High-Pressure NMR Spectroscopy

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The Chemical Shift Baseline for High-Pressure NMR Spectra of Proteins

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Abstract: *High-pressure (HP) NMR spectroscopy is an important method for detecting rare functional states of proteins by analyzing the pressure response of chemical shifts. However, for the analysis of the shifts it is mandatory to understand the origin of the observed pressure dependence. Here we present experimental HP NMR data on the ^{15}N-enriched peptide bond model, N-methylacetamide (NMA), in water, combined with quantum-chemical computations of the magnetic parameters using a pressure-sensitive solvation model. Theoretical analysis of NMA and the experimentally used internal reference standard 4,4-dimethyl-4-silapentane-1-sulfonic (DSS) reveal that a substantial part of observed shifts can be attributed to purely solvent-induced electronic polarization of the backbone. DSS is only marginally responsive to pressure changes and is therefore a reliable sensor for variations in the local magnetic field caused by pressure-induced changes of the magnetic susceptibility of the solvent.*

High-pressure (HP) NMR spectroscopy has become a major method for studying rare conformational states of proteins, which are crucial for the understanding of protein function[1] and are relevant for the development of a new type of inhibitory compounds called intrinsic allosteric inhibitors.[2] In this context, mainly the pressure (p) dependence of the ^{1}H, ^{13}C, and ^{15}N chemical shifts $\delta(p)$ of peptide backbone nuclei is analyzed in order to detect different conformational states of the protein in thermal equilibrium. The observed chemical shift changes are rather small, calling for the use of an internal standard. For measurements conducted at ambient pressure and in polar solvents 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) has been recommended by the IUPAC.[3] The presence of a polar solvent at HP may, however, lead to perturbations of the electronic structure of solutes that in turn can influence chemical shifts and, possibly, also coupling constants due to a change of dielectric properties and/or coordination patterns compared to ambient conditions. Consequently, an understanding of the pure solvent contribution to NMR parameters as a function of pressure is necessary in order to define a baseline that distinguishes inherent solvent-induced from conformational contributions in observed spectra.

Here we report the pressure sensitivity of chemical shifts of the most elementary fragment of a protein backbone, *N*-methylacetamide (NMA) as a prototypical compound from both an experimental and a computational point of view. NMA is structurally simple such that electronic effects can be distinguished effectively from conformational issues. We determined pressure-dependent NMR spectra to elucidate the intrinsic chemical shift dependence of the amide group nuclei, that is, ^{1}H, ^{13}C, and ^{15}N, where the latter isotope needed to be introduced synthetically. Since experimental spectra in protic solvents are commonly referenced to the methylsilyl group of the anion of DSS, the intrinsic pressure dependence of its shielding constant has to be known. Ideally this parameter is pressure-transparent, that is, it would mainly sense the variation of the local magnetic field arising from the pressure-dependent change in the magnetic susceptibility of the bulk solvent and not from changes related to its molecular structure. Therefore, it is necessary to determine the inherent pressure response of (isotropic) magnetic shielding constants σ of relevant DSS nuclei. This is even more pertinent since empirical scaling factors have been defined and recommended in the literature[3] that make it possible to indirectly reference nuclei other than hydrogen with respect to the proton resonance of the DSS methyl groups. Such parameters have been determined for ambient conditions, and in order to establish DSS as a good HP NMR standard in protic solvents the transferability of these parameters to extreme conditions must be examined. This is particularly important for accurate calculations in order to match the experimental and the computational chemical shift scale or, in other words, to understand what is really measured under extreme conditions.

Theory can supplement experiment with difficult to obtain data on shielding constants if two challenges are met: 1) A quantum-chemical (QC) methodology is required

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that properly accounts for HP solvent conditions in the electronic Schrödinger equation and yields reasonable results for NMR parameters in solution, and 2) the scaling factor approach must be circumvented for indirect referencing (vide infra). Issue (1) is addressed by employing the formalism of the "embedded cluster reference interaction site model" (EC-RISM).[4] This represents an integral equation-based approach to couple QC and liquid state theory that we have developed and applied over the past years and which has significant predictive advantages[5–7] over simpler continuum electrostatics approximations such as the polarizable continuum model (PCM).[8] In short, EC-RISM yields mutually self-consistent electronic and solvent structures around a solute by mapping the solvent charge distribution onto discrete background point charges that polarize the electronic Hamiltonian. Solving the Schrödinger equation yields a solvent-polarized electrostatic potential between solute and solvent sites used as input for a calculation of solvent site distribution functions around the solute from three-dimensional (3D) RISM integral equation theory.[9] An estimate of conformational populations in solution is directly possible since EC-RISM provides the free energy of each conformer as the sum of the electronic energy and the analytically available excess chemical potential. The 3D RISM part requires a property of the pure solvent as input, the so-called site–site "solvent susceptibility" χ. This function reflects the density response of the solvent upon insertion of a solute and contains intra- and (pressure-dependent) intermolecular solvent site distributions, representing essentially the quantity that allows for introducing pressure as a variable in QC calculations. χ can be derived either from 1D RISM calculations within a certain approximation (frequently the so-called "hypernetted chain" or HNC model) or taken directly from molecular dynamics (MD) simulations.[10] In this study both approaches were followed.

EC-RISM has been successfully applied separately in both realms, the computation of shifts of NMA in water under ambient conditions[7] and the characterization of pressure-induced intramolecular polarization on trimethylamine-*N*-oxide (TMAO).[10] The latter results were used to derive an effective pressure-dependent force field for TMAO/water mixtures that, employed within classical MD simulations, is able to reproduce hydration patterns from reference ab initio MD and experimental densities between 1 bar and 10 kbar. Here, shielding and shift predictions for NMA relative to DSS are combined with HP solvent conditions and compared with measured data to test the combination of EC-RISM-predicted NMR parameters with HP-induced polarization. An agreement between experimental and computational trends of pressure-dependent shifts would allow for conclusions about features of DSS as a universal HP NMR standard and the pure solvent-induced baseline shift of protein NMR.

We began our study by investigating properties of DSS in two representative solvents, water and methanol, covering a wide range of pressures. After exhaustively generating conformations and geometry optimization using PCM (default settings for water and methanol in Gaussian 09[11]) with the 6-31+G(d,p) basis set and the B3LYP functional (representing a reasonably efficient combination for EC-RISM-based NMR calculations),[7] only five unique relevant conformations remain for further analysis (Figure 1), justified from the corresponding populations derived from free energies (Tables S2 and S3 in the Supporting Information) as shown in Figure 1 for both water and methanol. PCM data reflect ambient (1 bar) conditions, which compare well with EC-RISM results at this pressure for both HNC- and MD-generated susceptibilities. The fully stretched conformation is most abundant in both solvents. We note in passing that water and methanol apparently accommodate DSS differently. While geometries and their energetic ordering are similar, the pressure effect on populations is inverted. The major difference between HNC and simulated (SIM) χ data is the larger population slope at lower pressures for the latter. Furthermore, SIM results in water show a remarkable discontinuity at around 4 kbar, which has also been identified for the TMAO dipole moment.[10] All further NMR-related observables were computed by Boltzmann averaging over the five dominant forms using EC-RISM free energies computed with the respective solvent models.

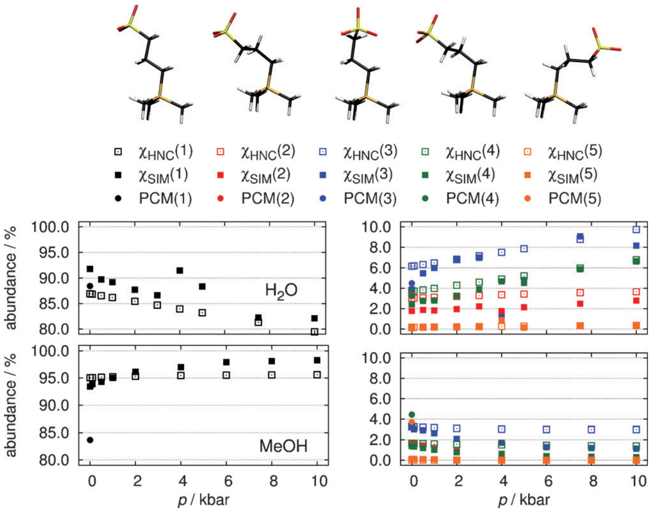

Figure 1. Pressure-dependent abundance of the predominant optimized DSS conformations (shown on top), left: principal form, right: other conformers, in aqueous and methanol solution from normalized Boltzmann factors considering the sum of electronic energy and excess chemical potential at 298.15 K, neglecting vibrational and rotational entropy contributions. B3LYP/6-31+G(d,p) calculations have been combined with different solvation models, PCM (corresponding to 1 bar only) and EC-RISM with susceptibilities χ from various sources (MD "SIM"ulation and HNC), numbers in parentheses reflect the conformer index. Filled circles represent PCM, filled and open squares EC-RISM data.

Results for pressure-dependent isotropic shielding constants of the DSS methyl group from "gauge-invariant atomic orbital" (GIAO) EC-RISM/QC calculations in water and methanol are presented in Figure 2 and in Tables S4 and S5. Most importantly, the absolute pressure dependence of both ^1H and ^{13}C are small throughout, even close to being negligible for protons (<0.01 ppm over 10 kbar in both water and methanol), rendering the experimental determination of such small magnetic-susceptibility-corrected shifts

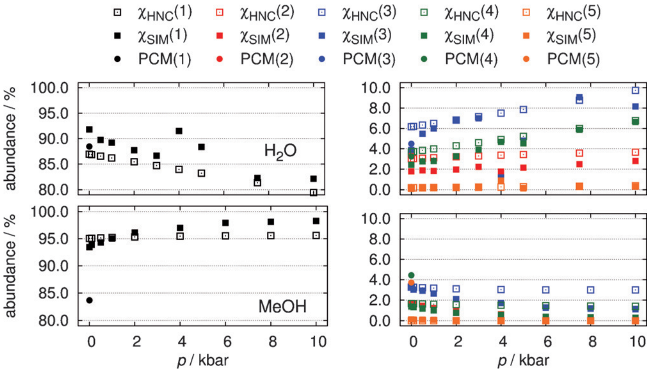

Figure 2. Pressure-dependent shielding constants of the DSS anion from GIAO/EC-RISM/B3LYP/6-31+G(d,p) calculations in water (top) and methanol (bottom) for ^{13}C (left) and ^{1}H (right) of the DSS methyl groups with χ taken from MD (orange) and HNC (blue). Data reflect arithmetic averages over equivalent nuclei.

very difficult. This is a first indication that DSS could indeed represent a good standard, if the major contribution to the solvent shift originates from the target species, in our case NMA. This can be examined by referencing NMA to DSS and studying the computational results (Table S6) in comparison with HP NMR experiments (Table S7), as depicted for aqueous NMA solutions in Figure 3.

The indirect referencing issue (2) mentioned above is related to the fact that QC calculations of chemical shifts benefit substantially from linear error compensation in computing the difference between shielding constants of target and reference compounds, the quantities directly accessible from QC. This advantage is lost when ratios (typically denoted by the symbol Ξ)[3] of NMR frequencies of non-protons and ^{1}H are determined. Here, this problem is resolved by reproducing computationally the experiments by which Ξ factors were determined.[3] For ^{13}C, NMA shieldings are referenced at all pressures to DSS methyl-C at 1 bar, that is, to σ_{ref}(1 bar);[12] for ^{15}N a two-step procedure is followed by first computing the shift relative to aqueous ammonia and then adding the experimentally known shift at 1 bar between dissolved and pure liquid ammonia of −19.4 ppm.[12,13] Due to the lack of nitrogen in DSS, there is no way to refer to a pressure-dependent standard, that is, $\sigma_{ref}(p)$. Such an approach essentially implies that the pressure dependence of the standard is a priori excluded. In the case of ^{13}C the validity of this assumption can be tested directly since this approach can be compared with the (direct) pressure-dependent reference to calculate ^{13}C DSS shielding constants. Similarly, NMA proton shieldings referenced to DSS at the respective pressure (as applied in experiments) can also be compared with referencing to 1 bar shieldings only. This comparison (middle column of Figure 3) clearly demonstrates that the inherent pressure-dependence of DSS ^{1}H shielding constants is negligible since the two approaches yield practically identical results (open vs. filled symbols). In this sense, DSS truly is a good standard, suitable for HP NMR setups as it is almost transparent to pressure changes. ^{13}C behaves only slightly worse which is not unexpected from Figure 2.

Table 1 (full data in Table S8) shows regression parameters for all experimental and computational shift data. The obtained prediction of the chemical shifts at ambient pressure is rather good in comparison to the quality of purely empirical shift prediction methods.[14] Theory mostly overestimates slopes (except for ^{15}N with χ_{HNC}), coming mainly from the low-pressure range in the case of χ_{SIM}, where simulated water distribution functions needed as input apparently vary more drastically with pressure than experimentally expected. HNC here performs quantitatively better, likely due to error compensation between an "underpolarizing" environment as seen earlier for TMAO in water[10] and an overpolarized electronic wave function within the chosen level of theory. Obviously, there is a need to develop better approximations for the pressure-dependent solvation contribution along with appropriate QC models. Overall, however, the computed slopes are positive throughout, in agreement with experiment, indicating that the

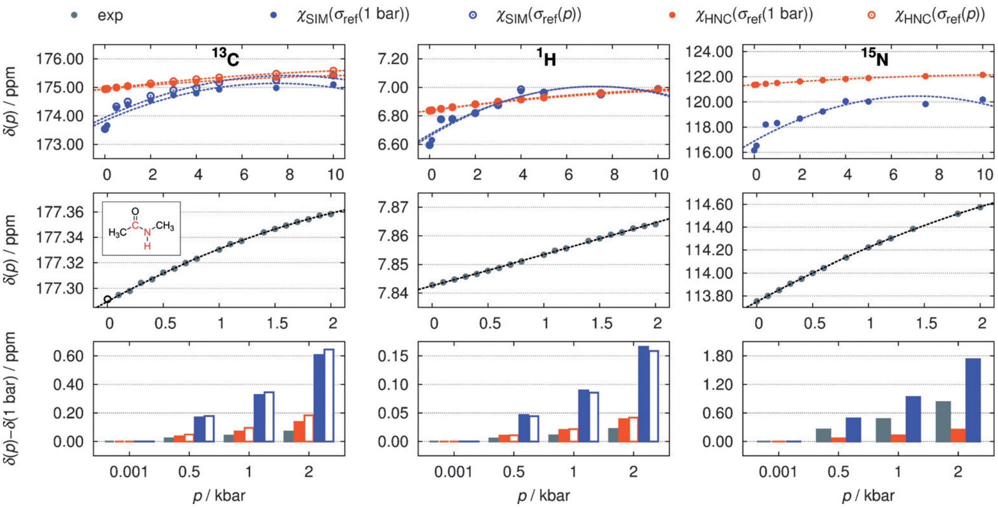

Figure 3. Pressure-dependent chemical shifts of the NMA amide nuclei ^{13}C (left), ^{1}H (middle), and ^{15}N (right) in water from theory (top) and experiment (middle) along with corresponding regression-corrected deviations from values at 1 bar (bottom). Calculation results using GIAO/EC-RISM/B3LYP/6-31+G(d,p) are shown in blue for χ taken from MD and in orange for HNC. Experimental data shown in gray are directly referenced to $\sigma_{ref}(p;{}^{1}H)$ for ^{1}H and indirectly to $\sigma_{ref}(1\ bar,{}^{1}H)$ for ^{13}C and ^{15}N; the open symbol for ^{13}C at 1 bar is the regression estimate for experimental data missing due to technical issues with the high-pressure system. Dashed lines correspond to fits of raw data by second-order polynomials with coefficients shown in Table 1 and Table S8. Filled and open symbols in the top and bottom rows reflect the reference shielding constants used for defining shifts, 1 bar for all pressures and the corresponding pressure pairs, respectively, which is possible for all nuclei except ^{15}N.

Table 1: Linear (B_1) and quadratic (B_2) coefficients obtained from fitting experimental and theoretical NMA shift data to second-order polynomials of the form $\delta(p) = \delta(0) + B_1 p + B_2 p^2$ corresponding to data shown in Figure 3. Full data including statistical uncertainties are reported in Table S8.

Nucleus	$B_1(^{13}C)$[a]	$B_2(^{13}C)$[b]	$B_1(^{1}H)$[a]	$B_2(^{1}H)$[b]	$B_1(^{15}N)$[a]	$B_2(^{15}N)$[b]
exptl	0.048	−0.007	0.011	0.000	0.53(1)	−0.05(8)
$\chi_{SIM}(\sigma_{ref}(1\ \text{bar}))$	0.35(0)	−0.02(3)	0.1(0)	−0.00(7)	1.(0)	−0.0(7)
$\chi_{SIM}(\sigma_{ref}(p))$	0.37(0)	−0.02(3)	0.0(9)	−0.00(6)	–	–
$\chi_{HNC}(\sigma_{ref}(1\ \text{bar}))$	0.07(4)	−0.003	0.021	−0.001	0.13(7)	−0.006
$\chi_{HNC}(\sigma_{ref}(p))$	0.09(9)	−0.004	0.023	−0.001	–	–

[a] In ppm kbar^{-1}. [b] In ppm kbar^{-2}.

theoretical approach presented here captures the essential physics of pressure-induced modulation of magnetic shielding properties. Moreover, predicted quadratic parameters, also summarized in Table 1, which reflect in proteins mainly large conformational fluctuations but are still a puzzle in random coil peptides, are in even better agreement with experiment, being negative throughout or negligible for ^{1}H.

In summary, the experimentally determined and computationally supported pressure dependence of NMA shifts with respect to DSS reveals an intrinsic, purely solvation-induced baseline shift contribution to observed shifts of protein backbone nuclei, independent of the reference originating from electronic polarization. DSS is by all measures a suitable NMR standard for HP conditions due to its pressure-transparent magnetic properties. Remarkably, the experimental ^{1}H, ^{13}C, and ^{15}N NMA slopes (0.011, 0.048, and 0.53 ppm kbar^{-1}) are in the same range as the values found earlier for random coil peptides. The average values (in ppm kbar^{-1}) are 0.038[15] and 0.052[16] for ^{1}H, 0.062[17] for ^{13}C, and 0.29[16] for ^{15}N, but show a large spread dependent on the type of amino acid studied. In proteins individual amino acids can show very different B_1 and B_2 values since they are also influenced by global conformational transitions.[18] The data here reveal that, significantly, also the solvent-induced electronic polarization contribute to pressure-dependent shielding constants. For a conformational interpretation of HP NMR on proteins it will therefore be necessary to study the coupled polarization effects induced by the solvent and by the protein background. These problems along with the development of quantitatively accurate QC/solvation models to allow for spectral assignments represent a substantial challenge to future combined experimental and theoretical efforts.

Experimental Section

NMR experiments were performed with synthetically obtained ^{15}N-enriched NMA in aqueous solution at 298 K (see the Supporting Information for details). Pure solvent χ functions for water were taken from earlier work;[10] a similar procedure was adopted for extracting data for a MeOH force field model.[19] EC-RISM calculations on DSS and NMA were performed following the strategy outlined for ambient conditions.[7] Full details are provided in the Supporting Information.

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