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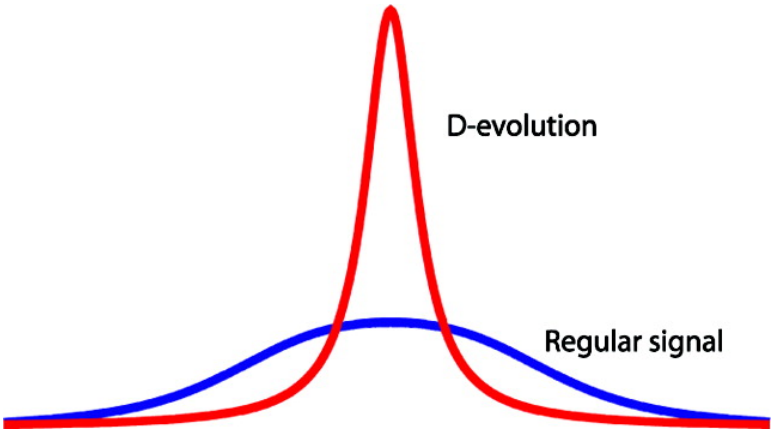


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# Divided Evolution: A Scheme for Suppression of Line Broadening Induced by Conformational Exchange

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One of the major limitations in biomolecular NMR is the inability to obtain high-quality spectra when a molecule is involved in a conformational exchange (CE) process occurring in the intermediate time scale. Significant line broadening and signal attenuation due to CE is often observed for globular proteins and is ubiquitous in NMR studies of protein−ligand complexes[1] and integral membrane proteins[2,3] as well as disordered and molten-globule protein states.[4] So far, the only recipe for improving the exchange-broadened spectra has been to make the studied system better suited for NMR spectroscopy. This approach, however, has a number of disadvantages. First, there is no general procedure for sample conditioning that guarantees success within the allowed range of sample parameters. Besides, the CE often reflects an important biological process and may itself constitute focus in a study.[5,6] Here, we suggest the first general signal acquisition technique that allows NMR adaptation to the systems with CE. While the sample conditioning aims to stabilize one predominant stage and/or shift the exchange time scale from the intermediate to the fast regime, the new approach allows separation of time scales of the CE and NMR chemical shift evolution. This reduces line broadening by avoiding the situation of intermediate exchange.

The transverse relaxation due to CE is suppressed during Carr-Purcell-Meiboom-Gill (CPMG) sequences,[7] provided that the radiofrequency pulses are applied with intervals shorter than the lifetime of the conformational states.[8,9] Unfortunately, CPMG quenches the chemical shift (CS) evolution as well and cannot be used directly to eliminate line broadening. Figure 1 shows a new acquisition scheme. The CS evolution is divided into a number of short intervals $\delta$; this is called D-evolution. The intervals $\delta$ are interlaced by the CPMG blocks of duration $T_{CPMG}$. The CS evolution and transverse relaxation due to CE are suspended during the CPMG block, while the physical transitions between conformational states naturally continue. In the limit $T_{CPMG}$, $\delta \ll 1/k$, the net effect of the scheme on a NMR line shape may be described as an apparent increase of exchange rates by $r_D = (T_{CPMG}+\delta)/\delta$ ratio. Here, $k$ is the fastest rate constant of the conformational exchange. Notably, the apparent rate of the spin system evolution caused by mechanisms, which are immune to refocusing by the CPMG pulses, increases by the same factor. For signals that are not affected by CE, the natural line width increases by $r_D$. This poses a practical limit to $r_D$, since the achieved line narrowing should be larger than the increased broadening due to amplification of other relaxation mechanisms and homonuclear scalar couplings (see simulations in the Supporting Information). For macromolecules, the D-evolution benefits from methods for reducing natural line widths, i.e., the TROSY and deuteration.[10,11]

Figure 2a,b demonstrates an implementation of the new detection scheme for the direct spectral dimension for two model systems

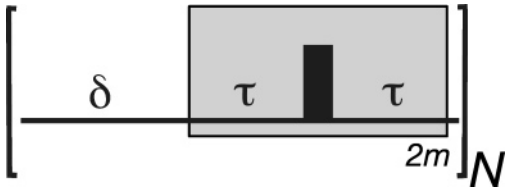


***Figure 1.*** D-evolution scheme. The period of chemical shift evolution is divided into $N$ equal intervals $\delta$ separated by the *CPMG blocks* (indicated by a gray bar). In this work, $\delta$ is equal to the inverse of the spectral width. The black bar flanked by delays $\tau$ indicates nonselective 180° pulses in the XY CPMG type sequence,[12] which is used to equally propagate X and Y components of the magnetization. The phases of the 180° pulses follow the XY4, XY8, and XY16 super cycles. The XY16 ($m$=8) phases are (x,y,x,y y,x,y,x, -x,-y,-x,-y, -y,-x,-y,-x), and the XY4 and XY8 use only the first $m = 2$ and $m = 4$ steps, respectively. In the 1D pulse-sequence with D-evolution in the directly detected dimension, the standard evolution is replaced by a period of explicit acquisition, where the data points are sampled at the end of every $\delta$ delay. In the indirect dimension, the D-evolution period replaces a standard evolution delay $\Delta$, so that $\Delta = N\delta$. NMR spectra were recorded on Varian Inova 600 and 800 spectrometers.

with a well understood exchange process. The chair−chair interconversion of a cyclohexane molecule is a classical case of a two-state conformational exchange.[13−15] Under our experimental conditions, the $^1$H line shapes for deuterated cyclohexane exhibit significant broadening with a line width of 109 Hz. Broadening of the $^{13}$C peak (4.5 Hz) in acetonitrile gives an example of motionally suppressed $J$-coupling as a result of a rapid spin-lattice relaxation of $^{14}$N nuclei. The carbon line shape can be described using the formalism of exchange between equally populated states corresponding to the individual components of the $^1J(^{13}C-^{14}N)$ coupling multiplet.[16] Figure 2 shows that the new acquisition scheme gives peaks that are 1.6 and 2.4 times narrower in comparison with the regular one-dimensional spectra of cyclohexane and acetonitrile, respectively.

Figure 2c demonstrates line narrowing for a selected signal from the $^{15}$N-$^1$H-TROSY-HSQC spectrum of $^{15}$N labeled 14 kDa globular protein azurin.[17] Here, the D-evolution scheme is used in the indirect nitrogen dimension. The figure depicts a cross-peak of Asn47, which is one of the few amides exhibiting conformational exchange in azurin.[18,19] Use of the D-evolution reduces the $^{15}$N line width 1.5 times relative to the regular TROSY spectrum and correspondingly improves the signal-to-noise ratio for the signal. Insufficiently short $\tau$ relative to $1/k$ can reverse the effect of the D-evolution and lead to significant signal broadening (see the Supporting Information).

The effect may explain line broadening for Gly90, Gly45, Ile87, and Thr48 signals (not shown) in azurin when the D-evolution is applied. Indeed, these residues are involved in a conformational exchange,[18,19] which is comparable or faster relative to the used CPMG intervals $\tau = 0.25$ ms. For the remaining azurin signals, the D-evolution increased the average peak line width from 7 to

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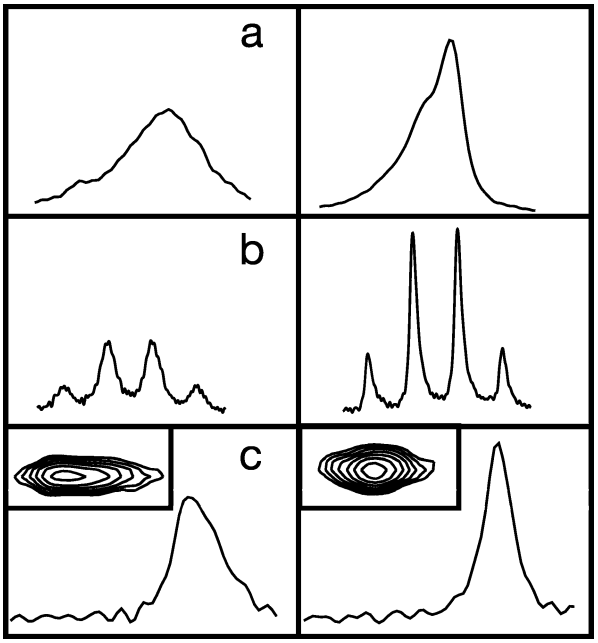


***Figure 2.*** Comparison of NMR spectra recoded using D-evolution (right) and regular evolution (left) schemes. *Direct dimension:* (a) residual $^{1}$H signal in deuterated cyclohexane, $r_D = 11$, $\tau = 0.04$ ms, $\delta = 0.2$ ms and (b) natural abundance $^{13}$C signal of C1 carbon of acetonitrile, $r_D = 5.5$, $\tau = 0.11$ ms, $\delta = 1$ ms. The multiplet for acetonitrile is due to $^{2}J(^{1}H\text{-}^{13}C)$ coupling with methyl protons. *Indirect dimension:* (c) amide cross-peak of Asn47 (127.1 ppm, 10.62 ppm) in the $^{15}$N,$^{1}$H-TROSY-HSQC spectrum of azurin is shown as cross sections along the $^{15}$N dimension and contour plots (inset). $r_D = 4$, $\tau = 0.25$ ms, $\delta = 1.25$ ms. Details about the samples can be found in the Supporting Information.

12 Hz, which reflects an apparent increase in the transverse relaxation by $r_D = 4$ and partial compensation of this effect by short acquisition time (125 ms) in the indirect spectral dimension. Within the experimental error, the line broadening corresponds to an observed drop of the average intensities and signal-to-noise by a factor of 1.8.

In order to check possible signal attenuation by our implementation of the D-evolution scheme, we systematically analyzed line-broadening for a system without conformational exchange: $^{15}$N/$^{2}$H labeled 7 kDa protein-L.[20] The $^{15}$N line widths exhibit linear increase, as the CPMG block gets longer, with the slope corresponding to $r_D$. With the $r_D$ equal to 9, the average $^{15}$N line width for the protein-L is 14 Hz. For azurin, which is two times larger than protein-L and not deuterated, the average width is 30 Hz ($r_D = 9$). The line widths obtained for protein-L and azurin are typical for $^{15}$N-HSQC spectra of globular proteins.

In this work we demonstrate a general principle of the D-evolution. Practical experiments may employ the scheme simultaneously in the direct and indirect dimensions and use methods for suppression of exchange induced relaxation during magnetization transfer periods.[21] The maximum CPMG rate is an important determinant of the effectiveness of the method, and means should be found to increase the affordable limits for the NMR probes, especially for the $^{15}$N channels. We can also envision applying different CPMG schemes. In particular for large molecules, it can be advantageous to partly preserve magnetization in the longitudinal polarization, which has a lower relaxation rate and is immune to CE relaxation. The D-evolution scheme shifts the apparent exchange time scale of the exchange process toward the fast regime. This improves the spectrum for a system in the intermediate-to-fast exchange but may worsen it if the exchange is in the slow-to-intermediate state or when the studied system experiences transitions between multiple states with broad distributions of exchange rates.

We introduce a powerful D-evolution scheme that opens avenues for improving the quality of spectra broadened by the conformational exchange in an unfavorable intermediate time scale. In a general sense, the method allows the separation of the evolution time scales of processes affected by the CPMG from those that are immune to the refocusing pulses. The method can be used for small molecules and macromolecular systems, in the direct and indirect spectral dimensions.

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**Supporting Information Available:** Theoretical consideration of the D-evolution scheme; results and description of numerical simulations of the D-evolution line shapes; effect of insufficiently short CPMG intervals $\tau$; and details about used NMR samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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