. The lower estimates from fluorescence quenching are not reliable yet because cosolvent and viscosity effects have not been separated in these complex nonlinear curves. Thus, our value for k, approx. 0.6, is quite a reasonable number. Consequently, the observed viscosity dependence is well accounted for by eq. 9. This strongly supports the original assumption we made about the exchange reaction taking place in the protein matrix phase. At this stage we have to make a point utilizing data in fig. 4. The viscosity dependence of the exchange of 30 of the fastest hydrogens in lysozyme could well be explained by a mechanism similar to that for Trp-63 which we have presented and accepted here. However, looking at the slower hydrogens, we see that a considerable number of them experiences rapidly increasing dependence of the rate on viscosity. For the slowest sites the derivative approaches a value of 3, not far from that predicted for total thermal unfolding. It is not possible to explain exchange kinetics from these sites by eq. 9. It clear that in order to explain the exchange from all sites at moderate pH and T values, we have to use both reverse-EX2 and EX2 mechanisms. For the hydrogen exchange method this means that both of the hotly contested models, partial unfolding and solvent penetration, are valid concepts

depending on pH, T and the relative strength of tertiary structure around the exchanging site.

We can now finally address the question as to what kind of a minimal physical picture of protein dynamics agrees with findings in this paper and with the collection of previous studies of hydrogen exchange and fluorescence quenching.

If a protein molecule proceeds along a gradient of temperature and we concurrently observe the exchange of hydrogens in a time window on the scale of seconds to minutes, we see at lower temperatures rapid exchange due to transient hydration of accessible regions [47] in the protein matrix. This matrix can be considered as a highly nonhomogeneous and anisotropic phase separate from but is equilibrium with the solvent. Regions containing well-fitting and strong secondary structures [1] have low probability for hydration and do not exchange. Such exchange has, of course, a small but finite probability to occur and can be observed at high catalyst concentrations. With increasing temperature, although we are still below the thermal transition point, the fluctuations of structure become larger and more cooperative, a phenomena well known for polymers in general [48]. Such fluctuations clearly resemble partial unfolding or melting. When the transition point is reached the protein phase is lost and the polypeptide dissolves in bulk solution. Such a picture [49] describes of course not all of the possible movements of the protein structure, but only a small subset, those involving changes in the degree of hydration. We believe that it is on the low-probability end of a scale of motion for protein [50].

For hydrogen exchange we have two clearly different regions of relevant motion.

(1) Transient hydration of part of the protein matrix: Low-enthalpy process of low cooperativity. Viscous damping of the exchange process. Compensation temperature * for hydrogen exchange is high.

* If the activation enthalpies of a number of rapidly or very slowly exchanging hydrogens of lysozyme are plotted as a function of the free energy of activation, straight lines of different slopes result [13]. The slopes have the dimension of temperature and are often referred to as the compensation temperatures.

(2) Transient hydration of a section of structure by solvation in bulk phase: High-enthalpy process of high cooperativity. Cosolvent effects by preferential interaction with different forms of protein structure. Compensation temperature is low, similar to thermal unfolding.

However, as stressed in section 1, the most interesting observation is the applicability of Kramers model for rate processes taking place in the protein matrix.

Hereto the most convincing argument for the usefulness of Kramers approach has been indirect from theoretical [51] and experimental [52] work on movement of polymer chains in solution. The direct observations of viscosity dependence of ligand binding to proteins [41] are very suggestive but the low activation energies for these reactions always raise the possibility that it is the ligand transport in the protein phase that we are observing and not the movement and friction along the reaction coordinate. The high activation barriers for our reaction reduce the probability of diffusion-limited rate process.

The question of internal friction and solvent viscosity is important because it has been directly linked to a general mechanism for enzyme catalysis [53,54]. If the movement of a protein matrix is essential to activation of a protein-substrate complex, its coupling to solvent has to be accounted for.

Appendix A

A.1. Derivation of the rate expression corresponding to the reverse-EX_2 mechanism

The reaction scheme (eq. 4) leads to the following system of differential equations:

$$\mathrm{d}[\mathrm{ND}]/\mathrm{d}t = -k_x[\mathrm{H}^+]^{\mathrm{in}}[\mathrm{ND}] + k_{-x}[\mathrm{D}^+]^{\mathrm{in}}[\mathrm{NH}] = -V_x + V_{-x} \quad \text{(A1)}$$

$$\mathrm{d}[\mathrm{D}^+]^{\mathrm{in}}/\mathrm{d}t = k_x[\mathrm{H}^+]^{\mathrm{in}}[\mathrm{ND}] - k_{-x}[\mathrm{D}^+]^{\mathrm{in}}[\mathrm{NH}] - k_3'[\mathrm{D}^+]^{\mathrm{in}} = V_x - V_{-x} - V_3 \quad \text{(A2)}$$

$$\mathrm{d}[\mathrm{D}^+]^{\mathrm{out}}/\mathrm{d}t = \mathrm{d}\left([\mathrm{ND}] + [\mathrm{D}^+]^{\mathrm{in}}\right)/\mathrm{d}t = -k_3'[\mathrm{D}^+]^{\mathrm{in}} \quad \text{(A3)}$$

This equation system can be solved through simplification. We can write $[ND]+[NH]=1$ and eliminate [NH]. The term $k_{-x}[D^+]^{in}[NH]$ becomes $k_{-x}[D^+]^{in}-k_{-x}[D^+]^{in}[ND]$. Now, if we further stipulate that $[H^+]^{in}$ remains constant and $k_{-x}[D^+]^{in} < k_x[H^+]^{in}$ we can write

$$d[ND]/dt = -k'_x[ND] + k'_{-x}[D^+]^{in} \quad (A4)$$

$$d[D^+]^{in}/dt = k'_x[ND] - (k'_{-x} + k'_3)[D^+]^{in} \quad (A5)$$

$$d[D^+]^{out}/dt = k'_3[D^+]^{in} \quad (A6)$$

This set of equations and the solution by truncation are similar to the Carlsberg set of equations for the EX_2 mechanism [34].

The nature of these truncations is more easily obvious if we instead make the stationary assumption $V_x = V_{-x} + V_3$ [32]. In such a case, we write directly from eq. A2

$$[D^+]^{in} = k_x[H^+]^{in}[ND]/\{k_{-x}([NH] + k'_3)\} \quad (A7)$$

The observed rate is given by eq. (A6) which now becomes

$$\text{rate} = \frac{k'_3 k_x [H^+]^{in}}{k_{-x}[NH] + k'_3}[ND] \quad (A8)$$

if $[H^+]^{in} = [H^+]^{out}K_{eq}$ the apparent rate constant becomes

$$k_{app} = \frac{k'_3 k_x k_{eq}[H^+]^{out}}{k_{-x}[NH] + k'_3} \quad (A9)$$

This is formally identical with the corresponding equation for the Carlsberg mechanism

$$k_{app} = \frac{k_1 k''_3 [H^+]^{out}}{k_2 + k''_3 [H^+]^{out}}$$

where k''_3 is from $k_3 = k''_3[H^+]$.

For the rapid opening and closing of the cleft containing Trp-63 indicated by the NMR result and the quite slow reaction $k_{-x}[NN]$ we can write

$$k'_3 \gg k_{-x}[NH]$$

Eq. A9 now reduces to

$$k_{app} = k_x k_{eq}[H^+]^{out}$$

Although as in the case of EX_2 we have again a product of an equilibrium constant and rate constant, the meaning of these constants is quite different.

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