NMR Study of Monomer−Dimer Equilibrium of Barstar in Solution

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Protein dimerization has increasingly attracted the attention of structural biologists recently as a cell regulatory mechanism,[1,2] an evolutionary step from monomeric to oligomeric proteins,[3] and as a phenomenon which might severely complicate the interpretation of NMR structural[4] and relaxation[5−7] data. Monomer−dimer equilibrium constants are usually obtained via methods such as analytical ultracentrifugation,[8] dynamic light scattering,[9] and pulse field gradient NMR.[10] However, the dimer interface and association−dissociation rate constants required for understanding the dimerization mechanism and useful for rational protein design cannot be obtained from these experiments.

This work suggests an alternative approach: ^{1}H−^{15}N NMR line-shape analysis, which provides a more detailed picture of the dimerization process. A small globular protein, barstar, an intracellular inhibitor of the ribonuclease barnase from *Bacillus amyloliquefaciens*, was chosen as the model system. Barstar, barnase, and their tight 1:1 complex are widely used as a model for protein−protein recognition studies by protein engineering, NMR, X-ray crystallography, and microcalorimetry.[11]

In the previous NMR study, substantial line broadening for a number of cross-peaks was observed in ^{1}H−^{15}N correlation spectra of C40/82A barstar. This was explained by a conformational exchange on the μs to ms time scale.[12] However, the number and intensities of the cross-peaks strongly depend on the protein concentration (Figure 1), that is self-association rather than conformational exchange is responsible for the cross-peak broadening.

Chemical exchange between two sites of different Larmor frequency has been well-studied by NMR.[14,15] The absorption line shape $I(\omega)$ in the case of exchange between monomer and symmetric dimer can be derived from principles previously outlined.[16] $I(\omega)$ is a function of $\Delta\omega$ − the difference in Larmor frequencies of a nucleus in monomer and dimer states, R_m and R_d − effective transverse relaxation rates, respectively, for monomer and dimer due to all mechanisms other than the chemical exchange, k_m and k_d − dimerization and dissociation rate constants, and C − protein concentration. R_m and R_d consider not only pure transverse relaxation mechanisms such as dipole−dipole (DD) or chemical shift anisotropy (CSA) but also the effect of magnetic field inhomogeneity, apodization procedure, and other contributions to the line width.

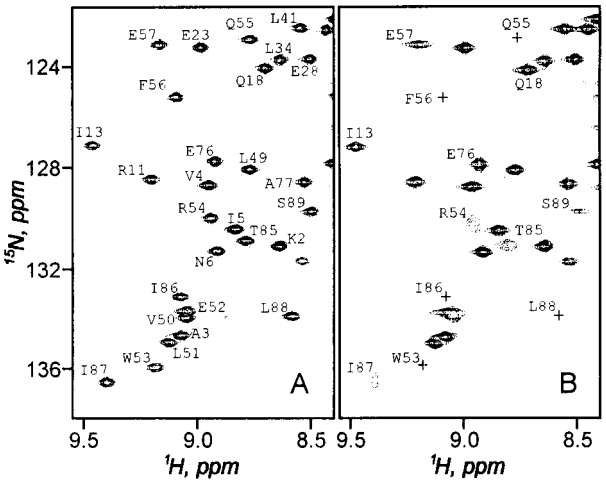

Figure 1. Fragments of ^{1}H−^{15}N HSQC spectra of 0.24 mM (A) and 2.31 mM (B) C40/82A barstar in 10 mM phosphate buffer (pH 6.5) at 298 K. The sequence-specific assignments[13] of cross-peaks are indicated by the single letter amino acid residue code and residue number. W53, Q55, F56 were assigned, and S89 was reassigned in this work. (B) Only cross-peaks with concentration-dependent line width are labeled. Cross-peaks broadened beyond detection are indicated by crosses.

^{1}H−^{15}N sensitivity-enhanced HSQC[17] spectra were recorded at 298 K using 600 MHz Unity Varian spectrometer. The samples contained C40/82A barstar at six concentrations between 0.24 mM (Figure 1A) and 2.31 mM (Figure 1B) in 10 mM phosphate buffer (pH 6.5). As one can see in Figure 1, some ^{1}H−^{15}N cross-peaks demonstrate substantial line broadening with the increase in protein concentration, while others remain practically unchanged. The line widths at half-height in ^{1}H and ^{15}N directions were measured for all nonoverlapped cross-peaks at all concentrations. Uncertainties of experimental line width were estimated as 0.5 Hz for ^{15}N direction and 1 Hz for ^{1}H direction, but for low-intensity peaks experimental uncertainties were increased up to 4 Hz in both directions. Theoretical line widths calculated from the line shape $I(\omega)$ were then fitted to the experimental line widths in order to obtain parameters $\Delta\omega$, R_m, R_d, k_m, and k_d. The fitting was accomplished by an iterative nonlinear least-squares procedure using a program specially written for this purpose. The adjusted parameters k_m and k_d were common for all resonances, while $\Delta\omega$ and R_m were fitted for every resonance in ^{1}H and ^{15}N

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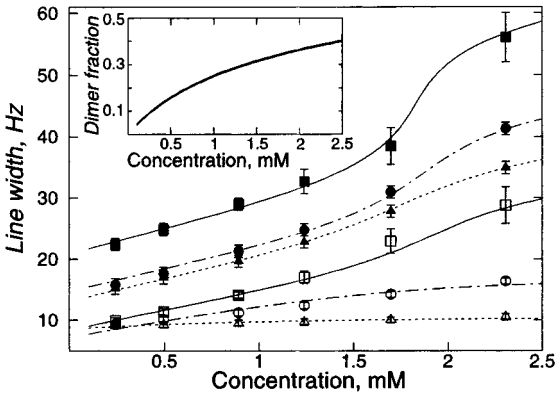

Figure 2. Experimental ^{1}H (opened symbols) and ^{15}N (filled symbols) line widths of W53 (squares), S59 (circles) and E57 (triangles), and fraction of dimer molecules (inset) plotted versus barstar concentration. Curves were obtained by nonlinear least-squares fit of experimental data.

directions. Model calculations showed a strong coupling between individual parameters R_d and $\Delta\omega$ when the dimer content is less than 50%, precluding simultaneous determination of these parameters. To avoid the coupling and to determine chemical shift differences $\Delta\omega$, it is necessary to estimate individual relaxation rates R_d. The main source of the difference between R_m and R_d is an increase in DD and CSA transverse relaxation rates due to slower rotational diffusion of the dimer. Available ^{15}N transverse relaxation data for barstar[12] show about 1 Hz dispersion of transverse relaxation rates among nuclei for which no exchange line broadening was observed. Moreover, when the dimer content is less than 50%, the uncertainty in R_d of 1 Hz causes less than 0.5 Hz uncertainty in theoretical line width. Thus, as the first approximation we used $R_d = R_m + \Delta_N$ for ^{15}N direction and $R_d = R_m + \Delta_H$ for ^{1}H direction, where Δ_N and Δ_H were the common fitting parameters for all resonances in ^{15}N and ^{1}H directions, respectively.

A total of 70 resonances in ^{1}H and 72 resonances in ^{15}N directions were included in the fitting procedure. The following values were obtained: $k_d = 54 \pm 3$ s^{-1}, $k_m = 24.2 \pm 0.9$ s^{-1} mM^{-1}, $\Delta_H = 15 \pm 1$ Hz, and $\Delta_N = 9.1 \pm 0.5$ Hz. The uncertainties were calculated from corresponding deviations of experimental data points from theoretical curves. Small errors for Δ_H and Δ_N imply that the first approximation for R_d is good enough. The final loss function in least-squares method χ^2 is 154 for 645 degrees of freedom. The small standard deviation of all parameters and low χ^2 value confirm the assumption that barstar forms a mixture of monomers and symmetric dimers, and heavier aggregates can be neglected. Representative examples of the fitted curves and the dimer content are plotted versus the barstar concentration in Figure 2.

The obtained $\Delta\omega$ values allow characterization of the dimerization interface. In this region, resonances of amide groups are expected to have large $\Delta\omega$ values. The backbone of the X-ray structure of C82A barstar dimer[18] is represented in Figure 3 as a "sausage" with radius proportional to the sum of $\Delta\omega$ in ^{1}H and ^{15}N directions. As one can see, the residues adjacent to the contact region have large $\Delta\omega$'s showing that NMR and X-ray deal with the same barstar dimer. According to previous NMR study,[12] the conformational exchange has been suggested for two regions: (1) residues P27-E32 (barnase binding loop) and N33-G43 (helix α2);

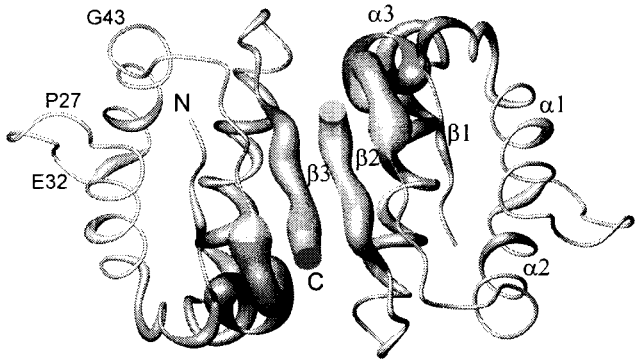

Figure 3. Sausage model of C82A barstar dimer as obtained by X-ray crystallography.[18] The radius of the sausage is proportional to the sum of $\Delta\omega$ in ^{1}H and ^{15}N directions for corresponding residue. N and C termini, barnase binding loop (P27-E32), α-helixes and β-strands are indicated. This figure was produced with the MOLMOL[19] program.

(2) helix α3, strands β2, β3 (Figure 3). Our data indicate, however, that broadening of cross-peaks in the second region is caused by barstar dimerization, and only the first one likely undergoes conformational exchange.

It is interesting to note that the dimerization interface is opposite to the barnase binding loop (P27-E32). A similar concentration dependence of line widths for residues on the barstar dimerization interface was observed in HSQC spectra of barstar-barnase complex (data not shown). This fact points to a dimerization of the barstar−barnase complex.

It is well-known that aggregation can severely complicate ^{15}N NMR relaxation data analysis. For example,[5] even 10% of dimers in the monomer/dimer mixture would lead to significant errors in internal motion correlation times τ_e and order parameters S^2 obtained by model free approach.[20,21] At the 2.0/3.5 mM barstar concentration used in the ^{15}N NMR relaxation study,[12] the dimer fraction is more than 30% (Figure 2). This explains the extremely high values of S^2 obtained in the study. Thus, the dimerization effects should be taken into account in any analysis of experimental data for both barstar and its complex with barnase.

It should be noted that information derived from line shape analysis could be also obtained from ^{15}N transverse relaxation measurements for a set of protein concentrations.[7] However, the latter approach is more time-consuming because several 2D spectra have to be acquired for each protein concentration. Besides that, the proposed method allows to obtain $\Delta\omega$'s for both ^{15}N and ^{1}H directions from the same experimental data set.

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Supporting Information Available: A more detailed description of the theory, data analysis, experimental methods, and calculated $\Delta\omega$ values for all residues (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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