Pressure Effects on Folded Proteins in Solution

HYDROGEN EXCHANGE AT ELEVATED PRESSURES*

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The observed rate constants for base-catalyzed hydrogen exchange reactions between solvent water and peptide nitrogen in lysozyme, ribonuclease A, oxidized ribonuclease A, and poly(oL-lysine) are all enhanced by an increase in pressure. Activation volumes have been calculated from the pressure effect on these rate constants. For the folded proteins lysozyme and ribonuclease A, \( \Delta V^f \) for base-catalyzed exchange changes from about +9 ml/mol at atmospheric pressure to -3 ml/mol at 2500 kg/cm\(^2\). The same quantity, determined for the random coil polypeptides oxidized ribonuclease A and poly(oL-lysine), does not show this dependence upon pressure. These effects can be understood either in terms of solvent penetration of the folded proteins or the onset of a small degree of pressure induced unfolding. Possible mechanisms by which such penetration could occur are discussed.

Conformational changes in enzymes dissolved in water can be caused by several variables, such as temperature, pH, solvent composition, and pressure, as well as by addition of substrates, inhibitors, and denaturants. Studies of these changes continue to advance the still incomplete understanding of how enzymes function.

The kinetics of proton exchange between solvent and protein is a powerful probe of the conformational properties of folded proteins since the protons on all but a few core peptide nitrogens will exchange with water under conditions such that the native state is much more stable than the denatured state (1-4). That water enters and leaves folded proteins is an experimental fact; how it does so seems more and more clearly to be tied to the conformational dynamics of protein structure and the role such movements play in enzyme catalysis.

Hydrogen exchange of folded proteins has been studied, utilizing all of the above-mentioned variables except pressure.

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The pressure coefficient of a rate constant yields the activation volume of the reaction, a quantity which can provide useful information about reaction mechanisms. Recognizing the importance of free volume and volume changes in protein function led us to measure the hydrogen-tritium exchange rates between water and the two enzymes lysozyme and ribonuclease A (RNase A) as a function of pressure.

To separate the pressure dependence of the exchange reaction itself from any pressure effect on the conformation of these folded proteins, we determined at various pressures the hydrogen-exchange kinetics of poly(oL-lysine) at pH 3.00 and 3.50 where only the base-catalyzed exchange reaction is kinetically significant and also at higher acidities where the acid-catalyzed reaction dominates. Under all our experimental conditions, polylysineexists in its random coil conformation. To further bolster this comparison of folded proteins with random coil polypeptides, we carried out the same type of experiment on oxidized RNase, a random coil heteropolypeptide (5).

Although there is no exact function for the variation of rate constant with pressure, there are several expressions which have been used (6-8). We have adopted the method favored by Whalley (8). The change in rate constant with pressure can be written as

\[
\ln \frac{k_2}{k_1} = \frac{\Delta V^f}{RT} \]

The activation volume represents the change in volume of a system as it progresses to the activated complex from unassociated reactants. For reactions in solution, it can be partitioned into an intrinsic part due only to the reactant(s) and a solvation part resulting from volume changes in solvation shell(s) of the reactants.

Integration of Equation 1, assuming \( \Delta V^f \) to be independent of pressure, gives

\[
\frac{\ln \left( \frac{k_2}{k_1} \right)}{P_2 - P_1} = -\frac{\Delta V^f}{RT} \]

By determining \( \Delta V^f \) using several different pressure ranges and plotting \( \Delta V^f \) versus \( P \), the mean of the pressure range over which \( \Delta V^f \) is obtained, one can check the assumption of the pressure invariance of \( \Delta V^f \). Extrapolation of this plot to 1 atm provides \( \Delta V^f \) atm if, as is often the case, \( \Delta V^f \) does change with pressure.

1 The abbreviation used is polylysine, poly(oL-lysine).
We have applied this technique to base catalyzed hydrogen exchange between water and the folded proteins lysozyme and RNase A as well as to both the acid- and base-catalyzed hydrogen exchange between water and the random coil polypeptides polylysine and oxidized RNase.

Our analysis of these results emphasizes the importance of peptide group solvation prior to exchange of hydrogen between peptide nitrogen and solvent. It is by a consideration of the equilibrium between solvated and unsolvated peptide bonds within a folded protein that we are able to reconcile the two major effects reported herein: that the activation volume of base-catalyzed hydrogen exchange is slightly more positive for folded proteins than for random coil polypeptides at 1 atm and that it is pressure-dependent for the former but not for the latter.

**EXPERIMENTAL PROCEDURES**

**Materials** — Lysozyme was Worthington Biochemical Corp. LYSF, salt-free. Bovine pancreatic ribonuclease A was Sigma protease-free type XII-A. The oxidized RNase used was a preparation by the method of Hirs (9) from bovine pancreatic RNase A (Sigma Type III-A), which had been frozen since its production. Poly(ε-lysine) with DP 260 was purchased from Sigma. Sephadex G-25 medium grade was obtained from A. B. Pharmacia. Tritiated water was purchased from International Chemiseal and Nuclear Corp. The scintillation mixture was 12.5% Beckman Bio-Solv BBS-3 in toluene, containing 4 gm/liter of PPO (2,5-diphenyloxazole) and 200 mg/liter of POPOP (1,4bis[2-(5-phenyloxazolyl)]benzene); PPO and POPOP were both obtained from Packard Instrument Co. All buffers were prepared from reagent grade chemicals.

**Methods** — The exchange rates were determined using the two-column separation technique described by Englander (10) with some modifications. Each macromolecule was equilibrated with tritiated water under conditions previously shown to affect complete in-exchange of tritium (see Wickett et al. (2) for lysozyme conditions, Woodward and Rosenberg (5) for oxidized RNase, Woodward and Rosenberg (11) for RNase A, and Englander and Poulsen (12) for polylysine). Each equilibrated solution of macromolecule in tritiated water is referred to as an "in-exchange." All experiments on a given macromolecule were done using the same in-exchange to ensure the validity of the experiments on polylysine and oxidized RNase, at 25°, the columns were at 6°. For the experiments on lysozyme and oxidized RNase, done at 25°, the columns were at 6°. For the experiments on polylysine and oxidized RNase, at 25°, the columns were at 6°.

Experiments with lysozyme and RNase A were carried out at pH 7.0 using Tris buffer to take advantage of the pressure independence of the pH of this buffer system (13). In the polylysine and oxidized RNase experiments, HCl/NaCl was used to adjust the pH.

All activation volumes are corrected for the change in (OH⁻) caused by solvent compression at elevated pressure using volume data for water obtained by Vedam and Holton (14).

**Results**

The hydrogen exchange reaction involves proton removal as the slow step when base catalysis is predominant (15), as shown in Equation 4. When acid catalysis predominates, protonation is considered the rate-determining step. This has traditionally been written as N-protonation (15), although more and more evidence indicates that peptide bonds are oxygen bases (Ref. 16 and references cited therein), in which case acid-catalyzed hydrogen exchange would occur as in Equation 5 with protonation presumably still the slow step.

\[
\begin{align*}
\text{N} + \text{H}_2\text{O} & \xrightarrow{\text{slow}} \text{HN}^+ + \text{OH}^- \\
\end{align*}
\]

The activation volumes for the base- and acid-catalyzed exchange reactions we write as \( \Delta V_{\text{base}} \) and \( \Delta V_{\text{acid}} \).

The experimental rate constants can be expressed as

\[
\begin{align*}
\text{rate base} &= \text{k}_\text{obs} \cdot \text{[H}_3\text{O}^+] \cdot \text{k}_\text{H}_2\text{O} \cdot \text{[OH}^-] \\
\text{rate acid} &= \text{k}_\text{obs} \cdot \text{[H}_3\text{O}^+] \cdot \text{k}_\text{H}_2\text{O} \cdot \text{[OH}^-] \\
\end{align*}
\]

while on the acidic side

\[
\begin{align*}
\text{k}_\text{obs} &= \text{[H}_3\text{O}^+] \\
\end{align*}
\]
At acidities where Equation 7 holds, the ionization volume of water, $\Delta V_w$, must be taken into account to obtain $\Delta V_{\text{app}}$ from the pressure effect on $K_w$, since (OH$^-$) is pressure-dependent. Hamann (17) found $\Delta V_w$ to be $-20.4 \text{ ml/mol}$. One can either use $\Delta V_w$ to adjust the acidity of each reaction so that (OH$^-$) is the same at each pressure or, as we chose to do, take advantage of the relationship expressed in Equation 9 which is shown under "Appendix" to obtain at acidities appreciably more basic than the pH$_{\text{pKw}}$. In Equation 9, $\Delta V_{\text{app}}$ is the apparent activation volume which results when $k_{\text{obs}}$ is used in Equation 2.

$$\Delta V_{\text{app}} = \Delta V_{\text{pp}} + \Delta V_{\text{w}}$$  \quad 9

The same complication does not arise on the acid side of the pH$_{\text{pKw}}$ where Equation 8 applies since (H$^+$) is so high that any pressure-induced change in $K_w$ produces an undetectable change in (H$^+$). Thus, in this pH range

$$\Delta V_{\text{app}} = \Delta V_{\text{pp}}$$  \quad 10

**Pressure Dependence of Exchange from Poly(mAlysine) and Oxidized Ribonuclease** — The rate constants for exchange between water and peptide hydrogen in polylysine were determined at 2.0$^\circ$, in 0.2 M NaCl at acidities between pH 0.51 and 3.50, and at pressures from 1 to 2500 kg/cm$^2$ (1 atm = 1.033 kg/cm$^2$). Fig. 1 shows semilogarithmic plots of the fraction of hydrogen remaining unexchanged per amide bond, $H_{\text{vam}}$ amide versus time for four pressures at pH 3.00. Rate constants obtained at other acidities were of the same quality as those calculated from these plots. We determined the pH$_{\text{min}}$ of our polylysine samples to be 1.8 to 1.9$^\circ$ so Equation 7 can be used for our results at pH 3.00 and 3.50 and Equation 8 is applicable for the pH 0.51 and 1.00 results.

From the rate constants recorded in Table I, $\Delta V_{\text{pp}}$ for polylysine was calculated as $-14 \pm 1 \text{ ml/mol}$ at pH 3.00 and 3.50. From Equation 9, $\Delta V_{\text{pp}}$ was $+6 \pm 1 \text{ ml/mol}$. From the work at pH 0.51 and 1.00, $\Delta V_{\text{pp}}$ can be estimated as $0 \pm 1 \text{ ml/mol}$.

Our study of oxidized RNase was carried out at 2.0$^\circ$, pH 2.5, conditions at which the exchange reaction was slow enough to be observable. This is the pH$_{\text{min}}$ for oxidized RNase (5) at which (see "Appendix")

$$\Delta V_{\text{pp}} = \frac{(\Delta V_{\text{pp}}^* + \Delta V_{\text{pp}}^* + \Delta V_{\text{pp}}^*)}{2}$$  \quad 11

The exchange reaction was followed at 1, 1000, 2000, and 3000 kg/cm$^2$. A plot of $H_{\text{vam}}$ versus time for the 1 kg/cm$^2$ experiment is slightly curved, very similar to the results of Woodward and Rosenberg (5). At higher pressures and resulting faster rates, the curvature becomes barely noticeable and can be approximated by straight lines within the precision of our experiments.

**Two experiments were carried out with a different in-exchange than was used for the other three experiments done at pH 3.0.**

Fig. 2 shows the pressure invariance of $\Delta V_{\text{pp}}$ at these conditions for oxidized RNase at three different values of $H_{\text{vam}}$. From this plot, a value of $-6 \pm 1 \text{ ml/mol}$ is obtained for $\Delta V_{\text{pp}}$ for oxidized RNase. Using the value for $\Delta V_{\text{pp}}$ obtained from the polylysine experiment and the known value for $\Delta V_{\text{pp}}$, we estimate from Equation II a value of $+8 \pm 2 \text{ ml/mol}$ for $\Delta V_{\text{pp}}$ from the oxidized RNase data. This is in fair agreement with either the polylysine value or the value obtained from the lysozyme and RNase experiments. There is more uncertainty in the oxidized RNase $\Delta V_{\text{pp}}$ than in the polylysine $\Delta V_{\text{pp}}$. The principal importance of the oxidized...
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RNase results, however, in the demonstration that the pressure invariance of $\Delta V_{\text{app}}^{+}$, found for polylysine is not a special feature of this homopolypeptide. Oxidized RNase can be thought of as a protein with no tertiary structure; it has the same diversity in amino acid sequence as RNase A, from which it was derived, yet $\Delta V_{\text{app}}^{+}$ determined using it is independent of pressure while the analogous experiment on native RNase A yields fundamentally different results (see below).

Pressure Dependence of Exchange from Lysozyme and RNase A - The exchange profiles of folded proteins are curved when displayed on a semilogarithmic plot because of the distribution of rates which extends over almost 6 orders of magnitude (3). Fig. 3 shows our RNase A results plotted in this way; the lysozyme data, also obtained at pH 7.0, in 0.05 M Tris buffer, 25°C, 1.5 mg/ml, look very similar. Apparent activation volumes at different values of $H_{\text{rem}}$ can be obtained from Fig. 3 and an analogous figure for lysozyme by determining the time required to reach a given $H_{\text{rem}}$ at two different pressures and substituting $\ln(t_2/t_1)$ for $\ln(k_2/k_1)$ in Equation 2. An alternative treatment which is completely equivalent, but easier to apply for the case in which the hydrogen exchange follows a simple power law in $t$, involves plotting $\log H_{\text{rem}}$ versus $\log t$. Fig. 4 shows our lysozyme data displayed in this way.

Extrapolation of the plots in Fig. 5 gives $\Delta V_{\text{app}}^{+}$ for lysozyme and RNase A of $-11 \pm 2$ ml/mol. Only the base-catalyzed reaction, Equation 4, is kinetically significant at pH 7.0 since the $pH_{\text{mi}}$ of the proteins is about 3. Consequently, Equation 9 applies and $\Delta V_{\text{app}}^{+}$ for both proteins is +9 ml/mol. The difference between this value and that obtained using polylysine is significant. Another difference between the results for the random coil polypeptides and the folded proteins in this study is seen by comparison of Figs. 2 and 5. At pressures above 1000 kg/cm², $\Delta V_{\text{app}}^{+}$ for the folded proteins

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**Fig. 2.** Pressure effect ($P$) on apparent activation volumes for oxidized RNase A at different values of $H_{\text{rem}}$. The rate constants used to determine the $\Delta V_{\text{app}}^{+}$ values were obtained at 25°C, 0.1 M NaCl, pH 2.50, 2.3 to 3.2 mg/ml.

**Fig. 3.** Pressure dependence ($P$) of out-exchange from RNase A, at 25.0°C, 0.05 M Tris, pH 7.00, 1.5 mg/ml.

**Fig. 4.** Pressure dependence ($P$) of out-exchange from lysozyme plotted as $\log H_{\text{rem}}$ versus $\log t$, at 25.0°C, 0.05 M Tris, pH 7.00, 1.5 mg/ml.

**Fig. 5.** Pressure effect ($P$) on apparent activation volumes for lysozyme (lower) and RNase A (upper) at different values of $H_{\text{rem}}$. 
becomes significantly more negative, while for polylysine and oxidized RNase, it remains constant as pressure is increased.

DISCUSSION

Before considering the implications our results have for protein conformation, a comparison of the activation volumes we have found for polylysine with those reported for similar reactions is in order. Neuman and co-workers (18, 19) have determined activation volumes for base-catalyzed hydrolysis of several esters. Le Noble (20) estimates activation volumes of about \(-10\) \text{ml/mol}\ for the bond formation part of each biomolecular reactions. The values reported by Neuman and co-workers (18, 19) are more positive than this by at least \(6\) \text{ml/mol}\ for all but one of their substrates, which they interpret as a decrease in solvation of hydroxide ion as the reaction proceeds to the transition state. This is quite reasonable since, as le Noble points out in his review (20), the volume contraction accompanying water dissociation \(\Delta V_\text{w} = -20.4\) \text{ml/mol}\ is not shared equally between the 2 ions produced; hydroxide ion is responsible for 12 to 15 \text{ml/mol}\ of the total, indicating that it is quite strongly solvated.

The biomolecular displacement of chloride ion from \(\text{NH}_2\text{Cl}^\text{-}\), \(\text{CH}_2\text{NHCl}\), and \((\text{CH}_3)_2\text{NCl}\) by hydroxide ion had \(\Delta V_\text{g} \approx -2\) to 0 \text{ml/mol}\, more positive by 7 to 10 \text{ml/mol}\ than for similar reactions in which carbon is the central atom which is thought due to a lesser electrostriction of solvent around the transition state when the central atom is nitrogen rather than carbon.

Thus, our own value of \(+6\) \text{ml/mol}\ for \(\Delta V_\text{g, RNase}\) from polylysine is not unreasonable; the catalyst is hydroxide ion, strongly solvated in the ground state, while the transition state has the negative charge shared by both the nitrogen and the carbonyl oxygen of the peptide group in addition to the oxygen of the hydroxide ion so the electrostriction of solvent in the transition state should be decidedly less than in the ground state. The value of \(+6\) \text{ml/mol}\ for \(\Delta V_\text{g, RNase}\) for polylysine, more negative by \(+6\) \text{ml/mol}\ than \(\Delta V_\text{g, chymotrypsin}\), also fits this picture; since hydronium ion does not have such a tightly constricted solvation shell as hydroxide, the solvation contribution to the activation volume will not be as negative in the acid-catalyzed as in the base-catalyzed reaction.

The oxidized RNase study serves to validate that the pressure invariance of \(\Delta V_\text{g, RNase}\) found in the case of the homopolymer polylysine also holds for heteropolymers and to substantiate the value of \(\Delta V_\text{g, RNase}\) found at 1 atm for polylysine. The data for oxidized RNase show somewhat larger scatter and the form of the plot of activation volume versus pressure is somewhat less well defined. We did not pursue this study further since oxidized RNase, although a good model for unfolded protein at 1 atm, has not been studied at higher pressures so we could not rule out the possibility of partial refolding. There could also be some refolding at higher basicieties. The oxidized RNase data support our use of polylysine as a model for the peptide group in the unfolded solvated state. The difference in \(\Delta V_\text{g, RNase}\) of polylysine and oxidized RNase at 1 atm, \(+6\) \text{ml/mol}\, is not statistically significant, and, as stated earlier, since the value obtained for polylysine is more precise we take \(+6\) \text{ml/mol}\ as the actual value of the activation volume of the base-catalyzed exchange reaction in the absence of conformation effects.

**Pressure Dependence of Exchange from Proteins** — For lysozyme and RNase A at pH 7.0, only the base-catalyzed exchange reaction, Equation 4, is kinetically significant since the \(\text{pH}_{\text{min}}\) of both these proteins is about 3. Extrapolation of the plots in Fig. 5 gives \(-11\) \text{ml/mol}\ for \(\Delta V_\text{g, lysozyme, RNase A}\) for both lysozyme and RNase A. Using Equation 9 gives \(\Delta V_\text{g, lysozyme} = +9\) \text{ml/mol}\ for both proteins, leaving a difference between the value for the proteins and that for polylysine of \(+3\) \text{ml/mol}\. A more significant result, however, is the effect of elevated pressure on \(\Delta V_\text{g, RNase}\) values calculated using Equation 9 for the two proteins. As shown in Table II, \(\Delta V_\text{g, RNase}\) changes from \(+9\) \text{ml/mol}\ at 1 atm to \(-5\) \text{ml/mol}\ at 2500 kg/cm\(^2\) for the proteins while it remains constant at \(+6\) \text{ml/mol}\ for polylysine and between \(+7\) and \(+10\) \text{ml/mol}\ for oxidized RNase. Since its pressure variation is so small in the case of the random coil polypeptide, it seems unlikely that \(\Delta V_\text{g, RNase}\) actually changes significantly with pressure in the case of the proteins; the observed change has to be due to some other pressure-sensitive process occurring in the proteins but not in polylysine or oxidized RNase.

The difference in behavior between random coil polypeptides and folded proteins could be due to pressure-enhanced solvent penetration of the proteins, or to the onset of pressure-induced reversible denaturation. Insofar as the results do not allow us to distinguish between them, both explanations are briefly discussed in the following sections.

**Pressure-enhanced Solvent Penetration of Folded Proteins** — Hydrogen exchange from buried peptide groups at pH 7.0 is base-catalyzed and involves the rate-determining production of an ion, \(-\text{CON}^-\). Stabilization of this ion may be accomplished by other polar groups of the protein itself in a few of the exchange events, but structural constraints limit this means of stabilization. It seems apparent, then, that the charge development in most of the reactions is stabilized by water. The solvation of \(\text{OH}^-\) is probably not larger than a single water molecule at the time that \(\text{OH}^-\) abstracts a proton from a peptide group, since recent fluorescence quenching studies\(^3\) (22) indicate that the size of molecules which penetrate to quench fluorescence of buried tryptophans in folded proteins such as RNase T\(_1\) and lactic acid dehydrogenase is strictly limited. Since in our experiments with lysozyme and RNase A the ratio of \(\text{H}_2\text{O}\) to \(\text{OH}^-\) concentrations is \(5.5 \times 10^8\) and since \(\text{H}_2\text{O}\) is smaller than \(\text{OH}^-\) (\(\text{H}_2\text{O}\)), water must penetrate to a buried peptide group many, many times for every time that an \(\text{OH}^-\) (\(\text{H}_2\text{O}\)) does. Thus, the exchange reaction can be written as a solvation step

\[
\text{CON}^- + \text{nH}_2\text{O} K \rightarrow \text{CON}^- (\text{H}_2\text{O})_\text{n}
\]

followed by rate-determining proton abstraction.

\[
\text{CON}^- (\text{H}_2\text{O})_\text{n} + \text{OH}^- (\text{H}_2\text{O}) K\text{H} \rightarrow \text{CON}^- (\text{THO}) (\text{H}_2\text{O})_\text{n+1}
\]

The rate constant for base-catalyzed exchange can then be written as \(k_\text{RNase} = kK\), and \(\Delta V_\text{g, RNase} = \Delta V_\text{e, RNase} + \Delta V_\text{h, RNase}\). Since the activation volume for base-catalyzed hydrogen exchange from the random coil polypeptides is pressure-invariant, it is not unwarranted to assume that \(\Delta V_\text{e, RNase}\) is also independent of

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3 M. Herschberger and R. Lumry, Private communication.
pressure and equal to the value obtained for polylysine of +6 ml/mol. The difference between $\Delta V_{\text{h}2\text{O}}$ for the proteins and polylysine is seen to be $\Delta V_k$, the volume change associated with solvating an exchangeable peptide group. At 1 atm, our results indicate that $\Delta V_k$ is small and positive, having a value of $-3 \pm 2$ ml/mol, but as the pressure is increased $\Delta V_k$ becomes more negative, reaching $-11 \pm 2$ ml/mol at 2500 kg/cm$^2$.

For $\Delta V_k$ to decrease with pressure, the compressibility of the product of the solvation reaction must be greater than the sum of the compressibility of the reactants. That is, for a given increment of pressure, $V'_{\text{CONT}(\text{H}_2\text{O})}$ must decrease more than does the sum of the volumes $V_{\text{CONT},i} + V'_{\text{H}_2\text{O}}$. This conclusion is not at odds with existing compressibility data. Liquid water has a low compressibility as compared to other liquids, losing only about 10% of its volume on going from 1 to 2500 kg/cm$^2$, and both lysozyme (23) and RNase A (24) are reported to have low compressibilities.

This finding implies that, since a solvated peptide group can occupy a smaller volume at elevated pressure than can a nonsolvated peptide group and the water necessary to solvate it, elevating the pressure on an aqueous protein solution will tend to force water into the protein. However, our 1 atm value of $\Delta V_k$ indicates that transfer of water into the protein is disfavored from a volume standpoint at atmospheric pressure. Examination of Fig. 5 and use of Equation 9 shows that for RNase A the movement of water into the protein has a favorable $\Delta V$ at pressures above about 1500 kg/cm$^2$. For lysozyme the turning point is at 1000 kg/cm$^2$.

Pressure-induced Unfolding - A second explanation for the pressure dependence of the activation volume for hydrogen exchange involves an increasing contribution from an unfolding mechanism of exchange with increasing pressure. There are two competing pathways for exchange: a low activation energy, solvent penetration process of the native state which is predominant at 1 atm, and under these experimental conditions of temperature and pH (2, 3), and a pathway involving an unfolding of the native protein which exposes the exchange site to the solvent. The solvent penetration process is described by Equations 12 and 13; however, in contradistinction to the previous explanation, the solvent penetration pathway is assumed to be independent of pressure. The competing unfolding mechanism may be written as:

$$-\text{CON}_{\text{N}} - \frac{k'}{k} - \text{CON}_{\text{U}} - \frac{k}{k'} - \text{CON}^- - (\text{THO})(\text{H}_2\text{O})_{\text{b}+}$$

where the subscripts N and U refer to the native and the unfolded states. The overall exchange constant for both pathways is then:

$$k_{\text{ON}} + k_{\text{ON}} = k'_{\text{ON}} + k'_{\text{ON}}$$

The observed activation volume is equal to:

$$\Delta V_{\text{app}} = \left( \frac{K}{K + K'} \right) \Delta V_{\text{ON}} + \left( \frac{k'}{K + K'} \right) \Delta V_{\text{ON}} + \Delta V_{\text{ON}}$$

The values of $\Delta V_k$ and $\Delta V_{\text{ON}}$ have been evaluated previously and are $+3$ ml/mol and $+6$ ml/mol, respectively. The $\Delta V_k$ of denaturation is generally in the range of $-20$ to $-50$ ml/mol. Since the equilibrium constant for solvation of the native protein has a small positive volume change associated with it, the value of $K$ will decrease slightly with increasing pressure. On the other hand, the pressure-induced unfolding constant, $K'$, will increase substantially with increasing pressure. Thus, an increasing fraction of the exchange will proceed via the unfolding pathway as the pressure is increased, and the apparent $\Delta V_{\text{h}2\text{O}}$ will decrease.

The pressure denaturation of lysozyme has been studied by Li et al. (25) and is a rather complicated phenomenon involving multiple domains. Thus, quantification via Equation 17 would be difficult. Brandts et al. (24) have studied RNase A at lower pH than the present experiments; extrapolation of their results to pH 7 would indicate that the $\Delta V_k$ for denaturation would be small and perhaps even positive. However, their experiments were carried out only up to 2300 kg/cm$^2$. Thus, although the data, as such, would exclude unfolding reaction as the basis for observed pressure effects and support the alternate based on increased solvent accessibility of the native state, the validity of our extrapolation and the absence of further pressure-dependent transitions has to be verified by additional experiments. The present study reveals unequivocally that the solvation process preceding the exchange of hydrogens at atmospheric pressure has a very small positive or no volume change associated with it. If we combine this with the observation that practically all hydrogens in the folded state of a protein exchange with such a low energy mechanism (2, 3), we see that the conformational substates responsible for structural motility of a protein appear to have very little volume work associated with it.

APPENDIX

The way we obtained $\Delta V_{\text{h}2\text{O}}$ and $\Delta V_{\text{h}2\text{O}}$ $\Delta V_{\text{app}}$ is as follows. Equation 6 can be simplified to

$$k_{\text{obs}} = k_{\text{h}+} + k_{\text{OH} K_{\text{w}}}/(H^+)$$

since the third term makes a negligible contribution to the observed kinetics. The apparent activation volume can be expressed as

$$\Delta V_{\text{app}} = -\frac{\Delta V_{\text{app}}}{k_{\text{app}}} = -\frac{RT\ln k_{\text{app}}}{(H^+)} = -\frac{RT\ln k_{\text{app}}}{(H^+)}$$

and since $\frac{\Delta V}{\Delta P}$ is pressure-invariant under our conditions we can write

$$\Delta V_{\text{app}} = -RT\ln k_{\text{app}}$$

where

$$\Delta V_{\text{app}} = -RT\ln k_{\text{app}}$$

The pressures of $\Delta V_{\text{h}2\text{O}}$ and $\Delta V_{\text{h}2\text{O}}$ have been evaluated previously and are $+3$ ml/mol and $+6$ ml/mol, respectively. The $\Delta V_k$ of denaturation is generally in the range of $-20$ to $-50$ ml/mol. Since the equilibrium constant for solvation of the native protein has a small positive volume change associated with it, the value of $K$ will decrease slightly with increasing pressure. On the other hand, the pressure-induced unfolding constant, $K'$, will increase substantially with increasing pressure. Thus, an increasing fraction of the exchange will proceed via the unfolding pathway as the pressure is increased, and the apparent $\Delta V_{\text{h}2\text{O}}$ will decrease.

The pressure denaturation of lysozyme has been studied by Li et al. (25) and is a rather complicated phenomenon involving multiple domains. Thus, quantification via Equation 17 would be difficult. Brandts et al. (24) have studied RNase A at lower pH than the present experiments; extrapolation of their results to pH 7 would indicate that the $\Delta V_k$ for denaturation would be small and perhaps even positive. However, their experiments were carried out only up to 2300 kg/cm$^2$. Thus, although the data, as such, would exclude unfolding reaction as the basis for observed pressure effects and support the alternate based on increased solvent accessibility of the native state, the validity of our extrapolation and the absence of further pressure-dependent transitions has to be verified by additional experiments. The present study reveals unequivocally that the solvation process preceding the exchange of hydrogens at atmospheric pressure has a very small positive or no volume change associated with it. If we combine this with the observation that practically all hydrogens in the folded state of a protein exchange with such a low energy mechanism (2, 3), we see that the conformational substates responsible for structural motility of a protein appear to have very little volume work associated with it.
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