Determination of the Activation Volume of the Uncatalyzed Hydrogen Exchange Reaction between *N*-Methylacetamide and Water

Stephanie A. Mabry, Bao-Shiang Lee, Tianshu Zheng, and Jiri Jonas*

Contribution from the Department of Chemistry, School of Chemical Sciences, and Beckman Institute for Advanced Science and Technology, University of Illinois, Urbana, Illinois 61801

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Abstract: The amide−water hydrogen exchange rates of the individual backbone amide protons are very sensitive to the local environment of the protons and can be used to probe fluctuations in the local conformations of proteins. Hydrogen exchange between *N*-methylacetamide (NMA) and water is important in that it serves as a model for the amide−water hydrogen exchange in peptides and proteins. The rate constants for amide−water hydrogen exchange between 16 mol % NMA and water at 66 °C were measured at pressures ranging from 1 bar to 5 kbar at three different pH values using the NMR magnetization transfer technique, and the apparent activation volumes of hydrogen exchange were obtained at each pH. The activation volume of the acid-catalyzed hydrogen exchange reaction was calculated to be $+1.7$ cm^3 mol^{-1}, and the activation volume of the base-catalyzed hydrogen exchange reaction was calculated to be $+11.0$ cm^3 mol^{-1}. The hydrogen exchange rate constants were also measured as a function of concentration and pH at ambient pressure at 66 °C so that the rate constants of the acid-, base-, and uncatalyzed hydrogen exchange reactions could be found. With these values, the activation volume of the uncatalyzed hydrogen exchange reaction was calculated to be equal to -9.0 cm^3 mol^{-1}. This quantity has not been previously determined and is required in hydrogen exchange studies of peptides and proteins at pressures above ambient pressure.

Introduction

The relationship between the amino acid sequence and the structure and dynamic properties of the native conformation of proteins represents a key problem in biochemistry and biology. Recently, increasing attention has been focused on denatured and partially folded states of proteins since determination of their structure and stability may help our understanding of the mechanisms of protein folding.[1−3] Most studies dealing with protein denaturation have been carried out at atmospheric pressure using temperature, pH, or denaturants as experimental variables. In contrast, the use of pressure[4,5] allows one to change, in a controlled way, the intermolecular interactions without the major perturbations produced by changes in temperature and/or chemical composition. In addition, by taking advantage of the phase behavior of water, high pressure can substantially lower the freezing point of aqueous protein solution. Therefore, by applying high pressure one can investigate in detail not only pressure-denatured proteins but also cold-denatured proteins in aqueous solutions.[6]

The hydrogen exchange method[7] represents a widely used approach of studying protein structure and dynamics. The overall hydrogen exchange rate constant (k) for each amide proton in peptides and proteins is pseudo first order and can be written as a sum of three terms

$$k = k_{H^+}[H^+] + k_{OH^-}[OH^-] + k_w \quad (1)$$

where k_{H^+} is the rate constant of the acid-catalyzed exchange reaction, k_{OH^-} is the rate constant of the base-catalyzed exchange reaction, and k_w is the contribution due to the pH independent exchange reaction, or uncatalyzed exchange reaction, assuming constant water concentration which is typical in peptide and protein studies. However, the contribution of the uncatalyzed exchange reaction actually has first-order dependence on the water concentration,[8] and thus the rate constant can be written in terms of the pH and water concentration as

$$k = k_{H^+}10^{-pH} + k_{OH^-}K_w10^{pH} + k_{H_2O}[H_2O] \quad (2)$$

where k_{H_2O} is the rate constant of the uncatalyzed exchange reaction, also called the water-catalyzed exchange reaction, and K_w is the ionization constant of water ($K_w = [H^+][OH^-]$).[8] The pH at which the minimum value of k occurs is referred to as the pH_{min}. The pH_{min} of polyalanine is at pH 3.0, and the pH_{min} value for proteins varies from pH 3.0 by ±1.0 pH units.[9]

The amide−water hydrogen exchange rates are very sensitive to the local environment of the protons; thus, fluctuations in the local conformations of proteins can be probed by measuring the hydrogen exchange rates of the individual backbone amide protons.[10,11] The effect of the local structure on the hydrogen exchange rate constants is typically quantified in terms of the protection factor, which is the ratio of the observed rate constant of each amide proton in the protein to a calculated rate constant

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for the amide proton in an analogous unfolded structure. The calculated unfolded rate constants are determined from the rate constant for the model compound, polyalanine, by factoring in side-chain effects on the acid-, base-, and water-catalyzed exchange rate constants and by using the activation energies of exchange to correct the rate constants for temperature differences.[12] In our laboratory, the amide−water hydrogen exchange rate constants of the individual amide residues of proteins are used to probe the local structure surrounding the residues in cold- and pressure-denatured proteins.[13,14] Thus, in addition to side-chain and temperature corrections, the effect of pressure on the rate constants must be included. Currently, the data analysis is difficult since information on the activation volume of the uncatalyzed amide−water hydrogen exchange reaction is lacking. The activation volumes of exchange for the acid- and base-catalyzed exchange reactions have been determined by Carter et al.,[15] who investigated the effect of pressures up to 2.5 kbar on hydrogen exchange in polypeptides and proteins using tritium−hydrogen exchange experiments. However, their study was incomplete in that it neglected the contribution of the uncatalyzed hydrogen exchange reaction to the rate constant.

Although the issue of the extent of the contribution of the uncatalyzed exchange reaction to the hydrogen exchange rate constants of peptides and proteins has not been definitively settled, several studies at ambient pressure have shown that the uncatalyzed exchange reaction is significant at the pH minimum.[8,12,16,17] Therefore, the idea that water catalysis might play a significant role in hydrogen exchange reactions under certain conditions should not be ruled out a priori since it is especially relevant to the hydrogen exchange investigations of cold-denatured proteins at high pressures in our laboratory.[13,14]

Hydrogen exchange between *N*-methylacetamide (NMA) and water is important in that it can serve as a simple model for the amide−water hydrogen exchange in peptides and proteins. The hydrogen exchange reaction between NMA and water has been studied at ambient pressure using both NMR line shape analysis[18] and magnetization transfer techniques.[8,19] The pH_{min} for the hydrogen exchange reaction between NMA and water occurs at pH 5.0.[8] By determination of the effects of pressure on the rate constants of the model hydrogen exchange reaction between NMA and water, a value of the activation volume of uncatalyzed hydrogen exchange has been obtained in this study which can be applied to protein amide−water hydrogen exchange investigations at high pressure.

Experimental Section

Materials. *N*-methylacetamide (NMA) was purchased from Sigma Chemical Co.

Sample Preparation. Samples for NMR hydrogen exchange experiments were made by combining appropriate volumes of NMA and double-distilled water. The volumes used were calculated using the densities, 0.956 g cm^{-3} (NMA) and 0.995 g cm^{-3} (H_2O), and ignoring the volume change of mixing. The pH of the samples was adjusted with small amounts of concentrated HCl and KOH and measured with a standardized glass electrode (Sigma, Trizma No. E 5259) and Corning pH meter (pH 103) at room temperature. For the high-pressure measurements, 20 mM maleic acid buffer and 20 mM Tris buffer were added to the samples at pH 2.75 and at pH 7.5 to take advantage of the pH pressure invariance of these buffers.

NMR Measurements. The hydrogen exchange rate constants of exchange between NMA and water at 66 °C were determined with the NMR magnetization transfer technique using inversion transfer proton NMR experiments. This technique can measure rate constants on the order of $10^{-2}-10^{2}$ s^{-1}. The hydrogen exchange rate constants were determined for samples containing 16 mol % NMA in water at pressures from 1 bar to 5 kbar at three different pH values, 2.75, 5.0, and 7.5, and also for a series of samples at 1 bar of varying concentration and pH.

All NMR experiments were made using a home-built FT-NMR spectrometer with a wide-bore (130 mm) Oxford 4.2 T superconducting magnet and interfaced to a GE 293D pulse programmer and GE/Nicolet 1280 data system.[20] The spectrometer frequency used for proton spectra was 180 MHz, and the nitrogen-14 decoupling frequency used was 13 MHz.

The pressure NMR probe used a single solenoid coil that offered increased sensitivity relative to a Helmholtz coil. Pressures up to 5 kbar were generated using a hydrostatic pressure generating system. The high-pressure vessel was fabricated from titanium alloy, and the pressurizing fluid used inside the vessel was carbon disulfide in order to eliminate interference with the proton signal of the sample solution. The sample was isolated from the CS_2 by a movable Teflon piston. The desired temperature of 66 °C was obtained using a circulating water bath (Brinkmann Instruments, RM20) connected to the exterior of the pressure vessel and was maintained to within ±0.2 °C as monitored by a digital thermometer (Omega) using a constantan/copper thermocouple inside the pressure vessel. The high-pressure probe was retuned and reshimmed after changes in pressure as needed. After every change of temperature, samples were allowed to equilibrate for several hours before spectra were collected.

The one-dimensional inversion transfer proton NMR experiments were performed using a simple selective inversion pulse sequence[21] and the following spectrometer parameters: 16K data points in the FID, a spectral width of 2000 Hz, 4 scans per spectrum, and an acquisition delay of 60 s. The spectrometer frequency was set onto the resonance of the exchange site to be inverted, the NMA amide or the water proton resonance. The selective inversion pulse used consisted of two $\pi/2$ pulses separated by a delay of length τ, such that $\tau = 1/(2\Delta\nu)$ where $\Delta\nu$ is the frequency difference between the NMA amide and water resonances.[21] This was followed by variable length delay ranging from 25 ms to 20 s and then a $\pi/2$ acquisition pulse. A complementary experiment where the other exchange site resonance was inverted was performed for each hydrogen exchange constant measurement.[19] The hydrogen exchange rate constants were then calculated by fitting the peak intensities from the resulting spectra to the McConnell equations using the MTNMR program obtained from H. Olin Spivey[22] (Figure 1). Error in the values of the rate constants was 10%.

Results

The rate constants for the hydrogen exchange reaction between 16 mol % NMA and water were determined as a function of pressure at pH 2.75, 5.0, and 7.5. The results of the three pressure experiments are shown in Figure 2. From the slopes of the plots in Figure 2, the apparent activation volume of hydrogen exchange ($\Delta V^{\ddagger}_{app}$) at each pH value was calculated from the equation

$$\Delta V^{\ddagger}_{app} = -RT(\partial(\ln k)/\partial(P))_T \qquad (3)$$

where R is the gas constant, T is temperature, and P is pressure.

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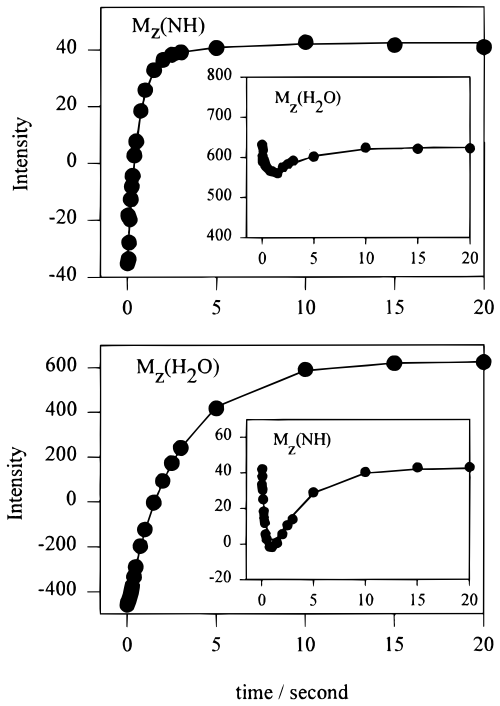

Figure 1. (top) Plot of the intensities of the amide (16 mol % NMA) and water proton resonances versus time at 66 °C, 1 bar, and pH 2.75 after inversion of the amide proton resonance. (bottom) Plot of the intensities of the amide and water proton resonances versus time after inversion of the water proton resonance. The fitting curves were drawn from the MTNMR program results.

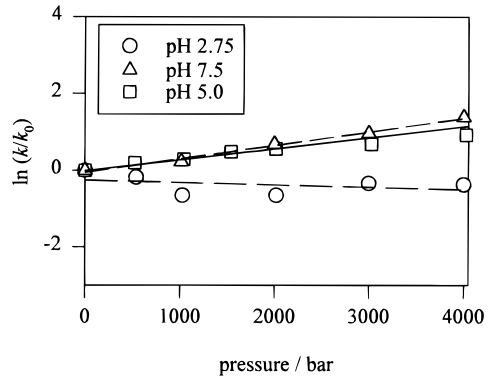

Figure 2. Plot of the natural log of the relative rate constant (k/k_0), where k_0 is the rate constant at 1 bar, versus pressure for the hydrogen exchange reaction between 16 mol % NMA and water at 66 °C and at pH 2.75, 5.0, and 7.5.

The values of $\Delta V^{\ddagger}_{app}$ determined were $+1.7 \pm 0.8$ cm^3 mol^{-1} at pH 2.75, -8.1 ± 0.8 cm^3 mol^{-1} at pH 5.0, and -10.0 ± 0.8 cm^3 mol^{-1} at pH 7.5. By application of the apparent activation volume equation (eq 3) directly to the rate constant equation (eq 2), an equation for the apparent activation volume is obtained which is the sum of contributions from the activation volumes of the three different hydrogen exchange reactions

$$\Delta V^{\ddagger}_{app} = (k_{H^+}10^{-pH}/k)(\Delta V^{\ddagger}_{H^+}) + (k_{OH^-}K_w10^{pH}/k)(\Delta V^{\ddagger}_{OH^-} + \Delta V_{K_w}) + (k_H[H_2O]/k)(\Delta V^{\ddagger}_{H_2O}) \quad (4)$$

where $\Delta V^{\ddagger}_{H^+}$ is the activation volume of the acid-catalyzed exchange reaction, $\Delta V^{\ddagger}_{OH^-}$ is the activation volume of the base-catalyzed exchange reaction, ΔV_{K_w} is the ionization volume of water, and $\Delta V^{\ddagger}_{H_2O}$ is the activation volume of the uncatalyzed exchange reaction.[15] At pH 2.75, the hydrogen exchange rate constant is dominated by the acid-catalyzed exchange reaction so that eq 2 reduces to $k = k_{H^+}10^{-pH}$ and then $\Delta V^{\ddagger}_{app} = \Delta V^{\ddagger}_{H^+} = +1.7$ cm^3 mol^{-1}. Likewise, at pH 7.5, the base-catalyzed exchange reaction dominates, so that $k = k_{OH^-}K_w10^{pH}$, and $\Delta V^{\ddagger}_{app} = \Delta V^{\ddagger}_{OH^-} + \Delta V_{K_w} = -10.0$ cm^3 mol^{-1}. Using the literature value of the ionization volume of water (-20.9 cm^3 mol^{-1})[23] gave $\Delta V^{\ddagger}_{OH^-} = +11.0$ cm^3 mol^{-1}. These values are reasonably comparable to the values of 0 ± 1 cm^3 mol^{-1} for the acid-catalyzed activation volume and $+6 \pm 1$ cm^3 mol^{-1} for the base-catalyzed activation volume determined by Carter et al.[15] for polylysine at 2 °C. The small difference in values may be attributed to the different systems studied and also possibly to the different temperatures used.

Table 1. Values of the Observed and Calculated Hydrogen Exchange Rate Constants at Various pH Values and Concentrations for Hydrogen Exchange between NMA and Water at 66 °C and at 1 bar

pH	[H_2O]/M	[NMA]/M	k_{obsd}/s^{-1}	k_{calcd}/s^{-1}
2.75	30.4	5.89	1.3220	1.3273
4.48	23.8	7.45	0.1029	0.0733
4.75	40.4	3.53	0.1603	0.1066
5.00	30.4	5.89	0.0930	0.0756
5.47	30.4	5.89	0.0959	0.0819
6.13	18.5	8.70	0.0289	0.0464
7.50	30.4	5.89	1.2990	1.8486
7.50	12.2	10.2	0.0862	0.0448
7.60	12.2	10.2	0.0563	0.0499
7.83	30.4	5.89	4.1530	3.8806

Next, the relative contributions of the three hydrogen exchange reactions at pH 5.0 and 66 °C were determined from the various values of the rate constants at 1 bar and various concentrations and pH values (Table 1). In order to use eq 2 to calculate the acid-, base-, and uncatalyzed hydrogen exchange rate constants, the value of the ionization constant has to be known for the different sample concentrations measured. Values of the ionization constant of pure water are available up to 60 °C.[24] Following the same procedure as Hvidt et al.,[8] the value of K_w for pure water at 66 °C was extrapolated to be 13×10^{-14} M^2. The concentration dependence of K_w was then given by[8]

$$K_w = (13 \times 10^{-14})([H_2O]/55.25)\exp[\alpha(\chi_{H_2O} - 1)] \quad (5)$$

where χ_{H_2O} is the mole fraction of water and α is a fitting parameter equal to $d \ln K_{H_2O}/d\chi_{H_2O}$, where K_{H_2O} is the dissociation constant of water. By fitting the values of the rate constants at 1 bar simultaneously to eqs 2 and 5, the values of the three hydrogen exchange rate constants and the α parameter at 66 °C were calculated to be $k_{H^+} = 7.11 \times 10^2$ M^{-1} s^{-1}, $k_{OH^-} = 4.78 \times 10^6$ M^{-1} s^{-1}, $k_{H_2O} = 2.07 \times 10^{-3}$ M^{-1} s^{-1}, and $\alpha = 11.25$. Comparing these values to those of Hvidt et al. for NMA at 78 °C ($k_{H^+} = 2.90 \times 10^3$ M^{-1} s^{-1}, $k_{OH^-} = 1.54 \times 10^7$ M^{-1} s^{-1}, $k_{H_2O} = 6.00 \times 10^{-3}$ M^{-1} s^{-1}, and $\alpha = 10.85$),[8] we observed that the values of the three rate constants are lower at the lower temperature as one would expect from the activation energies.

Using the value of α and eq 5, K_w was calculated to be 1.18×10^{-14} M^2 for 16 mol % NMA in water at 66 °C. The rate constant for 16 mol % NMA at 66 °C at pH 5.0 was then calculated using the values of the three hydrogen exchange rate constants and eq 2. The contribution of each of the three types of hydrogen exchange reactions to the total rate constant at pH

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5.0 was found by dividing each of the three terms in eq 2 by the total rate constant at pH 5.0, and the results were acid-catalyzed, 9.4%; base-catalyzed, 7.5%; and uncatalyzed, 83.1%. Hvidt et al. had found that the uncatalyzed exchange reaction contributes 75% of the exchange rate constant at pH 5.0 at 78 °C.[8] They concluded that since their value of the activation energy of exchange for the uncatalyzed exchange reaction is smaller than the activation energies of the acid- and base-catalyzed exchange reactions, the contribution of the uncatalyzed hydrogen exchange reaction to the hydrogen exchange rate constant would increase in importance as the temperature decreases.[8] Using Hvidt's values of the rate constants at 78 °C and the activation energies of exchange, we estimated that the contribution of the uncatalyzed hydrogen exchange reaction should increase from 75% at 78 °C to 81% at 66 °C, which agreed with our results.

The activation volume of the uncatalyzed hydrogen exchange reaction was calculated using eq 4 from the value of the apparent activation volume at pH 5.0 to be equal to -9.0 ± 1.8 cm^3 mol^{-1}. This is the first time that the value of the activation volume of uncatalyzed amide−water hydrogen exchange reaction has been determined.

Discussion

In addition to providing new information about the uncatalyzed hydrogen exchange reaction between NMA and water, this study has revealed significant information toward the analysis of pressure-dependent hydrogen exchange behavior. First, the current results confirm Hvidt's conclusion that the water-catalyzed exchange reaction increases in relative importance as the temperature decreases.[8] As mentioned previously, the extent to which the water-catalyzed, or uncatalyzed, exchange reaction contributes to the hydrogen exchange constant has not been entirely agreed upon. For example, an isotope exchange study by Englander et al.[16] determined that for polyalanine at 20 °C the pH independent exchange reaction term accounted for 40% of the exchange rate constant at the pH_{min}. Another study of polyalanine at 25 °C by Gregory et al.[17] determined that the water-catalyzed exchange reaction contributes 26%−38% of the rate constant near the pH_{min} depending on the type of isotope exchange used. This work also examined the temperature dependence of the exchange rate constants and concluded from values of activation energies of exchange that the contribution of the water-catalyzed reaction to the rate constant would increase and become more significant at high temperatures.[17] This conclusion was disputed by Roder et al., who measured the hydrogen exchange rate constants of the amide protons in unfolded BPTI proteins at 86 °C.[25] They determined that there was no contribution due to water catalysis at this temperature by fitting their hydrogen exchange data for unfolded BPTI at various pH values to the hydrogen exchange rate constant equation minus the water-catalyzed exchange term

$$k = k_{H^+}10^{-pH} + k_{OH^-}K_w10^{pH} \qquad (6)$$

and observing qualitatively that a good fit was obtained. The authors stated that the discrepancy between their observation of no water catalysis of amide−water exchange at high temperature and the previous observations by others of water catalysis at low temperatures could be explained if the activation energy of the water-catalyzed exchange reaction was lower than the activation energies of the acid- and base-catalyzed exchange reactions.[25] The authors of this paper referred to the study by Hvidt et al.[8] which indeed reported a lower activation energy for the water-catalyzed exchange reaction than for the acid- and base-catalyzed exchange reactions for amide−water hydrogen exchange between *N*-methylacetamide and water, indicating that any effect from water catalysis would be larger at lower temperatures and more likely to be measurable, but they did not discuss the implications of these results. Several other protein studies in the literature have used the Roder et al. paper to state that water catalysis can be neglected but do not provide any independent evidence that there is no effect by water catalysis. In addition, a recent study by Bai et al.[12] stated that for various dipeptides at 5 °C the water-catalyzed exchange term contributes approximately 25% of the exchange rate constant at the pH_{min} and is therefore not insignificant.

Secondly, the value of the activation volume calculated in this study can be used in hydrogen exchange studies of peptides and proteins at pressures above ambient pressure.[5] Again, this is the first time that the value of the activation volume of uncatalyzed amide−water hydrogen exchange reaction has been determined. The negative value of the water-catalyzed activation volume of exchange which we calculated indicates that the water-catalyzed reaction rate constant will increase with pressure becoming more important at high pressure. This is very relevant to hydrogen exchange studies of cold-denatured proteins which utilize both high pressures and low temperatures.[13,14]

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