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The effect of viscosity on the accessibility of the single tryptophan in human serum albumin

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Abstract

When reactions take place with one of the reactants tied to protein matrix, movements along the reaction coordinate towards the transition state can become coupled to structural fluctuations of the protein matrix. This investigation aims to test the assumptions underlying the arguments supporting such a coupling. A coupling is allowed only if the activation barrier is high and broad enough as shown to be the case for the proton catalyzed isotope exchange at Trp-63 of lysozyme. In the present investigation the activation barrier for the same reaction has been lowered radically in an effort to show that the coupling, as measured by the dependence of rate on solution viscosity, will diminish and ideally vanish, despite the unchanged effects of cosolvents on the chemical activities of all the reactants. The isotope exchange rate at the indole nitrogen of the single tryptophan residue of human serum albumin was measured with UV. This residue is rigidly held to the protein surface and the solvent access, although restricted, corresponds to a partially exposed residue. As a consequence, the isotope exchange rates and the bimolecular quenching rate of fluorescence by acrylamide, also measured, are high. The experiments were carried out at pH 5.2 where the molecule is in the N-form and the exchange is catalyzed by OH^- ions. The activation energy of the hydroxyl catalyzed reaction is 22 kJ lower than for the proton catalyzed process. Under these conditions the exchange rate is viscosity independent both in the case of glycerol and in ethylene glycol. This is in contrast to the proton catalyzed exchange at Trp-63 of lysozyme. These findings support the view that the kinetics of reactions where one of the reactants is part of the protein matrix can be represented by a Kramers type of kinetic model.

Keywords: Human serum albumin; Viscosity of glycerol, ethylene glycol; Hydrogen–deuterium exchange; Fluorescence quenching of acrylamide

1. Introduction

We have, for a long time, been interested in finding a biological function for structural fluctuations in proteins. We have, as a simple model for reactions influenced by structural dynamics and taking place in the protein matrix, used hydrogen isotope exchange kinetics. We recently determined the effect of viscosity on the exchange from a single site, Trp-63, in lysozyme. This slow, hydrogen ion catalyzed reaction with a free energy of activation of $\simeq 83$ kJ showed a dependence on solution viscosity. In fact the ex-

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change from all of the hydrogens of lysozyme with a rate equal to or slower than Trp-63 shows viscosity dependence [1]. These data could be best rationalized in the framework of the Kramers model for reaction kinetics [2], a model frequently used to show coupling between the viscosity of solution and the friction along the reaction coordinate [3,4]. Structural motion in proteins [5] and in polymers [6,7] has been suggested as the cause of such friction. In fact a good theoretical case has been made for the coupling of structural motion to enzyme catalysis [8] and in reactions of the condensed phase in general [9]. It has been difficult to produce convincing experimental support for such models and theories because the addition of viscogenic agents such as glycerol produces a plethora of effects besides the desired increase in viscosity. We have recently shown that the deacetylation of a thioester by subtilisin is viscosity dependent [10]. What could be common for those two reactions, hydrogen exchange and deacetylation, is a relatively high and broad activation barrier [11]. It would be highly desirable if we could show that if the activation barrier is substantially lowered, and the reaction becomes very much faster, the viscosity effect is radically reduced. The residency time on the top of the barrier would in such case be so short that the solvent and the segments of protein appear essentially as frozen. If the cosolvent effect we observe is due to factors other than viscosity an increase in rate should not abolish the observed cosolvent effect.

The reaction we have studied for this purpose is the hydrogen exchange kinetics at the single tryptophan of human serum albumin, HSA. We studied also the effect of glycerol on a second very fast reaction at the same residue, fluorescence quenching by acrylamide.

2. Materials and methods

2.1 Materials

Human serum albumin (HSA, fatty acid free), glycerol, acrylamide, L-tryptophan, deuterium oxide (99.8 atom% D), deuterium chloride (99 atom% D) all were purchased from Sigma Inc., St. Louis, MO. HSA was checked for the presence of dimers or tetramers by a Pharmacia FPLC-system, using a Superose 6 column. Absence of appreciable amounts of aggregated forms allowed us to use HSA without further purification. Sodium deuteroxide (40 wt% solution in D_2O) and ethylene glycol (spectrophotometric grade) were obtained from Aldrich Inc., L-tyrosine ethylester HCl was from Cyclo Chem. Corp. (Los Angeles, CA), and L-tryptophan-methylester HCl was from Mann Res. Lab., Inc. All buffer agents were of analytical grade, and the water used was first deionized and then glass distilled.

2.2 Methods

The methods of observations and analysis of steady state fluorescence quenching of HSA tryptophan fluorescence by acrylamide were the same as described previously [12].

Viscosity was measured at 15 °C using a Cannon 75 A-820 viscometer, measuring the time of the flowing through of buffer and protein solutions. HSA was dissolved in 0.1 *M* KCl and the pH was adjusted to the desired value with HCl. Intrinsic viscosity was calculated as

$$[\eta] = \lim_{c \to 0} \eta_{sp}/c$$

where $\eta_{sp} = (\eta/\eta_0) - 1$, and c is the protein concentration in g/ml.

The UV differential spectra of HSA and model amino acid mixtures resulting from solvent perturbation were measured in the presence of 20 wt% glycerol, as perturbant, according to Herskovits and Sorensen [13].

Hydrogen–deuterium exchange measurements of the proton belonging to the nitrogen of the indole ring of the single tryptophan in HSA were carried out using the UV method [14], as previously described in the studies of Trp-63 of lysozyme [15].

The protein was dissolved (150 mg/ml) in 0.1 *M* acetate buffer, pH 4.0 and then diluted with deuterium oxide to 65 mg/ml. The pH was adjusted to 3.0 with 1 *M* deuterium chloride or 40% sodium deuteroxide, and the solution was kept at

room temperature for 2 hours to complete the *in*exchange. L-Tryptophan was inexchanged by dissolving the dry substance in deuterium oxide, and keeping it at room temperature overnight. The solutions were membrane filtered before use. To measure the *out*exchange, 0.1 ml of a protein or tryptophan solution (inexchanged as described above) was transferred by a Hamilton syringe into the thermostatted cell of a Cary 219 spectrophotometer containing 2.5 ml of buffer or cosolvent containing buffer. A vibrating teflon device was used for 5–10 seconds to mix the solutions. During the preparation of the solutions and measurements the same precautions were taken as described by Somogyi et al. [15]. Change in the absorption at 290 nm (2.5 nm slit) was monitored as function of time, using a full scale range of 0.02 absorbance units. Experiments with HSA were run at 15 °C and with tryptophan at 10 °C to obtain outexchange reactions running with rates measurable with our experimental setup. The photometer was coupled to a Compaq "Deskpro" computer to collect and analyze the data. Absorbance changes as function of time were smoothed by a standard averaging routine, and the apparent first order rate constants were calculated by fitting the smoothed curves to $A - B\exp(-kt)$. The pH of each reaction mixture was determined after running the outexchange measurements. The readings of the glass electrode calibrated with standard buffers were corrected for the effect of glycerol and ethylene glycol [16]. The OH^--activity in glycerol was calculated taking into consideration that the ion product of water changes in the presence of cosolvent [17]. The ionization constants of water in cosolvents at different temperatures were from Wolley et al. [18] and Banerjee et al. [19]. The relative viscosities of glycerol and ethylene glycol containing solutions were calculated from published data [20,21].

3. Results

Before turning to the measurements of hydrogen exchange or presenting the fluorescence quenching data, we must determine the conformational state of the protein. We have to localize the N–F transition on the pH scale at the temperature and ionic strength we used using also the same buffer ions as in hydrogen exchange experiments. Figure 1 shows the results of the two experiments designed to detect the pH dependent transition: the first curve shows the change in the intrinsic viscosity of a 1 wt% HSA solution with increasing pH, the second curve shows the UV-difference spectra of 1×10^{-6} *M* HSA in 0.1 *M* KCl at 287 nm. Protein at the same concentration but at pH 2.5 was used as the blank. Both curves show that the transition at our experimental conditions takes place between pH 3 and 5. Consequently, we can assume that above pH 5 the protein exists in one, the electrophoretically normal, N state.

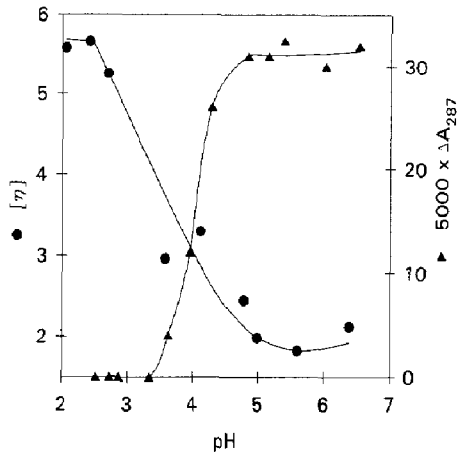

Fig. 1. The intrinsic viscosity $[\eta]$ and the differential changes of absorbtivity at 287 nm of a 1 wt% solution of HSA plotted as a function of the pH of the solution at 10 °C.

3.1 The hydrogen–deuterium exchange experiments

It is our intention to study the behavior of k_{OH}, the second order exchange rate constant for the OH^--catalyzed reaction. The first step is to determine the pH profile of the exchange. We measured the apparent first order exchange rate constant, k_{app},

$$k_{app} = k_0 + k_H[H^+] + k_{OH}[OH^-] \qquad (1)$$

where k_0 is the rate constant for direct exchange with water, and k_H and k_{OH} are the rate con-

stants for the H^+ and OH^- catalyzed reactions [22]. Figure 2 shows the pH dependence of k_{app} (min^{-1}) for HSA at 15 °C, in 0.1 *M* acetate buffer, and for tryptophan, Trp, at 22 °C (data for Trp are from Nakanishi et al. [14]). Because the N–F transition is essentially completed above pH 5 we chose pH 5.2 (very near the isoionic point) as the first pH where the exchange can be considered as being dominated by the OH^--catalyzed reaction and we have not yet reached regions of pH where additional pH effects might be present. The viscosity effect of glycerol and ethylene glycol on the exchange rate of HSA and L-Trp was measured at 10 °C, in 0.1 *M* acetate buffer, at pH 5.2 for HSA and at pH 5.5 for tryptophan. From the measured k_{app} values we calculated the second order rate constants, k_{OH}, using pH and K_w values corrected according to the procedure described in the methods section.

$$k_{app} = k_{OH} K_w / [H^+] \qquad (2)$$

Figure 3(a) shows the dependence of k_{OH} (M^{-1} s^{-1}) for L-tryptophan on the relative viscosity produced by the addition of glycerol and ethylene glycol. Figure 3(b) shows the same kind of data for HSA.

3.2 Fluorescence quenching experiments

We have recently shown that the steady state quenching by acrylamide of the fluorescence from the single tryptophan residue in HSA is dominated by static quenching and that cosolvents have a large effect on the formation of complexes between the quencher and the ground state tryptophan residue. Thus the data for dynamic

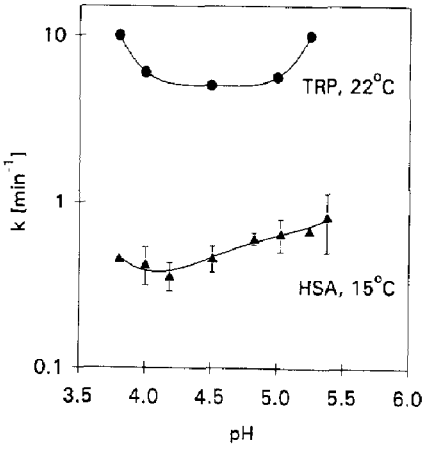

Fig. 2. The apparent first order rate constant for the exchange of the hydrogen at the indole nitrogen in tryptophan and HSA plotted as a function of the pH of the reaction mixture.

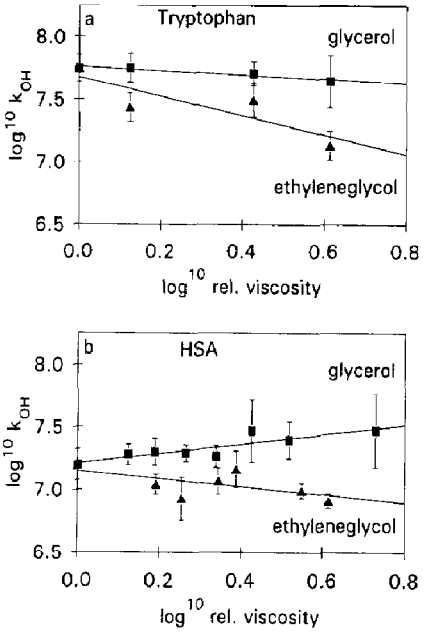

Fig. 3. The viscosity dependence of the second order rate constant for OH^--catalyzed exchange of the hydrogen at the indole nitrogen of (a) tryptophan measured at pH 5.5 and 10 °C and (b) the indole nitrogen in HSA determined at pH 5.2 and 10 °C.

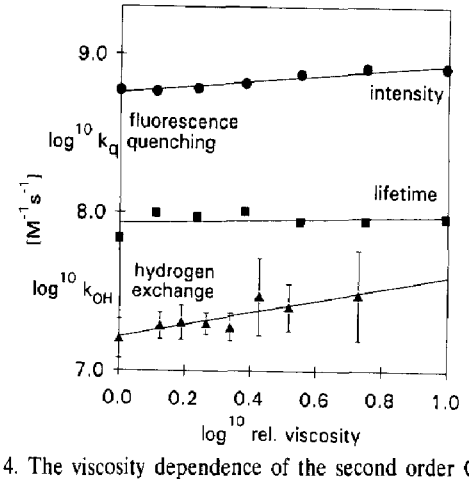

Fig. 4. The viscosity dependence of the second order OH^--catalyzed exchange rate constant of the hydrogen at the indole nitrogen in HSA, k_{OH} and the viscosity dependence of the second order acrylamide quenching rate constant, k_q, of fluorescence from the same residue.

quenching represent not more than 25% of total quenching. We have used and plotted in Fig. 4 lifetime data in the form of k_q the second order collisional quenching constant from our previous unpublished work.

4. Discussion

When studying structural dynamics of the lysozyme molecule we observed that the hydrogen isotope exchange kinetics of the Trp-63 residue showed dependence on solution viscosity although the exchange from the free indole residue was viscosity independent. The effect was the same whether glycerol or ethylene glycol was used as cosolvent [15]. Studies of protein stability in presence of cosolvents have shown that ethylene glycol and glycerol have different effects on the thermal equilibrium. However, as it is very unlikely that the exchange from the unfolded state contributes anything to the observed exchange, it should be, and we have shown by calculation, that the transfer free energies and enthalpies for tryptophan peptides from water to solvent mixtures were quite different for glycerol and ethylene glycol [15]. We concluded that the rate constant for exchange we observed, 6×10^2 s^{-1} M^{-1}. represented a slow reaction with a high enough activation barrier allowing time enough for the coupling of structural movement to movement along the reaction coordinate [4]. The local frictional coefficient is sufficiently high for the high viscosity case of the Kramers model [2] developed for polymers [6] and for reactions in solution in general [9]. These observations are important for us because using hydrogen exchange as a simple model for reactions taking place in protein matrix we argue that in enzyme catalysis some of the steps subsequent to ligand binding represent a similar type of reaction. In fact we have recently shown that the deacetylation step in subtilisin catalyzed ester hydrolysis is viscosity dependent [10]. If the model for coupling between the reaction coordinate and structural motion is real we should be able to show that a similar reaction taking place in the protein matrix but with considerably lower activation barrier should not be viscosity dependent. If not, the cosolvent effects we see must be due to other, unrecognized effects on the protein structure. We are fortunate in so far that the hydrogen exchange rates are, when catalyzed by hydroxyl ion instead of hydrogen ion about four orders of magnitude faster [23,24]. We choose human serum albumin, HSA, for our studies because this single tryptophan protein belongs to a family of proteins whose interactions with cosolvents have been studied in detail. This is important because the charged intermediate in hydroxyl catalyzed exchange is sensitive to solvent composition as studies of L-Trp showed. We are also able to study the collisional quenching reaction taking place at the same residue. The fluorescence quenching by acrylamide is diffusion limited with a very low activation barrier for the reaction. The data we present in Fig. 4 are in the form of comparable second order rate constants, k_{OH} and k_q. Before presenting models that relate these quantities to structural motion we will briefly summarize what is known about the HSA molecule and its structural properties. This will help us to justify the use of a relatively simple model for the exchange reaction we are going to use.

Although a unique three dimensional structure of HSA was published in 1988 [25], the HSA molecule goes through four different conformational changes in solution between pH 2 and pH 10 [26]. The so-called N–F transition that takes place around pH 4.3 is well known from the works of Foster and others [27,28]. The definition of N- and F-states came from the different electrophoretic mobility of the two forms: normal (N), and fast (F). The transition in bovine serum albumin is correlated with the separation of the domain III from the rest of the molecule and the subdomains of III from each other [29]. The pH defined midpoint of the N–F transition of HSA at different temperatures has been determined by optical rotation [30]. It was found at pH 4.24 both at 11 °C and 20 °C. It increased slightly with increasing temperature. At the pH and temperature used in our experiments the protein is in the N-state.

The fluorescence spectra of HSA obtained with the excitation wavelength of 280 nm is dominated

by the single Trp (Trp214 located in the domain II), in spite of the presence of 18 tyrosines/mole protein [31]. The immediate vicinity of the Trp in the amino acid sequence is totally hydrophobic: (–Arg–Ala–Phe–Lys–Ala–*Trp*–Ala–Val–Ala–Arg–) [32]. The position of the emission maxima (342 nm) suggests that the single Trp is located near the surface of the protein and remaining in limited contact with water [33].

Our solvent perturbation spectra obtained in the presence of 20 wt% glycerol show that the Trp contribution measured at 290 nm has exactly the same form and magnitude as the mixture of L-Trp-methyl ester HCl and L-Tyr-ethyl ester HCl in a molar ratio of 1:18, a result expected for a not too well-buried tryptophan.

We know from fluorescence anisotropy measurements that the Trp in the native conformation of HSA is held rigidly by the protein matrix [31]. The time resolved emission anisotropy data indicate that the residue has almost no rotational freedom at low temperature [34].

This picture of the molecule helps to explain why the pH dependence of the exchange seen in Fig. 2 is little influenced by the large N–F transition seen in Fig. 1. Most of the change during the transition takes place around domain III whereas our residue is in the hydrophobic pocket in domain II.

We see directly from Figs. 3(a) and 3(b) that the exchange rate for the tryptophan residue in HSA is about 10 fold slower than the exchange we see for the free tryptophan. In view of the possible spread of hydrogen exchange rate constants in proteins over at least 7 orders of magnitude [23,24] our results point to a partially accessible residue, again in agreement with the accepted picture of HSA.

We can express the exchange rate constant for the OH^--catalyzed exchange reaction for the single residue we observe as

$$k_{OH} = \beta k_3 \qquad (3)$$

where k_3 represents the exchange rate in tryptophan (Fig. 3a) and β represents the attenuation of the rate due to the location of the exchanging residue in the protein matrix. This constant can be interpreted as representing some type of equilibrium process leading to transient exposure of the residue to the solvent. The two possible general mechanisms for such a transient access for the hydroxyl ion, EX_2 and the reverse-EX_2 [15] represent local unfolding equilibria and solvent penetration equilibria, respectively. Although we have provided in previous publications strong arguments in favor of the reverse-EX_2 mechanism as the dominating exchange pathway for proteins, the specific point we are trying to make with this investigation does not depend on any specific mechanism as long as β represents a contribution due to the protein matrix. A very similar simple formal model has been proposed for the collisional quenching of fluorescent residues in the protein matrix [35]. The processes are formally similar and the constant representing rate attenuation could be common for both reactions. We have in Fig. 4 plotted both the rates of acrylamide quenching of fluorescence and the exchange rates taking place at the same residue under the same conditions.

In order to compare the matrix effects of the two different solvents, in the case of hydrogen exchange, we have to correct the observed rate constant for the effect of cosolvent on the intrinsic rate constant k_3 in equation (3) using data for tryptophan exchange in Fig. 3(a). This is not as simple as it looks; we cannot insert the values of k_3 measured for tryptophan, the solvent conditions are not identical. The protein molecule is known to interact preferentially with water, thus excluding part of glycerol from the hydration shell. Consequently, the effective concentration of glycerol or ethylene glycol at the exchanging site is considerably less than in solution. The preferential interaction parameter

$$\{\delta g_3/\delta g_2\}_{T,\mu_1,\mu_2}$$

where g_3 and g_2 stand for the cosolvent and protein content at equilibrium has been determined experimentally for BSA [36–38]. We can then, departing from the known degree of hydration of the protein in cosolvent free solution, calculate the effective concentration of the cosolvent in the hydration sphere of the protein [38].

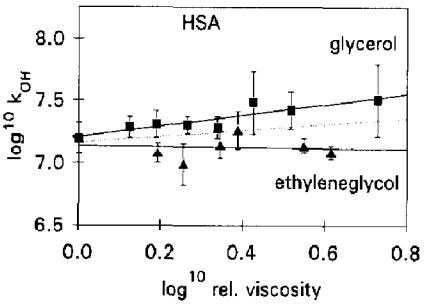

Fig. 5. The viscosity dependence of the exchange rate constant k_{app} of eq. (3), corrected for cosolvent affect on k_3 of eq. (3). The viscosity dependence now attributable to conformational effects was determined both with glycerol and ethylene glycol as cosolvents.

We can use that effective cosolvent concentration, determined for BSA, to correct [1] the k_3 values in eq. (3). The results plotted in Fig. 5 show k_{OH} corrected for the cosolvent effect on the chemistry of the exchange reaction. The results are quite clear cut, although one might argue that the effects of glycerol and ethylene glycol are not totally identical; neither cosolvent shows viscosity dependence of the exchange reaction. The data could, as seen from the dotted line in Fig. 5, well be fitted to a single line.

The striking similarity between the hydrogen exchange results and the fluorescence quenching of the same residue by acrylamide, Fig. 4, is gratifying. However, we have to remember that the bimolecular quenching constant k_q we compare to the k_{OH} of the exchange reaction represents a reaction that is diffusion limited. The collisional quenching in the case of HSA is mostly carried out by acrylamide molecules in the protein sphere (our unpublished results). In this case the activity of acrylamide in solution changes with addition of the cosolvent. Our data on the preferential interaction of acrylamide with the protein are not precise enough to allow us, in contrast to the [OH^-] values in hydrogen exchange, to correct for changing activity of acrylamide in the protein volume. Consequently, the good agreement with the hydrogen exchange data may be fortuitous.

The major conclusion from our work is quite clear—a lowering of the activation barrier for the exchange reaction by 23 kJ results in rates by four orders of magnitude faster than the hydrogen ion catalyzed rates we observed in lysozyme. This in turn leads to the absence of viscosity effects on the reaction. This conclusion rests on the premise that the cosolvent effects observed, after correction for changes in OH^- ion activity, would mirror the absence or presence of changes in viscosity. One can argue that the apparent viscosity effects are always overwhelmed by effects on chemical activities and that such effects, in the case of lysozyme [15] and in the present case, just happen to point in different directions. If we were dealing with a single cosolvent the argument may have some merit, but we chose glycerol and ethylene glycol because their effects on the activity of tryptophan and tryptophanyl peptides have been shown to be very different [39,40]. Thus the probability that these effects on two occasions should accidentally be similar to glycerol effects becomes quite small.

The data also strongly support the explanation for the observed viscosity dependence we suggested as a result of observing the exchange at Trp-63 of lysozyme [15]. We argued that if the activation barrier for the reaction taking place in the protein matrix is substantial, as in the case of the Trp-63 of lysozyme, the passage along the reaction coordinate is coupled to the movement of the protein matrix, which in turn is influenced by the viscosity of the bulk solution. In the case where the reaction is fast and the barrier low, as in the case of the Trp of HSA, such coupling does not appear. This is in good agreement with models for the kinetics of reactions in condensed phase [8,9]. It is the protein matrix that provides the local high viscosity necessary for a non-transition state model to be valid.

[1] The corrected values plotted Fig. 5 as straight lines: $y = 0.42x + 7.21$, $r^2 = 0.81$ for glycerol; $y = -0.028x + 7.14$, $r^2 = 0.44$ for ethylene glycol and $y = 0.24x + 7.17$, $r^2 = 0.10$, the broken line fitted to all the data.

Acknowledgement

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