Effect of a Hydrophobic Environment on the Hydrogen Exchange Kinetics of Model Amides Determined by ^{1}H-NMR Spectroscopy

Leo Spyracopoulos and Joe D. J. O'Neil*

Contribution from the Department of Chemistry, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada

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Abstract: In proteins, backbone amide hydrogen exchange rates can reveal important information about protein structure and dynamics. In order to assess the possible effects of detergent on the hydrogen exchange rates of detergent-solubilized proteins, we have synthesized a series of model aliphatic amides and measured their amide proton exchange rates in water and sodium dodecyl sulfate (SDS) micelles. Hydrogen exchange was measured using steady-state saturation-transfer proton nuclear magnetic resonance (NMR) spectroscopy. The extent of interaction of the model compounds with SDS was determined by measuring the longitudinal relaxation times, chemical shifts, and temperature coefficients of the amide protons. The sensitivity of the amide proton chemical shift to hydrogen bonding was found to be a particularly useful indicator of the extent of interaction of the amides with the hydrophobic core of the micelle. It is argued that the measured hydrogen exchange parameters reflect the dynamics of exchange of the molecules between bulk solvent and the surface and core of the micelle. Two major effects of the micelle on hydrogen exchange were measured: First, an electrostatic effect due to the negatively charged sulphate groups of SDS causes a decrease of the local pH at the micellar surface. This effect increases with the affinity of the amides for the micelle and enhances acid-catalyzed exchange and decreases base-catalyzed exchange. Second, a hydrophobic effect of the core of the micelle causes a depression of the minimum rate of exchange, which, for the most nonpolar molecule, is 25-fold. This effect is similar in magnitude to the slowing of exchange by hydrogen bonding reported by Perrin *et al.* (*J. Am. Chem. Soc.* **1990**, *112*, 3122–3125). The hydrophobic effect is likely to be an important factor in the slowing of exchange in the solvent-excluded interior of water-soluble proteins as well as in the exchange of detergent-solubilized peptides and proteins.

Introduction

The proton exchange of backbone amides in proteins has been of interest since it was first recognized that exchange rates reveal structural and dynamic information about proteins.[1-3] Generally, hydrogen exchange in folded proteins is considered to depend on structural fluctuations that expose buried and/or hydrogen-bonded amide protons, allowing for their exchange with solvent.[4] Recently, a wealth of hydrogen exchange information has become available with the introduction of multidimensional, multinuclear NMR[5] spectroscopy, which has permitted the measurement of exchange rates for individual amides in small proteins. The high-resolution maps of hydrogen exchange rates have uncovered dynamic processes such as fraying at the end of the N-terminal helix in cytochrome *c*[6] and fraying of the SDS-solubilized M13 coat protein helix.[7] Hydrogen exchange measurements have also found their way into protein solution structure determination where slowly exchanging amide protons are constrained to be hydrogen bonded in distance geometry and simulated annealing programs.[8,9] A powerful new tool in the characterization of protein-folding intermediates is pulse-labeled hydrogen exchange measurement.[10,11] The protein, initially in D_2O-denaturant, is rapidly diluted and allowed to fold for a variable time period, after which it is exposed to a pulse of H_2O. The number of amide sites protonated and the extent of protonation depend upon the time scale of the folding process for various regions of the polypeptide.

In order to determine the physical and chemical variables that influence exchange rates in the absence of protein structure, the hydrogen exchange kinetics of several model systems have been investigated.[4] For example, the pH and temperature dependence of the hydrogen exchange rates of secondary amides such as *N*-methylacetamide in aqueous solvent were measured in order

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(5) Abbreviations used: δ, chemical shift in parts per million from DSS; δ_{H2O}, chemical shift in parts per million from DSS in aqueous solution; δ_{SDS}, chemical shift in parts per million from DSS in detergent solution; DSS, disodium 2,2-dimethyl-2-silapentane-5-sulfonate; FAB, fast atom bombardment; f^C, fraction of time amide spends in micellar core; f^S, fraction of time amide spends at micellar surface; f^W, fraction of time amide spends in bulk solvent; k, Boltzmann constant; k_H, amide hydrogen exchange rate *via* acid catalysis; k_{min}, hydrogen exchange rate at the pH of minimum exchange; k_{OH}, amide hydrogen exchange rate *via* base catalysis; k^D_H, amide hydrogen exchange rate *via* acid catalysis in detergent solution; k^D_{min}, hydrogen exchange rate at the pH of minimum exchange in detergent solution; k^D_{OH}, amide hydrogen exchange rate *via* base catalysis in detergent solution; $k^D_{H,N}$, acid catalyzed exchange rate in detergent normalized the rate in water; $k^D_{OH,N}$, base-catalyzed exchange rate in detergent normalized to the rate in water; k^W_H, amide hydrogen exchange rate *via* acid catalysis in aqueous solution; k^W_{min}, hydrogen exchange rate at the pH of minimum exchange in aqueous solution; k^W_{OH}, amide hydrogen exchange rate *via* acid catalysis in aqueous solution; NMR, nuclear magnetic resonance; ppm, parts per million; pH_{min}, pH at the minimum rate of hydrogen exchange; PROXYL, 2,2,5,5-tetramethyl-1-pyrrolidine-*N*-oxyl; SDS, sodium dodecyl sulfate; T_1, longitudinal relaxation time; T_2, transverse relaxation time; $T_{1,aq}$, longitudinal relaxation time of solubilizate in the aqueous phase; $T^p_{1,aq}$, longitudinal relaxation time of solubilizate in the aqueous phase in the presence of paramagnetic ion; $T_{1,det}$, longitudinal relaxation time of solubilizate in the presence of SDS; $T^p_{1,det}$, longitudinal relaxation time of solubilizate in the presence of SDS and paramagnetic ion.

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to gain an understanding of hydrogen exchange in unfolded peptides and proteins.[12,13] Substituent and inductive effects on exchange were measured for secondary amides such as acyl-substituted amides and *N*-methylacetamides with various degrees of halogen substitution.[14] The inductive effects of nearest neighbor peptide bonds and the side chains for all twenty amino acids were also measured in small peptides.[15,16] In order to model the apolar environment of the interior of a protein, the exchange rates of model amides were measured in a dioxane–water mixed solvent;[12,17] the rate of exchange in this apolar environment was decreased due to an increase in pK_w. Electrostatic interactions were studied by comparing the rates of exchange of homopolymers such as neutral poly(DL-alanine) and positively charged poly(DL-lysine).[18] On the basis of the observed exchange rates, it was concluded that the charged lysine groups condense hydroxyl ions and raise the pH at the surface of the polymer. The effect of intramolecular hydrogen bonding was studied on a specially-designed molecule.[19] The molecule contained two primary amide groups constrained in a conformation that allows for the formation of an intramolecular hydrogen bond. Since the amide groups are primary, there are also two chemically equivalent amide protons which are not hydrogen bonded and thus provide a reference. It was found that catalysis of exchange by base was retarded 30-fold and catalysis by acid was relatively unaffected for the particular protons being studied.

SDS has long been used to solubilize peptides and proteins in order to facilitate their study by NMR in a membrane-mimetic environment.[20–22] If exchange rates in detergent solution are to be interpreted unambiguously, the effect of the micellar environment must be fully taken into account. To address this question, we have synthesized a series of model secondary amides of varying hydrophobicity in order to probe the SDS micelle. These studies have enabled us to correlate the measured rates of exchange with the extent and nature of interaction of the amides with SDS.

Experimental Section

Materials. SDS, DSS, 3-carboxy-PROXYL, and 5-amino-1-pentanol were purchased from Sigma Chemical Co. (St. Louis, MO). Perdeuteriated acetic acid was purchased from MSD Isotopes (Point Claire, Dorval, PQ, Canada). D_2O, 2-ethylhexanoyl chloride, isovaleryl chloride, valeryl chloride, *tert*-octylamine, hexylamine, and butylamine were purchased from Aldrich Chemical Co. (Milwaukee, WI). Isopropylamine was purchased from Eastman Kodak Co. (Rochester, NY).

Methods. Synthesis and Purification of Amides. The synthesis of molecule **1** (Figure 1) was initiated by addition of 5-amino-1-pentanol to anhydrous benzene which contained chlorotrimethylsilane. Valeryl chloride was added to an ice-cold solution of the silylated product in pyridine to produce *N*-(5-(trimethylsilyl)pentyl)pentanamide. Following desilylation with dilute HCl, *N*-(5-hydroxypentyl)pentanamide in cold methylene chloride was added to a solution of chlorosulfonic acid in methylene chloride, followed by treatment with methanolic sodium hydroxide to produce **1**. The solvent was evaporated under vacuum, and the product was purified by reverse-phase flash chromatography on a C_{18}-silica gel column using 70:30 H_2O–methanol for elution. The purity of **1** was checked by ^{1}H-NMR spectroscopy. The correct mass of the parent ion was checked with FAB mass spectrometry. All other amides were prepared by the addition of the appropriate acid chloride to an ice-cold solution of the primary amine in pyridine. The reaction products were extracted using a 1:1 water–ethylacetate mixture and purified by silica gel flash chromatography. The exception was amide **2**, which was purified by silica gel flash chromatography after evaporation of the reaction solvent on a rotary evaporator. The mobile phase was 75:25 hexane–ethylacetate for **2**, 65:35 hexane–ethylacetate for **3**, 60:40 hexane–ethylacetate for **4**, 75:25 hexane–ethylacetate for **5**, and 90:10 hexane–ethylacetate for **6**. The amides were characterized, and their purity was checked by ^{1}H- and ^{13}C-NMR spectroscopies.

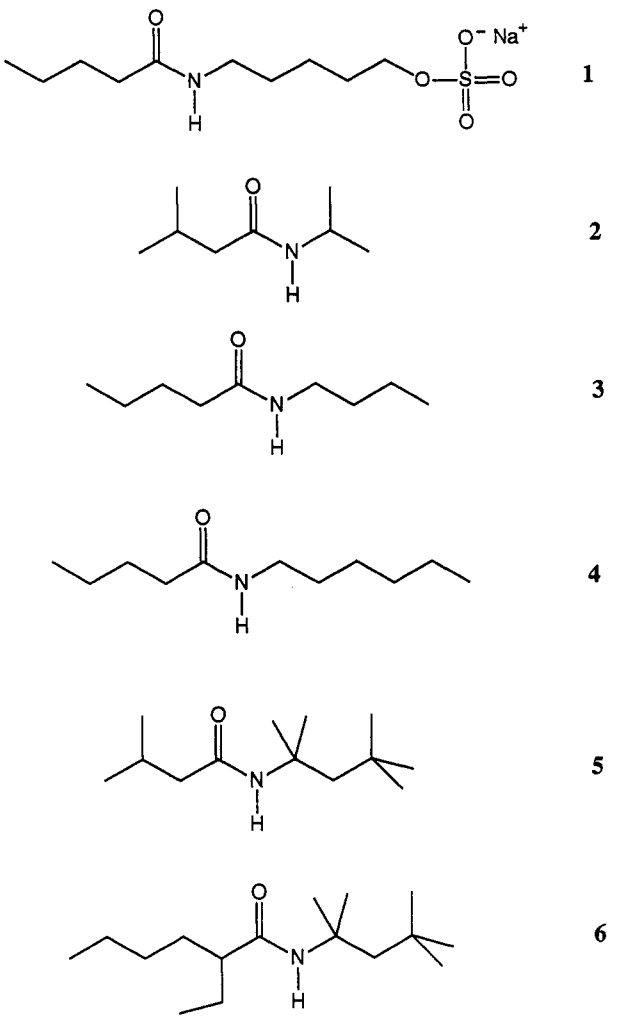

Figure 1. Structures of the amides employed in this study. *N*-(5-(sulfooxy)pentyl)pentanamide (**1**), *N*-isopropyl-3-methylbutanamide (**2**), *N*-butylpentanamide (**3**), *N*-hexylpentanamide (**4**), *N*-(1,1,3,3-tetramethylbutyl)-3-methylbutanamide (**5**), *N*-(1,1,3,3-tetramethylbutyl)-2-ethylhexanamide (**6**).

Saturation-Transfer Measurements of Amide Hydrogen Exchange. The method of H_2O saturation transfer was employed to determine the hydrogen exchange kinetics of the amides.[23–25] When the rate of exchange of amide protons with aqueous solvent is on the order of, or greater in magnitude than, the longitudinal relaxation rate, the saturation of the H_2O resonance will be transferred to the amide, resulting in a diminution of the amide intensity compared to the amide intensity in the absence of exchange. For pH titrations, solutions in 5-mm NMR tubes contained 5 mM amide in 90% H_2O/10% D_2O, 82 mM acetic acid-d_4, 30 mM phosphate, and DSS as the chemical shift reference. Proton spectra were acquired with a Bruker AM300 NMR spectrometer at 27 °C. The H_2O resonance was preirradiated for 2 s, the acquisition time was 2 s, the observe pulse was a composite pulse consisting of four 90° pulses with widths of 5.3 μs, and 64 scans were usually acquired. The pH titrations

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in detergent contained 50 mM SDS, ensuring that the surfactant concentration was well above the critical micelle concentration. The longitudinal relaxation times of the various amide protons in water and SDS solutions, at their respective pH_{min}, were measured with the standard inversion–recovery experiment incorporating presaturation of the water resonance. The T_1's were determined with 12 delays and 128 scans per acquisition.

Measurement of the Distribution Coefficient of Amide 2 in SDS. The mole fraction of amide **2** in the micellar phase was evaluated using an NMR paramagnetic method.[26] This method requires the measurement of solubilizate T_1's in water and SDS solutions in the presence and absence of paramagnetic ions. The method relies on the assumption that the observed T_1 of solubilizate in SDS solution is a weighted average due to rapid exchange between the aqueous and micellar phases. It is essential that the paramagnetic ion used to enhance the relaxation rates does not associate with either the solubilizate or the micelle. For this reason, 3-carboxy-PROXYL is used in SDS solutions since it is anionic and will be repelled by the sulfate groups of SDS. Proton T_1's for the low-frequency methyl resonance of amide **2** were measured in 5-mm NMR tubes with the standard inversion–recovery sequence on a Bruker AM300 spectrometer at 27 °C with a proton-selective probe. The T_1's were determined using 12 delays. The errors in the T_1 values are the standard deviations of duplicate measurements. The 90° pulse width was 5.3 μs, the acquisition time was 2 s, and 64 scans were usually acquired. Solutions contained 44 mM amide in D_2O. The concentration of SDS was 270 mM, and the concentration of 3-carboxy-PROXYL was 2 mM. The chemical shift reference was DSS. Solutions which contained the paramagnetic salt were adjusted to pH > 7 to ensure that 3-carboxy-PROXYL bore a negative charge.

Results

^{1}H NMR Characterization of the Amides. The structures of the various amides synthesized in this study are shown in Figure 1. The amide proton regions of the 300-MHz ^{1}H-NMR spectra of the aliphatic amides dissolved in water and SDS solutions are shown in Figure 2 and summarized in Table 1. All of the amide protons, except amides **5** and **6**, have similar spectra in water characterized by a broad peak approximately 15 Hz wide at half the peak height and a chemical shift of 7.9 ppm. Amides **5** and **6** are not soluble enough in H_2O to acquire an NMR spectrum. Addition of SDS has little effect on the amide proton resonance of molecule **1**. However, there are dramatic changes in the amide resonances of the rest of the aliphatic amides upon solubilization in SDS. The chemical shift of the amide proton of **2** moves 0.23 ppm to lower frequency in SDS solution (Figure 2). In addition, the resonance narrows to approximately 5 Hz wide at half-height and appears as a doublet due to resolved coupling to the methine proton. Similarly, the amide protons of **3** and **4** shift to lower frequency by 0.49 and 0.64 ppm, respectively, upon solubilization in SDS. The amide proton resonances of molecules **3** and **4** become sharper, with widths at half-height of about 4 Hz. The coupling to the methylene protons is observed, resulting in a splitting of the signals into triplets. The amide protons of amides **5** and **6** appear as singlets in SDS solution at 6.60 and 6.28 ppm, respectively. Since the differences in structure between amides **4**, **5**, and **6** are not greater than the differences among amides **1–4**, we assume that the resonance frequences of amides **5** and **6** in water are similar to the other amides at approximately 7.90 ppm. This suggests a detergent-induced shift of 1.30 ppm for amide **5** and 1.60 ppm for amide **6**. The 5.8-Hz line width of the amide resonance of **6** is slightly greater than that of the other amides (Table 1). In deuteriated chloroform all amides resonate at 5.6 ppm (data not shown).

In peptides and proteins, the hydrogen-bonding state of amide protons can often be determined by measuring the sensitivity of the amide chemical shift to temperature. The temperature coefficients for the aliphatic amide protons in water and SDS solutions are given in Table 1. In aqueous solution, the temperature coefficients of the amide protons are similar, having

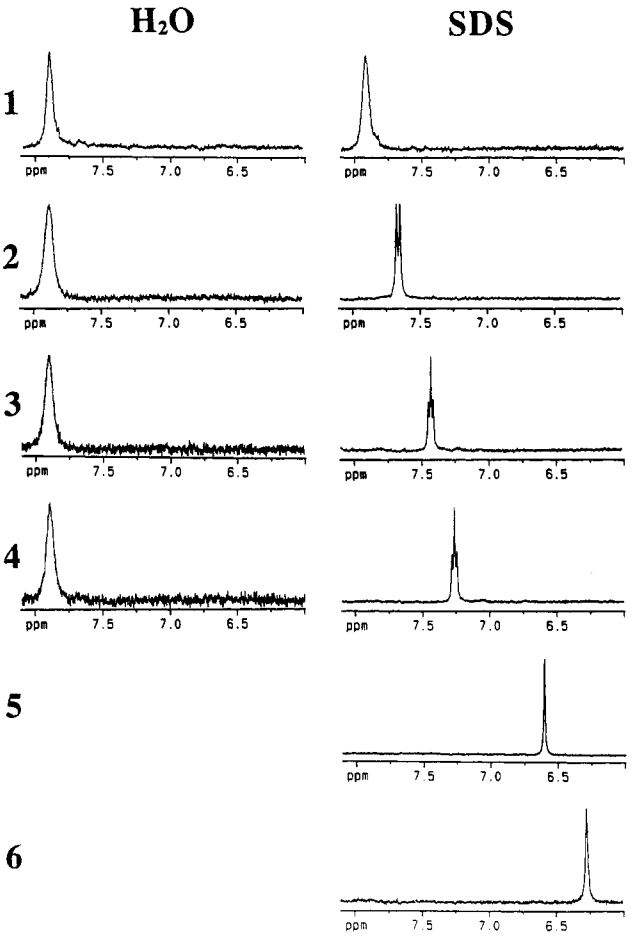

Figure 2. NH regions of 300-MHz ^{1}H-NMR spectra of the aliphatic amides at 27 °C. All spectra were recorded at the hydrogen exchange pH_{min}. Amide concentrations were approximately 5 mM. H_2O indicates spectra acquired in 90% H_2O–10% D_2O, 82 mM acetic acid-d_4, 30 mM phosphate; SDS indicates the addition of 50 mM detergent. The chemical shift reference was DSS. All spectra are the average of 64 scans and were processed with 0.3-Hz line broadening. Amides **1–6** are identified in Figure 1.

a value of approximately −10 to −12 ppb K^{-1}. In SDS, the temperature coefficients for amides **1–5** range from −9 to −12 ppb K^{-1}, similar to their respective coefficients in water. Interestingly, the −14.6 ppb K^{-1} temperature coefficient for amide **6** in SDS solution is larger than the coefficients for the other amides.

The amide proton longitudinal relaxation times, T_1, of amides **1–6** in water and SDS solutions are listed in Table 2. In water, the T_1's of the amide protons of **1–4** range from 0.7 to 1 s, with an apparent tendency toward shorter T_1 as molecular size increases. Except for amide **1**, the T_1's shorten when the molecules are solubilized in SDS. Just as in water, the T_1's tend to be shorter in SDS as molecular size increases; the amide proton T_1 of amide **6** in SDS micelles is the shortest of all the amides. The proton T_1's of SDS were also measured in the presence and absence of each of the amides. The measured T_1's in the presence of the amides ranged from 0.6 to 0.7 s for the α-CH_2, β-CH_2, and n-CH_2 resonances and was approximately 1.2 s for the terminal methyl group of SDS. The proton T_1's of SDS in the absence of amide were 0.6–0.8 s for the α-CH_2, β-CH_2, and n-CH_2 resonances and 1.3 s for the terminal methyl group.

During the pH titrations of amide intensity (see below), a dependence upon pH of the amide chemical shifts in SDS solution was observed (Figure 3). This effect was not observed in aqueous solution. For all of the amides except **1**, the chemical shift of the amide proton moves to higher frequency as the pH is lowered. The effect is most evident for the amide protons of **5** and **6**, which

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Table 1. Summary of the Chemical Shifts, Line Widths, and Temperature Coefficients of the Aliphatic Amide Protons in Water and SDS

amide		δ (ppm)[a]	$\Delta\delta$ (ppm)[b]	line width (Hz)[a]	mol wt	temp coefficient (ppb K^{-1})[c]
1	H_2O	7.93		15.61		−9.9 ± 0.1
			0.03		266	
	SDS	7.90		15.47		−9.9 ± 0.1
2	H_2O	7.89		20.33		−12.0 ± 0.2
			0.23		143	
	SDS	7.66		4.21		−11.6 ± 0.3
3	H_2O	7.91		18.25		−10.3 ± 0.1
			0.49		157	
	SDS	7.42		3.79		−9.6 ● 0.2
4	H_2O	7.90		16.82		−10.2 ± 0.1
			0.64		185	
	SDS	7.26		4.22		−8.9 ± 0.1
5	H_2O	(7.90)[d]		ND		ND
			1.30		213	
	SDS	6.60		3.40		−12.2 ± 0.3
6	H_2O	(7.90)[d]		ND		ND
			1.62		255	
	SDS	6.28		5.77		−14.6 ± 0.3

[a] Chemical shifts and line widths of the aliphatic amide resonances shown in Figure 1. [b] $\Delta\delta = \delta_{H_2O} - \delta_{SDS}$. [c] Temperature coefficients for the aliphatic amides were measured for samples prepared similarly to those used for pH titrations of the amide proton intensity, described in Methods. 300-MHz ^{1}H-NMR spectra were acquired in the temperature range 22–52 °C, in 5-deg steps. The chemical shifts of the amide protons were plotted as a function of temperature. A least-squares fit to the equation of a straight line was used to calculate the slope. The errors are the standard deviations of the fits. [d] Estimated chemical shift in H_2O; see text.

have measurable intensity at a pH less than 2.5 in SDS solution, unlike the other amides. The effect appears to increase in the order **1** ≪ **2** < **3** < **4** < **5** < **6** (see Figure 3).

To quantify the interaction between amide **2** and SDS, the time-averaged mole fraction of **2** in SDS micelles was measured via an NMR paramagnetic method.[26] The T_1 measured for the low-frequency methyl protons of **2** in water was 1.37 ± 0.01 s ($T_{1,aq}$) and was reduced to 0.80 ± 0.02 s ($T^p_{1,aq}$) by addition of the paramagnetic salt 3-carboxy-PROXYL. In the presence of SDS micelles, the low-frequency methyl proton T_1 was 0.79 ± 0.01 s ($T_{1,det}$), and this was reduced to 0.70 ± 0.02 s ($T^p_{1,det}$) by the addition of 3-carboxy-PROXYL. From the T_1 measurements and eq 3 in ref 26, the distribution coefficient of amide **2** was calculated as 0.67 ± 0.08, indicating that it is accessible to solvent about 33% of the time.

Hydrogen Exchange Rates. To determine the hydrogen exchange properties of the amides, pH titrations of amide intensity were carried out in water and SDS solutions, as described in the Methods section. The pH titrations are shown in Figure 4, and the hydrogen exchange kinetics of the model aliphatic amides are summarized in Table 2. Table 2 and Figure 4 show that the hydrogen exchange parameters, k_H, k_{OH}, k_{min}, and pH_{min} are all similar for the water-soluble amides **1**, **2**, **3**, and **4** in aqueous solution. Since amides **5** and **6** are similar in structure to amides **1**–**4**, we assume that their rates of hydrogen exchange, though not measurable by ^{1}H-NMR, are similar to those measured for amides **1**–**4**. Therefore, the hydrogen exchange parameters listed in Table 2 for amides **5** and **6** in water are the calculated averages of amides **1** to **4**.

In contrast to the situation in water, there are marked differences in the hydrogen exchange properties of the different amides in SDS. Figure 4D shows the hydrogen exchange curves for amide **4** in water and SDS solutions. The acid- and base-catalyzed limbs of the pH titration in SDS are shifted to higher pH compared to the titration in water. Solubilization in SDS causes a 42-fold increase in k_H and a 33-fold decrease in k_{OH}, resulting in an increase of the pH_{min} by 1.56 (Table 2). Since the changes in k_H and k_{OH} are about equal in magnitude but opposite in direction, there is little change in k_{min}. Detergent also enhances the acid-catalyzed exchange of amide **3** and suppresses its base-catalyzed exchange. But as Figure 4C shows, the effect on the acid-catalyzed limb is greater than the effect on the base-catalyzed limb; thus, the rate of acid catalysis is enhanced 25-fold and the rate of base catalysis is decreased only 3-fold, so that ΔpH_{min} is 0.97 (Table 2). The apparent 7.5-fold difference in the changes of k_H and k_{OH} causes an apparent enhancement of k_{min} by 2.7-fold.

Although the acid- and base-catalyzed limbs shift to higher pH for both amides **3** and **4**, it should be noted that k_H and k_{OH} for amide **3** are affected to a smaller extent than those for amide **4** in SDS. Similarly, the rates of acid and base catalysis of amide **2** in detergent are not affected to the same extent as those for amide **3**. Figure 4B shows that the base-catalyzed limb of amide **2** apparently does not shift upon detergent solubilization, though the acid-catalyzed limb is shifted to higher pH. Since k_H is enhanced 12-fold relative to the rate in water, and k_{OH} remains unchanged, k_{min} is elevated 3.9-fold (see Table 2 and Figure 4B). Amide **2** shows the largest k^D_{min}/k^W_{min} and the smallest ΔpH_{min} since detergent solubilization affects k_H to a greater extent than k_{OH} and this differential effect is the largest for amide **2** compared to amides **3** and **4**. The hydrogen exchange properties of amide **1** are the least affected by SDS (see Figure 4A). There is little effect of SDS on k_H, although k_{OH} is slightly enhanced (3-fold), the net effect being only a small change in k_{min}.

Whereas the effect of detergent on amides **2** and **3** is to enhance k_H to a greater extent than k_{OH} is depressed, the opposite effect is observed for amides **5** and **6** (see Figure 4E,F). Table 2 shows that the detergent depresses the k_{OH} of amide **5** by about 600-fold and enhances the k_H by only about 30-fold. The pH_{min} is thereby elevated by 2.13 units and the k_{min} is depressed by 4-fold. The greatest effect of detergent is on amide **6**. Figure 4F shows that the acid-catalyzed limb is moved from its expected position in water to slightly higher pH in SDS. The k_H for amide **6** in SDS solution is enhanced only 7-fold. On the other hand, the base-catalyzed limb is shifted to higher pH by at least 2 pH units. There is some uncertainty in the position of the midpoint of the base-catalyzed limb. This is because the SDS–amide complex precipitates from solution at about pH 12. At approximately pH 11.8, 75% of the amide intensity is still present and it is not certain if the observed diminution is due to precipitation or saturation transfer. From the pH titration in Figure 4F, we have calculated an upper limit for the k_{OH} of amide **6**, by assuming 50% exchange at pH 12, and included it in Table 2. From this value, it is possible to estimate a minimum depression of k_{OH} by detergent of 3300-fold, a minimum elevation of pH_{min} of 2.2 units, and a minimum depression of k_{min} by 25-fold.

Discussion

On the basis of the currently accepted model of aqueous micelles, it is likely that three distinct micellar regions are capable of influencing amide hydrogen exchange.[27,28] These are the diffuse Guoy–Chapman layer surrounding the micelle, the surface and Stern layer, and the hydrophobic core. In order to interpret the effect of SDS on amide hydrogen exchange rates, we must measure the extent and nature of interaction of solubilized molecules with the different regions of the micelle.

Amide Proton Longitudinal Relaxation Times. Except for amide **1**, all the aliphatic amide protons have shorter T_1's in SDS than in water (Table 2). However, the relaxation mechanisms in the two states is likely to be different. Since the amide protons are bound to a quadrupolar nucleus (^{14}N), their relaxation in water will have contributions from scalar relaxation of the second kind as well as from dipolar relaxation. If the correlation time

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Table 2. Summary of the Hydrogen Exchange Kinetics Measured by Saturation Transfer for the Aliphatic Amides at 27 °C

amide		T_1 (s)[a]	k_H (M^{-1} s^{-1})[b]	k^D_H/k^W_H[c]	k_{OH} (M^{-1} s^{-1})[b]	k^D_{OH}/k^W_{OH}[c]	k_{min} (s^{-1})[d]	k^D_{min}/k^W_{min}[c]	pH_{min}[d]	ΔpH_{min}[e]
1	H_2O[f]	0.69 ± 0.04	830 ± 51		$(2.2 \pm 0.2) \times 10^6$		0.009		5.29	
				1.3		3.0		1.2		−0.19
	SDS[g]	0.66 ± 0.03	1060 ± 98		$(6.7 \pm 0.7) \times 10^6$		0.011		5.10	
2	H_2O	1.04 ± 0.01	850 ± 140		$(2.1 \pm 0.3) \times 10^6$		0.008		5.30	
				11.7		1.1		3.9		0.52
	SDS	0.69 ± 0.04	$(1.0 \pm 0.1) \times 10^4$		$(2.4 \pm 0.2) \times 10^6$		0.031		5.82	
3	H_2O	0.98 ± 0.02	605 ± 66		$(2.0 \pm 0.2) \times 10^6$		0.007		5.24	
				24.8		0.3		2.7		0.97
	SDS	0.66 ± 0.06	$(1.5 \pm 0.2) \times 10^4$		$(5.9 \pm 0.8) \times 10^5$		0.019		6.21	
4	H_2O	0.71 ± 0.02	480 ± 40		$(3.5 \pm 0.3) \times 10^6$		0.008		5.07	
				41.7		0.03		1.1		1.56
	SDS	0.46 ± 0.02	$(2.0 \pm 0.2) \times 10^4$		$(1.1 \pm 0.1) \times 10^5$		0.009		6.63	
5	H_2O	ND	[688][h]		$[2.2 \times 10^6]$[h]		[0.008][h]		[5.25][h]	
				{29.1}[i]		{1.6×10^{-3}}[i]		{0.25}[i]		{2.13}[i]
	SDS	0.31 ± 0.01	$(2.0 \pm 0.3) \times 10^4$		3600 ± 600		0.002		7.38	
6	H_2O	ND	[688][h]		$[2.2 \times 10^6]$[h]		[0.008][h]		[5.25][h]	
				{7.0}[i]		{3×10^{-4}}[i]		{0.04}[i]		{2.21}[i]
	SDS	0.20 ± 0.01	4800 ± 900		574[j]		3×10^{-4}[j]		7.46[j]	

[a] The errors in the T_1 values are the standard deviations of duplicate or triplicate measurements. [b] The pH dependence of amide proton intensity due to saturation transfer was fit to eq 2 (ref 38) and the T_1 values given in the table using a nonlinear least-squares fitting procedure in order to determine the second-order rate constants k_{OH} and k_H. [c] D indicates detergent; W, water. [d] The pH_{min} and k_{min} values were calculated using eqs 4 and 5 (ref 39), respectively. The errors are the standard deviations of the fits. [e] $\Delta pH_{min} = pH_{min}(SDS) - pH_{min}(water)$. [f] H_2O indicates measurements carried out in buffered 90% H_2O–10% D_2O. [g] SDS indicates measurements carried out in a similar fashion but with the addition of 50 mM SDS. [h] Numbers in square brackets are the averages of the exchange parameters for amides **1**–**4** in water (see text). [i] Numbers in curly brackets are values calculated using the experimentally measured exchange parameters for amide **5** or **6** in SDS and the averages of the exchange parameters of amides **1**–**4** in water. [j] The value of k_{OH} for amide **6** in water was calculated by assuming 50% exchange at pH 12 (see Figure 4F). This value was then used to calculate the k_{min} and pH_{min} (see text).

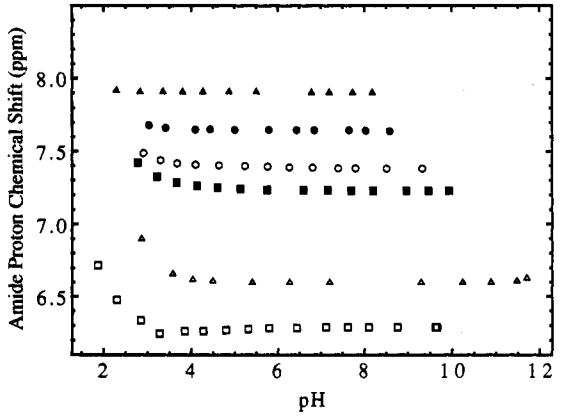

Figure 3. The pH dependence of amide proton chemical shifts for the aliphatic amides in SDS. The chemical shifts of amides **1** (▲), **2** (●), **3** (○), **4** (■), **5** (△), and **6** (□) were measured from spectra acquired during pH titrations of the amide intensity (see Figure 4).

of the molecule is lengthened by binding to a micelle, the ^1H and ^{14}N nuclei will become decoupled, due to the shortened T_1 of nitrogen, thereby removing any contributions to the proton relaxation by scalar relaxation. Thus, it is likely that the contribution to T_1 by scalar relaxation is removed by solubilization in SDS micelles and that the T_1 is shorter in amides **2**–**6** because of more efficient dipolar relaxation in SDS compared to that in aqueous solution. The enhancement of the amide proton relaxation rates in detergent solution is therefore evidence of an interaction of the amides with micelles which results in a lengthening of their rotational correlation times. This is consistent with the generally accepted view of micellar structure in which a spherical aggregate of about 100 detergent monomers can persist in solution for time scales on the order of milliseconds,[29] which would be expected to have a slow tumbling rate compared to small molecules in water. The effect of SDS on amide proton T_1 (Table 2) suggests a ranking of the molecules in terms of their increasing affinity for the micelle accordingly: **1** ≪ **2** < **3** < **4** < **5** < **6**. The longitudinal relaxation times of the proton resonances of SDS are approximately the same in the presence and absence of amide, suggesting that none of the amides cause a large change in micellar structure or dynamics.

Amide Proton Chemical Shifts and Line Widths. A more informative indicator of the extent of interaction of the various amides with the detergent micelle is the chemical shift differences between the amide protons in water and detergent solutions. The resonance frequencies of the aliphatic amide protons shift to lower frequency in SDS solution, the magnitude of the changes dependent on the size, and presumably the hydrophobicity, of the amide (see Figure 2 and Table 1). These chemical shift changes are reminiscent of the shifts to lower frequency of the hydroxyl proton of ethyl alcohol upon dilution with carbon tetrachloride, first observed by Arnold and Packard.[30] The effect is likely due to the dilution of hydrogen-bonding interactions, which can deshield exchangeable hydroxyl and amide protons. This suggests that the differences in SDS-induced chemical shift changes among the amides depend upon the relative affinities of the amides for the micellar core. If the amide is very hydrophobic, it will be primarily associated with the micellar core, diminishing hydrogen bonding with aqueous solvent and moving the amide chemical shift to lower frequency. On this basis, it would appear that the hydrophobicities of the model aliphatic amides, and their affinity for the micellar core, increase in the order **1** ≪ **2** < **3** < **4** < **5** < **6** (see Table 1). For example, the amide proton chemical shift of molecule **1** does not differ between aqueous and detergent solutions, indicating that because of its negative charge, this amide does not interact with SDS. Amide **6** is the most insoluble of the molecules in water. In addition, the resonance frequency of its amide proton in SDS solution is the lowest of all the amides. This suggests that amide **6** interacts only weakly with water and has a strong affinity for the nonpolar core of the micelle. In fact, this was suggested above by the T_1 measurements (Table 2). Thus, amide proton chemical shifts are apparently a highly sensitive indicator of the accessibility to water of the detergent-solubilized molecules.

Interestingly, most of the amide proton resonances in detergent are narrower than in aqueous solution (Figure 2). In water, scalar coupling of the amide protons to ^{14}N results in line broadening of the amide resonance due to quadrupolar relaxation of the

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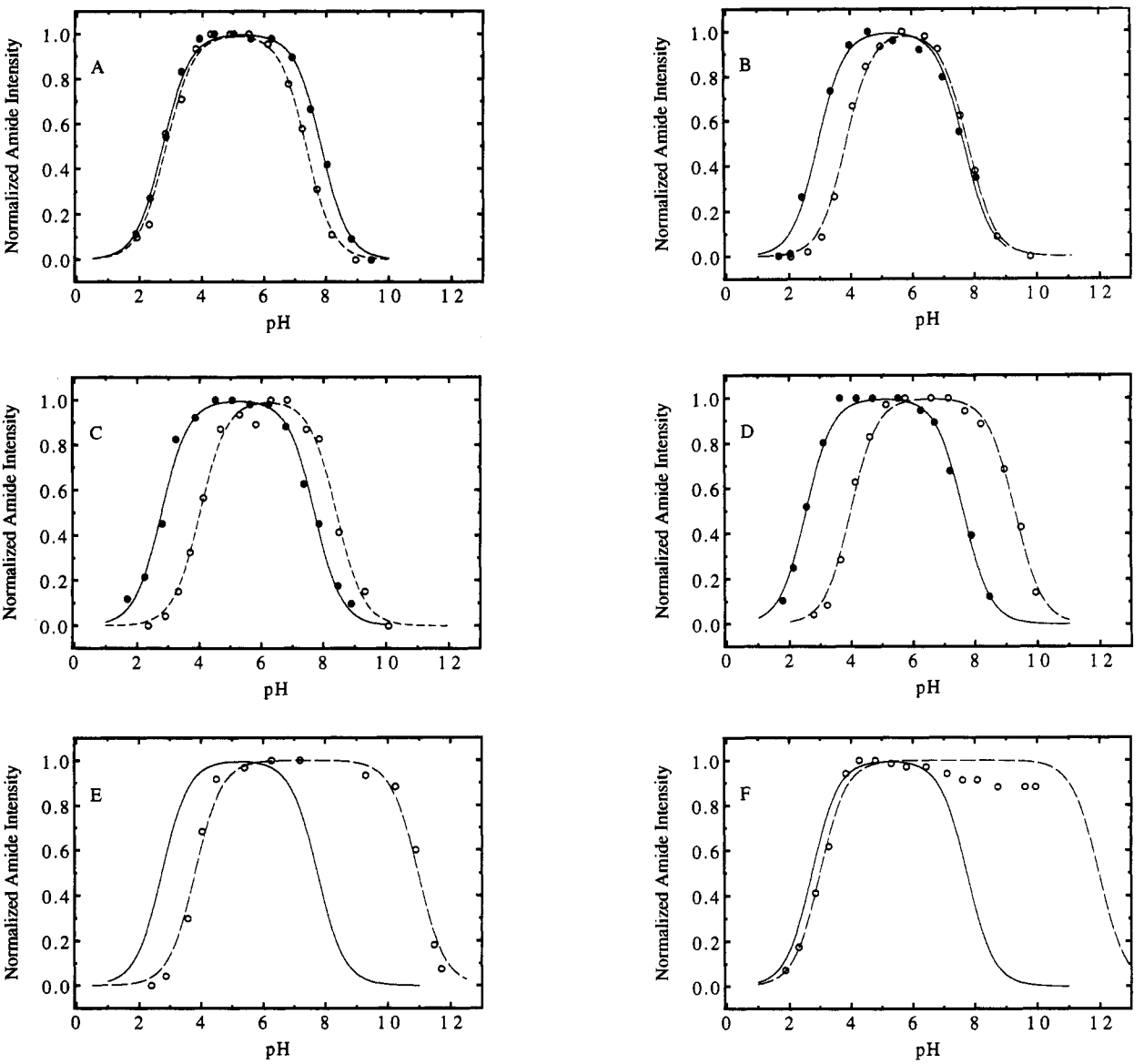

Figure 4. The pH dependence of amide proton intensity due to saturation transfer for the aliphatic amides (A) **1**, (B) **2**, (C) **3**, (D) **4**, (E) **5**, and (F) **6** in buffered H_2O (–●–) and with the addition of 50 mM SDS (--○--). The solid and dashed lines through the points are nonlinear least-squares fits of the data using eqs 1 and 2 in ref 38 and the data given in Table 2. The solid curves in E and F are calculated using the average values of k_H and k_{OH} of amides **1**–**4** in water. The dashed curve in F is a least-squares fit of the data combined with the assumption that amide intensity is reduced to half at pH 12.0.

nitrogen.[31,32] This was confirmed by the 300-MHz proton NMR spectrum of amide **2** in 90:10 H_2O–D_2O at 5 °C, in which the amide proton resonance becomes sharper and the coupling to the methine proton is resolved (data not shown). In SDS, provided that the association of amide with detergent is intimate, the rotational correlation time of the amide molecules may be significantly lengthened. In this case ^{14}N relaxation is enhanced, effectively decoupling the ^1H from its attached ^{14}N, thereby sharpening the amide proton resonances in SDS (see Figure 2). The amide proton line width of molecule **1** is nearly the same in aqueous and detergent solutions, indicating that it does not interact with SDS.

Temperature Coefficients of Amide Proton Chemical Shifts. The temperature dependence of hydrogen-bonded proton resonances has been attributed to an energy separation between hydrogen-bonded and non-hydrogen-bonded states of the order of kT.[33,34] In the limit of fast exchange between the two states a single peak is observed, whose resonance frequency is a weighted average of the resonance frequencies of the two states. A change in temperature will alter the populations of the hydrogen-bonded and nonbonded states, giving rise to changes in the chemical shift of the resonance of interest.

The measured temperature coefficients for the model amides are given in Table 1. The temperature coefficients for most of the amides in water and SDS solution fall near the range –9 to –12 ppb K^{-1}. In proteins and peptides temperature coefficients in this range are usually taken as evidence that backbone amides are not intramolecularly hydrogen bonded but interact with water.[35] This interpretation suggests that none of the model amides experience significant intermolecular hydrogen bonding in either water or SDS solutions. However, the chemical shift of amide **6** in SDS is more sensitive to temperature than any of the other amides (Table 1). On the basis of the chemical shift and T_1 of the amide proton of **6** in detergent solution, it was

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argued that this amide interacts strongly with the micellar core and is less extensively hydrogen bonded to water than the other model amides. The large, negative temperature coefficient for **6** suggests that it participates in hydrogen bonding that is weak in comparison with the other amides employed in this work.

Distribution Coefficient for Amide 2 in SDS Micelles. A quantitative measure of amide interaction with SDS micelles was obtained for molecule **2**. The distribution coefficient of 0.67 ± 0.08 may be compared to the values of 0.37 ± 0.07 and 0.67 ± 0.05 reported for 1-propanol and benzyl alcohol, respectively, in SDS solution.[26] The distribution coefficient for amide **2** indicates that this amide spends an appreciable amount of time in both the micellar phase (67%) and in bulk water (33%). It was not possible to measure the distribution coefficients for amides **3**, **4**, **5**, and **6** in SDS due to their low solubilities in water. However, the very low solubilities of amides **5** and **6** in water (see Figure 2) suggests that their distribution coefficients are near 1, whereas the higher solubilities of **3** and **4** in water (see Figure 2) suggests that their distribution coefficients are between 0.7 and 1. This conclusion is in broad agreement with the amide proton T_1 and chemical shift data which suggest a progressive transfer of amide from the bulk solvent to the detergent micelle as amide hydrophobicity increases from **1** to **6**.

Steady-State Measurements of Amide Proton Hydrogen Exchange Rates in Water and SDS Solutions. The second-order rate constants for specific acid and base catalysis, k_H and k_{OH}, respectively, for the water-soluble amides in aqueous solution are within the range expected for small secondary amides (Table 2). The rates of acid catalysis for amides **1**–**4** in aqueous solution are elevated relative to the values of 113 and 360 M^{-1} s^{-1} for *N*-methylformamide and *N*-methylacetamide, respectively, at 25 °C, ionic strength 0.1 M reported by Molday and Kallen.[14] Generally, electron-donating groups cause an increase in the rate of catalysis by acid by stabilizing the acid-catalyzed transition state, which bears a positive charge in the *N*-protonation mechanism. It has been suggested that the *N*-protonation mechanism for acid-catalysis in water is favored for secondary amides which bear electron-donating alkyl substituents.[36] The acid-catalyzed rates of exchange for amides **1**, **2**, **3**, and **4** in water are similar within the errors of the fits (see Table 2). This is not surprising since substituent effects are expected to be small due to the similarity of the alkyl groups for these amides. The electron-withdrawing effect of the sulfate group of amide **1** does not appear to depress the rate constant for acid catalysis of exchange in aqueous solution relative to the k_H's of amides **2**, **3**, and **4**. The sulfate group is seven bonds away from the amide group in this molecule, so it is likely that it does not have a large inductive effect on the measured k_H.

The second-order rate constants for specific base catalysis are equivalent within error for the secondary amides in aqueous solution (Table 2). The electron-donating effect of the alkyl substituents serves to decrease k_{OH} relative to the values of 3.39 × 10^7 and 4.19 × 10^6 M^{-1} s^{-1} for *N*-methylformamide and *N*-methylacetamide, respectively, at 25 °C, ionic strength 0.1 M,[14] by destabilizing the base-catalyzed transition state, which bears a negative charge.[36] The sulfate group of amide **1** does not appear to significantly affect the measured k_{OH} in water. In accordance with the results for acid catalysis, the differences in substituent effects on k_{OH} are small for the various amides in aqueous solution. On this basis, it is reasonable to use the averages of the rate constants of amides **1**–**4** to estimate the k_H and k_{OH} of amides **5** and **6** in water (see Table 2).

The effect of aqueous micelles on organic reaction rates has been studied in some depth.[37] For example, it is well-known that reagent solubilization in the vicinity of an anionic micellar surface will enhance a reaction involving hydronium ions and, conversely, depress any reaction involving hydroxide ions. For hydrogen exchange the net effect will be an elevated pH_{min} but no change in the k_{min} and has been observed previously.[38,39] Table 2 shows that interaction with SDS elevates the pH_{min} of the amides, the extent of which appears to depend on their hydrophobicity, which in turn is reflected in the amide proton chemical shifts and T_1's in SDS. Thus, the order of the ΔpH_{min} is **1** ≪ **2** < **3** < **4** < **5** < **6**. The hydrophobic core of a detergent micelle might also be expected to restrict the access of charged catalysts and water to nonpolar solubilizates. In the hydrophobic core of the micelle it is possible that pK_w is increased and formation of the charged hydrogen exchange intermediates is unfavorable. This would have the effect of depressing k_H, k_{OH}, and k_{min}. The k_{OH} and k_{min} values of amides **5** and **6** are significantly depressed, suggesting that they interact strongly with the hydrophobic core of the micelle. However, the k_H values of amides **5** and **6** are enhanced compared to the rates of exchange of amides **1**–**4** in water. This can be explained by a combination of two micellar effects on the exchange rates: First, k_H is elevated and k_{OH} is depressed due to the electrostatic effect of the micellar surface. This raises the pH_{min} and leaves the k_{min} unchanged. Second, k_H and k_{OH} are both depressed by solvent exclusion in the hydrophobic core of the micelle. The net effect is a large depression of the k_{OH} and a small change in k_H, since the two effects partially cancel each other out.

That an electrostatic effect and solvent exclusion effect are most likely operating together for amides **5** and **6** points out that the measured exchange parameters are the weighted averages of exchange occurring in different environments. To illustrate the effects of dynamics of the measured exchange parameters, we will consider exchange to occur from bulk solvent (k^W), the micellar surface (k^S), and the micellar core (k^C) and ignore any minor effects that monomer detergent or the diffuse Guoy–Chapman layer might have on exchange. Then the observed rates of exchange catalyzed by acid (k^D_H) and base (k^D_{OH}) in detergent can be expressed in terms of their rates in each environment and the fraction (f) of time spent there:

$$k^D_{H,N} = f^W k^W_{H,N} + f^S k^S_{H,N} + f^C k^C_{H,N} \quad (1)$$

$$k^D_{OH,N} = f^W k^W_{OH,N} + f^S k^S_{OH,N} + f^C k^C_{OH,N} \quad (2)$$

In eqs 1 and 2 each of the exchange rates are normalized to their rates in water, *e.g.* $k^D_{H,N} = k^D_H/k^W_H$. Our results (Table 2) and earlier calculations[38] suggest that the pH at the surface of an SDS micelle is about 2 units lower than that in the bulk solvent, yielding estimates of $k^S_{H,N} = 100$ and $k^S_{OH,N} = 0.01$. Our data also indicate that the interior of a micelle can significantly reduce the concentrations of charged catalysts, suggesting estimates of $k^C_{H,N} = 0.0001 = k^C_{OH,N}$; this implies a 4-pH-unit difference between bulk solvent and the interior of the micelle. Substitution of these values into eqs 1 and 2 shows how partitioning of the amides between bulk solvent and the surface and core of the micelle could affect the measured exchange parameters. The results in Table 3 duplicate the enhancement of pH_{min} for all amides as well as the depression of k_{min} for amides **5** and **6** (compare k^D_H/k^W_H and k^D_{OH}/k^W_{OH} in Tables 2 and 3). They also illustrate why k_{min} is elevated for amides **2** and **3**. The micelle enhances k_H more than it depresses k_{OH} because acid-catalyzed exchange occurs predominantly when the amide is at the micellar surface, whereas base-catalyzed exchange occurs mainly in bulk solvent for molecules in rapid exchange between the three environments.

Although the partitioning of the amides suggested in Table 3 is in qualitative agreement with the measured effects of SDS on the chemical shifts, T_1's, and temperature coefficients of the amide

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Table 3. Enhancements for Various Distributions of the Aliphatic Amides among the Micellar Regions[a]

amide	f^W	f^S	f^C	$k^D_{H,N}$	$k^D_{OH,N}$
2	0.7	0.15	0.15	15	0.7
3	0.3	0.24	0.46	24	0.31
4	0.03	0.42	0.55	42	0.03
5	0.001	0.29	0.71	29	0.004
6	0.0001	0.06	0.94	6	0.0007

[a] The normalized hydrogen exchange rate constants were calculated using eqs 1 and 2 in the text and the fractions (f) listed in the table. The fractions were chosen which best reproduced the measured enhancements (k^D_H/k^W_H and k^D_{OH}/k^W_{OH}) for each of the amides listed in Table 2.

protons, they are not unique in explaining the exchange parameters. For example, localization of amide **4** at the surface of the micelle would enhance k_H and depress k_{OH} equally without affecting k_{min} (see Table 2). Just such as explanation was physically reasonable for cationic peptides[38] but seems less likely in this case. Another example is amide **2** since its paramagnetic distribution coefficient in water (0.33) is difficult to reconcile with the exchange data ($f^W = 0.7$). If we constrain f^W to 0.33, then $f^S = 0.12$ and $f^C = 0.55$ could approximate the exchange of amide **2**. However, it is difficult to see how the the water-soluble amide could spend more time in the core of the micelle than at the surface.[40] It should be pointed out though that the conditions in which the two measurements were carried out were significantly different (see Methods). There is also a discrepancy between the predicted enhancement of k_{OH} for amide **2** in Table 3 and the measured value in Table 2. This difference may be related to the 3-fold enhancement of k_{OH} observed for amide **1** in SDS, although it does not appear to interact with micellar detergent. Since amide **2** is quite water soluble, it is reasonable to expect to observe a similar effect on it. Finally, we have not attempted to account for the possibility that k^D_{OH} could be depressed to a greater extent than k^D_H in the micellar core, which would be the case if catalysis of exchange by acid proceeded *via* the imidic acid mechanism involving a neutral transition state.[41] As long as k^D_H and k^D_{OH} are both significantly depressed by the core of the micelle, differences in their depression would be difficult to detect.

pH Dependence of Amide Proton Chemical Shift. We have assumed that there are no significant changes in amide–detergent interactions during the pH titrations of amide proton intensity. This assumption seems reasonable since the amide molecules contain no ionizable groups and the detergent molecules become protonated below the pH range examined in this study. In water, over the pH range 3–11, the lack of change in the amide proton chemical shifts of molecules **1–4** is strong evidence that the above assumption is valid. However, a significant change in chemical shift of the amide protons of **5** and **6** and smaller changes in the shifts of amides **2–4** occur below pH 3.5 (Figure 3). The shift to higher frequency suggests that the amides become more exposed to water as the pH is lowered below 3.5. The possibility exists that the acid-catalyzed exchange, particularly of amides **5** and **6** (Figure 4E,F), occurs from a different time-averaged environment than that which exists at pH's above 3.5. This possibility would lead to an underestimation of the depression of k_H by the micellar core. On the other hand, there is no change in the chemical shifts of amides **5** and **6** (Figure 3) until 50% of acid-catalyzed exchange (Figure 4E,F) has occurred. This suggests that the change in the time-averaged environment below pH 3.5 for amides **5** and **6** does not significantly affect the determination of k_H.

Conclusions

The model compounds used in this study, and their amide protons in particular, are likely to be generally useful probes of the different microenvironments of detergent micelles. They might also prove useful as probes of lipid bilayers in structures such as small unilamellar vesicles. The results for amide **6** show for the first time that solvent exclusion by the hydrophobic interior of a detergent micelle can slow amide hydrogen exchange by at least 25-fold (see k^D_{min}/k^W_{min} for amide **6** in Table 2). Clearly, a possible slowing of backbone amide hydrogen exchange due to detergent must be kept in mind when interpreting hydrogen exchange measurements of peptides and proteins solubilized with SDS. However, the results also have implications for the interpretation of protein hydrogen exchange data. In proteins, hydrogen exchange is generally considered to depend on structural fluctuations that expose hydrogen-bonded and/or buried amide protons to solvent catalysts. The effect of a hydrogen bond has been quantified by Perrin *et al.*,[19] who showed that an intramolecular hydrogen bond can depress k_{OH} about 30-fold but that the effect on k_H is small. Herein, we have quantified the solvent-exclusion effect of a detergent micelle on hydrogen exchange and found it to be similar in magnitude to the hydrogen-bonding effect. However, by comparison a micelle is a much less stable entity than a folded protein, which suggests that the retardation of exchange by solvent exclusion is likely to be even greater in magnitude in the "interior" of globular proteins than in the core of a detergent micelle. For example, several very slowly exchanging amides, apparently not involved in intramolecular hydrogen bonding, have been observed to be deeply buried in the interior of an antibody V_L domain.[42]

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