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Improved HSQC experiments for the observation of exchange broadened signals

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Summary

We present here HSQC experiments with improved sensitivity for signals in the presence of exchange broadening. During periods of coherence transfer through scalar coupling the experiments employ CPMG-derived pulse trains to reduce loss of dephasing of spin coherence due to chemical exchange. ^{15}N-^{1}H gradient CPMG-HSQC and SE-CPMG-HSQC experiments have been developed and applied to complexes of *lac* repressor headpiece with operator DNA. Improved sensitivity is demonstrated for many protein backbone and side-chain resonances in the complex, markedly for signals of protons located at the protein–DNA interface. In addition, a significant increase in intensity is observed for arginine guanidino groups undergoing conformational exchange.

It is a well-known phenomenon that transverse magnetization can be efficiently relaxed by chemical exchange (see for example Kaplan and Fraenkel, 1980). In general, in the absence of rf radiation the contribution of chemical exchange to the relaxation is dependent on the rate of the exchange process, the chemical shift differences between participating sites and the populations thereof. The contribution of chemical exchange to the transverse relaxation rate is maximal near coalescence, i.e. when the rate of the exchange process is on the order of the chemical shift difference. Although the study of exchange processes has been succesfully applied to obtain valuable information about the physical chemistry involved, chemical exchange is generally detrimental in NMR spectra and it is therefore desirable to search for ways to minimise or eliminate the effect of such processes. In some cases adjustment of sample conditions or field strength may reduce exchange broadening, but such changes may not be desirable or possible and improvements in the experiment are necessary.

There are several methods known to preserve spin coherence in the presence of chemical exchange. First, the magnetization can be aligned along a large effective field, either by applying a strong spin-lock field on-resonance (Meiboom, 1961; Deverell et al., 1970) or by applying a more moderate spin-lock field off-resonance (Desvaux et al., 1995). In both cases the exchange contribution to the relaxation is reduced if the effective field strength exceeds the rate of the exchange process. Alternatively, it was recognized that dephasing of spin coherence by exchange processes can be reduced by CPMG-like pulse trains if the rate of the 180° spin-echoes is on the order of or exceeds the rate of the exchange process (Luz and Meiboom, 1963; Allerhand and Gutowsky, 1964). Such an approach should prove fruitful in biomolecular NMR, as exchange processes are often present in biomolecules. Recently, Müller and co-workers (1995) have succesfully used CPMG-derived spin-echoes for ^{15}N-^{1}H coherence transfer in isotopically enriched RNA. In their work CPMG-INEPT was combined with a NOESY experiment which allowed indirect observation of NOEs with protons of the slowly rotating amino groups. In this paper we explore the use of CPMG-INEPT as a general tool in heteronuclear NMR to optimize spin coherence transfer in systems with chemical exchange.

In the presence of chemical exchange multidimensional NMR experiments suffer from dephasing of spin coherence during periods of evolution and coherence transfer

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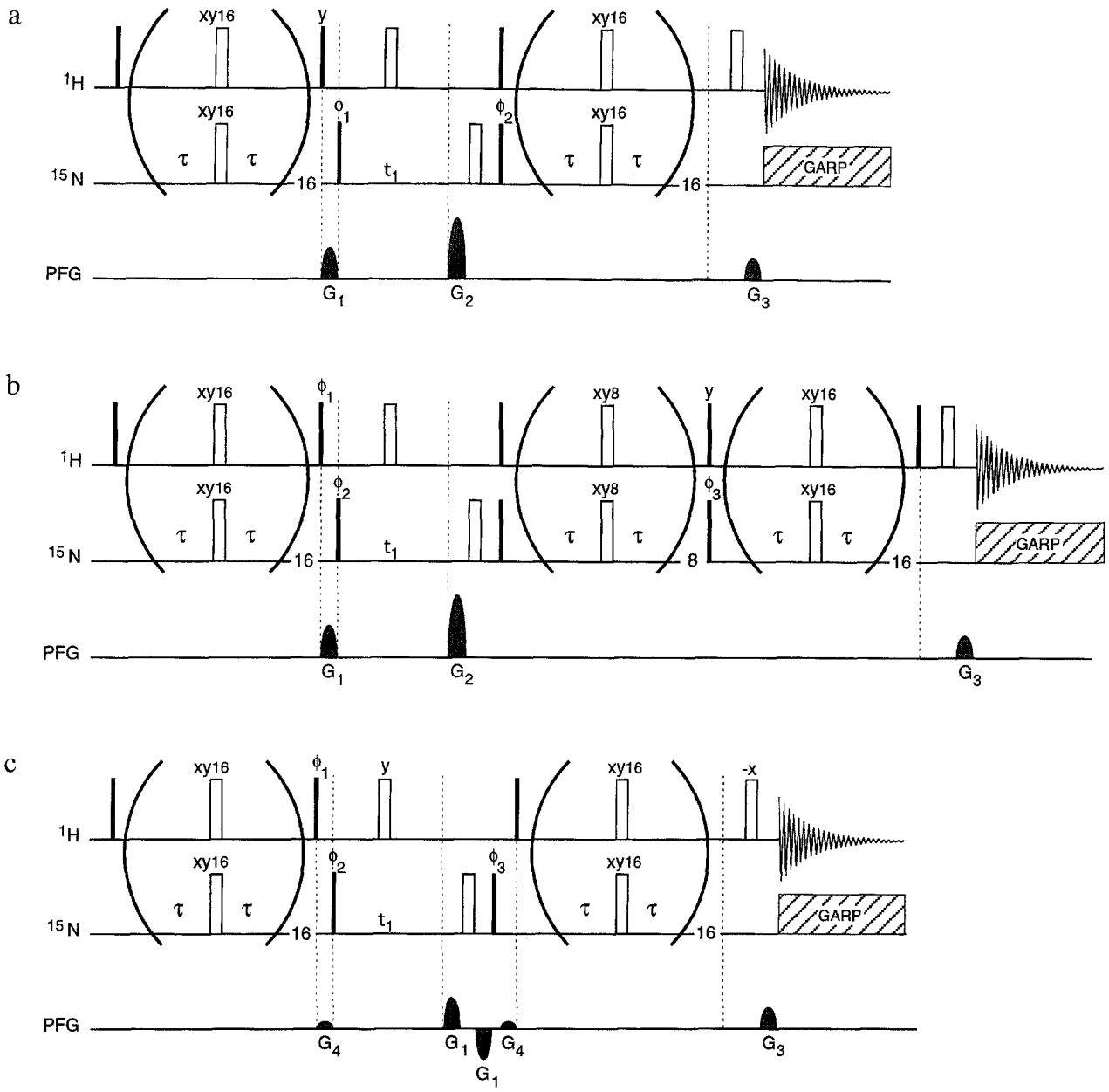

Fig. 1. (a) Pulse scheme of the gradient ^{15}N-^{1}H CPMG-HSQC experiment. (b) Pulse scheme of the gradient ^{15}N-^{1}H SE-CPMG-HSQC experiment. (c) Pulse scheme of the gradient ^{15}N-^{1}H CPMG-HSQC experiment, designed to minimize saturation transfer. In all schemes narrow (filled) and wide (open) rectangles represent 90° and 180° pulses, respectively. Unless indicated, the pulses are applied along the x-axis. The delay τ was chosen approximately 140 μs such that the total length of the XY-16 and XY-8 spin-echo pulse trains equalled 5.55 ms and 2.78 ms, respectively. The phase modulation scheme for XY-16 is x, y, x, y, y, x, y, x, –x, –y, –x, –y, –y, –x, –y, –x and for XY-8 only the first eight phases are used. The following phase cycling was used: (a) ϕ_1 = x, –x; ϕ_2 = 2(x), 2(–x); ϕ_{rec} = x, –x, –x, x; (b) ϕ_1 = 2(y), 2(–y); ϕ_2 = x, –x; ϕ_3 = y; ϕ_{rec} = x, –x, –x, x; (c) ϕ_1 = 4(y), 4(–y); ϕ_2 = x, –x; ϕ_3 = 2(x), 2(–x); ϕ_{rec} = x, –x, –x, x, –x, x, x, –x; Heteronuclear decoupling during acquisition was achieved by GARP. For each t_1 increment axial peaks were shifted to the sides of the spectrum by inversion of ϕ_1 (a) or ϕ_2 (b,c) in concert with the receiver phase, similar as in States-TPPI (Marion et al., 1989). All sequences employ sine-bell shaped pulsed field gradients (PFG) along the z-axis. To yield absorption mode spectra P- and N-type coherence selection is achieved by inversion of gradient G_2 for each t_1 increment (a), by inversion of G_2 in concert with inversion of ϕ_3 (b) or by inversion of G_3 (c). The duration of all gradients was 400 μs, followed by a 400 μs recovery delay. Gradient strengths were G_1 = 37.5 G cm^{-1}; G_2 = 75.0 G cm^{-1}; G_3 = 7.5 G cm^{-1}; G_4 = 2.2 G cm^{-1}.

by scalar coupling (J-transfer). The result of chemical exchange during periods of evolution is a broadening of the resonance lines, while it leads to loss of magnetization during periods of coherence transfer and therewith signal intensity. The net result is very weak or even unobservable signals. However, concerted spin-echoes on J-coupled nuclei during coherence transfer preserve the evolution of scalar coupling and at the same time reduce dephasing of spin coherence of the transverse spin. In this way dephasing of spin coherence during coherence transfer periods can be minimized. The resultant coherence transfer block, previously described by Müller et al. (1995), is termed

CPMG-INEPT and is the basic building block employed in the pulse sequences described below. As an alternative to CPMG-INEPT, cross-polarisation techniques may be used for coherence transfer in the presence of chemical exchange (Simorre et al., 1995; Yamazaki et al., 1995). However, periods of coherence transfer by cross-polarisation are generally longer than INEPT periods in HSQC sequences, which is unfavourable for biomolecules with short T_2 relaxation times.

2D ^{15}N-^{1}H HSQC pulse sequences designed to minimize the loss of spin coherence during periods of evolution of scalar coupling are shown in Figs. 1a–c. The pulse sequence in Fig. 1a is an adaptation of the standard HSQC experiment (Bodenhausen and Ruben, 1980) that employs gradients for P- and N-type coherence selection to yield absorption mode spectra (Davis et al., 1992). The INEPT and reverse- INEPT periods for coherence transfer through scalar coupling (Morris and Freeman, 1979) are modified by the inclusion of a phase-modulated spin-echo pulse train. The phase modulation of spin-echo pulses compensates off-resonance effects and cumulative pulse errors for all components of magnetization. The retention of both x- and y-components of magnetization is necessary, as J-coupling involves evolution of in-phase to anti-phase magnetization, which are mutually orthogonal. In our experiments XY-8 and XY-16 pulse trains were used (Gullion et al., 1990). The second experiment is a straightforward extension of the first, including sensitivity enhancement by selection of both coherence transfer pathways after t_1 evolution (Cavanagh and Rance, 1993)

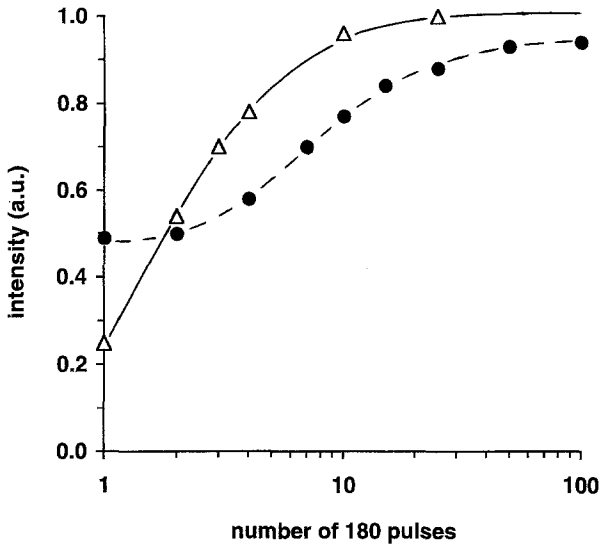

Fig. 2. Plot of the arginine η-NH_2 signal intensity as a function of the number of proton spin-echo pulses. The curves correspond to 'moderately fast exchange' (308 K; filled circles) and 'moderately slow exchange' (278 K; open triangles) for rotation of the side chain η-NH_2 groups about the N^{ε}-C^{ζ} partial double bond. The NMR sample used contained 0.1 M arginine (L-arginine monohydrochloride, BDH biochemicals 98.5%) at pH 4.9, dissolved in H_2O/D_2O 90:10 (v/v). NMR experiments were recorded on a Bruker AMX-500 spectrometer.

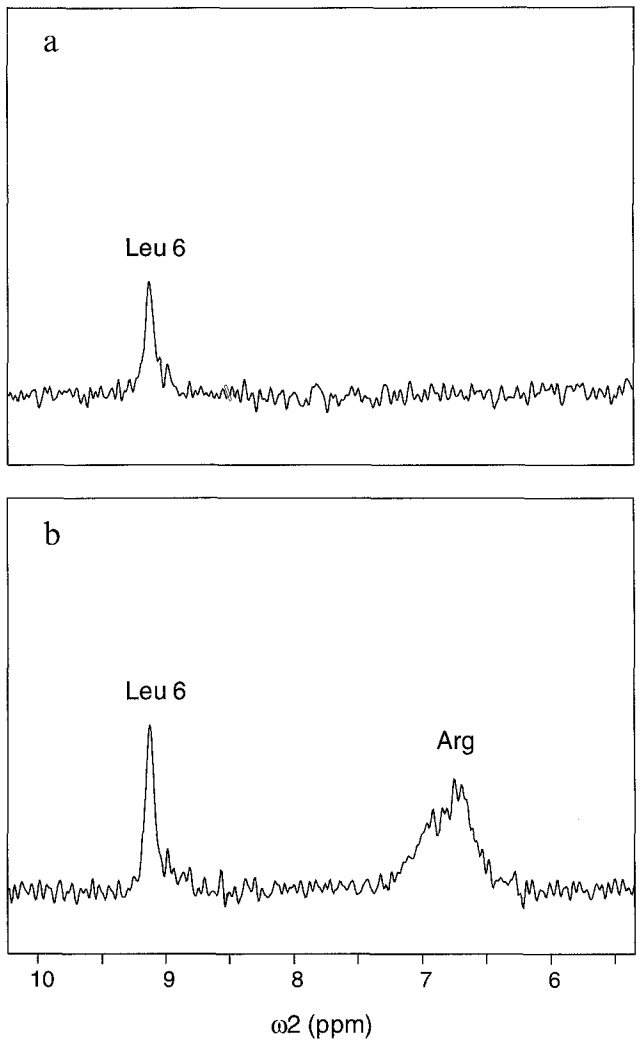

Fig. 3. 1D traces from the 2D HSQC spectra of the *lac* HP56/*lac* 11 bp half operator complex; (a) using ^{15}N-^{1}H gradient HSQC; (b) using ^{15}N-^{1}H gradient CPMG-HSQC (sequence 1a). NMR samples of *lac* HP56 complex contained 3 mM protein and DNA in buffer consisting of 200 mM KCl, 5% [D_8-]glycerol and 10 mM KPi at pH 6.0 in H_2O/D_2O 95:5 (v/v). Experiments were performed on a Bruker AMXT 600 spectrometer, operating at 25 °C. Spectral widths were 10 000 and 1818 Hz, to acquire 512 and 64–80 complex points in the ^{1}H and ^{15}N directions, respectively. All spectra were processed using the in-house TRITON software package on Silicon Graphics computers. Spectra were processed to 1024 by 1024 data matrices using sine-bell window functions, shifted by π/3 and π/5 in t_1 and t_2, respectively. No phase corrections were applied in the indirect frequency domain, but the first complex point was halved prior to Fourier transformation (Otting et al., 1986).

by use of gradients (Kay et al., 1992). Sensitivity enhancement is achieved for both NH and NH_2 cross peaks by setting the reverse coherence transfer periods to (1/4J) and (1/2J), respectively (Schleucher et al., 1994). Experiment 1c is designed to minimize saturation transfer of labile protons with solvent water (Grzesiek and Bax, 1993). Such experiments can be performed with or without solvent presaturation to assess hydrogen-exchange characteristics. The experiment employs slightly modified phase-

cycling with respect to sequence 1a and two weak gradients bracket the nitrogen evolution period to avoid radiation damping. Furthermore, the phases of the pulses are set in a way to flip water back along the +z axis prior to acquisition (Stonehouse et al., 1994). In this way the water signal is kept in a well-defined state during the entire experiment, which assures minimal water saturation. For convenience we use CPMG-HSQC and SE-CPMG-HSQC as shorthand notations for the sequences described.

In biomolecular NMR a clear example of chemical exchange is given by arginine guanidino groups. At room temperature arginine side chain η-NH_2 signals are often very broad or even unobservable in protein ^{15}N-^{1}H HSQC spectra. This is due to conformational exchange as a result of the rotation of the η-NH_2 groups about the N^{ε}-C^{ξ} partial double bond (Henry and Sykes, 1995). Furthermore, in proteins also backbone and other side-chain resonances are extensively broadened or are missing as a consequence of exchange processes. For example, the equilibria involved in complexes of biomolecules constitute an inherent source of chemical exchange from which line broadening and loss of intensity may result.

To assess the quality of CPMG-INEPT to reduce loss of coherence due to chemical exchange we recorded spin-echo spectra of an arginine sample (experimental conditions are summarized in the caption of Fig. 2). The experiment consisted of a 90°x pulse to create transverse magnetization, followed by a variable number of evenly spaced spin-echo 180°y pulses of constant total time. The number of spin-echo pulses was increased in concert with a decrease in the CPMG time τ (defined as half the spin-

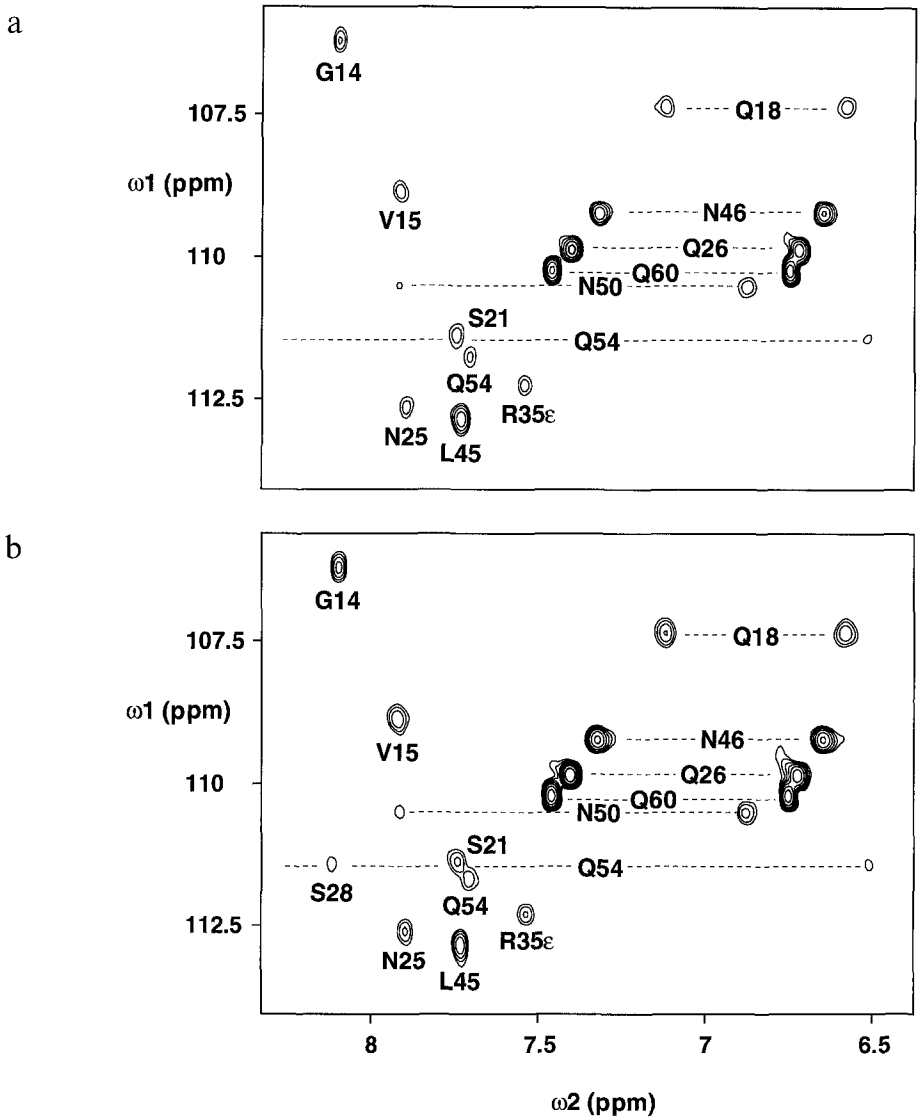

Fig. 4. Contour plots of the 2D HSQC spectra of the *lac* HP62/*lac* 22 bp operator complex; (a) using ^{15}N-^{1}H gradient SE-HSQC; (b) using ^{15}N-^{1}H gradient SE-CPMG-HSQC (sequence 1b). NMR samples of *lac* HP62 complex contained 2.8 mM protein and 1.4 mM DNA in buffer consisting of 15 mM KCl, 0.4% [D_8-]glycerol and 9 mM KPi at pH 6.1 in H_2O/D_2O 90:10 (v/v). Experiments were performed on a Bruker AMXT 600 spectrometer, operating at 50 °C. Processing parameters were as described for Fig. 3.

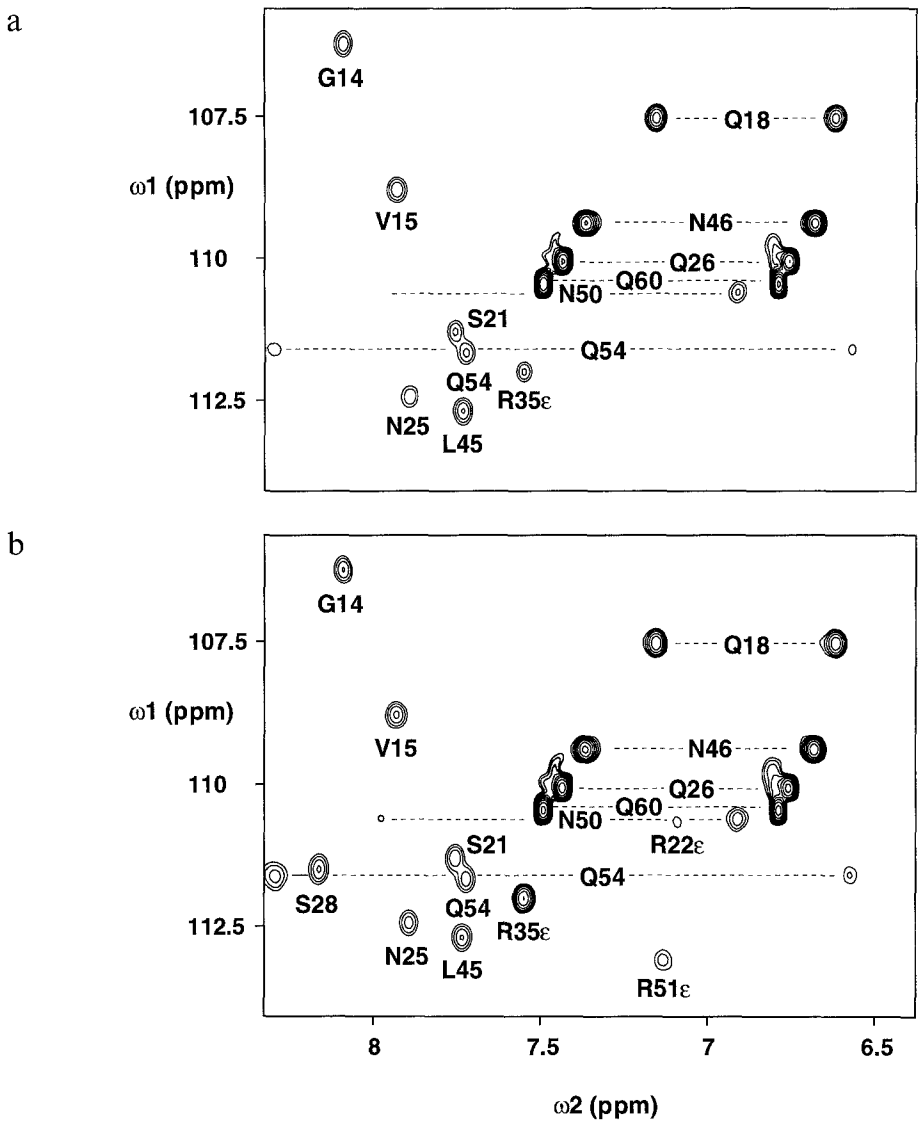

Fig. 5. Contour plots of the 2D HSQC spectra of the *lac* HP62/*lac* 22 bp operator complex using ^{15}N-^{1}H gradient CPMG-HSQC (sequence 1c). Experiments were performed (a) with, or (b) without water presaturation during the relaxation delay. Experimental conditions were pH 6.0 and 42 °C. Other experimental and processing parameters are as described for Fig. 4.

echo inter-pulse delay time) in a way to keep the proton magnetization on the transverse plain during a constant period of 5 ms. No phase modulation of the spin-echo pulses was employed as only the y-component of the magnetization needed to be retained. Peak intensities were scaled to a reference spectrum (single 180°y pulse with $\tau = 0$) and plotted as a function of the number of 180° pulses in Fig. 2. Results are shown at two temperatures, corresponding to 'moderately fast exchange' (308 K) and 'moderately slow exchange' (273 K). In both cases a severe loss of intensity for the arginine η-NH_2 protons is observed when a single 180° pulse is used at the center of the 5 ms period, corresponding to a conventional INEPT period. For example, near coalescence (290 K) reduction of as much as 90% of the peak intensity was observed (data not shown). In contrast, in the presence of a large number of spin-echo pulses the magnetization could be largely retained. These results stress the need for CPMG-derived coherence transfer periods in multidimensional heteronuclear NMR pulse sequences, where the presence of chemical exchange in several coherence transfer steps may lead to considerable loss of intensity.

2D gradient ^{15}N-^{1}H HSQC experiments were performed on two complexes of uniformly ^{15}N-labeled *lac* repressor headpiece with operator fragments. Complexes consisted of *lac* headpiece 1–56 (*lac* HP56) complexed 1:1 with an 11 bp *lac* half-operator fragment and *lac* headpiece 1–62 (*lac* HP62) complexed 2:1 with a 22 bp *lac* operator fragment, respectively.

A comparison of the performance of gradient ^{15}N-^{1}H HSQC and CPMG-HSQC experiments is given in Fig. 3, where a trace along the ω_2 (^{1}H) axis is shown for several broadened resonances of the HP56 complex, which is a complex in 'moderately fast exchange'. Signals correspon-

ding to Thr[5] and Leu[6] of the first helix of HP56, which are all in close proximity of DNA, show considerably higher intensity in the CPMG-HSQC spectrum than in the standard HSQC spectrum. Furthermore, signals of the arginine guanidino groups (HP56 contains three arginines) have become observable. This is of particular importance, since arginines are involved in specific binding (Chuprina et al., 1993). The broadening of the resonances in the HP56 complex is probably due to equilibria between different HP56/*lac* half-operator complexes (Scheek et al., 1983). Furthermore, conformational exchange may be present, which is the case for the arginine η-NH_2 groups. Comparison of the HSQC and CPMG-HSQC spectra showed that for most unbroadened resonances both sequences work equally well and that no significant signal loss occurred as a result of adding many rf pulses.

2D gradient ^{15}N-^1H SE-HSQC and SE-CPMG-HSQC spectra were acquired on the HP62 complex, which is a complex in 'moderately slow exchange'. Figure 4 shows a portion of the spectra. Obviously, the CPMG-derived sequence shows improved sensitivity for almost all resonances, resulting in an average signal enhancement of 25%. The enhancement for weak signals is of pronounced importance again for the system under study. Several of the weak signals belong to residues at the protein–DNA interface. For example, residues Gln[18], Ser[21], Asn[25] and Asn[50] are involved in important protein–DNA interactions (Chuprina et al., 1993) and are difficult to observe in spectra of multidimensional NMR experiments based on standard INEPT coherence transfer.

Hydrogen exchange characteristics of the HP62 complex were investigated through measurement of saturation transfer with sequence 1c. Figure 5 shows portions of the spectra obtained with (Fig. 5a), or without (Fig. 5b) water presaturation during the relaxation delay between scans. The use of the CPMG-HSQC experiment allowed saturation transfer to be measured reliably for many signals that would otherwise not even be observable, such as Ser[28]. Thus, the combination of CPMG-HSQC with methods which avoid water saturation lead to considerably improved intensities of labile protons under conditions of high pH and/or temperature, as can be concluded by comparing Figs. 4a and 5b.

In this communication we have described the use of CPMG-derived sequences as a building block for coherence transfer in the presence of chemical exchange in two-dimensional heteronuclear NMR experiments. The extension of CPMG-HSQC and SE-CPMG-HSQC experiments to other multidimensional heteronuclear NMR pulse sequences is straightforward. Particularly beneficial applications can be experiments employing multiple coherence transfer steps and coherence transfers involving small J-couplings. Furthermore, the use of the experiments presented here may be used as a tool to unequivocally demonstrate the presence of chemical exchange or as a tool to study exchange kinetics in biomolecules. Such investigations are currently under study in our laboratory.

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