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Identification of Protein Surfaces by NMR Measurements with a Paramagnetic Gd(III) Chelate

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Experimental methods for the identification of solvent-exposed regions of a protein are of great interest for the detection of intermolecular contact sites in protein–ligand complexes and protein-multimers. One popular method measures the effect of paramagnetic additives on nuclear magnetic resonance (NMR) parameters of the protein, in particular its ^{1}H relaxation times. Most often, TEMPOL is used as the paramagnetic agent.[1] Here we demonstrate that a gadolinium-based paramagnetic relaxation agent, Gd–diethylenetriamine pentaacetic acid-bismethylamide (Gd(DTPA-BMA)), yields more predictable results.

Like TEMPOL, Gd(DTPA-BMA) is uncharged and highly water-soluble. As very small binding affinities to the protein already cause excessive linebroadening effects,[2] one of the main advantages of Gd(DTPA-BMA) is its lesser hydrophobicity. The chances of binding are further reduced by its stronger paramagnetism ($J = {}^{7}/_{2}$ for Gd^{3+} versus $S = {}^{1}/_{2}$ for nitroxyl radicals)[3] which allows the use of lower concentrations. DTPA-BMA acts as an octadentate ligand, leaving a ninth coordination site filled with a water molecule.[4] This water molecule is difficult to replace by carboxyl or amino groups and therefore does not present a site for protein binding.[5] Finally, Gd(DTPA-BMA) is stable over a wide range of pH and against redox-active compounds in solution. An early investigation reported the preferential binding of $Gd(DTPA)^{2-}$ to carboxyl and amide groups of ubiquitin.[1a] This effect did not occur with Gd(DTPA-BMA) (Figure 1), but could be reproduced by the addition of 25 μM $GdCl_3$ to the solution (data not shown). The absence of binding of Gd(DTPA-BMA) was confirmed using three criteria: (i) resonances were attenuated, but none of them disappeared; (ii) no significant chemical shift changes were observed upon addition of the relaxation agent; (iii) Dy(DTPA-BMA), expected to act as a shift reagent,[6] did not cause chemical shift changes.

In the absence of specific binding, the paramagnetic agent is expected to enhance the relaxation rates of the protein protons as a function of their surface exposure and distance from the surface. In principle, the phenomenon should best be described by a diffusional relaxation model.[7] Predictions based on such a model, however, correlated rather poorly with the experimental data (Supporting Information), possibly because the model assumes spherical-shaped molecules and neglects intermolecular forces between the electrostatic dipole moments.

The best description of the relaxation enhancements caused by Gd(DTPA-BMA) on ubiquitin seems to be offered by a "second-sphere interaction" model, where the relaxation agent is assumed to form a nonspecific, yet rotationally correlated, complex with the protein in which the dipolar coupling between the electronic spin J and the ^{1}H spin is modulated by the molecular rotation of the protein (correlation time τ_R), the electron relaxation (T_{1e}) and the lifetime of the intermolecular adduct (τ_M).[8,9] In this model, the T_1 relaxation rate of the protons, R_1, is:

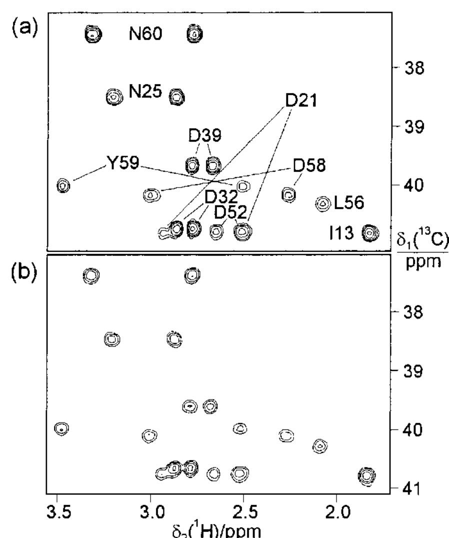

Figure 1. Region of ^{13}C HSQC spectra of human ubiquitin at natural isotopic abundance without (a) and with (b) 4 mM Gd(DTPA-BMA) present in solution. Parameters used: protein concentration 2 mM in 90% H_2O/10% D_2O, pH 4.7, 25 °C, experimental time 66 h per spectrum, ^{1}H NMR frequency 600 MHz, Bruker DMX-600 NMR spectrometer. Cross-peaks from β-protons are labeled.

$$\frac{1}{T_1} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_I^{\,2}(g_J\mu_B)^2 J(J+1)}{r^6}\left(\frac{3\tau_c}{1+\omega_H^{\,2}\tau_c^{\,2}} + \frac{7\tau_c}{1+\omega_S^{\,2}\tau_c^{\,2}}\right) \tag{1}$$

where μ_0 is the induction constant, γ_H the ^{1}H gyromagnetic ratio, g_J the Landé factor, μ_B the Bohr magneton, r the distance between the electron and ^{1}H spin, and ω_H and ω_S the Larmor frequencies of the ^{1}H and electron spin, respectively. The correlation time τ_c is given by:

$$\frac{1}{\tau_c} = \frac{1}{T_{1e}} + \frac{1}{\tau_M} + \frac{1}{\tau_R} \tag{2}$$

The effective distance r was determined by a grid search (grid point spacing 1 Å), where the NMR structure of ubiquitin[10] was used to identify all sites within 10 Å of the protein which are accessible to Gd(DTPA-BMA) (represented as a sphere of 3.5 Å radius). For each protein proton, the distance r_i to each grid point i was calculated and the average value of $1/r_i^6$ computed to determine the effective distance r. The relaxation rates were predicted for each of the NMR conformers and the results averaged and scaled by the occupancy of the grid points which was calculated from the known concentration of the relaxation agent.

Predicted and experimental relaxation enhancements correlate (Figure 2a) and suggest a τ_c value of about 0.5 ns. Assuming τ_R = 4 ns[12] and T_{1e} = 10 ns,[13] τ_M becomes about 0.6 ns (eq 2). Using

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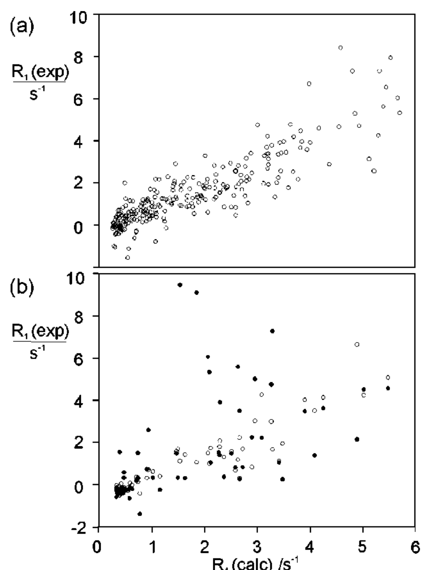

Figure 2. (a) Experimental proton T_1 relaxation enhancement, R_1, versus predicted R_1 values. T_1 relaxation enhancements of ubiquitin by 4 mM Gd(DTPA-BMA) were measured by comparing the cross-peak heights in ^{13}C HSQC experiments preceded by a nonselective 180°(^{1}H) pulse and a recovery delay.[11] Spectra were recorded with recovery delays of 5 and 100 ms, in the presence and absence of Gd(DTPA-BMA). Sample conditions and experimental parameters were the same as in Figure 1, except that the ubiquitin concentration was 5 mM and each spectrum was recorded in 33 h. The predicted R_1 values were calculated using eq 1 with $\tau_c = 0.5$ ns. The plot shows the result for 250 protons. (b) Comparison between the proton T_1 relaxation enhancements, R_1, of the methyl groups of ubiquitin by 4 mM Gd(DTPA-BMA) (open circles) and 25 mM TEMPOL (filled circles), plotted versus the R_1 values calculated using eq 1 with the same parameters as in (a).

$\tau_M = \overline{x^2}/6D$, where D is the diffusion coefficient of Gd(DTPA-BMA) (ca. 3×10^{-10} m^2 s^{-1}), the diffusion distance x required for Gd(DTPA-BMA) to leave the interaction complex becomes about 10 Å. This value appears to be of the correct order of magnitude, considering that any direct interactions become negligeable beyond this distance.

A completely analogous analysis can be performed to describe the enhancement of the transverse relaxation rate R_2 of the protein protons. R_2 values were determined by measurement of the increase in ^{1}H NMR line widths due to Gd(DTPA-BMA). These data also correlated with the predicted values, but the spread was larger than in Figure 2a (Supporting Information). The slope of experimental versus predicted R_2 relaxation rates suggests a τ_c value of about 0.5 ns, in agreement with the R_1 relaxation data.

Equations 1 and 2 predict that Dy(DTPA-BMA) should increase R_1 much less than Gd(DTPA-BMA) because of the rapid electronic relaxation of Dy^{3+} ($T_{1e} \approx 1$ ps).[14] This was confirmed experimentally (Supporting Information).

The deviations observed between experimental and predicted relaxation enhancements (Figure 2a) may thus arise from shortcomings of the relaxation model used but may also reflect the limited sensitivity available in the experiments and, probably, the non-force free interaction between the protein and Gd(DTPA-BMA) which may be uncharged, but still has an electric dipole moment. Most importantly, the relaxation enhancements were reliably big for highly surface-exposed ^{1}H spins and small for the deeply buried protons (Figure 2a).

For comparison, we also measured the T_1 relaxation enhancement of ubiquitin induced by 25 mM TEMPOL. This concentration was required to yield relaxation enhancements of the same magnitude as with 4 mM Gd(DTPA-BMA). Figure 2b shows that the relaxation enhancement caused by TEMPOL did not correlate as well with the predictions based on eqs 1 and 2 as did the data obtained with Gd(DTPA-BMA). We attribute this effect to transient specific binding interactions with ubiquitin which are also evidenced by chemical shift changes (data not shown). Small chemical shift changes were also observed with the commercial formulation of Gd(DTPA-BMA) (sold by Nycomed as an MRI contrast agent under the tradename Omniscan) which contains 5% NaCa(DTPA-BMA) to capture any free Gd^{3+}. However, no chemical shift changes were observed with a sample of Dy(DTPA-BMA) which did not contain Ca(DTPA-BMA)$^-$. Omniscan still induced smaller chemical shift changes than TEMPOL which is a significant advantage in the comparison of experiments with and without relaxation agent. In a preliminary application, Gd(DTPA-BMA) was used successfully to identify the homodimer interface in a 51 kDa protein dimer.[15]

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Supporting Information Available: Additional figures and correlation plots (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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