Using NMR to Distinguish Viscosity Effects from Non-specific Protein Binding under Crowded Conditions

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Materials and Methods

Protein Expression. ^{15}N enriched protein was expressed and purified as described.[1, 2] BSA was purchased from Sigma-Aldrich and used without further purification.

NMR Relaxation and Diffusion Measurements. R_1 and R_2 data were acquired on a Varian Inova 600 MHz spectrometer equipped with standard HCN triple resonance probe. The temperature for all spectra was 25 °C. The pulse sequences are from the Biopack software supplied with the instrument. The ^1H dimension was acquired with a sweep width of 12000 Hz and comprised 1024 complex points. The ^{15}N dimension was acquired with a sweep width of 2500 Hz and consisted of 80 and 128 complex increments for CI2 in BSA and in buffer, respectively. The data were processed with NMRPipe and NMRDraw. [3]

Diffusion experiments were performed on a 600 MHz Varian Inova spectrometer equipped with a triple resonance probehead and xyz gradients using 0.4 mM CI2 in 200 mM sodium acetate buffer pH 5.4 in 10% D_2O. The long ^{15}N T_1 was exploited to determine the diffusion of CI2 in BSA by using a heteronuclear stimulated echo (X-STE).[4] Eight 1D HSQC spectra were collected as a function of gradient strength. Gradient strengths ranged from 480 G/m to 5800 G/m. The integrated area between 8 and 10 ppm was used to monitor the signal decay as a function of gradient strength. The intensities were normalized to the experiment with weakest gradient strength. Data were processed and analyzed by using VNMR (Varian) and NMRpipe. The logarithm of the intensities were plotted versus gradient strength, and fitted to the following equation

$$\ln(I_t) = \ln(I_o) - \gamma^2 G^2 \delta^2 (\Delta - \delta/3)$$

where Io is the initial intensity, γ is the gyromagnetic constant, G and δ are the magnitude and duration of the field-gradient pulses, and Δ is the delay between gradient pulses. The slope of the line is the self-diffusion coefficient. The results are shown in Table S1.

Hydrodynamic Calculations. The program Hydro++ [5, 6] along with the diffusion coefficient ratio

from Table S1 were used to calculate the correlation times of CI2, CI2●CI2 homodimer and the CI2●BSA heterodimer. As no structural information about the CI2●CI2 homodimer and the CI2●BSA heterodimer are available, the radii of 1.610 nm for CI2 and 3.592 nm for BSA from x-ray structure of CI2 (PDB: 1CIQ) and human serum albumin (HSA, 80% sequence identity to BSA,[7] PDB: 1O9X) were used in the calculation. ^{15}N relaxation times at a Larmor frequency 600 MHz were then calculated using the Model Free approach, assuming an order parameter of 0.90 and an internal correlation time of 10 ps.[8, 9] As no additional cross peaks of CI2 are observed in the HSQC spectrum (Fig. S1), the exchange between the CI2 monomer and the dimer (hetero or homo) is fast on NMR chemical shift time scale. Thus, we used following equation to calculate the relaxation rate,

$$R_{obs}=R_{mono} \bullet X_{mono} + R_{dimer} \bullet (1-X_{mono})$$

where X_{mono} is the mole fraction of monomer and R is R_1 or R_2.

Table S1: Diffusion of CI2 in glycerol and in BSA.

	200g/L BSA	42% glycerol
Macroviscosity (cP)	2.5[10]	4.0[11]
$D_{buffer}/D_{crowded}$	2.05[a]	3.95

[a] measured for 0.4 mM and 1.2 mM CI2. The difference in the ratio between the two CI2 concentration is within the measured uncertainty (~3.5%). The X-STE pulse sequence[4] was used to measure the diffusion coefficient.

HSQC spectra of CI2 in the presence and absence of BSA

Figure S1(a) shows the ^{1}H-^{15}N HSQC of 1.2 mM ^{15}N-enriched CI2 in the presence and absence of

200 g/L BSA. The BSA increases the line widths 3.5 to 4.0 fold compared to dilute solution. Except for small chemical shift changes for a few resonances from CI2's flexible loop and turn (Glu26, Val31, Leu32, V38, Thr39, Met40, Glu41, Val60 and Arg62) which are surface exposed, most resonances show no shift change in the presence of BSA. As shown in Figure S1 (b), the chemical shift

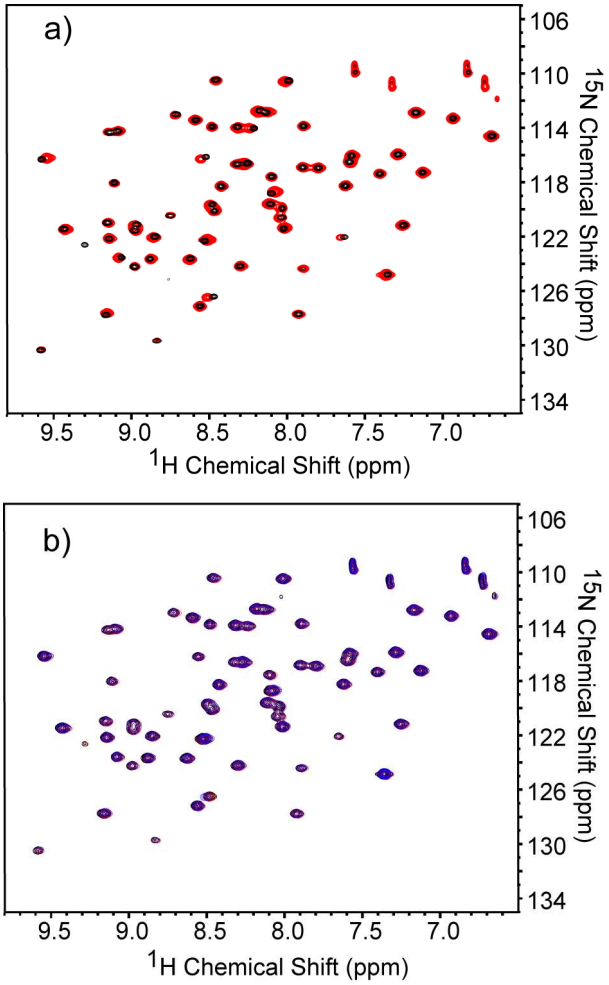

Figure S1. Two-dimensional ^{15}N-^{1}H HSQC spectra of CI2 in sodium acetate buffer, pH 5.4. a) 1.2 mM CI2 at 25 °C in dilute solution (black) and in 200 g/L BSA (red). b) 0.4 mM (black), 0.8 mM (red) and 1.2 mM (blue) CI2 in 200 g/L BSA.

changes are independent of the CI2 concentration in our experimental range. Although some small chemical shift changes of CI2 are detected in BSA, this experiment does not tell us whether the changes reflect CI2-BSA binding, CI2-CI2 binding, or just small structural perturbations induced by crowding.

CI2 Diffusion

The translational diffusion coefficient is also widely used to characterize protein binding and aggregation in dilute solutions.[12-14] As shown in Table S1, the viscosity of a 200 g/L BSA solution is approximately 2.5 times that of water,[10, 15] and the diffusion coefficient of the CI2 in 200 g/L BSA decreases two fold. The diffusion coefficient of CI2 decreases almost four fold in 42% glycerol,[11] which has a viscosity four times that of water. Diffusion is dominated by the solution viscosity.

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