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Communication

Measuring protein self-diffusion in protein–protein mixtures using a pulsed gradient spin-echo technique with WATERGATE and isotope filtering

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Abstract

Here we report a modified pulsed gradient spin-echo (PGSTE) pulse sequence to measure diffusion coefficients. This approach incorporates WATERGATE combined with isotopic filtering into a standard PGSTE experiment. Doing this eliminates much of the disadvantages from the combination of diffusion encoding and heteronuclear selection intervals and allows for facile modification of the diffusion pulse sequence with flexibility of the time period between RF pulses. The new diffusion pulse sequence is demonstrated using an ^{15}N-labeled peptide and an ^{15}N-labeled protein in a mixture with a protein of similar size.

1. Introduction

Growing interest in structurally defining protein–protein and protein–ligand interactions has stimulated development of various heteronuclear NMR filtering and editing techniques [1–3]. Heteronuclear filtering is traditionally employed to simplify NMR spectra and to determine intermolecular NOEs between units in a complex [4–10].

Usually as a prerequisite to studying molecular complexes, an initial titration experiment is performed in which a series of ^{15}N HSQC spectra is recorded as a function of protein and/or ligand concentration [11] and chemical shift perturbations are used to indicate specific interactions between molecules in the complex. However, from these data alone, it may not be possible to deduce the aggregate state (dimers or higher order species) or the life time of the complex. Such information may be had by performing diffusion pulsed gradient spin-echo (PGSTE) NMR measurements (for recent reviews, see [12–16]). The diffusion coefficient, which is inversely proportional to the hydrodynamic radius of a species diffusing in solution, can be used to estimate the size of the complex formed. Measuring the dependence of the diffusion coefficient on concentration, pH or diffusion time can yield detailed information about the association process, e.g., estimation of association constants, life time of associates, etc. [17–23]. Because diffusion measurements can be performed relatively quickly, they can be easily done in conjunction with initial HSQC titration studies. Incorporation of heteronuclear filtering in PGSTE experiments allows diffusion coefficients of different molecules mixed together in solution to be measured individually. If these molecules differ significantly in size, they diffuse at different rates and other methods that rely on the difference in diffusion coefficients [24,25] or relaxation times (idea realized in Water-PRESS [26]) can be effectively employed. However, as two molecules become similar in size, the error in separating their diffusion coefficients increases, and heteronuclear filtering becomes one of the most efficient ways to determine their individual, yet dependent, diffusion coefficients.

Several diffusion pulse sequences that use heteronuclear filtering have been reported [27–29]. The efficiency of these sequences depends on the system being studied [28]. In general, these sequences have one common

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feature: the "diffusion part" of the pulse sequence is combined with heteronuclear coherence selection. This feature can be disadvantageous for two reasons. First, time intervals used for diffusion encoding and heteronuclear coherence selection are strictly related. Sometimes, however, it is desirable to have flexibility in the time period between radiofrequency (RF) pulses when the magnetization is in the transverse plane, such as when investigating a complex system displaying a distribution of relaxation times [30]. Second, the combination of the "diffusion part" of the sequence with coherence selection complicates utilizing some of the modifications of the basic diffusion experiment reported in the literature.

In a diffusion experiment, water suppression presents yet another problem, especially in the case of ^{15}N-labeled proteins. In choosing an appropriate water suppression method, it is desirable to maintain signals from exchangeable protons in the protein and avoid radiation damping effect of the water during the diffusion time. The ideal solution here would be to flip water magnetization back along the $+z$ axis during the course of pulse sequence [31]. In practice, residual water is always present and suppression methods like WATERGATE [32] should be used. The introduction of WATERGATE in a diffusion experiment provides an excellent opportunity to combine heteronuclear filter with the WATERGATE sequence because the $1/2J$ time period is sufficiently long to provide good water suppression. Separating heteronuclear coherence selection in this way makes for facile modification of the basic diffusion experiment, including measuring the time dependences of diffusion coefficients.

In the present communication, we report an improved PGSTE pulse sequence that employs water flip-back and WATERGATE combined with a heteronuclear filter. We call this modified diffusion experiment the WIF-(WATERGATE Isotope-Filtered)-PGSTE pulse sequence. To demonstrate the usefulness of this pulse sequence, we have measured diffusion coefficients of a peptide and for a mixture of two homologous proteins with similar molecular weights.

2. Materials and methods

A peptide having the amino acid sequence GFSKAELAKARAAKRGGY was synthesized using standard Fmoc solid-phase methodology on either a Milligen/Millipore Excell automatic peptide synthesizer or on an Applied Biosystems 431A Peptide Synthesizer and was purified by HPLC using a linear acetonitrile/water gradient as described by Idiyatullin et al. [33]. Residues A5 and L7 were ^{15}N-enriched (CIL, Cambridge). Peptide purity was checked by MALDI- TOF mass spectrometry and analytical HPLC on a C18 Bondclone (Phenomenex) column. The proteins PF4 and IL-8 were expressed and purified as described in [34].

For NMR measurements, freeze-dried samples were dissolved in a H_2O/D_2O (95/5) (v/v) mixture. Peptide/protein concentration was determined from the dry weight of freeze-dried samples. The pH was adjusted by adding microliter quantities of NaOD or DCl. NMR experiments were performed on Varian Inova-600 NMR spectrometer equipped with triple-resonance probes.

The WIF-PGSTE pulse sequence reported here is illustrated in Fig. 1. The "diffusion part" of the sequence consists of three 90° pulses and basically represents the well-known stimulated-echo sequence [35]. Two magnetic field gradients of amplitude g and duration δ are used to "encode diffusion." The spacing between the

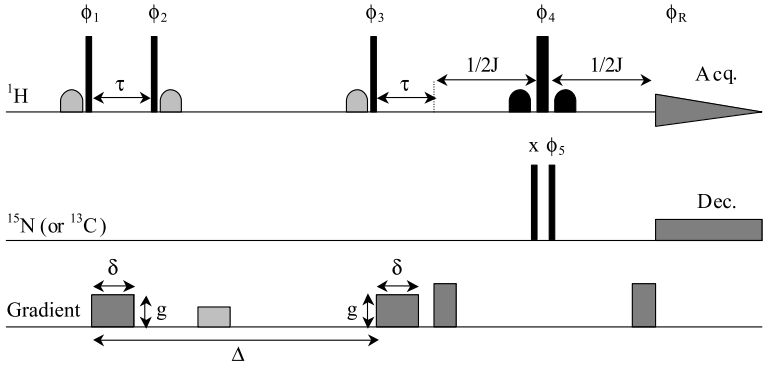

Fig. 1. The WIF-PGSTE pulse sequence for diffusion measurements using water flip-back and heteronuclear filtrating combined with WATERGATE [32]. Black bars represent non-selective RF pulses of 90° and 180°. Selective pulses applied at the water resonance are shown as dotted rounded bars (flip-back) and black rounded bars (part of WATERGATE scheme). Gradients for the diffusion measurements of duration δ and amplitude g are shown as wide hatched bars. Phase cycling is: $\phi_1 = 8(x), 8(-x)$; $\phi_2 = x, y, -x, -y$; $\phi_3 = x, y, -x, -y, -x, -y, x, y$; $\phi_4 = 4(x), 4(-x)$; $\phi_5 = x, -x$; $\phi_R = 4(-x), 8(x), 4(-x)$. Selective "water" pulses are 180°-shifted relative to the following or preceeding non-selective pulse. A ^{13}C 180°-pulse in the middle of WATERGATE period and carbon decoupling during acquisition time should be applied if double, ^{15}N–^{13}C, labeled samples are studied.

front edges of these two gradients determines the diffusion time, Δ. Prior or next to high-power 90° pulse, a low-power selective 90° pulse of opposite phase is applied at the frequency of water. Each pair of selective and non-selective pulses returns water magnetization along the $+z$ direction for most of the duration of the pulse sequence. Because of this, exchangeable protons contribute to the spin-echo, and maintaining water magnetization along the $+z$ axis eliminates the problem of radiation damping that can be severe due to the relatively large separation between the second and third 90° pulses [36]. To dephase magnetization remaining in the xy plane, a spoiler gradient can be applied during the diffusion delay, with the magnitude of this gradient being chosen to avoid refocusing effects from other gradients. The "diffusion part" of the sequence is then followed by WATERGATE [32]. WATERGATE is used to remove the residual water signal that is always present due to field inhomogeneities and imperfections in selective pulses. This provides mild saturation because most of the water is flipped back during the pulse sequence. If x and y gradients are available, it is also possible to use MEGA instead of WATERGATE [36,37]. WATERGATE is combined with an isotope half-filter, consisting of two ^{15}N pulses. Alternation of the phase of a second ^{15}N-pulse inverts the sign of heteronuclear antiphase magnetization, $2H_xN_z$, created from protons coupled to ^{15}N, whereas the sign for other proton magnetization remains the same. Subtraction of these two datasets selects the signal from protons bound to ^{15}N, thus providing ^{15}N-filtration.

The diffusion attenuation of the spin-echo from which the diffusion coefficient, D, of the molecule of interest can be estimated by using

$$A(g^2) = A(0)\exp[-\gamma^2\delta^2 g^2 D(\Delta - \delta/3)], \quad (1)$$

where γ is the gyromagnetic ratio for protons.

3. Results and discussion

Initially, the WIF-PGSTE pulse sequence was tested on a small 18-residue peptide that was selectively ^{15}N-labeled at residue positions 5 and 7. The resulting diffusion attenuation curve is shown in Fig. 2 (open circles). For comparison, the diffusion attenuation acquired using the standard stimulated echo pulse sequence with bipolar gradients [38] is also shown (solid circles). These two diffusion attenuation curves recorded using different diffusion pulse sequences coincide very well and yield the same diffusion coefficient of $(1.47 \pm 0.02) \times 10^{-6}$ cm^2/s. This comparison validates use of the new WIF-PGSTE pulse sequence to measure diffusion coefficients.

Next, the WIF-PGSTE pulse sequence was used to determine the diffusion coefficient of PF4-M2, an N-terminal chimera of the protein platelet factor-4 [34] in solution with a protein of similar size, interleukin-8 (IL-8). PF4-M2 and IL-8, which both belong to the family of α-chemokine proteins that are involved in the regulation of angiogenesis, inflammatory processes, and wound healing, show approximately 60% sequence homology, have nearly the same structural folds as monomers [34,39], and interact with each other to form heteroaggregates [40]. At millimolar concentration in aqueous solution, pure IL-8 forms homodimers [39], whereas pure PF4-M2 forms mostly homotetramers (a dimer of dimers) with some homodimers and monomers that exist in relatively slow exchange on the chemical shift time scale [34]. In PF4-M2/IL-8 heteroaggregates, therefore, PF4-M2 subunits could be found in heterodimers and/or in heterotetramers.

When both proteins are present together in solution, the small difference in their monomer molecular weights (700 Da), as well as aggregation and spectral overlap, makes determination of their individual diffusion coefficients using the standard PGSTE diffusion pulse

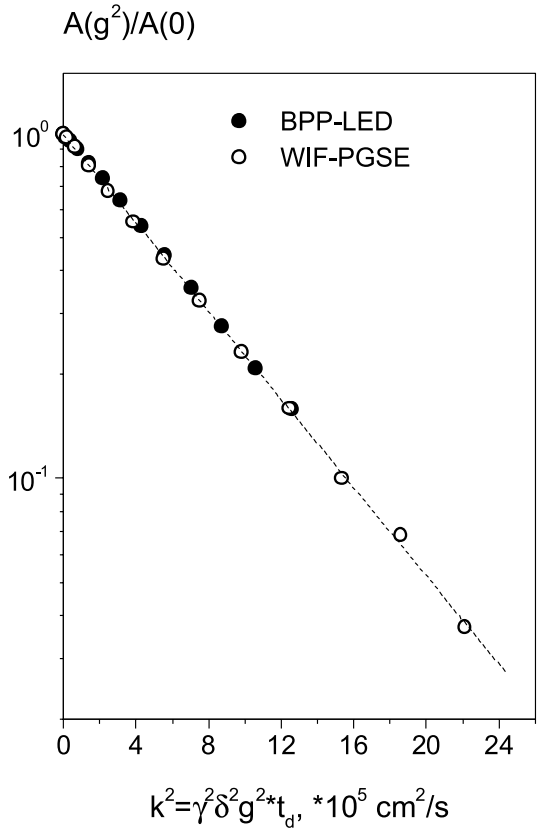

Fig. 2. Diffusion decays recorded using BPP-LED [38] (solid circles) and WIF-PGSTE (open circles) pulse sequences are illustrated. Measurements were performed on an 18-residue α-helix-forming peptide dissolved in 95/5 (v/v) H_2O/D_2O at 10 °C. Time delays were: $\delta = 4$ ms; $\tau = 4.2$ ms; $\Delta = 43.1$ ms (BPP-LED) and 34.2 ms (WIF-PGSTE). Gradients were varied in amplitude from 1 to 33 G/cm (BPP-LED) and from 1 to 56 G/cm (WIF-PGSTE).

sequence essentially impossible. This difficulty may be obviated by having one of the two proteins in the hetero complex isotopically labeled, for example, ^{15}N-labeled PF4-M2 subunits, and by using the WIF-PGSTE diffusion pulse sequence. To demonstrate this, diffusion coefficients for ^{15}N-labeled PF4-M2 in solution were determined before and after addition of unlabeled IL-8. For pure ^{15}N-labeled PF4-M2, the derived diffusion coefficient is $(1.12 \pm 0.017) \times 10^{-6}$ cm^2/s (Fig. 3, filled-in circles), which according to the Stokes–Einstein equation, $D = kT/6\pi\eta R$ (k is the Boltzman constant, η is the viscosity of pure solvent, and R is the radius of a spherical particle) is consistent with PF4-M2 being mostly in the homotetramer state as reported earlier [34]. As IL-8 is added to this solution and the molar ratio of IL-8–PF4-M2 is increased, the ^{15}N-labeled PF4-M2 diffusion coefficient increases from $(1.12 \pm 0.017) \times 10^{-6}$ cm^2/s to $(1.49 \pm 0.02) \times 10^{-6}$ cm^2/s at the molar ratio of 1:2 (PF4-M2:IL-8) (Fig. 3, opened circles). By itself, this indicates a change in the aggregation state of PF4-M2. According to the Stokes–Einstein equation, a diffusion coefficient of 1.49×10^{-6} cm^2/s is consistent with the PF4-M2/IL-8 heteroaggregates being close to that of a dimer. This information is not available from HSQC titration studies. The accuracy of this diffusion information is also crucial to estimating heteroaggregate association constants as accurately as possible.

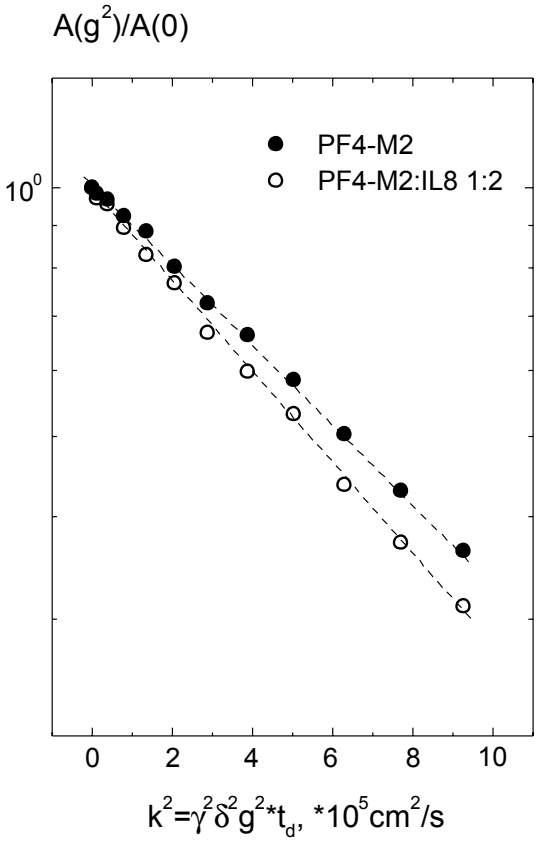

Fig. 3. Diffusion decays recorded for ^{15}N-labeled PF4-M2 in pure solution (solid circles) and in solution to which unlabeled IL8 was added at a molar ratio of 1:2 (PF4-M2:IL8) (open circles). Experiments were performed in a solution of 95/5 (v/v) H_2O/D_2O, 20 mM NaCl at pH 5.0 and 40 °C. Time delays were: $\delta = 4$ ms; $\tau = 4.2$ ms; $\Delta = 51.5$ ms. Gradients were varied in amplitude from 1 to 52 G/cm.

In conclusion, with so many available modifications of diffusion experiments, it is necessary to decide which pulse sequence to use for each particular system. A pulse sequence that is advantageous for one system may be less suitable for another. The WIF-PGSTE pulse sequence reported here is most useful when studying a mixture of proteins of similar size. However, there are two other distinct advantages: (1) it uses an arbitrary duration of the gradient coding time and spacing between RF pulses; (2) it has a "block structure" that allows easy modification by simply substituting existing variations of pulsed gradient sequences for diffusion experiments. For example, if diffusion measurements are performed in low viscosity solutions at high temperature, induced convection in the sample could contribute to signal decay and make it appear as though molecules were diffusing more rapidly [41–44]. In this case, a double-stimulated-echo [42] could be easily integrated into the "diffusion part" of the pulse sequence to compensate for convection artifacts. Although the pulse sequence reported here has been designed for and applied to ^{15}N-labeled proteins/peptides, it can be easily modified to measure diffusion coefficients of biomolecules labeled with other NMR-active isotopes.

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