Modeling Amino Acid Side Chains in Proteins: ^{15}N NMR Spectra of Guanidino Groups in Nonpolar Environments

Yaowu Xiao[§] and Mark Braiman*

Department of Chemistry, Syracuse University, Syracuse, New York 13244-4100

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Natural-abundance ^{15}N NMR spectroscopy on dodecylguanidine reveals solvent and protonation effects that model those that could occur for the arginine side chain in proteins. Our results demonstrate that the ^{15}N chemical shifts of the terminal guanine nitrogens strongly depend on the solvent chosen for measurements. A polar H-bond-donating solvent like water has strongly deshielding effects on the neutral guanidine group (with the latter acting predominantly as an H-bond acceptor). As a result, a substantial upfield shift occurs when neutral guanidine is dissolved instead in a non-H-bonding solvent (chloroform). These solvent effects can be as large as those induced by protonation changes. This limits the ability of ^{15}N chemical shifts to distinguish the protonation state of the arginine side chain, at least without specific knowledge of its environment. These results help to reconcile previous interpretations about the protonation state arg-82 in the M state of bacteriorhodopsin based on FTIR and ^{15}N NMR spectroscopy. That is, contrary to earlier conclusions from solid-state NMR, the side chain of arg-82 could undergo a deprotonation between the bR and M states, but only if it also experienced a significant decrease in the H-bonding character and polarity of its environment. In fact, the average ^{15}N chemical shift of the two N_η of arg-82 in bacteriorhodopsin's M intermediate (from the previous NMR measurements) is 17 ppm upfield from the corresponding value for the deprotonated arginine side chain in aqueous solution at pH >14, but only 3 ppm upfield from the value for deprotonated dodecylguanidine in chloroform.

Introduction

The guanidinium side chain of arginine is the least acidic cationic group among the 20 natural amino acids, with a pK_a value above 12. Under physiological conditions, the arginine side chain is therefore expected to be essentially 100% protonated, except possibly within very unusual environments in the interior of proteins. Recently, transiently deprotonated arginine residues at pH values near 7 have been hypothesized to play an important role in a few proteins, e.g., bacteriorhodopsin[1] and bovine cytochrome *c* oxidase.[2] Experimental attempts to directly detect deprotonated arginine side chains have given ambiguous results, in part due to the absence of clear NMR spectroscopic signals that would signal a deprotonation. Here we show, however, that this is largely due to the incorrect expectation that guanidine groups in aqueous solutions at high pH accurately model the NMR spectrum expected for a deprotonated arginine sequestered in a nonpolar environment within a protein.

In previous investigations of the deprotonation of arginine side chains, ^{15}N NMR experiments have been carried out using water or water−DMSO mixtures as solvent.[3,4] Unlike the alkylguanidinium side chain of arginine, which has a single tautomeric form (with multiple electronic resonance structures), the uncharged alkylguanidine group exists as three tautomers in equilibrium. An ^{15}N-shielding titration curve in aqueous solution thus shows simultaneous changes in the chemical shift values of all three nitrogens.[3] Although this curve has insufficient data points at very high pH values to accurately define the pK_a, it provides clear evidence of the effects of deprotonation on the ^{15}N NMR spectrum: downfield shifts of ∼15 and ∼3 ppm for N_η and N_ϵ, respectively.[3]

Applicability of the results from arginine in aqueous solution to arginine side chains in proteins is acceptable in most cases, because arginine is most likely found in a hydrophilic environment at or near the protein surface. However, in rare protein structures arginine is found buried in the interior, in an environment that is not well modeled by aqueous solution. Unfortunately, ^{15}N NMR studies of arginine in nonpolar solvents have never been reported. This is mainly due to the fact that arginine itself, with three ionizable groups in one molecule, has insufficient solubility in nonpolar solvents for ^{15}N NMR measurements. In contrast, strong solvent effects on ^{15}N chemical shifts have extensively been demonstrated for many compounds,[5−12] including pyridine[9] and simple alkylamines.[11,12]

On the other hand, vibrational spectra modeling the arginine side chain in various protein environments have recently been measured using alkylguanidinium salts under various solvent conditions.[1,13] These spectra model a range of possible structural changes for buried arginines in proteins, such as deprotonation, counterion substitution, and desolvation. Here we extend this work by measuring ^{15}N NMR spectra of dodecylguanidinium chloride and dodecylguanidine in different solvents, including the rather nonpolar chloroform.

Our results indicate that the ^{15}N chemical shift value of the guanidine nitrogens strongly depends on choice of solvent. Specifically, dodecylguanidine in a nonpolar solvent (C^2HCl_3) does not show the large ^{15}N chemical downshifts that are characteristic of its deprotonation in a hydrophilic environment. Instead, it exhibits ^{15}N chemical shifts that are remarkably

* To whom correspondence should be addressed. E-mail: mbraiman@syr.edu.

§ Current address: Department of Physics and Astronomy, Vanderbilt University, Station B #351807, Nashville, TN 37235.

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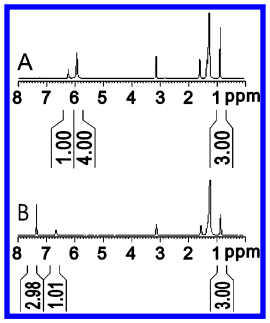

Figure 1. ^{1}H NMR spectra of saturated solutions of (A) dodecylguanidinium chloride in C^2HCl_3 and (B) dodecylguanidine in C^2HCl_3. The ~0.9 ppm bands in both spectra represent the signal from the terminal methyl group and are used as reference for integration. Chemical shift values were measured relative to TMS.

similar to those of the dodecylguanidinium ion in a polar (dimethyl sulfoxide, DMSO) environment. This indicates that great caution must be taken when trying to infer the protonation state of the arginine side chain from observed ^{15}N chemical shift values.

Experimental Procedure

Sample Preparation. Dodecylguanidinium chloride was prepared from the corresponding acetate salt (trivial name dodine), the major component of the commercial fungicide Syllite 65W, through extraction and recrystallization using 2-propanol. The acetate ion was then replaced by chloride to simplify the FTIR spectrum, which was used to determine the protonation states of the model compound. (Acetate and uncharged guanidine show overlapping vibrational bands near 1550 cm^{-1}.) The Cl^- replacement was carried out by dissolving the solid acetate salt in saturated aqueous NaCl, followed by extraction with $CHCl_3$. The latter solvent was evaporated to obtain dodecylguanidinium chloride. These steps (beginning with dissolution in aqueous NaCl solution) were repeated 2−3 times, yielding >95% counterion exchange as determined by the absence of a signal from the acetate methyl group in the ^{1}H NMR spectrum (see Figure 1 below), which would appear at ~2 ppm relative to TMS.

Dodecylguanidine (the deprotonated species) was produced by redissolving dodecylguanidinium chloride, to a concentration of 0.5 M (~9% w/w) in C^2HCl_3. This solvent had been preequilibrated with an equal volume of 1 M aqueous NaOH, and the two phases were mixed vigorously throughout the addition of the dodecylguanidinium chloride. The equilibrated C^2HCl_3 layer, containing most of the dodecylguanidine, was then separated from the aqueous phase. The C^2HCl_3 phase was dried for 2 days with anhydrous Na_2SO_4 to remove trace amounts of H_2O. The C^2HCl_3 solution was then partially evaporated under dry N_2 to give a saturated (~1 M) solution of the dodecylguanidine, which was used directly for ^{1}H and ^{15}N NMR measurements. High purities of dodecylguanidine and the dodecylguanidinium chloride could be verified by their ^{1}H NMR spectra, shown in Figure 1.

To obtain samples of dodecylguanidine in dry deuterated DMSO, ~0.5 mL of the latter was added to ~0.5 mL of the above saturated C^2HCl_3 solution. Thereafter, a dry N_2 stream was used to evaporate the C^2HCl_3, which could be removed essentially quantitatively. The remaining ~1 M solution of dodecylguanidine in DMSO was used directly for ^{15}N NMR measurements.

NMR Measurements. ^{1}H and ^{15}N NMR spectra were recorded of natural-isotope-abundance samples at 22 °C in 5-mm tubes, using a 600-MHz Bruker ADVANCE spectrometer (operating at 60.8 MHz for direct ^{15}N detection). For ^{15}N measurements, an external reference of saturated $(^{15}NH_4)_2SO_4$ (Cambridge Isotope Laboratories, Inc.) in H_2O was used. Normal operating conditions included a 3.5 μs pulse width and a 10 s pulse delay, with broadband ^{1}H decoupling.

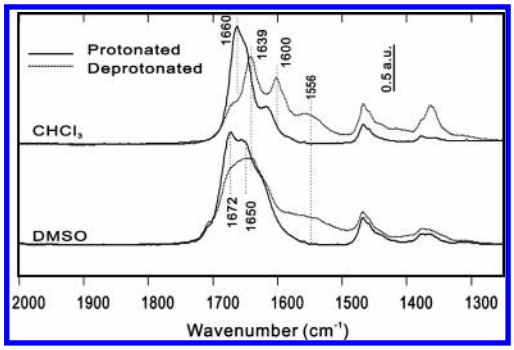

Figure 2. FTIR spectra of dodecylguanidinium (solid lines) and dodecylguanidine (dotted lines) in C^2HCl_3 (top) and d_6-DMSO (bottom). Labels and vertical lines indicate positions of band maxima.

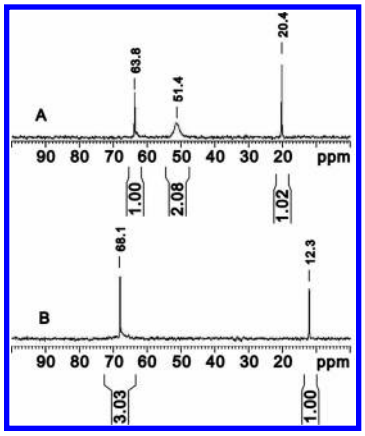

Figure 3. ^{15}N NMR spectra of saturated arginine at pH 7 (A) and 14.1 (B) in 4:1 $H_2O/^2H_2O$. Chemical shift values were measured relative to $(^{15}NH_4)_2SO_4/H_2O$.

FTIR Measurements. FTIR spectra (Figure 2) were obtained primarily to confirm the protonation states of the NMR samples. Solution spectra were measured on a Nicolet 860 spectrophotometer with 2-cm^{-1} resolution, in a demountable liquid cell with BaF_2 windows and 10 μm path length, and using pure solvent at 5 μm path length as a background. As a final correction to reduce residual solvent bands, an absorbance spectrum of pure solvent was added or subtracted with an adjustable scaling factor, until the baseline was essentially flat.

Spectra of arginine in the deuterated water used for our NMR work are strongly affected by H/D exchange with the deuterated NMR solvent, and are therefore not directly comparable to those in the nonexchanging solvents presented in Figure 2. However, FTIR measurements on similarly prepared arginine samples in undeuterated water at pH 7 and 14 (not shown) exhibited the same features as in previously published IR spectra of aqueous arginine.[14]

Results

^{15}N NMR Spectra of Arginine in Aqueous Solution. Figure 3 shows the ^{15}N NMR spectra of saturated aqueous l-arginine solution at two different pH values. At pH 7 (Figure 3A), arginine shows three ^{15}N resonances, assignable to the α-amino nitrogen (20.4 ppm), the two terminal (N_η) guanidinium nitrogens (51.4 ppm), and the internal (N_ϵ) guanidinium nitrogen

(63.8 ppm). When the pH is increased to ~14.1 (Figure 3B), the α-amino ^{15}N shifts upfield 8 ppm to 12.3 ppm, due to the increased diamagnetic shielding generally expected for a primary amine upon deprotonation. In contrast, the 17- and 4-ppm downfield shifts seen upon deprotonation of the guanidino nitrogens (N_η and N_ϵ, respectively) are most likely due to a change in the second-order paramagnetic effects on the neutral guanidinyl nitrogens.[3] These downfield shifts agree with previously reported values of 15 and 3 ppm for N_η and N_ϵ, respectively, in 1 M aqueous l-arginine measured at the same two pH values.[3] The slight differences are most likely due to use of different l-arginine concentrations.

In both guanidinium and guanidine forms of l-arginine, a single averaged peak for the two N_η was observed. For the guanidinium form, the chemical equivalence of these two nitrogens indicates that rotation about the C−N_ϵ bond is rapid on the NMR time scale at room temperature. Likewise, in the guanidine form, the observation of only a single narrow band at ~68 ppm assignable to the two N_η, and a broader overlapping band assignable to the internal nitrogen (N_ϵ), provides clear evidence for rapid interconversion of the three possible similar-energy tautomers on the NMR time scale. The nearly-equal contribution of all three tautomers is demonstrated in particular by the closeness of the two observed ^{15}N resonances, overlapping at ~68 ppm but with different bandwidths (Figure 3B). If one specific guanidine tautomer were formed preferentially, a ~100 ppm chemical shift gap would be expected between the sp^2-hybridized nitrogen in the C═N bond and the sp^3-nitrogen in the C−N bond, as observed previously in pentamethylguanidine, for example.[4,15]

NMR Spectra of Dodecylguanidinium and Dodecylguanidine. The presence of a long carbon chain in dodecylguanidinium chloride and dodecylguanidine raises these compounds' solubility in C^2HCl_3 sufficiently high, without lowering their solubility in DMSO too much, to permit natural-abundance ^{15}N NMR measurements in both solvents. (However, the alkyl chain and the absence of other polar groups reduces the solubility of dodecylguanidine in water or 1:1 water/DMSO, two solvents previously used for ^{15}N NMR of arginine,[3,4] to a level too low for natural-abundance ^{15}N NMR measurements.) Thus pure DMSO and C^2HCl_3 have the right properties to be of value in comparing the size of guanidino-group ^{15}N chemical shift differences resulting from changes in protonation state to those resulting purely from large changes in solvent properties such as dielectric constant and H-bond accepting capability.

Figure 4A shows the ^{15}N NMR spectrum of dodecylguanidinium chloride in C^2HCl_3. The two terminal nitrogens are chemically identical on the NMR time scale, and give rise to a single resonance. This is in agreement with the ^1H NMR spectrum of the same solution (Figure 1A), which shows that four of the five guanidinium protons are chemically equivalent, a result that is most consistent with rapid rotation about all three C−N bonds on the NMR time scale. The averaged chemical shift value of the two N_η of dodecylguanidinium chloride in C^2HCl_3 is 49.3 ppm—somewhat upfield from the value for the N_η of arginine (51.4 ppm). By contrast, dodecylguanidinium in DMSO (Figure 4B) shows three distinct ^{15}N resonances: at 66.0 ppm due to the internal nitrogen (analogous to N_ϵ of arginine) and at 53.8 and 56.7 ppm due to the two terminal nitrogens (analogous to N_η of arginine).

The appearance of distinct resonances for the latter two nitrogens indicates that the internal C−N bond rotation in DMSO is significantly slower than in $CHCl_3$. It is also notable that DMSO moves the average chemical shift of the two terminal

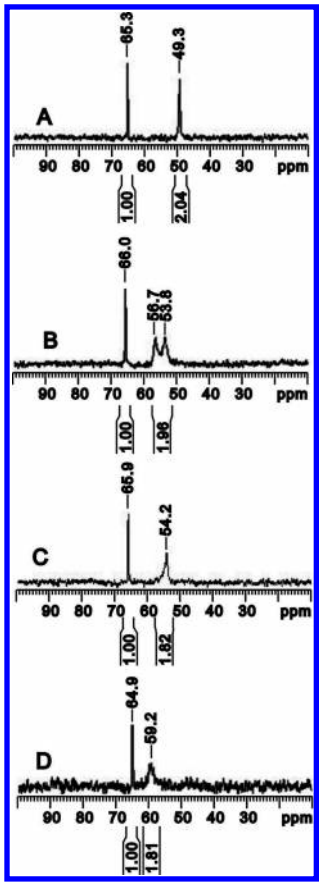

Figure 4. ^{15}N NMR spectra of saturated dodecylguanidinium chloride in (A) C^2HCl_3 or (B) DMSO and dodecylguanidine in (C) C^2HCl_3 or (D) DMSO. Chemical shift values were measured relative to $(^{15}NH_4)_2SO_4/H_2O$.

nitrogens to 55.3 ppm, i.e., substantially downfield from both the value of 51.4 ppm observed for arginine in water (Figure 3A) and the value of 49.3 ppm observed for dodecylguanidinium chloride in C^2HCl_3 (Figure 4A). Both the splitting of the resonance and its downfield shift are most likely related to the strong H-bond accepting properties of DMSO (see Discussion for details).

In either C^2HCl_3 (Figure 4C) or DMSO (Figure 4D), deprotonation of dodecylguanidinium causes only a 4−5-ppm downfield shift for the terminal nitrogens, and almost no change for the internal nitrogen. The average ^{15}N chemical shift of the terminal guanidine nitrogens is 59.2 ppm in DMSO and 54.2 ppm in C^2HCl_3. By comparison, for deprotonated arginine in H_2O, the averaged chemical shift value for the terminal nitrogens is 68.1 ppm (Figure 3B), almost 17 ppm downfield from the value for protonated arginine.

Thus, for the two terminal nitrogens in dodecylguanidine, the ~5-ppm solvent-induced differences between the ^{15}N chemical shifts in DMSO and $CHCl_3$ are as large as or larger than the 4−5-ppm differences induced by protonation changes in the same solvents. As a result, the average N_η chemical shift value for protonated dodecylguanidium in DMSO, 55.3 ppm (Figure 4B), is actually slightly downfield from the value for deprotonated dodecylguanidine in C^2HCl_3, 54.2 ppm (Figure 4C). The assigned chemical shift values are summarized in Table 1.

Due to the extremely high basicity of guanidine, our procedures (involving neutralization with NaOH and partial air-drying with dry N_2, as described in the Experimental Procedure) may not be sufficient to produce 100% deprotonation of dodecylguanidine. The extent of the dodecylguanidinium ion

TABLE 1: ^{15}N Chemical Shift Values of Guanidino Nitrogens, in ppm Relative to $(^{15}NH_4)_2SO_4/H_2O$

	protonated (chloride salt)			deprotonated		
	water[a]	DMSO[b]	chloroform[b]	water[a]	DMSO[b]	chloroform[b]
terminal $-NH_2$ (av value)	51.4	55.3[c]	49.3	68.1	59.2	54.2
internal $-NH-$	63.8	66.0	65.3	68	64.9	65.9

[a] Values measured using arginine (Figure 3). [b] Values measured using dodecylguanidine (Figure 4). [c] Average of two distinct resonances detected at 53.8 and 56.7 ppm (Figure 4B).

impurity becomes worse for the DMSO solvent, because an additional air-drying process has been employed. However, ^1H NMR (Figure 1) and FTIR spectra (Figure 2) clearly indicate that the predominant product is dodecylguanidine, at least for the $CHCl_3$ solvent. While our reported ^{15}N chemical shifts may not represent the absolute values for alkyl-substituted guanidine of 100% purity, it is still safe to state that the range of ^{15}N chemical shifts of neutral guanidine in very nonpolar environments overlap those of guanidinium ion in polar H-bonding environments.

Discussion

The previously described ~15-ppm deprotonation-induced downfield shift of the two terminal nitrogens of the arginine side chain in aqueous solution[3] has been reproduced (Figure 3). A similar, but smaller (~5 ppm) downfield shift is also induced by deprotonation of a simple alkylguanidine in both a less-polar solvent C^2HCl_3 and an aprotic polar solvent DMSO (Figure 4). However, in these solvents at least, the chemical shift value of the terminal nitrogens is not an unambiguous indicator of the protonation state of the guanidino group, because solvent effects are as large as the 4−5-ppm effect of the protonation change. Specifically, the deprotonated guanidine species in the less-polar solvent ($CHCl_3$) exhibits a similar ^{15}N chemical shift for the two terminal nitrogens (54.2 ppm) as observed for the protonated guanidinium species in DMSO (55.3 ppm), and only ~3 ppm away from that of the protonated arginine side chain in water (51.4 ppm).

An intuitive explanation is that in a polar solvent—either water or DMSO—strong H-bonds of the guanidinium ion with acceptors in its environment weaken all five N−H covalent bonds. The overall effect on diamagnetic shielding is to simulate the nearly stoichiometric loss of a single proton. This moves the average ^{15}N chemical shift value close to where it is for the deprotonated species (guanidine) in a non-H-bonding solvent, $CHCl_3$.

Several Solvent Parameters Required To Account for Observed Spectra. A single parameter, e.g., dielectric constant, by itself is inadequate to account for the solvent dependence of the chemical shift values of the protonated and deprotonated guanidino group (Table 1). There is, roughly speaking, a monotonic upfield trend in ^{15}N chemical shifts of deprotonated guanidine as the dielectric constant is decreased from the value of water (80) to that of DMSO (48) to that of chloroform (4.8). However, for protonated guanidinium, such monotonicity is not observed.

Solvent parameters relevant to NMR spectra have previously been rationalized in terms of three scales:[16−18] a π^*-scale of polarity−polarizability describes the ability of the solvent to stabilize a charge or a dipole by virtue of its dielectric properties; an α-scale of hydrogen bond donor acidities measures the solvent's ability to donate a proton in a solvent-to-solute hydrogen bond; and a β-scale of hydrogen bond acceptor basicities provides an index of the solvent's ability to accept a proton in a solute-to-solvent hydrogen bond. These π^*, α, and β values are 1.09, 1.10, and 0.14−0.47 for H_2O;[16,17] 1.00, 0, and 0.76 for DMSO;[16−18] and 0.5−0.76, 0.22, and 0 for $CHCl_3$.[16,18] In other words, H_2O is the strongest H-bond donating solvent and DMSO is the strongest H-bond accepting solvent, while both have a similar capability for dielectric stabilization.

Using these scales, the upfield shift of the terminal nitrogens of dodecylguanidinium chloride in $CHCl_3$, in comparison with arginine in H_2O (Figure 3A and Table 1), is most likely the result of the absence of H-bonding accepting capability of the solvent. As previously discussed,[13] the partially anionic character of the O atoms in H-bond-accepting solvents results in electron density being pushed away from the nitrogens and into the π-bonding system, thereby increasing the C−N bond order. At the same time, the stiffening of the H-bonds results in an increase in the C−N−H bending mode frequencies, which couple with ν_{C-N} and thereby increase the frequency of the latter. A combination of both effects explains the high ν_{C-N} frequencies for monoalkylguanidinium chlorides in H-bond-accepting solvents: 1672 cm^{-1} for the most intense IR band of dodecylguanidinium chloride in DMSO, as shown in Figure 2; and previously observed frequency of ~1670 cm^{-1} for arginine in water (depending somewhat on the pH).[14] These are both somewhat higher in frequency than ν_{C-N} for dodecylguanidinium chloride in pure chloroform, ~1660 cm^{-1}, as shown in Figure 2. (Note that the latter frequency is also ~10 cm^{-1} lower than that previously observed[13] for ethylguanidinium chloride in chloroform containing 3% methanol, which can serve as an H-bond-acceptor.) In chloroform, guanidinium halides probably exist as metastable H-bonded ion pairs, as concluded previously based on IR comparisons of the chloride, bromide, and iodide salts of ethylguanidinium in $CHCl_3$:MeOH 97:3.[13] Nevertheless, the polarization of the π-bond system and other H-bonding effects of a single bound Cl^- appear to be weaker than those produced by multiple H-bond-accepting solvent molecules containing O atoms. As a result, ν_{C-N} for monoalkylguanidinium chlorides is fairly well correlated with the H-bond-accepting character of the solvent. As mentioned above, the higher ν_{C-N} frequency in H-bond-accepting solvents is consistent with a lower electron density on the nitrogens. The β-scale of hydrogen bond acceptor basicities can thus largely account for both the progressively higher ν_{C-N} (Figure 2 and ref 14) and the progressively greater ^{15}N deshielding (Table 1) of monoalkyl guanidinium chlorides (including arginine) in $CHCl_3$, H_2O, and DMSO.

The magnetic inequivalence of the terminal nitrogens of dodecylguanidinium in DMSO (Figure 4B) is attributable to the absence of rapid isomerization about the internal C−N bond. This is possibly due to strong H-bonding of all five guanidinium N−H bonds to DMSO molecules (stronger than in chloroform or water), providing the greatest hindrance to bond rotation. This hypothesis is suggested by the high β value for DMSO, and is supported by other aspects of the spectrum mentioned in the preceding paragraph. In particular, the average of the chemical shifts of the two terminal nitrogens in DMSO (55.5 ppm; Figure 3B) is 4 ppm downfield, compared with the corresponding average for arginine in aqueous solution (51.4 ppm; Figure 3A). A similar DMSO-induced downfield shift was

also observed for the unsubstituted guanidinium ion $[C(NH_2)_3]^+$.[19] These 4-ppm deshieldings in DMSO, compared with H_2O, are likely caused by the extremely strong H-bonding between guanidinium ion and DMSO. Partial proton transfer of the solute to a solvent that acts as such a strong H-bond acceptor is estimated to be 10−30%.[20]

In contrast to alkylguanidiniums such as arginine at physiological pH, deprotonated alkylguanidine exhibits predominately an H-bond accepting capacity, although in theory it may also act as an H-bond donor. Therefore, the greatest deshielding effect on the side chain of arginine in H_2O at pH 14.1, with an average chemical shift of ∼68 for three nitrogens (Figure 3B), is likely associated with the strong polarization and H-bond donating ability of the water solvent, acting in combination on the lone pairs of the guanidine nitrogens to lower their electron densities.

Thus, of the three solvents examined, $CHCl_3$ exhibits the smallest solvent-induced deshielding effect, with an average chemical shift of ∼58 ppm for three nitrogens of dodecylguanidine (Figure 4C), due to its minimal dipolar stabilization and H-bond donating capabilities. DMSO shows an intermediate solvent effect, with an average chemical shift of ∼61 ppm (Figure 4D), because it possesses a similar polarization capability as water, but without any H-bonding donating ability.

Different Solvent Effects on Imino vs Amino Nitrogens. A different paradigm for explaining the results shown in Figures 3 and 4 is based on a consideration of molecular electronic structures. Monoalkylguanidine contains two enamino (═C−NR_2) nitrogens and one imino (R−N═C) nitrogen. Considering direct effects on the respective lone pairs, protonation is generally expected to produce a small amount of deshielding at the enamino-like nitrogens, as observed for an enamine or pyrrole,[8] and a large shielding effect on the latter, as observed for an imine or pyridine.[9] The rapid tautomerization of the deprotonated guandine means that a near-cancellation of the protonation effects on the imino- and enamino-like nitrogens is expected to result in a relatively small average chemical shift change upon protonation.

However, the degree of cancellation of the two effects can vary significantly, depending on solvent. For *N*-methylimidazole,[8] protonation causes a net average upfield shift for the two nitrogen atoms, of 34 ppm in H_2O and 27 ppm in $CHCl_3$. For monoalkylguanidine, the protonation-induced effect is, averaging over all three nitrogen atoms, 13 ppm upfield in water (see Figure 3) and only 3.5 ppm in $CHCl_3$ (see Figure 4). One possible explanation for the much smaller shielding of monoalkylguanidine, as compared to *N*-methylimidazole, is that guanidine has two enamine-like nitrogens, rather than one in imidazole, producing more protonation-induced deshielding that cancels the large shielding effect caused by protonation of the imino nitrogen.

Implications for Vibrational and NMR Spectroscopic Measurements on Arg-82 in the M State of Bacteriorhodopsin. Time-resolved FTIR spectroscopy indicates that arg-82 transiently deprotonates at the M stage of the proton-pumping photocycle of bacteriorhodopsin (bR). This conclusion is based primarily on an observed transient decrease in absorbance at ∼1660 cm^{-1}, characteristic of protonated guanidinium, and the concomitant appearance of a new absorbance feature near 1556 cm^{-1}, characteristic of deprotonated alkylguanidine compounds (ref 13; see also Figure 2). Both features show the expected sensitivity to specific ^{15}N isotope substitution of arginine.[1,21]

In contrast, solid-state NMR measurements of ^{15}N-arginine-labeled bR[22] have continued to be interpreted as arguing against arg-82 deprotonation in the M state of bR. The average ^{15}N chemical shift for the two η nitrogens of arg-82 in the M photoproduct state prepared at −44 °C was observed to be ∼48 ppm [referenced to 5.6 M $^{15}NH_4Cl$], i.e., nearly the same as in the unphotolyzed state, and also nearly the same as all six other arginines in bR. These results were originally interpreted as indicating that all seven arginines in bR must remain protonated in the M state.[22]

However, from the discussion above, the ^{15}N chemical shifts of guanidinium and guanidine are very sensitive to solvent environment. This limits the ability of the ^{15}N NMR spectroscopy to differentiate protonation states of the guanidyl group in buried protein environments. Specifically, the reported ∼48-ppm average chemical shift value for the η nitrogens of the perturbed arg-82 in the M state of bacteriorhodopsin—which actually corresponds to a value of ∼52 ppm relative if our reference $(^{15}NH_4)_2SO_4$ is used[23]—could be indicative of an arginine guanidinium group in a hydrophilic environment, or a deprotonated guanidine side chain in a very nonpolar environment.

That is, there is only a 3−4 ppm chemical shift difference between N_η of arg-82 in M and the terminal nitrogens in deprotonated dodecylguanidine in C^2HCl_3 (Figure 4C), taking into account the chemical shift scale difference that results from the use of two different ammonium salt reference compounds. We have not yet modeled a specific solvent environment for a deprotonated alkylguanidine that would produce an average $^{15}N_\eta$ chemical shift value for the two terminal nitrogens that closes the 3−4 ppm gap to reach the exact value found for the M state. However, this might indicate that in the M state trapped for the NMR measurements at −44 °C, arg-82 is located in a very nonpolar environment, which can be modeled only by a solvent even more nonpolar than $CHCl_3$, e.g., hexane or CCl_4, in which we cannot obtain adequate solubility of an alkylguanidine to measure its ^{15}N chemical shift using natural isotope abundance samples. Such a highly nonpolar environment may seem unlikely for an ionizable residue in the interior of a protein. However, the unusually high 1761-cm^{-1} frequency for the carboxylic acid C═O stretch vibration of the nearby residue asp-85 in the M state can also be modeled only by a nonpolar environment, i.e., the frequency is nearly matched by propionic acid in CCl_4.[24]

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