The Solvent Dependence of Hydrogen Exchange Kinetics of Folded Proteins*

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SUMMARY

The effects of ethanol, ethylene glycol, dioxane, and other organic co-solvents upon the hydrogen exchange rates of randomly coiled oxidized RNase, native RNase, and native trypsin have been measured. The exchange rate of oxidized RNase, the model compound for the proton transfer step in hydrogen exchange, is decreased by all of the co-solvents studied at temperatures in the range 3-20°C. This has been ascribed to the combined effects of the disruption of peptide bond solvation due to a reduction in the concentration of water, and of changes in [OH⁻] ion concentration due to changes in the acid dissociation constant of water, K₆w.

The solvent dependence for both native RNase and native trypsin is similar in all of the solvents studied. At a low temperature (3-20°C), the exchange rates go through a minimum as the solvent concentration is increased. At higher temperatures (20-35°C) the exchange rates are increased at all concentrations of the co-solvent.

The apparent rate minimum at lower temperatures is due to two opposing effects. Co-solvents decrease the rate of exchange that occurs directly from the folded molecule. At higher concentrations and higher temperatures, exchange rates are increased due to the increase in equilibrium concentration of unfolded protein resulting from the lowering of the thermal unfolding transition temperature. The decrease in rates for exchange directly from folded protein is primarily due to the effects on the proton transfer step, and not to bonding or the solvent effects on protein structure.

The solvents used in this study have no apparent effect on conformational processes contributing to the hydrogen exchange process in folded proteins.

In folded proteins, the exchange of labeled peptide amide protons with solvent water occurs by one of two processes which can be differentiated by their temperature and solvent dependence.

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from the 3° data (Fig. 1) using 20 Cal per mol of activation energy.)

Interpretation of the results of Fig. 1 depends in part upon the pH-rate profile both with and without co-solvent (Ref. 4 and below). Fig. 2 gives the pH dependence of the exchange rate of fully tritiated trypsin. The pH of minimum rate, pH_min = pH 2.3. The curves in Fig. 2 were hand-drawn through the data points for the pH values 1.7, 2.0, and 2.3. The data for pH values 3.0 and 3.5 are shown on the same graph.

An identical dependence is observed in 20% ethanol, but the rate at pH_min is lower. Fig. 3 shows the exchange kinetics in 20% ethanol at pH_min and on either side of pH_min.

The effect of 30% ethylene glycol on partially tritiated trypsin was also examined (Fig. 4) giving results similar to those in 20% ethanol (Fig. 1).

To test the generality of the co-solvent effects on folded proteins exchange kinetics, the ethanol dependence of exchange from partially tritiated RNase was studied (Fig. 5). Very similar decreases in RNase exchange rates were observed with 20% butanol and 30% ethylene glycol. The RNase solvent effects are likewise similar to those of trypsin in Figs. 1 and 4.

The model for random coiled protein, oxidized RNase (5), also exhibits a similar solvent effect of ethanol, dioxane, and ethylene glycol (Figs. 6 and 7).

**DISCUSSION**

A priori, a decrease in the low $E_{app}$ exchange rates upon the addition of ethanol to trypsin (Fig. 1) and to RNase (Fig. 5) may arise from the solvent effects on either the chemical exchange (protein transfer) step, or on the conformational properties of the protein. Effects on the chemical exchange step may be separated by the use of oxidized RNase, the model system for the chemical exchange step (5). Exchange rates of oxidized RNase are influenced by co-solvents (Figs. 6 and 7) hence the origin of these effects must be analyzed before proceeding to native proteins.

**Solvent Dependence of Chemical Exchange Step**

In analyses of protein hydrogen exchange mechanisms, the chemical exchange step for random coil polypeptides is assumed to be identical with that described by Berger et al. (6) for N-methylacetamide. For this mechanism the chemical exchange rate $k_{cx}$, is given by

$$k_{cx} = k_H[H^+] + k_{OH}[OH^-]$$

where $k_H$ and $k_{OH}$ are the rate constants for direct exchange with water, acid-catalyzed exchange, and base-catalyzed exchange, respectively. Because the rate is both acid and base-catalyzed, there is a pH of minimum rate, pH_min. The rate constant at pH_min is $k_{min}$ (4). Berger et al. (6) as well as others (4) conclude that direct exchange with water is negligible and does not contribute to the overall observed rate.

As pointed out by Leichtling and Klotz (4), one needs a full pH rate profile in order to interpret solvent effects, because at a single pH an apparent decrease in rate can reflect a shift in pH_min even if there is no change in $k_{min}$. Comparison of rates at a single pH cannot be interpreted. On the other hand, changes in $k_{min}$ and pH_min may be interpretable in terms of environmental and conformational influences (4, 7).

Although a pH rate profile is easy to obtain for amides or homopolymers, in which all of the exchanging protons have the same $k_{cx}$, in heteropolymers, in which there is a distribution of $k_{cx}$ values, the over-all pH_min cannot be obtained to within less than 0.5 pH unit. Considering the very large distribution of exchange rate constants for folded proteins (1), the determination of pH_min to ± 0.5 unit is significant.
FIG. 3 (left). Ethanol dependence of fully tritiated trypsin, 10°, in the region of pHₘᵢₙ. □—□, no ethanol; ○—○, 20% ethanol.

Fig. 4 (center). Ethylene glycol dependence of out-exchange from fully tritiated trypsin, pH 2, at 3 and 10°. □—□, no ethylene glycol; X—X, 30% ethylene glycol.

FIG. 5 (right). Ethanol dependence of out-exchange of partially tritiated native RNase, pH 3.1, at 2 and 10°; ■—■, no ethanol; △—△, 12% ethanol; ○—○, 20% ethanol. The curves are hand drawn through the data points. The upper curve is for the 12% ethanol data.

FIG. 6 (left). Ethanol dependence of out-exchange from oxidized RNase, 3°.

FIG. 7 (right). Out-exchange of oxidized RNase, 3°, in the presence of 50% dioxane and 30% ethylene glycol.

The pHₘᵢₙ of oxidized RNase has previously been shown to be pH 2.0 to 2.5 (1). We find no measurable change in pHₘᵢₙ in the presence of 20% ethanol. However, there is a decrease in the value of kₘᵢₙ in the presence of 20% ethanol (Fig. 6). Thirty per cent ethylene glycol and 50% dioxane also decrease the observed rates of oxidized RNase at pH 3.1 (Fig. 7). In addition, a small decrease in rate with 3 M urea and with 5 and 10% trifluoroacetic acid have been observed for oxidized RNase at pH 3.1.

There are four solvent properties which can decrease the value of kₘᵢₙ. These are the change in Kₑ with added solvent, the decrease in the mole fraction of water with added solvent, the relationship of the hydrogen ion activity to the pH meter readings in binary aqueous solvents, and the change in the dielectric constant with solvent. Our results are explained by a combination of the first two effects; the last two are apparently negligible. The arguments are as follows:

A change in the acid dissociation constant of water, Kₑ, can change the observed rate because

1 C. K. Woodward, and A. Rosenberg, unpublished results.
assuming $k_0$ (Equation 1) is negligible. A solvent which increases the value of $K_w$ will shift both $k_{\text{min}}$ and $p\text{H}^{\text{min}}$ to higher values (6).

The calculated effects of a change in $K_w$ on the exchange kinetics of oxidized RNase is shown in Fig. 8. These curves are computed as before (1) using the model system described by Molday et al. (8). The calculations take into account the effects of a change in $K_w$ on both $k_0$ and $k_{\text{OH}}$, and therefore also on $k_{\text{min}}$ and $p\text{H}^{\text{min}}$.

As can be seen (Fig. 8) in the absence of other influences, raising the value of $pK_w$ to 14.3 (approximately equal to that of 20% ethanol) should result in a decrease in the observed rates. Lowering the value of $pK_w$ to 13.7 (approximately equal to that of 30% glycol) should result in an increase in the observed kinetics. Because a decrease in exchange rates is observed in both solvents (Figs. 6 and 7), it is clear that influences other than the change $K_w$ must be operating.

Table 1 lists the mole fraction of water and the literature values of $pK_w$ and dielectric constant for the solvents used in these studies.

Artifacts in pH arising from the measurement of hydrogen ion concentration in mixed solvents must be considered. The relationship of the pH values to the hydrogen ion activity of aqueous alcohol solutions obtained from a standard pH meter is a complicated function of alcohol and buffer, and the same glass electrode pH meter reading in pure water and in aqueous alcohol indicates lower hydrogen ion activity in the latter (9). However, this correction is small for 20% ethanol (9). Furthermore, if there were a systematic decrease in $[\text{H}^+]$, due to the fact that the 0 and 20% solutions are maintained at the same pH meter reading, then we would expect an increase in the base-catalyzed rate, a decrease in the acid-catalyzed rates, and a shift of the apparent $p\text{H}_{\text{min}}$ to lower pH values. However, the rates are decreased on both sides of the $p\text{H}_{\text{min}}$ (Figs. 3 and 6) indicating that both the acid- and the base-catalyzed reactions are decreased.

Lowering the dielectric constant should decrease both the acid- and base-catalyzed reactions because each proceed through a charged intermediate, the formation of which may be suppressed in a lower dielectric medium. This does not have a very large effect on oxidized RNase exchange rates because solvents of widely divergent dielectric constants (20% ethanol and 50% dioxane) give approximately the same rates (Figs. 6 and 7 and Table I).

Thus, the decrease of water mole fraction ($X_{\text{H}_2\text{O}}$) remains as an explanation of the decrease in $k_{\text{ex}}$ with mixed organic solvents. The effect of $X_{\text{H}_2\text{O}}$ on $[\text{H}^+]$ and $[\text{OH}^-]$ ion concentrations has already been taken into account by our choice of $K_w$ values.  

In Table I, the dissociation constant of water, $K_w$, is equal to $K_{a/a}$ reported by Woolley et al. (11).

$$k_{\text{ex}} = k_{\text{H}} [\text{H}^+] + k_{\text{OH}} [\text{OH}^-] / [\text{H}^+]$$

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$$K_w = K_{a/a} = [\text{H}^+] [\text{OH}^-] (y_x) / a_w$$

where $y_x$ is the mean activity coefficient for the solute ions and $a_w$ is the activity of water. Thus, the effect of changes in the activity of water upon the solute ion concentrations is already taken into account in the calculations. In our experiments, we adjust the glass electrode pH meter readings of the binary solvent equal to that of the aqueous solvent. Therefore, $[\text{H}^+]$ ion activity is

Therefore, we suggest that the lowering of $X_{\text{H}_2\text{O}}$ decrease the peptide bond solvation and that it is this that accounts for the attenuation of exchange rates in organic solvents. The chemical exchange step, i.e. the proton transfer step, requires previous solvation of the peptide amide bond (1).

A combination of $K_w$ and solvation effects can account for the decrease in rates observed in all of the solvents. $X_{\text{H}_2\text{O}}$ is decreased in all cases (Table I). The resultant attenuation of exchange rates is further increased by co-solvents that lower $K_w$ (ethanol and dioxane) and partially compensated by solvents that raise $K_w$ (ethylene glycol and urea) (Table I). This could explain why the observed exchange rate of oxidized RNase decreases more in 20% ethanol and 50% dioxane than in 30% ethylene glycol (Figs. 6 and 7) and in 3 M urea.  

Hydrogen Exchange from Folded Proteins—It is characterized by $E^{\text{app}} \approx 20$ Cal per mol (1). This fact has been utilized to define the conditions for the measurement of solvent effects upon the same in all our experiments and $[\text{OH}^-]$ concentration readjusts according to Equation 3.
the process of exchange directly from the folded molecule (1). We find that the solvent dependence of exchange from folded proteins is qualitatively similar to that of the random conformation protein, i.e. no detectable change in $pH_{\text{eq}}$ and a small decrease in $k_{\text{on}}$ (Figs. 1 to 5). This indicates that the decrease in exchange rates from folded proteins is due primarily to bulk solvent effects upon the chemical exchange step and not to binding or solvent effects upon protein conformation. As discussed above, the effect of bulk solvent on the chemical exchange (proton transfer) reaction arises from the combined effect of a reduction in peptide bond solvation due to a reduction in $\lambda_{\text{H}_2\text{O}}$ and changes in $[\text{OH}^-]$ concentration due to changes in $K_s$.

These results support our model for hydrogen exchange from folded proteins (1–3). We have argued (1) that the characteristic differences between exchange kinetics in folded proteins versus that of random conformation proteins, i.e. the increase in distribution and decrease in value of the exchange rate constants, are due primarily to the decrease in solvent accessibility in the hydrophobic region of the folded protein which in turn reduce peptide bond solvation. The same effect on the distribution and value of the rates from random coil proteins is observed when the peptide bond solvation is reduced by the addition of organic co-solvents (Figs. 6 and 7). In Figs. 6 and 7, as well as in Figs. 1 to 5, it can be seen that the distribution of rates is increased, i.e. there is no measurable effect upon the fastest rates whereas the lowest rates are attenuated.

Because ethanol has the same effect on folded protein and on randomly coiled protein, any conformational processes which account for solvent accessibility, as measured by hydrogen exchange kinetics, are not affected by ethanol. A breathing mechanism, involving localized, reversible unfolding accompanied by the breaking of several intramolecular hydrogen bonds, has been proposed to account for hydrogen exchange kinetics (13). A mechanism involving an equilibrium between amide-amide and amide-solvent hydrogen bonds would be expected to be sensitive to the nature of the solvent (14).

The qualitative similarity between the effect of organic co-solvents on exchange rates of oxidized RNase and the effect of aqueous solvent accessibility upon exchange rates in native proteins also argues against intramolecular amide-proton exchange in the native protein.

**Solvent Water Clathrates**—Clathrates similar to those around apolar molecules have been proposed for the solvent water in contact with the apolar side chains on the surface of the protein (15). It has been suggested that these may contribute to the structural stability of folded proteins (15, 16) and that the structure of the clathrates may be strengthened or disrupted by certain co-solvent systems (16). The solvents in Table I consist of both water structure breakers, e.g. 20% ethanol, urea, and ethylene glycol, and water structure makers, e.g. 12% ethanol at low temperature (17). Because these experiments measure the exchange of NH protons with the surrounding solvent, one might expect an indication of the perturbation of local water structure to be reflected in the hydrogen exchange kinetics. No such effect was detected, that all of the co-solvent systems have qualitatively the same effect on exchange rates.

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