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Covalently Bound Azido Groups Are Very Specific Water Sensors, Even in Hydrogen-Bonding Environments

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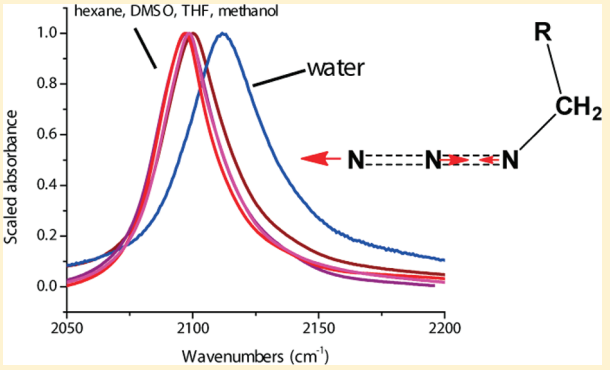
Supporting Information

ABSTRACT: Covalently bound azido groups are found in many commercially available biomolecular precursors and substrates, and the NNN asymmetric stretching band of these groups is a strong infrared absorber that appears in a spectral region clear of other signals. In order to evaluate comprehensively the solvatochromism of the asymmetric azido NNN stretching band for site-specific use in biomolecular contexts, infrared spectra of the model compounds 5-azido,1-pentanoic acid and 3-(*p*-azidophenyl),1-propanoic acid were acquired in a large variety of nonpolar, polar, and hydrogen-bond-donating solvents, as well as mixed aqueous-organic solvents. Spectra in pure solvents indicated that the aliphatic NNN stretching frequency maximum does not depend on solvent polarity, while the aromatic NNN frequency displays a weak but nonzero sensitivity to polarity. In both cases, the NNN frequency exhibits a blue-shift in H-bond-donating solvents, but the frequency in water is higher than in any other H-bond-donating solvent including solvents that are stronger H-bond donors. In nonfluorinated H-bond donor solvents, the frequency blue shift scales with the density of H-bond donors. This sensitivity to the presence of water was further explored in several mixed solvent environments, with the conclusion that this vibrational mode is a highly specific sensor of hydration, even in environments containing other H-bond donors like amides and alcohols, due to the very high local density of H-bond donors in water. The relatively uncomplicated (compared to nitriles, for example), water-specific response of this vibrational mode should lead to its adoption as a site-specific probe of hydration in many different possible systems in which the presence and role of molecular water is of primary interest.



INTRODUCTION

The site-specific incorporation of novel functional groups into proteins and other biomolecules has seen much recent growth in the area of probe moieties designed to serve as chromophores for vibrational spectroscopy. Vibrations based on amino acid side chains or other variable sites that appear in clear frequency regions of the biomolecular vibrational spectrum can report on their local structural environment in a number of different ways.[1−5] The utility of such probe groups is generally associated with the small size of vibrational chromophores relative to bulkier probe groups for other spectroscopies and the resulting lack of large perturbations in the nearby structure due to the presence of a novel functional group. Most artificial or isotopically edited amino acids used as vibrational probes must be incorporated into proteins via total protein synthesis or semisynthesis,[1,2,4,6] with notable exceptions involving chemical ligation at naturally occurring amino acids[3,7−10] or site-directed mutagenesis facilitated by stop codon suppression.[11−13] Other vibrational chromophores have been incorporated synthetically into active-site inhibitors of enzymes[3,14,15] or into other biomolecules like model nucleotides.[16−18]

The most common vibrations targeted as site-specific infrared chromophores in proteins are the nitrile C≡N stretch (including bound ∼C−C≡N, ∼O−C≡N and ∼S−C≡N moieties), the C-D stretch, and the asymmetric N═N═N stretching motion of covalently bound azide (∼C−N═N═N). Of these three, the azido asymmetric stretch has the strongest infrared oscillator strength and extinction coefficient,[1] and its relatively strong signal has led to recent reports of its use as a vibrational probe in proteins,[13,19−21] natural-sequence aggregating peptides,[1] artificial enzyme inhibitors,[15,22] and model nucleic acids.[16,18,23] Solvent-dependent studies designed to determine vibrational solvatochromism of nitriles and azides due to the vibrational Stark effect,[24,25] as well as model peptide[1] and nucleic acid[16,18] studies, have documented the frequency sensitivity of the azido asymmetric stretch to water vs a few non-H-bonding organic solvents. There appears to be a very small Stark tuning rate (meaning a lack of sensitivity to electric fields as compared to other chromophores) for this vibration.[25] The line shape and dynamic sensitivity of the typically broad and asymmetric azido NNN band are largely unexplored.

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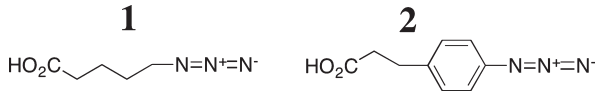

Figure 1. Structures of 5-azido-1-pentanoic acid (**1**) and 3-(*p*-azidophenyl)-1-propanoic acid (**2**).

Advances in bio-orthogonal conjugation chemistry driven by Bertozzi,[26] Sharpless[27] and others have led to a recent proliferation of commercially available biomolecular substrates and synthetic precursors containing covalently bound azido groups. In proteins, five azido-labeled amino acids are currently commercially available, and two of these have been successfully incorporated into proteins using different site-directed mutagenesis strategies.[11,13] Azido-derivatized saccharides have been successfully targeted to cell surface receptors for cell imaging purposes.[26] Following these early examples of azido incorporation and anticipating many more associated with "click chemistry", we fully expect a proliferation of vibrational studies of proteins and many other biomolecular systems using the N=N=N asymmetric stretching band as a site-specific probe.

To aid the interpretation and design of such future studies, we present here the most comprehensive consideration to date of the solvatochromism of this vibration (which has been considered in organic solvents and mixed water/organic solutions previously[1,16,18−20]), with somewhat surprising new results. Compared to prior solvatochromic studies on azido-containing compounds, this work includes a larger range of non-H-bonding solvents (including nonpolar alkanes relevant to possible application in lipids), as well as a systematic set of H-bond donor solvents not previously investigated. The model azide-containing solutes used here are 5-azido,1-pentanoic acid (species **1** in Figure 1) and 3-(*p*-azidophenyl),1-propanoic acid (species **2** in Figure 1), which are each soluble in nearly any solvent of interest due to a mixture of hydrophobic, polar, and charged functional groups. The broad-spectrum solubility of each of these compounds is greater than that of either unsubstituted amino acids or the fmoc-protected versions of these amino acids (which are not particularly alkane-soluble), and the lack of amino acid-based or protecting functional groups ensures that there will be little or no intramolecular interaction between the azido moiety and other potentially noninnocent functional groups. The current work is designed to examine the response of the azido moiety to isotropic solvent media. Thus, **1** and **2** are each more systematically useful for solvatochromic studies than the peptide or nucleic acid-based models used previously, and we expect our results to be applicable to any system with covalently bound azido groups. The best direct comparison of **1** to an existing azido-containing vibrational probe moiety is the azido group of azidohomoalanine,[19] due to the methylene group at the β position to the azido-functionalized carbon, and **2** is intended to best reproduce the spectral response of *p*-azidophenylalanine.[13,20,21] We are particularly interested in clearly documenting the sensitivity of this band (or lack thereof) to the following factors: local polarity and electric fields, fs−ps time scale solvent dynamics, hydrogen bond donor strength and local density, and the presence or absence of water in the probe moiety's solvation sphere.

EXPERIMENTAL METHODS

Materials. **1** was purchased from Bachem (>97%) and used without any further purification. 3-(4-Aminophenyl)-propionic acid (97%) and sodium azide were obtained from Aldrich. All solvents for infrared experiments were from either Aldrich, Baker, or Pharmco-Aaper, of HPLC or higher quality, and used without further purification. All water was doubly deionized with a resistivity of at least 17.8 MΩ/cm.

Synthesis of 2. Following a published procedure for the conversion of anilines to phenyl azides,[28] 0.79 g of 3-(4-aminophenyl)propionic acid (Aldrich, 97%) was dissolved in 49.5 mL of 6 M HCl and cooled to 0 °C. After 12 min of cooling, 0.81 g of sodium nitrite was added, and the mixture released gas and turned yellow. After stirring for 10 min at this temperature, 1 g of sodium azide was added and the solution was stirred under N_2 for 2 h. The resulting aqueous solution and 50 mL of ethyl acetate were then shaken in a separatory funnel, removing the orange-colored **2** to the organic phase. The organic layer was removed and washed with brine, then dried over sodium sulfite. Almost all of the solvent was evaporated using a rotary evaporator, then **2** (an orange oily solid) was further dried overnight under applied vacuum. Purity was established by ^{1}H NMR; remaining traces of ethyl acetate were removed from IR samples by dissolution in each solvent of choice and evaporation before redissolution in the chosen solvent. ^{1}H NMR (Varian 500 MHz, $CDCl_3$): δ 10.85 (bs, 1H); 7.23 (d, 2H); 6.96 (d, 2H); 2.94 (t, 2H); 2.67 (t, 2H).

Infrared Spectroscopy. Most infrared spectra were collected using a Thermo Scientific Nicolet 6700 infrared spectrometer (detector: DTGS) with 32 scans at a resolution of 1.0 cm^{-1}; the aqueous spectrum of **2** was collected using a Vertex 70 infrared spectrometer (detector: photovoltaic MCT) with 1024 scans at 1 cm^{-1} resolution. Separate spectra were collected of all media with no **1** or **2** added for subtraction purposes. Samples were placed in a Harrick demountable liquid cell with polished CaF_2 windows and a Teflon spacer. The path length used was 50 or 25 μm for organic solvents, 100 μm for water, and 12 μm for the sugar solution experiments (see below). All spectra were collected at 298 K, except for the temperature dependent spectra, in which case the temperature was controlled using a temperature jacket and a circulating water bath. All solutions of **1** were ∼45 mM, and solutions of **2** were ∼10−50 mM except for the water spectrum, which was closer to 2 mM in a saturated solution. **2** is photosensitive to ambient light conditions on the time scale of hours in solution, so fresh solutions were mixed for all experiments. As a solid, **2** is not particularly photosensitive, but it was stored in a dark refrigerator at −20 °C.

Lineshape parameters for each NNN asymmetric stretching band were calculated generically, without assumptions about the shape of the distribution. The mode frequency (the frequency at maximum intensity) was determined by visual inspection followed by interpolation (in Origin 8) using data points within 5 cm^{-1} of the visible maximum. The maximum of the interpolated function is reported as the mode, and does not depend on any functional assumption about the line shape. The mean and standard deviation were calculated using data between 2030 and 2170 cm^{-1} and the following equations:

$$\text{mean} = \langle\tilde{\nu}\rangle = \frac{\sum_{\tilde{\nu}} \tilde{\nu} \times I(\tilde{\nu})}{\sum_{\tilde{\nu}} I(\tilde{\nu})} \quad (1)$$

$$\text{standard deviation} = \sigma_{\tilde{\nu}} = \left(\frac{\sum_{\tilde{\nu}} \tilde{\nu}^2 \times I(\tilde{\nu})}{\sum_{\tilde{\nu}} I(\tilde{\nu})} - \langle\tilde{\nu}\rangle^2 \right)^{1/2} \quad (2)$$

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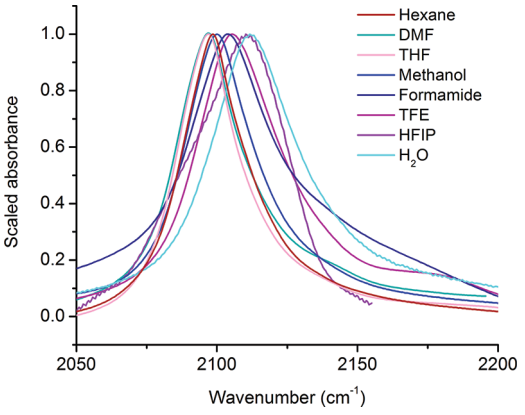

Figure 2. Asymmetric NNN stretching bands for **1** in pure solvents.

where $\tilde{\nu}$ is the wavenumber and $I(\tilde{\nu})$ is the absorbance intensity for each spectral data point.

Mixed Organic−Aqueous and Water−Sugar Samples. Mixed solvent solutions of THF/water and DMSO/water were prepared by volume without correction for nonideal mixing. For the sugar spectrum labeled "sucrose:water A", a stock sugar solution of 250 mg of sucrose in 500 μL of H_2O was prepared by heating at 50−55 °C until the sucrose was fully dissolved. Then 15 μL of this sugar stock solution was added to 300 μL of H_2O along with 1−2 μL of **1**. A small amount of this resulting solution was placed on a single CaF_2 plate and heated at ca. 50 °C until the solution became viscous and near-glassy, then the still-viscous sample was pressed under another CaF_2 window. For the spectrum labeled "sucrose:water B", the same procedure was performed using an initial sugar stock solution of 500 mg of sucrose in 500 μL of H_2O. The sample "trehalose:water" used a sugar stock solution of 500 mg of trehalose in 500 μL of H_2O.

RESULTS AND DISCUSSION

The asymmetric NNN stretching band of **1** in representative pure solvents at room temperature is shown in Figure 2 (see Supporting Information for all spectra), with numerical analysis of all bands in Table 1. The same band for **2** in selected pure solvents at room temperature is shown in Figure 3 (with all spectra available in the Supporting Information file), with numerical analysis of all bands in Table 2. All azido stretching lineshapes are analyzed as generic distributions, with no assumptions made about symmetry or other line shape qualities. The asymmetric azide frequency of **1** is about 10−15 cm^{-1} lower than that reported for the same vibration in model peptide[1] and nucleic acid[16] compounds and quite comparable to that reported for azidohomoalanine.[19] There is a significant inductive effect on the frequency from the presence of different functional groups β to the azido-bound carbon, and prior reports show that the presence of a ring at the azido-bound carbon introduces Fermi resonances in this band from both aliphatic and aromatic azido groups. However, all frequency shifts due to solvent observed in **1** scale similarly with those previously reported for the other biomolecular model compounds, and conclusions drawn from Figures 2 and 3 and Tables 1 and 2 are expected to be general to the behavior of all aliphatic or aromatic azides.

In Figure 2, the N═N═N bands can be broken into two clear groups based on both the mean and mode (peak) frequencies:

Table 1. Calculated Lineshape Parameters for all NNN Asymmetric Stretching Bands for 1[a]

solvent	mode frequency	mean frequency	standard deviation
hexane	2098.5	2107.9	20.6
heptane	2098.5	2107.5	23.2
tetrahydrofuran (THF)	2098.2	2104.3	26.0
methylene chloride (CH_2Cl_2)	2100.1	2103.4	16.6
chloroform	2100.6	2112.8	44.1
acetonitrile	2101.0	2100.1	19.3
dimethyl sulfoxide (DMSO)	2097.7	2100.9	34.8
dimethylformamide (DMF)	2096.7	2103.2	31.0
N-methylacetamide (NMA)	2098.2	2096.5	27.5
methanol	2099.6	2098.3	22.3
formamide	2104.0	2102.4	52.9
trifluoroethanol (TFE)	2105.4	2099.4	34.6
hexafluoro-2-propanol (HFIP)	2110.2	2091.2	39.4
water	2111.7	2142.7	53.7

[a] Units for all values are cm^{-1}.

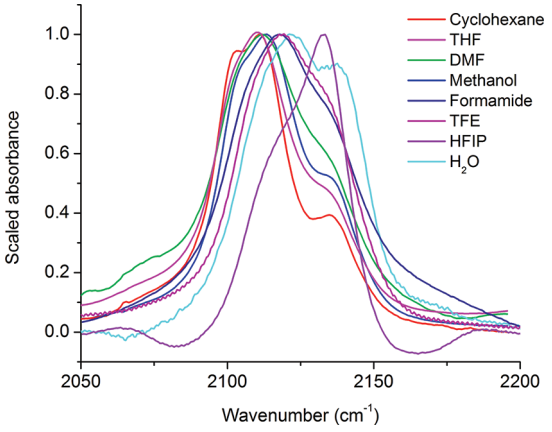

Figure 3. Asymmetric NNN stretching bands for **2** in pure solvents.

Table 2. Calculated Lineshape Parameters for all NNN Asymmetric Stretching Bands for 2[a]

solvent	mode frequency	mean frequency	standard deviation
cyclohexane	2111.2	2109.1	24.1
THF	2110.2	2109.7	31.2
chloroform	2111.8	2113.6	22.4
acetonitrile	2111.8	2114.5	30.6
DMF	2111.9	2111.5	29.1
DMSO	2112.2	2113.2	13.9
NMA	2111.8	2121.3	62.6
methanol	2112.6	2112.7	26.5
formamide	2117.7	2122.5	25.4
TFE	2117.8	2119.9	22.2
HFIP	2133.4	2124.7	9.7
water	2121.2	2127.3	19.5

[a] Units for all values are cm^{-1}.

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those in solvents with H-bond donors, and those in non-H-bond donating solvents. The lowest-frequency bands occur in polar solvents that are not H-bond donors, and some nonpolar solvents yield a slightly higher frequency but not with any systematic variation. The great similarity in frequency between all non-H-bonding solvents agrees qualitatively with the very small Stark tuning rate reported for this particular vibrational mode.[29] In an averaged, Onsager-like picture, polar solvents apply a stronger local electric field and thus are more able to stabilize the transition dipole of the vibrational transition when compared to nonpolar solvents. Compared to nitriles, the frequency sensitivity due only to solvent polarity of the azido stretching motion (i.e., a shift of only 0.3 cm^{-1} from alkane solvents to THF) is vanishingly small. This is not a particularly surprising result given prior published Stark effect results,[25,29] but it shows clearly and comprehensively that aliphatic azide will never be a good local electric field sensor. Within the non-H-bonding group of solvents, there are widely varying dipolar relaxation times.[30,31] There is no systematic variation of azido band's frequency or width in **1** due to dipolar solvent dynamics, which is also not surprising given the poor frequency sensitivity of this mode to solvent dipoles.

The asymmetric azido stretching mode of **1** in most solvents displays a mean at higher frequency than the mode, which is an indication of the asymmetry of this vibrational band, which is usually skewed with a prominent tail at higher frequencies. This asymmetry, which is clear in several nonpolar and non-H-bonding solvents, is *not* apparently due to hydrogen bonding or any other intermolecular effect; rather, it is likely due to some underlying physical property of the vibration such as a weak Fermi resonance or other possible anharmonic complications of the potential surface for this mode. Clear baseline subtraction of this vibrational band is problematic, even at the relatively high concentrations of these experiments, due to both the band's asymmetry and its very long tails in both the red and blue directions. Reported linewidths and other calculated parameters for this band are expected to vary greatly based on fidelity of solvent subtraction and assumptions about the baseline around this band, so the calculated standard deviations reported in Table 1 are not expected to be definitive. Since quantitative analysis of the line shape of this band will usually be problematic, the clearest experimental observable for reproducible comparisons is expected to be the mode frequency. Interpretation of difference experiments (for example, those following a dynamic protein unfolding event) is also expected to be nontrivial for this vibration due to these line shape issues.

In Figure 3, the response of the azido band of **2** to solvent is slightly more complicated than for **1**, mainly due to the clear appearance of a second band at a higher frequency that is most likely due to a Fermi resonance.[32] Interestingly, the band in cyclohexane displays evidence of *three* transitions, including an additional one at slightly lower frequency; the resolution of subbands is apparently enabled by narrower transitions in the alkane solvent. All non-H-bonding solvents lead to NNN bands from **2** that have lower mode frequencies than any H-bonding solvent, qualitatively matching the behavior seen for **1**. Despite limited frequency variability due to polarity, the aromatic NNN frequency is also mainly determined by the presence of H-bond donors. The Fermi resonance, however, significantly complicates the spectral response. As the NNN frequency shifts to the blue, the relative intensity of the higher-frequency spectral component increases. This solvent-dependent effect is likely due to increased

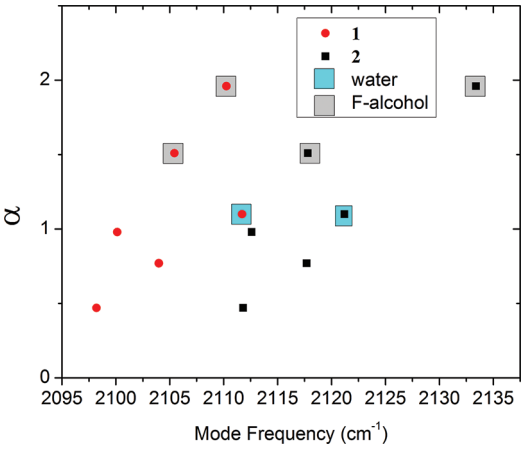

Figure 4. The NNN asymmetric stretching mode frequency (in cm^{-1}) compared to the Kamlet–Taft H-bond donor strength parameter α,[33] for **1** and **2** in H-bond-donor solvents. The points from H_2O and fluorinated alcohol solvents are indicated by blue and gray squares, respectively.

Fermi resonance between the NNN asymmetric mode and its (unassigned) Fermi resonance partner, which may not shift as much as the NNN stretching mode due to the formation of H bonds to the azido N atoms. In hexafluoro-2-propanol (HFIP), the strongest H-bond donor, the high-frequency spectral component is the most intense and defines the mode of **2**′s NNN band, while in all other solvents the mode is defined by the lower-frequency component. Pieces of the complicated spectral response of this multicomponent band are spread between 2060 and 2150 cm^{-1} (indeed, significant difference features were previously observed for this band across that entire window[13,20]), making subtraction of solvent peaks particularly difficult. The mean frequencies for **2** qualitatively track the means and modes for **1** in the same solvents, but the calculated standard deviations vary quite widely and nonsystematically due to the multicomponent nature of this band and the squared-difference weighting of frequencies far away from the mean. The broad and variable shape of this band will always pose significant challenges for background subtraction despite its strength, and the calculated line shape parameters for **2** are not expected to be definitive (as for **1** above, but with even greater challenges for clear characterization of the complicated lineshapes). Since the NNN band is quite strong in the infrared, its mode will nearly always be the most accessible spectral quantity, and thus the mode will be the major subject of much of the following discussion. There are clear trends in the spectral mode due to the presence of hydrogen bond bonors.

In Figures 2 and 3, nonaqueous H-bond donor solvents lead to blue shifts of varying magnitude, depending somewhat weakly on the H-bond donor strength of the solvents as quantified by the widely used Kamlet–Taft empirical scale[33] (see Figure 4). Taking into account the nearly identical frequencies in all non-H-bonding solvents, the asymmetric N═N═N band is clearly much more sensitive to H-bonding than to any other local electrostatics, and thus it might be used as a local reporter of H-bonding groups without the complication of competing red- and blue-shifts of similar magnitudes and opposite signs that is a reported problem for the CN stretching vibration of nitriles.[31]

However, the blue shift due to H-bonding from water is by far the largest induced by any of the H-bonding solvents,

dramatically beyond what might be predicted based on water's empirical H-bond donor strength. The mode frequency shift in water compared to any solvent with protein-like functional groups is at least +8 cm^{-1} for **1** and +10 cm^{-1} for **2**; the only H-bond-donor solvents that come close to water's effect on the mode frequency are the artificially strong H-bond donors trifluoroethanol (TFE) and HFIP. From Figure 4, it is clear that, for some reason that goes beyond simple H-bonding to a single site on the azide moiety, the azido asymmetric stretching vibration is specifically sensitive to water, despite the similar donor strengths of alcohols and amides and the greater donor strength of TFE and HFIP. The frequency of the NNN band is thus a clear and uncomplicated vibrational indicator of "hydration", even in environments expected to contain many other possible H-bonding groups in the local vicinity of the azido group.

Why is this mode so specifically sensitive to water? While simple calculations by Brewer[16,18] indicate that a single water molecule can produce a blue shift in the otherwise gas-phase NNN asymmetric stretching frequency, such results depend strongly on the choice of functional and basis set (see Supporting Information) and are not directly applicable to a probe molecule in a liquid-phase solvent. Explicit-solvent molecular dynamics simulations of azidomethane and azidoalanine in water by the Cho group[34] indicate that the azido moiety is capable of accepting H-bonds from a variety of angles at both the terminal N and the carbon-linked N atoms of the azido group, with varying predicted effects on the asymmetric NNN frequency calculated using quantum mechanical methods. Recent molecular dynamics-based frequency calculations[35] attempting to explain the strong folding sensitivity of azido-homoalanine reported by Raleigh[19] also predict major influences on the azido frequency from the distributions of water molecules at both ends of the NNN moiety. Those protein-based experiments and subsequent MD simulations take place in a highly anisotropic environment compared to the more isotropic solvent-dependent work presented here, but the primary effect of water on the NNN asymmetric stretching frequency is quite clear in both cases.

Something specific about water appears to bias the frequency distribution in both **1** and **2** resulting from accepted H-bonds strongly in one particular direction as compared to other H-bonding solvents. This could derive from the ability of water, whose density of H-bond donors is certainly higher than any of the other solvents studied here, to form H-bonds at both ends of the azido group simultaneously at almost all times.

The number and orientation, rather than the strength, of incoming H-bonding interactions appears to have the largest effect on the asymmetric NNN stretching frequency. Figure 5 plots the mode frequencies from Figure 4, but instead the comparison is to the density of possible hydrogen bond donors in each solvent rather than the empirical α measure of their strength, which was derived mainly from solvent effects on the electronic transitions of H-bond-sensitive dye molecules.[36,37] Aside from the artificially strong (and biomolecularly not meaningful) TFE and HFIP, the mode frequencies from **1** and **2** in H-bonding solvents follow a nearly linear trend in Figure 5 when compared to the H-bond density of the solvent. The particular sensitivity of the NNN stetching band to water appears to come only from the extremely high H-bond density in liquid water, which is more than twice as high as for any of the other H-bond-donating solvents. It is likely that the fluorinated alcohols form long-lived 1:1 complexes with the azido moiety, as they do with nitriles,[31] and this would explain their ability to cause a more substantial blue shift as compared to the weaker H bond donors.

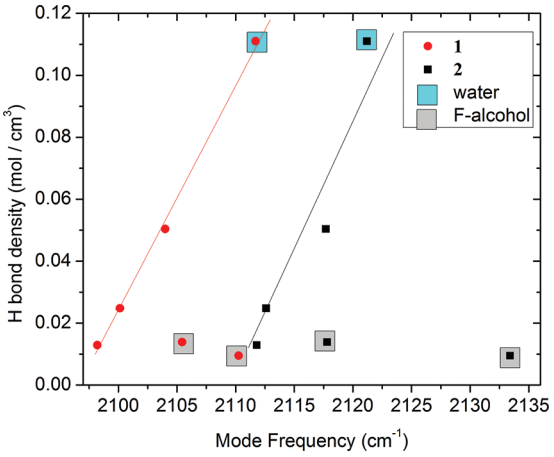

Figure 5. NNN asymmetric stretching mode frequency (in cm^{-1}) compared to the density of hydrogen bond donor groups in H-bond-donor solvents. The points from H_2O and fluorinated alcohol solvents are indicated by blue and gray squares, respectively. Trend lines for **1** and **2** in solvents relevant to biomolecular sensitivity are drawn as a visual guide.

Figure 5 indicates that in biomolecular contexts free of artificially strong H-bond donors, the NNN frequency reports the local density of hydrogen bond donors, which is highest in the presence of substantial numbers of local water molecules. However, in environments where sequestered water molecules might persist in non-bulk-like conditions and a strong network of H-bond donors is not present, the blue shift of the azido stretching band compared to a non-H-bonding environment might be substantially smaller than in the cases observed here. In addition, if an azido group is primarily tied up in a single H-bond to a non-network group of water molecules, its frequency might also appear lower than an azido group that is more "saturated" by H-bonds from many local water molecules. In any case, since liquid water presents such a high density of H-bond donors, the NNN stretching vibration is predicted to be an excellent and highly specific water sensor, even in environments that might otherwise be dominated by the presence of other biomolecule-based H-bond donors.

The temperature-dependence of this band was investigated for both **1** and **2** in water. (See the Supporting Information file for this data.) For **1**, only very small changes in standard deviation (about 2 cm^{-1} out of more than 30 cm^{-1}) and no significant change in the frequency were observed over a temperature range of 5−75 °C. The lack of temperature dependence in **1** indicates that there are *not* H-bonded subpopulations around the NNN group that are more thermodynamically favorable than others, meaning that the azido group is a passive observer of the water structure rather than a strongly shaping influence on the direction of H_2O's orientation. The aqueous spectrum of **2** is similarly temperature-independent, with no clear changes in mode or standard deviation over the range of 5−50 °C.

Azido moieties similar to that in **2**, including a phenyl azido group[13,20] and a nonaromatic, β- disubstituted ring-constrained azido group,[16,18] have been placed in mixed aqueous/organic solvents to demonstrate their sensitivity to varying amounts of local water, resulting in complicated lineshapes that likely derive from solvent-dependent Fermi resonances depending on the overlap between the solvent-dependent NNN frequency and the less-malleable frequency of the unassigned Fermi resonance

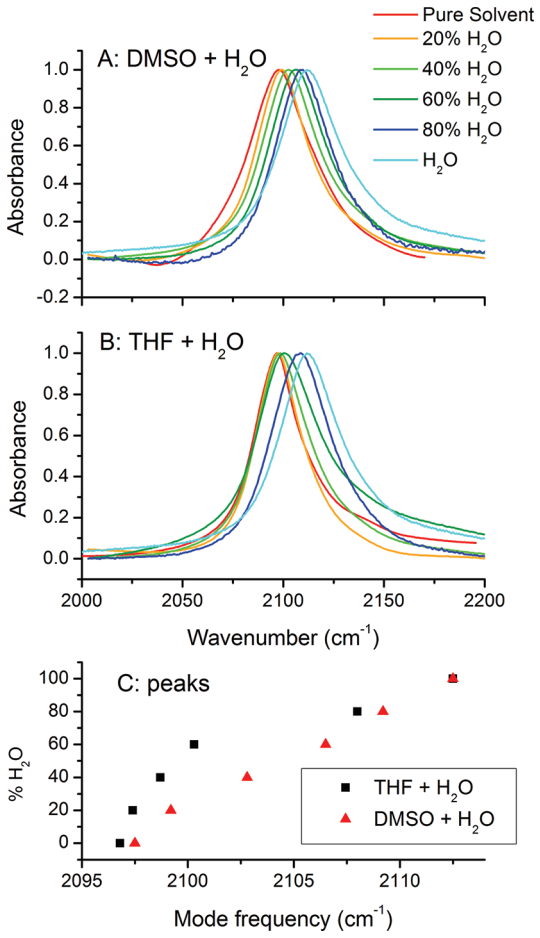

Figure 6. (A) NNN asymmetric stretching bands for **1** in DMSO/water mixed solutions from pure water to pure DMSO. (B) Same bands for **1** in THF/water mixed solutions. (C) Comparison of the mode frequencies for the mixed solvent spectra in parts A and B with the percent water in the mixed solvent.

partner. Our expectation based on data from Figure 3, prior solvent-dependent studies,[16,18,20] and a recent attempt to remove Fermi resonances by selective isotopic substitution of each azido N atom,[32] is that aromatic and α-disubstituted azido groups will always exhibit complicated solvatochromism due to solvent-dependent Fermi resonances in aqueous solution and aqueous/organic mixtures. Furthermore, this complicated solvatochromism will be systematically difficult to analyze without substantial high-level DFT calculations that include possible Fermi resonance partners in each particular case of interest.

However, the aliphatic azido band of compounds like **1** is much "cleaner" of large-scale Fermi resonances and has been examined much less thoroughly in mixed solvents. To further explore the idea of sensitivity to "hydration", **1** was placed in two different solvent mixtures: DMSO/water (Figure 6A), and THF/water (Figure 6B). DMSO is a high-dielectric, very polar cosolvent often used as a solubility aid for peptides. THF is a relatively nonpolar, low-dielectric solvent sometimes used as a proxy for "protein interior-like" environments.[4,7,17] Each set of spectra exhibits a systematic blue shift in the NNN band on moving from a less aqueous environment to the pure water environment, but the patterns depend differently on the volume % of the nonaqueous solvent (Figure 6C). Since the solvents were

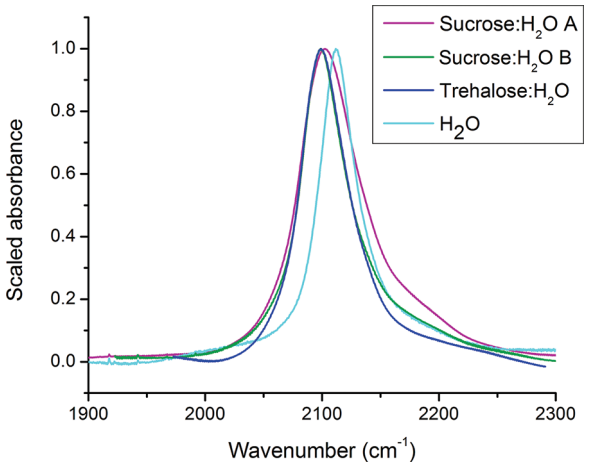

Figure 7. Asymmetric NNN stretching band for **1** in heated, then cooled, sugar solutions of varying stock concentrations (see Experimental Methods for details), with the NNN band for **1** in pure water for reference. The "Sucrose:H_2O A" sample started with approximately half as much sucrose as the "Sucrose:H_2O B" sample.

mixed by volume, the aggregate H-bond density is approximately the same in samples with identical % H_2O.

The shift in DMSO/water mixtures is much more monotonic than in the THF/water mixtures, possibly indicating that there are substantive microscopic differences between **1**'s environments in the two mixed solvents. Atom-level differences between the two solvent mixtures are not surprising, given that THF/water displays behavior sometimes interpreted as partial phase segregation[38,39] across its compositional phase diagram while DMSO/water is much more uniform with very strong nonideal attractive interactions between the two solvents.[40−42] Since the N═N═N infrared band is so specifically sensitive to the local presence of water, the data in Figure 6 suggest that at the molecular level, the THF/water mixtures are much less homogeneous and more likely involve selective clustering of THF around the solute molecules at intermediate concentrations. The more steady frequency shift in DMSO/water, without large line width variations, indicates that the solute's solvation shell becomes proportionally more aqueous as the water content is increased, in keeping with a molecularly homogeneous and nonsegregated solvent mixture. Such conclusions about local solvation and clustering of water or nonwater species are expected to be possible using aliphatic azides like **1**, but drawing similar conclusions might be more problematic in aromatic or ring-based azides like **2** due to H-bond-dependent Fermi resonance complications.

The possibilities for extending this water-specific sensitivity to other mixed aqueous solvation environments, especially in more complicated multiphase systems like lipid/water mixtures and carbohydrate/water materials, are quite broad. Solutions of simple sugars in water can create solvent conditions with widely varying mechanical, osmotic, and dynamic properties, and these properties are the basis for a plethora of both naturally occurring and human-created carbohydrate-based biomaterials.[43] To investigate the dependence of the asymmetric NNN mode on both large dynamic changes and changes in mean solvent hydration in high sugar-content environments, **1** was placed into concentrated sugar-water solutions with near-glassy properties at sugar concentrations used to immobilize peptides and proteins. Figure 7 shows the results from these samples.

One similar feature between the data in Figure 2 and Figure 7 is immediately obvious: the asymmetric azide band can clearly tell the difference between water-saturated and nonwater-based H-bond donating environments. In all sugar mixtures, the line shape is very broad and spans frequencies from more water-like solvent environments to those observed in nonaqueous solvents, indicating heterogeneous H-bonding solvent environments around the azido groups. A comparison between Figure 7 and Figure 6A suggests that there may be some dynamic dependence of the line shape on the solvent relaxation rate (which for this vibration would not come from dipolar relaxation, but rather mainly from solvent H-bond exchange and the resulting fluctuations in the N═N═N frequency). In Figure 7, the bands in the very viscous sugar solutions are very broad, while in DMSO/water in Figure 6A, a more "averaged" yet still asymmetric band shape appears for the intermediate DMSO/water solutions. The heterogeneity of the microscopic solvent structure evident in the sugar solutions suggests that the N═N═N asymmetric mode might be an excellent probe of the site-specific, atom-level "hydration distribution" in polysaccharide biomaterials, for which a dynamic picture of water motion has been proposed indirectly by molecular dynamics simulations[44,45] to explain less site-specific experimental observations.

Recent nonlinear vibrational spectroscopy experiments focused on the azido moiety[15,21−23] have indicated both the feasibility of the NNN band as a chromophore for 2D infrared photon echo experiments and its dynamic sensitivity to what appears to be the dynamics of bulk-like water. It is likely that further nonlinear vibrational experiments in mixed and heterogeneous media using this band as a probe group will shed specific light on the dynamics of water on the time scale of a few picoseconds, which is the typical time scale for H-bond exchange in liquid water. One word of caution for these studies, which involve infrared pulses with finite bandwidths, is that 2D-IR signals for all azido NNN bands are expected to reflect a convolution of the intensity spectrum of the infrared pulses with the very broad and typically asymmetric NNN stretching bands, and care should be taken to deconvolute the pulse shape from the actual spectral signals (which typically have very long spectral "wings" in all of our data). A clear explanation for the Fermi resonance in **2** might also be discovered through coupling patterns between spectral sub-bands in 2D IR spectra as it was recently for the similarly plagued aromatic OCN moiety.[10]

Given the growing variety of possible placements of the azido group, the future for the NNN asymmetric vibration as a specific water sensor is quite bright. Its weaker sensitivity to other, lower-density H-bond donors suggest that it will also be useful inside proteins where water is more sparse and amide backbone- and side-chain-based donors are the majority species.

CONCLUSIONS

The comprehensive solvent-dependent study of two model aliphatic azido- containing molecules reported here shows clearly that the frequency of the asymmetric azido stretching band is a specifically sensitive detector for the presence of water in the solvation sphere of the probe moiety, mainly due to water's uniquely high density of H-bond donors. The frequency response of this spectral band is largely uncomplicated by other factors such as local electric fields, especially for the aliphatic azido moiety. The water sensitivity applies surprisingly even in the presence of other H-bond donors of varying empirical strength. Questions remain about the dynamic dependence of the NNN bands' asymmetric and broad bandshapes, and these might be best addressed via 2D-infrared or other echo-based, time-resolved, nonlinear vibrational techniques. Given the increasing commercial availability of azido-containing starting materials, it is anticipated that the aliphatic azido vibrational probe group can be applied quite broadly as a relatively simple, site-specific molecular sensor for the presence of water *without* accompanying complications from other local electrostatic forces.

ASSOCIATED CONTENT

Supporting Information. Infrared spectra for **1** and **2** in all solvents and temperature-dependent infrared spectra of **1** and **2** in water, as well as density functional frequency calculations of azido/single water molecule complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Oh, K. I.; Lee, J. H.; Joo, C.; Han, H.; Cho, M. *J. Phys. Chem. B* **2008**, *112*, 10352–10357.
(2) Cremeens, M. E.; Fujisaki, H.; Zhang, Y.; Zimmermann, J.; Sagle, L. B.; Matsuda, S.; Dawson, P. E.; Straub, J. E.; Romesberg, F. E. *J. Am. Chem. Soc.* **2006**, *128*, 6028–6029.
(3) Fafarman, A. T.; Webb, L. J.; Chuang, J. I.; Boxer, S. G. *J. Am. Chem. Soc.* **2006**, *128*, 13356–13357.
(4) Getahun, Z.; Huang, C. Y.; Wang, T.; De Leon, B.; DeGrado, W. F.; Gai, F. *J. Am. Chem. Soc.* **2003**, *125*, 405–411.
(5) McMahon, H. A.; Alfieri, K. N.; Clark, K. A. A.; Londergan, C. H. *J. Phys. Chem. Lett.* **2010**, *1*, 850–855.
(6) Waegele, M. M.; Tucker, M. J.; Gai, F. *Chem. Phys. Lett.* **2009**, *478*, 249–253.
(7) Bischak, C. G.; Longhi, S.; Snead, D. M.; Costanzo, S.; Terrer, E.; Londergan, C. H. *Biophys. J.* **2010**, *99*, 1676–1683.
(8) Degani, Y.; Neumann, H.; Patchornik, A. *J. Am. Chem. Soc.* **1970**, *92*, 6969–6971.
(9) Jo, H.; Culik, R. M.; Korendovych, I. V.; DeGrado, W. F.; Gai, F. *Biochemistry* **2010**, *49*, 10354–10356.
(10) Tucker, M. J.; Kim, Y. S.; Hochstrasser, R. M. *Chem. Phys. Lett.* **2009**, *470*, 80–84.
(11) Kiick, K. L.; Saxon, E.; Tirrell, D. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 19–24.
(12) Schultz, K. C.; Supekova, L.; Ryu, Y. H.; Xie, J. M.; Perera, R.; Schultz, P. G. *J. Am. Chem. Soc.* **2006**, *128*, 13984–13985.
(13) Ye, S. X.; Huber, T.; Vogel, R.; Sakmar, T. P. *Nat. Chem. Biol.* **2009**, *5*, 397–399.
(14) Fang, C.; Bauman, J. D.; Das, K.; Remorino, A.; Arnold, E.; Hochstrasser, R. M. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 1472–1477.
(15) Nydegger, M. W.; Dutta, S.; Cheatum, C. M. *J. Chem. Phys.* **2010**, *133*, 134506.

(16) Gai, X. S.; Fenlon, E. E.; Brewer, S. H. *J. Phys. Chem. B* **2010**, *114*, 7958–7966.
(17) Watson, M. D.; Gai, X. S.; Gillies, A. T.; Brewer, S. H.; Fenlon, E. E. *J. Phys. Chem. B* **2008**, *112*, 13188–13192.
(18) Gai, X. S.; Coutifaris, B. A.; Brewer, S. H.; Fenlon, E. E. *Phys. Chem. Chem. Phys.* **2011**, *13*, 5926–5930.
(19) Taskent-Sezgin, H.; Chung, J. A.; Banerjee, P. S.; Nagarajan, S.; Dyer, R. B.; Carrico, I.; Raleigh, D. P. *Angew. Chem. Int. Ed.* **2010**, *49*, 7473–7475.
(20) Ye, S. X.; Zaitseva, E.; Caltabiano, G.; Schertler, G. F. X.; Sakmar, T. P.; Deupi, X.; Vogel, R. *Nature* **2010**, *464*, 1386–1389.
(21) Thielges, M. C.; Axup, J. Y.; Wong, D.; Lee, H. S.; Chung, J. K.; Schultz, P. G.; Fayer, M. D. *J. Phys. Chem. B* **2011**, *115*, 11294–11304.
(22) Dutta, S.; Rock, W.; Cook, R. J.; Kohen, A.; Cheatum, C. M. *J. Chem. Phys.* **2011**, *135*, 055106.
(23) Tucker, M. J.; Gai, X. S.; Fenlon, E. E.; Brewer, S. H.; Hochstrasser, R. M. *Phys. Chem. Chem. Phys.* **2011**, *13*, 2237–2241.
(24) Andrews, S.; Boxer, S. *J. Phys. Chem. A* **2002**, 469–477.
(25) Andrews, S. S.; Boxer, S. G. *J. Phys. Chem. A* **2000**, *104*, 11853–11863.
(26) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007–2010.
(27) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004–2021.
(28) Hu, M. Y.; Li, J. Q.; Yao, S. Q. *Org. Lett.* **2008**, *10*, 5529–5531.
(29) Suydam, I. T.; Boxer, S. G. *Biochemistry* **2003**, *42*, 12050–12055.
(30) Horng, M. L.; Gardecki, J. A.; Papazyan, A.; Maroncelli, M. *J. Phys. Chem.* **1995**, *99*, 17311–17337.
(31) Maienschein-Cline, M. G.; Londergan, C. H. *J. Phys. Chem. A* **2007**, *111*, 10020–10025.
(32) Lipkin, J. S.; Song, R.; Fenlon, E. E.; Brewer, S. H. *J. Phys. Chem. Lett.* **2011**, *2*, 1672–1676.
(33) Kamlet, M. J.; Abboud, J. L. M.; Abraham, M. H.; Taft, R. W. *J. Org. Chem.* **1983**, *48*, 2877–2887.
(34) Choi, J. H.; Oh, K. I.; Cho, M. H. *J. Chem. Phys.* **2008**, *129*, 174512.
(35) Choi, J. H.; Raleigh, D.; Cho, M. *J. Phys. Chem. Lett.* **2011**, *2*, 2158–2162.
(36) Taft, R. W.; Kamlet, M. J. *J. Chem. Soc., Perkin Trans. 2* **1979**, 1723–1729.
(37) Taft, R. W.; Pienta, N. J.; Kamlet, M. J.; Arnett, E. M. *J. Org. Chem.* **1981**, *46*, 661–667.
(38) Hao, J. K.; Cheng, H.; Butler, P.; Zhang, L.; Han, C. C. *J. Chem. Phys.* **2010**, *132*, 154902.
(39) Yang, C.; Li, W.; Wu, C. *J. Phys. Chem. B* **2004**, *108*, 11866–11870.
(40) Kirchner, B.; Reiher, M. *J. Am. Chem. Soc.* **2002**, *124*, 6206–6215.
(41) McLain, S. E.; Soper, A. K.; Luzar, A. *J. Chem. Phys.* **2007**, *127*, 174515.
(42) Rasmussen, D. H.; Mackenzie, A. P. *Nature* **1968**, *220*, 1315–1317.
(43) Morra, M., Ed. *Water in Biomaterials Surface Science*; John Wiley & Sons: New York, 2001.
(44) Almond, A.; Brass, A.; Sheehan, J. K. *J. Phys. Chem. B* **2000**, *104*, 5634–5640.
(45) Ramadugu, S. K.; Chung, Y. H.; Xia, J. C.; Margulis, C. J. *J. Phys. Chem. B* **2009**, *113*, 11003–11015.