Journal of Magnetic Resonance **137,** 39–45 (1999)
Article ID jmre.1998.1614, available online at http://www.idealibrary.com on IDEAL®

Computer Assisted Assignment of ^{13}C or ^{15}N Edited 3D-NOESY–HSQC Spectra Using Back Calculated and Experimental Spectra

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Received May 1, 1998; revised September 15, 1998

A new tool, for the simulation of ^{15}N or ^{13}C edited 3D-NOESY–HSQC spectra using the complete relaxation matrix approach, has been developed and integrated in the program AURELIA. This tool should be particularly useful for the fast and reliable computer assisted assignment of 3D-NOESY–HSQC spectra by comparing back-calculated and experimental spectra in an iterative process. Folded spectra are sometimes used to enhance the digital resolution in the indirect dimensions of multidimensional spectra. However, these spectra are usually difficult to analyze. To simplify this assignment process we have incorporated the simulation and automated annotation of folded peaks into the program. It is hereby possible to simulate multiple folding in all three dimensions of 3D ^{15}N- or ^{13}C-NOESY–HSQC spectra. By comparing experimental 3D-NOESY–HSQC spectra with spectra back calculated from a single trial structure or a set of trial structures, a user can easily check if the final structures explain all experimental NOEs. The new feature has been successfully tested with the histidine-containing phosphocarrier protein HPr from *Staphylococcus carnosus.*

Key Words: **3D-NOESY–HSQC; back calculation; computer assisted assignment; full relaxation matrix approach; histidine-containing phosphocarrier protein HPr.**

INTRODUCTION

The use of ^{15}N or ^{13}C edited 3D-NOESY–HSQC (*1*) spectra is one of the standard methods for the structure determination of proteins in solution. However, the necessary assignment of these spectra is quite time consuming and often the time limiting step in the structure determination process. Even after the sequence specific assignment is complete, one has to extract distance constraints from the experimental spectra by assigning almost all of their signals. This can be a tedious and error-prone process. One possible strategy to speed up the assignment process is the iterative combination of back-calculated and experimentally determined NOESY spectra. During the past few years, a number of groups have developed computer programs such as CORMA (*2*), BCKCALC (*3*), X-PLOR (*4*), BIRDER (*5*), and RELAX (*6*) for the calculation of NOESY cross-peak volumes by complete relaxation matrix analysis. Programs such as IRMA (*7*) and MARDIGRAS (*8*) use relaxation matrix calculations together with molecular-dynamic simulations in an iterative approach for structural refinements. Recently, new approaches were proposed to combine three-dimensional structure calculations more closely with the assignment problem of NOESY spectra. In one of these approaches experimental NOESY spectra are automatically assigned by feedback filtering and self-correcting distance geometry (*9*). In another approach structures are calculated with ambiguous assignments, where all assignments are allowed that correspond to the provided chemical shift table (*10*). However, to our knowledge all of these programs are limited to the simulation or evaluation of homonuclear 2D-NOESY or 3D-NOESY–NOESY spectra (*11*). For larger proteins the use of heteronuclear three-dimensional spectra becomes more and more important. The method described in this paper is a new feature of our program RELAX (*6*), which is capable of calculating homonuclear ^{1}H–^{1}H 2D cross-peak volumes based on the complete relaxation formalism. We have now incorporated the back-calculation of hetero nuclear 3D ^{15}N- or ^{13}C-NOESY–HSQC peak volumes.

One of the problems associated with three-dimensional spectra in general is the insufficient resolution in the indirect dimensions. One possible solution is the use of spectral folding in one or more dimensions. A disadvantage of folding in experimental spectra is that they are usually difficult to analyze since the resonance frequencies of the observed cross peaks are not unambiguous. However, if a folded NOESY-type spectrum is simulated from a given structural model, then all resonance frequencies in this spectrum are known exactly together with their assignments (peak labels). By comparison of the experimental and simulated spectra, the assignment process is greatly simplified and folding of the peaks is easily revealed. The usefulness of the new routines were tested for the assignment

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1090-7807/99 $30.00

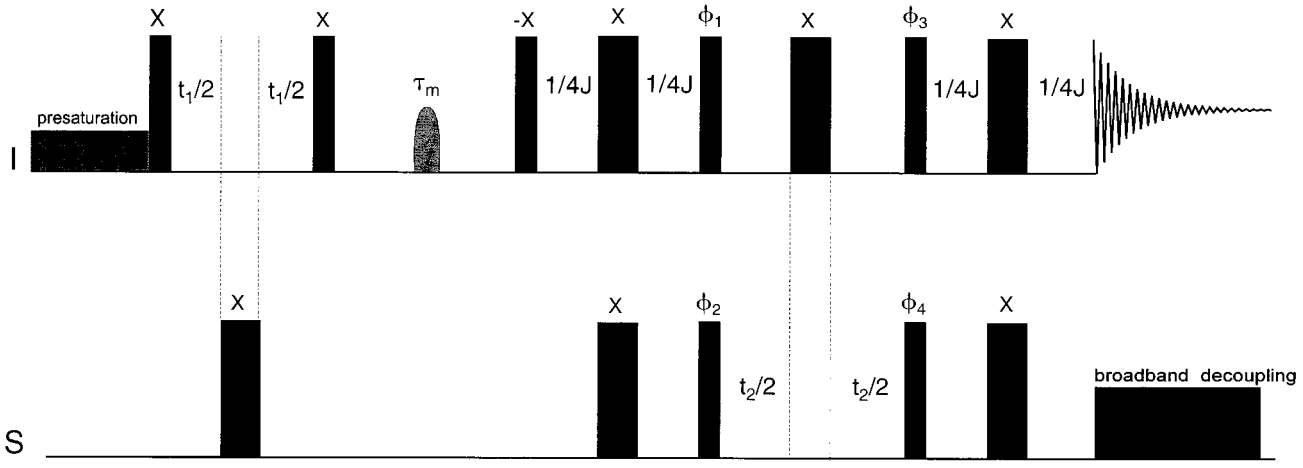

FIG. 1. Pulse sequence for a 3D-NOESY–HSQC experiment. The phase cycling is $\phi_1 = y, -y$; $\phi_2 = 2(x), 2(-x)$; $\phi_3 = 4(x), 4(-x)$; $\phi_4 = 8(x), 8(-x)$; and receiver $= -x, x, x, -x, x, -x, -x, x, x, -x, -x, x, -x, x, x, -x$. Presaturation is used for water suppression. The $180°_S$ pulse at the midpoint of t_1 is often replaced by broadband decoupling of the heteronuclei during t_1. The influence of the heteronuclei can be completely removed by from the NOESY part by additional broadband decoupling during the NOE mixing time. A *z*-gradient at the midpoint of the mixing time is used to remove residual magnetization in the *x*–*y* plane.

of 3D ^{15}N edited NOESY–HSQC spectra of the histidine-containing phosphocarrier protein (HPr). HPr from *Staphylococcus carnosus* is a medium-sized protein of 88 residues. Its three-dimensional structure was recently solved by NMR methods (*12*) and shows a well-defined tertiary structure consisting of a four stranded antiparallel β-sheet and three α-helices.

MATERIALS AND METHODS

The program module RELAX-3D is written in ANSI-C and has been integrated into the most recent version of the program package AURELIA (*13*), which is available from Bruker, Karlsruhe. It can also be obtained as part of the stand-alone program RELAX from the authors. ^{15}N-enriched HPr-protein from *S. carnosus* was obtained from W. Hengstenberg, Bochum. The sample contained 3.1 mM HPr in 95% H_2O/5% D_2O, pH 7.2. The NOESY–HSQC spectra were recorded with an AMX-500 NMR spectrometer (Bruker, Karlsruhe) with the pulse sequence given in Fig. 1. Two different data sets were used as example in this paper; a folded (A) and an unfolded (B) NOESY-HSQC spectrum. Spectrum (A) consists of 512 $\times$ 128 $\times$ 350 real data points in ω_1-, ω_2-, and ω_3-directions and spectrum (B) of 448 $\times$ 256 $\times$ 512 real data points with total acquisition times of 43 and 61 h, respectively.

THEORETICAL CONSIDERATIONS

The *IS* ($I = {}^1$H, $S = {}^{13}$C or ^{15}N) NOESY–HSQC experiment, whose pulse sequence is illustrated by Fig. 1, is basically a concatenation of a homonuclear ^{1}H-NOESY and a heteronuclear *IS* HSQC experiment. Under ideal conditions the net magnetization transfer during the entire experiment can be calculated by multiplying the net polarization transfers of the two basic experiments. Usually, the NOESY-parts of the 3D pulse sequences differ slightly from the standard homonuclear NOESY pulse sequence, since it is advantageous to decouple the heteronuclei during the evolution period t_1. This is often done by an additional $180°_S$ pulse on the midpoint of t_1 (Fig. 1). Alternatively, the *S*-spin can be decoupled during the evolution time by broadband decoupling; this means that the initial value of the *S*-magnetization is in the ideal case zero. Finally, the influence of the *S*-spin can be completely removed from the NOESY part by additional broadband decoupling during the NOE mixing time. Since it is a general property of the Fourier transformation that the integral of the frequency domain signal is only dependent on the magnitude of the time domain data point at time zero, the relaxation matrix formalism has only to take into account the initial values of the *S*- and *I*-magnetization at the beginning of the mixing time. In the following we discuss the standard case, where a $180°_S$ pulse is used during t_1 and both the *I*- and *S*-magnetizations are inverted at the beginning of the mixing time.

For a NOESY experiment the evolution of the deviation of longitudinal magnetization from thermal equilibrium $\Delta\mathbf{M}_z$ is described by the generalized Solomon equation (*14*)

$$\frac{d}{dt}\,\Delta\mathbf{M}_z(t) = -\mathbf{D}\Delta\mathbf{M}_z(t). \qquad [1]$$

The dynamics matrix $\mathbf{D}$ that governs the time evolution of the cross-peak intensities in a 2D-NOESY experiment is given by

$$\mathbf{D} = \mathbf{R} + \mathbf{K}. \qquad [2]$$

K is the kinetic matrix that describes chemical and or conformational exchange (*15*), while **R** is the relaxation matrix (*16–18*). If the effects of chemical shift exchange are neglected, as in the current version of RELAX, the solution of Eq. [1] simplifies to

$$\Delta\mathbf{M}_z(t) = \Delta\mathbf{M}_z(0)\exp(-t \cdot \mathbf{R}), \qquad [3]$$

wherein $\Delta\mathbf{M}_z$ (0) is the deviation of the longitudinal magnetization from thermal equilibrium at time zero. For dipolar homo- or heteronuclear relaxation and spin $I = \frac{1}{2}$ the rates of autorelaxation R_{ii} and the cross-relaxation R_{ij} between two spins i and j are given by

$$R_{ii} = \sum_{j \neq i} q_{ij}[J_{ij}^0(\omega_i - \omega_j) + 3J_{ij}^1(\omega_i) + 6J_{ij}^2(\omega_i + \omega_j)] \qquad [4]$$

and

$$R_{ij} = q_{ij}[6J_{ij}^2(\omega_i + \omega_j) - J_{ij}^0(\omega_i - \omega_j)] \qquad [5]$$

with $J_{ij}^n(\omega)$ ($n = 0, 1, 2$) being the spectral densities for n-quantum transitions characterizing the motion of a vector connecting spin i and j relative to the $\mathbf{B}_0$ field. The dipolar interaction constants q_{ij} are given by

$$q_{ij} = (1/10)\gamma_i^2\gamma_j^2\mathrm{h}^2(\mu_0/4\pi)^2, \qquad [6]$$

where γ_i and γ_j are the gyromagnetic ratios of spins i and j, respectively.

The time required for performing the back calculations for medium-sized proteins such as HPr is usually only a couple of minutes. If even faster calculations are desired or if less accuracy is necessary for a special task, it is possible to neglect the heteronuclear relaxation during the calculations. By doing so it is possible to reduce the needed CPU time by a factor of 2–3. In most cases only a small error is introduced in the calculation of the cross-peak intensities if the heteronuclear relaxation is neglected. The relaxation rate between a ^{1}H nucleus i and a ^{15}N- or ^{13}C-nucleus j is proportional to the squares of the gyromagnetic ratios of the two nuclei (Eqs. [5] and [6]). Compared to ^{1}H nuclei the magnetogyric values for the ^{15}N and ^{13}C nuclei are smaller by a factor of 0.101 and 0.25, respectively. This means that the heteronuclear relaxation rates are much smaller than the proton–proton relaxation rate, assuming the same distance r_{ij} between the corresponding nuclei i and j.

A second reason for this fact becomes obvious if one looks at the most basic spectral density function describing the motion of a rigid isotropically diffusing body with τ_c being the overall rotational correlation time (*19*):

$$J_{ij}^n(\omega) = \frac{1}{r_{ij}^6}\frac{\tau_c}{1 + \omega^2\tau_c^2}. \qquad [7]$$

For homonuclear relaxation the Larmor frequencies ω_i and ω_j of the two interacting nuclei can be approximated to be equal. Thus, for $\omega^2\tau_c^2 \gg 1$, which is valid for long τ_c or high $\mathbf{B}_0$ fields, like those found in practical applications to protein NMR, the zero quantum transitions $J(\omega_i - \omega_j) \approx J(0)$ dominate the homonuclear relaxation because the delimiter in Eq. [7] approaches its minimum value for $\omega = 0$. However, in the case of heteronuclear ^{1}H–^{15}N or ^{1}H–^{13}C relaxation this is not true. As a conclusion, the rate of the heteronuclear ^{1}H–^{15}N or ^{1}H–^{13}C relaxation is much smaller than the homonuclear ^{1}H–^{1}H relaxation rate at the same distance. By the same reasoning, as described for heteronuclear dipolar relaxation, the influence of the heteronuclear chemical shift anisotropy (CSA) on the proton magnetization via heteronuclear relaxation is neglected. In addition, interference effects between dipolar coupling and CSA (*20*) are neglected as well. As discussed above, sometimes the 180°$_S$ pulse during t_1 is replaced by broadband decoupling. If broadband decoupling is used during the NOESY mixing time, the heteronuclear relaxation should be switched off in the back calculations.

Ideally, the HSQC part of the 3D-pulse sequence only reflects the polarization transfer produced by the NOESY part of the sequence. However, the signal amplitude is modified by several factors during the HSQC sequence. For the quantitative analysis, the only factors of importance are those which change the relative intensities of the NOE signals. Two main sources cause such changes: the differences in the indirect *IS*-spin coupling constants which are essential for the INEPT-transfer and the differences in individual transversal relaxation times. In principle, the *IS* coupling constants could be determined independently and the transversal relaxation times could be calculated for every individual *IS* spin pair from the three-dimensional structure and the motional parameters. A practical solution to the problem is the determination of the individual transfer factors from a two-dimensional data set recorded with a vanishing NOESY mixing time and a constant value of $t_1 = 0$. This method works fine under almost all conditions. However, if the *I* and *S* resonance frequencies of two cross-peaks in the two-dimensional HSQC spectrum are identical, one cannot determine the individual contributions of the two cross-peaks. In first approximation, though, one can assume the same intensity decay for the two signals and weight the data with the average HSQC intensity.

IMPLEMENTATION

So far only the NOESY part of the ^{15}N edited 3D-NOESY–HSQC experiment has been described. As discussed above, the most elegant form to take effects of the HSQC part into account is a scaling of the simulated 3D intensities with the

intensity factors obtained from a two-dimensional version of the three-dimensional pulse sequence with a NOESY mixing time of zero. This can be done automatically in the RELAX-3D routines. If no suitable 2D spectrum is available a constant scale factor of 1 will be applied to all calculated intensities.

The input files required are an extension of those already used in the original version of our back calculation program. The input *spt*-file now contains, in addition to the proton assignments, ^{15}N or ^{13}C assignments and a new class which describes for the heteronuclei the parameters of the applied dynamical model. The output of the program is a list of simulated peaks with their corresponding chemical shift values, labels, and intensities. In addition, it is possible to calculate a simulated ^{15}N- or ^{13}C-edited 3D-NOESY–HSQC spectrum in BRUKER submatrix format which can used directly by AURELIA (*13*). To calculate a spectrum, parameter files are required which can be obtained from an experimental spectrum. In the current version, the BRUKER processing files from the experimental spectrum to be compared with are read for automatically obtaining the spectral widths, the data sizes, and the spectral reference. The simulated spectrum will then have the same spectral widths, resolution, and referencing as the measured one. However, these data can also be changed manually. The lineshapes of the peaks and the linewidths in three dimensions have to be selected by the user. It is possible to select a Gaussian or Lorentzian lineshape with a suitable linewidth. To display the peak labels within AURELIA the appropriate data is automatically created. In cases where the experimentally determined spectrum is folded in one or more dimensions, the simulated spectrum will be folded in the same way as the experimental spectrum. The signals will be either mirror imaged on the nearest side of the spectrum or aliased from the opposite edge, depending on the used type of phase incrementation or direct detection (essentially simultaneous or sequential acquisition modes of the complex data). This information has to be supplied by the user. The program will automatically determine if one or more shifts of the *spt*-file are outside of the spectral widths as specified in the parameter files. Because of the folding process, phase distortions can be introduced into the spectrum. In the case of sequential data acquisition and provided that the zero- and first-order phase corrections are equal to 90° and zero, respectively, one will yield a positive sign for genuine signals and signals that are folded an even number of times. A negative sign is obtained for signals that are folded an odd number of times. For simultaneous data acquisition, discrimination between folded and nonfolded signals can be achieved by introducing a frequency-dependent phase shift. For example, phase alterations of 180° between folded and nonfolded signals, in one of the indirect dimensions, can be accomplished by setting the first increment to half of the dwell time (*21*). For reasons of clarity the program was written in a way that all simulated folded peaks have a positive sign. The peak labels for all folded peaks are marked with an *f*.

Since a single trial structure may only explain a limited number of NOEs as a possible option, a set of structures can be supplied by the program and an average spectrum can be calculated by averaging the relaxation matrices. Such an averaging would correspond to a fast exchange between the different conformational states represented by this set of structures. In this case, the chemical shift values given the program represent the ensemble averaged shifts.

RESULTS AND DISCUSSION

The program can be used to help assigning a new spectrum when the chemical shifts are known and a model structure is available. It was tested for the assignment of ^{15}N-NOESY–HSQC spectra of the histidine-containing phosphocarrier protein HPr.

Figure 2 gives an example how a slice of a folded ^{15}N 3D-NOESY–HSQC experimental spectrum can easily be assigned using the corresponding back-calculated spectrum. The AURELIA correlations menu was used for performing this task. Please note that the spectral width in the indirect proton dimension is only 9 ppm, while the spectral width in the acquisition domain is 14.0 ppm. The spectrum is therefore slightly folded in the indirect proton dimension. At left in Fig. 2 is the back-calculated NOESY trace for residue 30 of HPr, while the corresponding experimental trace is shown at right. At lower left in Fig. 2, an expansion of the region around the folded diagonal peak of residue 30 is shown. Because of the small spectral width in the indirect proton dimension, the amide diagonal peak of residue 30 is folded in that dimension and occurs at 7.33 ppm, while the true chemical shift is at 11.31 ppm. For easy identification all simulated folded peaks are marked with an *f*. A comparison of the simulated peaks of residue 30 with the corresponding experimental peaks allows fast assignment of the latter. An unfolded spectrum would have required a spectral width of 14.00 ppm in both proton dimensions. By reducing the spectral width of the indirect proton dimension to 9.00 ppm, a gain of 36% in digital resolution could be achieved, assuming in each case the same number of points. A scan through the whole spectrum (data not shown) showed that with a spectral width of 9 ppm in the indirect proton dimension almost no additional overlap was introduced into the spectrum. This is because no densely populated regions were folded.

Figure 3 gives an example of assigning overlapping resonances with the help of a back-calculated spectrum. Figure 3A displays a slice of the experimental 3D-NOESY–HSQC spectrum of HPr. In Fig. 3B, the corresponding simulated spectrum is shown. All peaks that arise from NOEs to the amide protons of residues 48 and 55 are displayed with their assignments. The ^{15}N and HN shifts of these two residues are overlapping in both 2D-NOESY and 3D-NOESY–HSQC spectra, and therefore their NOEs are difficult to assign in the experimental spectra. However, by comparing back-calculated and experimental

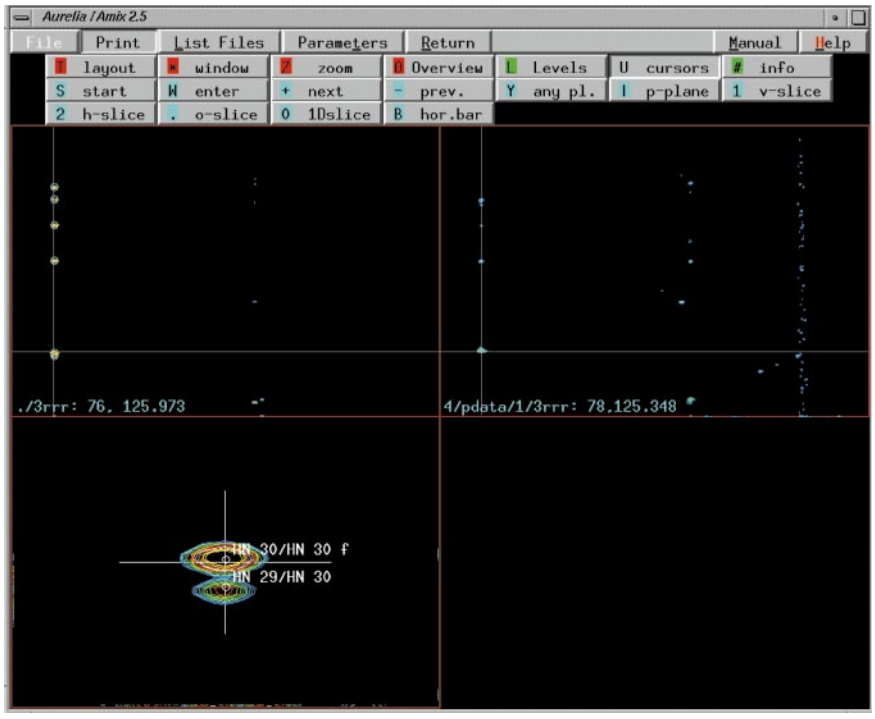

FIG. 2. At left is the proton–proton slice 76 at an amide chemical shift of 125.9 ppm of the back-calculated 3D-NOESY–HSQC spectrum of HPr. The corresponding experimental slice is displayed at right. Because of the small spectral width of 9.00 ppm in the indirect proton dimension, the amide diagonal peak of residue 30 is folded in that dimension and occurs at 7.33 ppm, while the true chemical shift is at 11.31 ppm. An expansion of the region around the diagonal peak of residue 30 is displayed at lower left. The folded diagonal peak of residue 30 is marked with an *f*.

spectra it is relatively easy to obtain unambiguous NOE assignments for these two residues. Another useful application for the program is as a very easy and fast check of the quality of the calculated structures. It is obvious that the calculated spectrum shows a cross-signal between the amide protons of residues 54 and 55 which is missing in the experimental spectrum. This can be an indication for a possible problem with the structure which was used for the simulations. It should be noted here that no finally refined structures were available at this point.

The described routines are most useful when the sequential assignment has been completed and the user is in the process of identifying long-range NOEs. If the program is used in an iterative fashion one can start by identifying additional NOEs with the help of the back-calculated spectrum. Next, refined structures can be calculated with the increased number of NOEs. Based on the refined structures an improved simulated spectrum can be calculated, and by comparing the new simulated spectrum with the experimental one, more NOEs can be identified. This process can be repeated until no further improvements are possible. At the beginning of this iteration it may be useful to rely on a set of trial structures instead of a single one. The advantage of such a procedure is that the bias toward the initial starting structure is reduced since, because of the r^{-6} dependence of the NOEs, only a few close contacts in the set of spectra will give rise to nonnegligible cross-peaks. Another useful application is the optimization of NMR parameters before starting the actual experiments. Examples are the estimation of the optimal NOE mixing time for observing a selected spin pair or, if folding is wanted for improving the digital resolution, the optimal spectral widths causing minimal superpositions of signals.

A particularly interesting possible application of the back calculation of NOESY–HSQC spectra is the study of conformational changes of an enzyme that arise from its interaction with a small ligand or another protein. If the enzyme but not the ligand is ^{15}N labeled, it should be possible to record a NOESY–HSQC spectrum of the enzyme–ligand complex which will contain only signals of the enzyme. Because the conformational changes will affect only a few residues on the contact surfaces of enzyme and ligand, it should be possible to assign the cross-peaks of the NOESY–HSQC spectrum in minimal time. This procedure is of course also applicable in the case of an unlabeled enzyme and a labeled ligand to study conformational changes of the ligand if the spectrum of the free ligand has been assigned already.

During the revision of this manuscript we became aware of a paper by Zhu *et al.* (*22*) which describes the program SPIRIT for the calculation of 3D NOESY–HSQC spectra. It has some similarities with the routines described here. As an interesting feature, the HSQC transfer is also simulated in an approximate way. Such a simulation would be useful if an experimental

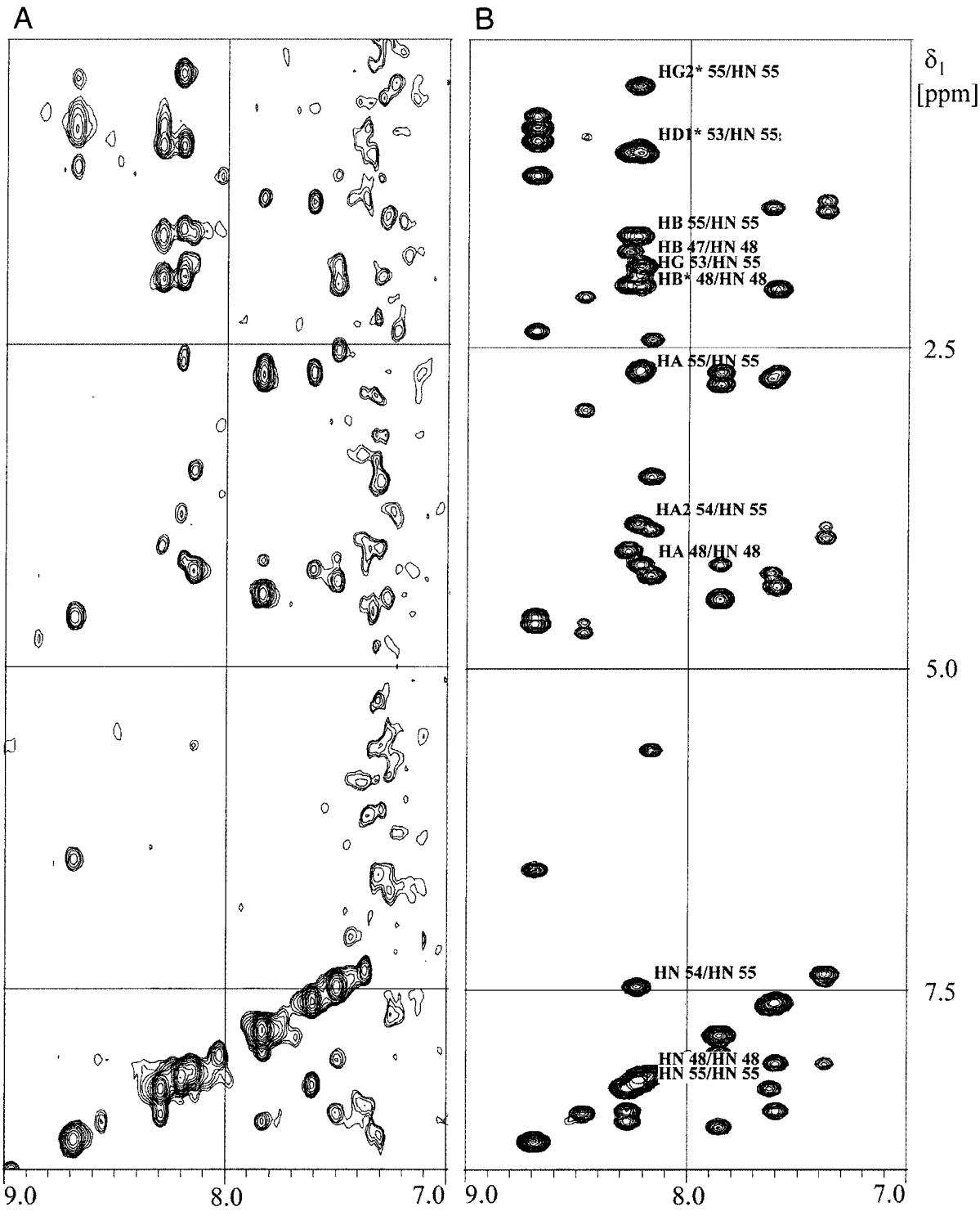

FIG. 3. Example of how a back-calculated 3D-NOESY–HSQC spectrum can aid the assignment process of the corresponding experimental spectrum. (A) A proton–proton slice of an experimental 3D-NOESY–HSQC spectrum of HPr at an amide chemical shift of 120.1. (B) The corresponding simulated spectrum. All back-calculated peaks that arise from NOEs to the overlapping amide peaks of residue 48 and 55 are displayed with their labels. Spectral widths and digital resolution are the same for both spectra.

two-dimensional HSQC at time $t_1 = 0$ were not available and could give a better idea of the real situation.

In summary we feel that the program is a useful tool for the assignment of the spectra of larger proteins.

ACKNOWLEDGMENTS

We thank Werner Kremer, M. Geyer, and Till Maurer for critically reading of the manuscript. We thank W. Hengstenberg for providing the ^{15}N-enriched sample of HPr protein and K. C. Holmes for continuous support of our investigations. This work was supported by a biotechnology grant from the European Union.

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