Vibrational Spectroscopy

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Toward Extreme Biophysics: Deciphering the Infrared Response of Biomolecular Solutions at High Pressures

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Abstract: *Biophysics under extreme conditions is the fundamental platform for scrutinizing life in unusual habitats, such as those in the deep sea or continental subsurfaces, but also for putative extraterrestrial organisms. Therefore, an important thermodynamic variable to explore is pressure. It is shown that the combination of infrared spectroscopy with simulation is an exquisite approach for unraveling the intricate pressure response of the solvation pattern of TMAO in water, which is expected to be transferable to biomolecules in their native solvent. Pressure-enhanced hydrogen bonding was found for TMAO in water. TMAO is a molecule known to stabilize proteins against pressure-induced denaturation in deep-sea organisms.*

An ever-increasing number of microorganisms are known that flourish only when subjected to extreme environmental conditions, including high pressures in the kilobar regime.[1–4] In fact, the deep sea, the sub-seafloor, and the continental subsurface together provide the largest microbial habitats on Earth.[5] Laboratory experiments in the multi-gigapascal (10 kbar) range even point to survival at higher pressures than achieved on Earth.[6,7] Although it is well-known that organisms living in such extreme conditions must have strategies to adapt to those conditions, the underlying molecular adaptation mechanisms still remain largely unknown.[1–4] One such strategy involves the intracellular accumulation of small molecules, termed piezolytes, that stabilize protein structures at extreme pressures.[6,7] Trimethylamine *N*-oxide (TMAO), in particular, is known to efficiently offset the perturbing effects of high hydrostatic pressure in deep-sea animals.[8,9] Although the consensus view on the stabilizing effect of TMAO under *ambient* conditions implies that it functions as a so-called molecular crowder that depletes from protein surfaces,[10–12] there exists no coherent molecular picture of the stabilizing effect of TMAO under extreme-pressure conditions, despite the fact that experiments have revealed its strong potency also at high pressures.[13] Moreover, even the molecular-level effect of TMAO on the pressure-dependent structure and dynamics of water remains unknown.[14]

It is the realm of an emerging field, "extreme biophysics", to elucidate the (bio)molecular foundations that allow for life far away from the usual physiological conditions which govern ambient life.[15–23] Although temperature effects have been studied extensively over the last 30 years, extreme pressures are much more difficult to access in the laboratory. This difficulty constitutes a critical bottleneck because pressure, being a crucial thermodynamic variable next to temperature and concentration, could otherwise be used as a gateway to gain fundamental insight into the structure, dynamics, and hence function of these systems.[15–23]

In a thrust to push forward extreme biophysics, we focus herein on Fourier transform infrared (FTIR) spectroscopy as a probe to reveal the molecular response of piezolytes in water and—only at a later stage—their interactions with biomolecules at high pressures.[15–24] Information obtained by direct structural probes, for example, by measurement of the structure factors of a solution by X-ray and neutron scattering, may not be sensitive enough at extreme conditions.[14,25] However, in stark contrast to the increasing number of high-pressure FTIR studies on biomolecules,[24] truly quantitative FTIR spectroscopic studies on cosolvents effects lag behind, but are mandatory to fully explore the effect of pressure on the spectral properties of complex multicomponent solutions of biomolecules in future studies. An in-depth combined experimental FTIR and computational approach is required, including ab initio molecular dynamics (AIMD)[26] to predict IR spectra along with comprehensive molecular-level information and liquid-state integral-equation-based solvation models[27] capable of reproducing pressure-induced spectral shifts. Such an approach is demonstrated herein with a small yet important piezolyte, and we expect that it will stimulate a multitude of insightful studies on the pressure response of complex biomolecular processes at extreme pressures.

In this study, we investigated the piezolyte $(CH_3)_3NO$ in water, TMAO(aq), at room temperature in a pressure range from 1 bar up to several kilobars. FTIR spectra were measured for a 0.5 M TMAO solution in pure water at 300 K from ambient pressure up to approximately 12 kbar by using a membrane-driven diamond anvil cell equipped with an automated pneumatic pressure controller (Diacell iGM Controller, Almax easylab). The molecular-level picture was provided by AIMD[26] of one TMAO and 107 H_2O molecules in a cubic supercell by using the RPBE-D3 functional[28,29] as implemented in CP2k.[30,31] This approach has been shown to

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provide an excellent description[25] of the pressure response of pure water at 300 K up to 10 kbar. The reported mid-IR spectra were obtained from 32 independent NVE trajectories of 20 ps in length with initial conditions sampled from a 250 ps NVT trajectory at 300 K by using massive Nosé–Hoover chains[26] (see the Supporting Information). We stress that all reported AIMD spectra are neither empirically scaled nor adjusted in an effort to match experimental spectra and therefore display the typical errors in absolute frequencies for the employed class of density functionals (on the order of 10%). An alternative to such demanding AIMD simulations are embedded cluster reference interaction site model (EC-RISM)[32] calculations. As an integral equation theory for the solvent coupled to a quantum-chemical treatment of the solute,[27] EC-RISM provides computationally efficient access to piezolyte properties as a function of pressure.[33,34] Thus, it is possible to dissect the vibrational pressure response into contributions stemming from the deformation of the electronic energy surface in solution (E_{sol}) and from the effective forces exerted by the solvent as measured by the change in the excess chemical potential (μ^{ex}) as a function of displacement. Herein, the pressure-dependent spectral shifts of TMAO(aq) are approximated by EC-RISM computations of effective pressure-dependent force constants relative to 1 bar along the normal mode vectors from a polarizable continuum model (PCM)[35] based on B3LYP/6-311+G(d,p) (see the Supporting Information for full details).

Before addressing the pressure response of the mid-IR spectrum of TMAO in water, our first step was the full assignment of the experimental reference spectrum at 1 bar (Figure 1a; see Figure S1 in the Supporting Information for a comparison of the AIMD spectra at 10 kbar and 1 bar). Under ambient conditions, such spectral analysis at the corresponding finite temperature by either the static PCM embedding approach,[35] which yields harmonic normal modes, or the dynamical AIMD technique, which provides generalized normal modes,[36,37] is routine. Both methods lead to the same assignment in terms of the vibrational modes, as depicted in Figure 1b (see Figure S2), in agreement with another independent study.[38] We note that the antisymmetric C–N stretching mode has a vanishingly small oscillator strength according to calculations and is therefore barely visible in the experimental 1 bar spectrum (at roughly 970 cm^{-1}; see Figure 1a), whereas it systematically gains some intensity (and is also blueshifted) upon compression (see inset in Figure 1a).

Next, the experimental mid-IR spectrum of TMAO(aq) was found to feature significant frequency shifts upon compression. Owing to the modulated background absorption as a result of the diamonds of the anvil cell, four peaks can be followed up to about 12 kbar (Figure 2); the pressure dependence of the N–O stretching peak is depicted in the inset of Figure 1a. Broadly speaking, all these vibrational resonances were found to shift systematically towards higher frequencies as the pressure increased. An overall increase of several wavenumbers was observed at the highest pressure. The first and foremost question now was whether our computational methods were able to reproduce the experimentally observed frequency shifts. Indeed, both EC-RISM and AIMD correctly predicted the pressure-induced blueshifts of all peaks by about the right order of magnitude: The N–O, CH$_3$ def-low, CH$_3$ def-middle, and CH$_3$ def-high modes

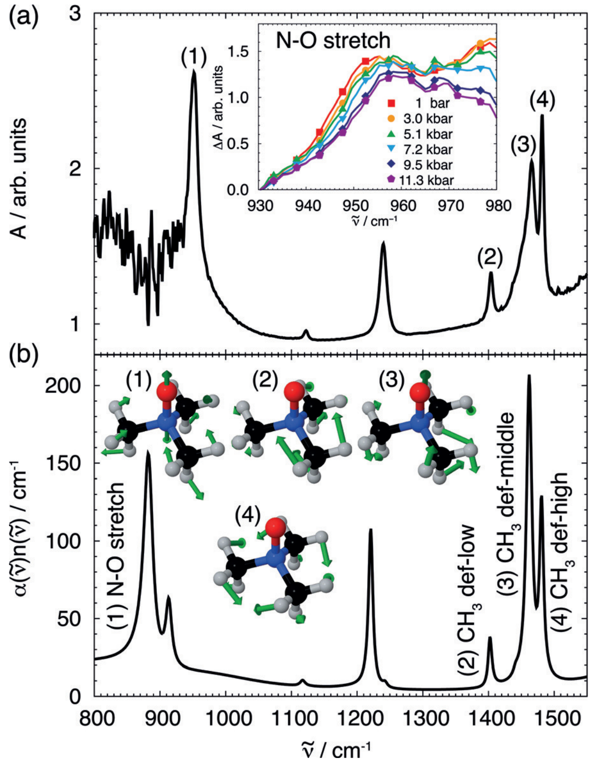

Figure 1. a) Experimental FTIR absorption overview spectrum obtained for a 0.5 M TMAO solution at ambient pressure and 300 K by using a standard CaF$_2$ cell setup. The inset shows the N–O stretching peak observed when a diamond anvil cell was used at the indicated pressures; note that the resonance close to 980 cm^{-1} is due to a residual diamond anvil cell absorption after subtraction of the pure water spectrum. b) AIMD spectrum at 10 kbar and 300 K with assignments of the four relevant peaks (see text) and illustrations of the corresponding mode displacements (green arrows); only one of the degenerate modes is shown for CH$_3$ def-low and CH$_3$ def-middle.

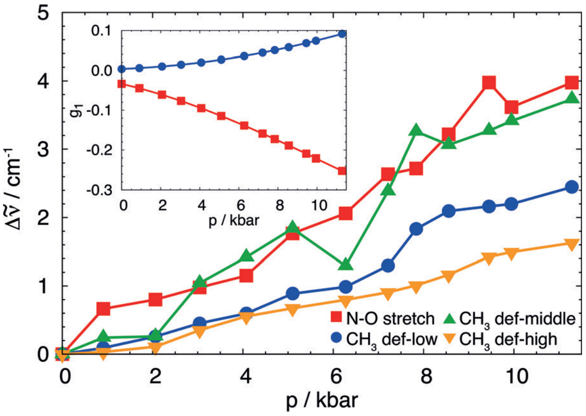

Figure 2. Experimental pressure-induced frequency shifts of the four peaks in Figure 1. The inset shows the skewness g_1 (see text) for the N–O stretching and CH$_3$ def-low peaks.

shifted 5/22, 8/21, 6/33, and 6/19 cm^{-1} from 1 bar to 10 kbar according to AIMD/EC-RISM. Furthermore, EC-RISM unanimously showed that these blueshifts increase monotonically with pressure from 1 bar to 10 kbar, in accord with experiment (see Table S1 in the Supporting Information for details). However, the EC-RISM equilibrium solvation approach overestimates both experimental and AIMD shifts by roughly 10–20 cm^{-1}, with an absolute uncertainty of approximately 10 cm^{-1} at 10 kbar, as determined from numerical force-constant discrepancies for degenerate modes, in line with PCM estimates under equilibrium displacement conditions as compared to non-equilibrium environments.[35] Concerning the accuracy in terms of wavenumbers, shifts of 5 cm^{-1} correspond to errors of less than 1% for frequencies in the order of 1000 cm^{-1}. Remarkably, the observed shift direction contradicts the simple electrostatic picture attributed to the “vibrational Stark effect”,[39,40] which would predict redshifts when switching from lower- to higher-polarity solvents, in our case from ambient to high-pressure water. This observation already hints at a dominant impact of H-bonding on the spectra,[41] as confirmed by AIMD analysis (see below). Dissection of the EC-RISM results (see the Supporting Information) shows that only a smaller fraction of the shifts originates from a pressure-dependent modulation of the intramolecular energy, whereas the larger part is the consequence of enhanced solute–solvent interactions. This latter contribution is a direct consequence of increased electronic solute polarization, which can be shown by comparison with the results of a simpler model. The assumption of an unchanged electronic energy surface and a fixed-charge TMAO force field displaced along normal modes yields shifts on the order of 1 cm^{-1} only for 10 kbar. This result clearly demonstrates that solvent-induced polarization is the key factor for rationalizing theoretically the pressure-induced blueshifts observed in both experiment and AIMD simulations.

But which are the vibrational displacements that underlie these pressure dependencies? AIMD enables the extraction and assignment of such solute modes also at extreme pressures. Indeed, the mode displacement patterns of TMAO shown in Figure 1b are qualitatively similar to those at 1 bar (see Figure S2). All these factors seem to suggest—at first glance—that compression of this piezolyte aqueous solution does not lead to any interesting effect even at extreme pressures.

In stark contrast, careful inspection of the experimental high-pressure spectra reveals characteristic pressure-induced shape changes. Although the peaks appear to be rather symmetric at ambient conditions, they pick up significant distortions upon compression, as observed for the N–O peak in the inset of Figure 1a. In particular, it not only broadens but becomes skewed, which is a systematic effect as quantified by the normalized skewness, $g_1 = \langle(\tilde{\nu} - \langle\tilde{\nu}\rangle)^3\rangle / \langle(\tilde{\nu} - \langle\tilde{\nu}\rangle)^2\rangle^{3/2}$, in which $\langle\cdots\rangle$ represents the intensity-weighted average, in the inset of Figure 2. Similarly, the CH_3 def-low peak also becomes skewed as a result of compression, yet to a much lesser extent as compared to the N–O stretch (Figure 2), whereas the other two deformation peaks are too close in frequency to be disentangled for this analysis.

Is there a molecular basis for the skewness phenomenon? A recent AIMD investigation[25] into the structure of TMAO(aq) predicted a significant pressure-induced change of the interfacial water network at 10 kbar as compared to 1 bar. At ambient pressure, the oxygen site of TMAO accepts on average three H-bonds from surrounding water molecules (Figure 3c). In stark contrast, in roughly half of the time there are four such H-bonds found when TMAO(aq) is compressed to 10 kbar (Figure 3d). Disentangling of the N–O stretch resonance in Figure 3 for three- and fourfold H-bonded TMAO species (by the separate computation of their IR spectra by the approach described in Section II.B of the Supporting Information) revealed that the experimentally observed N–O peak skewness is due to these distinct TMAO solvation patterns: At 10 kbar, the fourfold-coordinated state was found to be redshifted by approximately 10 cm^{-1} as compared to the threefold-coordinated species at 10 kbar, since the covalent N−O bond is weakened slightly by the increased H-bonding upon compression. However, there is an additional opposite blueshift of the threefold-coordinated TMAO species, again of roughly 10 cm^{-1}, upon compression from 1 bar to 10 kbar (Figure 3a,b). Thus, the total N–O stretching peak is not only expected to shift, but also to be greatly distorted at 10 kbar, since at high pressure its line shape stems from a superposition of the two distinct solvation patterns. Indeed, the resulting skewness of the total N–O peak is found to increase from about −0.357 at ambient pressure to −0.464 at 10 kbar according to AIMD, that is, by −0.107, which is in accord with the value of −0.187 found by experiment (see the inset of Figure 2), whereas the skewness of the two individual threefold- and fourfold-coordination peaks at 10 kbar (red and blue lines of Figure 3b), being −0.330 and −0.317, remains comparable to that at 1 bar and, moreover, is remarkably similar for the two solvation complexes of TMAO.

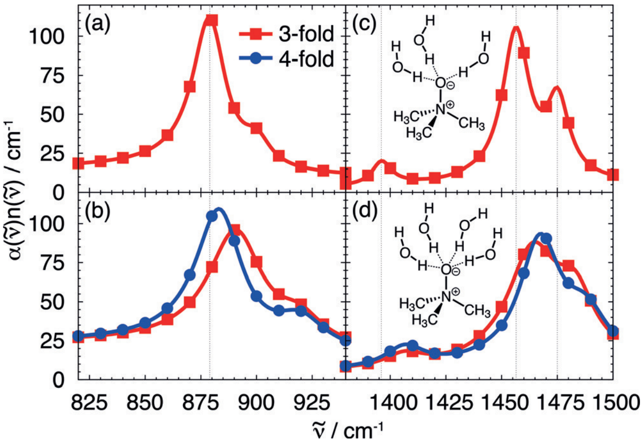

Figure 3. Line shape of the N–O stretching (left column) and the three CH_3 deformation peaks (right column) according to AIMD analysis carried out separately for the threefold and fourfold H-bonded TMAO species at 1 bar (top) and 10 kbar (bottom). The relevant threefold and fourfold H-bonded TMAO molecules are depicted schematically.

This analysis leads to the consistent molecular explanation that the pressure-induced skewness increase of the N–O peak is not due to line-shape changes of a single solvation complex as a result of compression, but is due to the overlapping of the spectra of the two distinct H-bond complexes observed at high pressures. In the case of the lowest-deformation CH_3 peak, the blueshifts of the two resonances due to three- and fourfold-coordinated species are very close to each other (Figure 3d), so that the pressure effect on the skewness of the total peak is much less pronounced than that of the N–O peak; the change in g_1 from 1 bar to 10 kbar is only $+0.070$ and $+0.009$ for experimental and AIMD spectra, respectively. This result is not unexpected given that the water molecules close to the three bulky CH_3 groups do not form H-bonds but rather straddle these hydrophobic groups.[25] The discovered peak skewness of the N–O stretch is therefore a pressure-induced heterogeneous broadening effect that directly probes the distinct changes in the H-bonding pattern of the TMAO molecule in the three-dimensional H-bonded water network as a result of extreme compression.

In conclusion, we have demonstrated that FTIR spectroscopy tightly combined with electronic-structure-based computer simulations is a quantitative tool for research into extreme biophysics along the pressure axis. We scrutinized a small molecule of high relevance to biophysics at extreme pressures: TMAO, the most potent piezolyte known to stabilize proteins against pressure-induced denaturation in deep-sea organisms. The observed peak shifts are of the same order of magnitude as those that allow one to distinguish α-helix from β-sheet conformations of proteins[42] or to probe cosolvent perturbations of proteins.[43] For TMAO, our analyses directly connect pressure-induced changes in its IR spectrum to a locally enhanced H-bonding network at high compression. Moreover, it is experimentally known that TMAO increases the attractive part of protein–protein interactions significantly at high pressures,[44] an effect that has been suggested to be due to changes in the H-bonding network of the solvent. In fact, our findings provide molecular evidence for such changes induced by TMAO. The next steps, therefore, will be applications to small peptides and aqueous piezolyte/protein cosolvent systems to decipher their properties at extreme compression. Beyond these specific examples, our multimethod approach will provide unique molecular insight into biomolecular function at pressures relevant to extant life on Earth under extreme conditions and into hypothetical extraterrestrial habitats, which are otherwise not accessible.

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