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ARTICLE

Estimation of $^3J_{HN-H\alpha}$ and $^3J_{H\alpha-H\beta}$ coupling constants from heteronuclear TOCSY spectra

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Abstract ^3J proton–proton coupling constants bear information on the intervening dihedral angles. Methods have been developed to derive this information from NMR spectra of proteins. Using series expansion of the time dependent density matrix, and exploiting the simple topology of amino acid spin-systems, formulae for estimation of $^3J_{HN-H\alpha}$ and $^3J_{H\alpha-H\beta}$ from HSQC-TOCSY spectra are derived. The results obtained on a protein entailing both α-helix and β-sheet secondary structure elements agree very well with J-coupling constants computed from the X-ray structure. The method compares well with existing methods and requires only 2D spectra which would be typically otherwise recorded for structural studies.

Keywords Density matrix · HSQC-TOCSY · Isotropic mixing · J-coupling · Proteins · TOCSY

Introduction

J-coupling constants between protons separated by three bonds ($^3J_{H-H}$ coupling constants) depend on the intervening torsion angle according to Karplus equation (Karplus 1959). Measurement of such coupling constants may therefore convey information on conformation which can be used in the determination of structure and dynamics of molecules in solution. Such task may be accomplished by different methods: (i) measuring frequency separation (Kim and Prestegard 1989; Montelione and Wagner 1989; Kay and Bax 1990; Billeter et al. 1992; Griesenger et al. 1985; Ludvigsen et al. 1991; Szyperski et al. 1992); (ii) measuring peak intensities or ratios whose analytical dependence on J-coupling constants is known (Neri et al. 1990; Archer et al. 1993; Vuister and Bax 1993; Bax et al. 1994); (iii) and in general fitting different experimental data whose dependence on coupling constants is (empirically or theoretically) known (Stonehouse and Keeler 1995; Wang et al. 1997).

Among the many methods published to date the quantitative J correlation method of Vuister and Bax (Vuister and Bax 1993) is by far the most accurate and most used although the methods based on frequency separation can also be convenient when the ECOSY principle (Griesenger et al. 1985) is implemented in heteronuclear-resolved experiments (Schmieder et al. 1991), as shown, for totally different purposes, by application to residual dipolar coupling estimation (Ottiger et al. 1998; Ding and Gronenborn, 2003).

The direct fitting of TOCSY peak intensities evolution or ratios for the estimation of J-coupling constants has been proposed by us and others many years ago (Fogolari et al. 1993; Archer et al. 1993; van Duynhoven et al. 1992). Coherence transfer from one proton to a coupled proton at short mixing times is proportional to the square of the product of the J-coupling by the mixing time. Thus the measurement of the auto- and cross-peak intensities allows, in principle, the estimation of the relevant J-coupling constant, no matter how complex is the coupling network. For higher order terms in the series expansion of the peak intensities it is unfortunately not easy to disentangle all contributions bearing information on J-coupling constants. However whether or not the expansion is truncated at the

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initial terms, for short mixing times the amplitudes of auto- and cross-peaks are not affected significantly by different relaxation damping, which makes unnecessary the corrections that are instead required when comparing in-phase and antiphase auto- and cross-peaks as with the quantitative J correlation and related methods (Vuister and Bax 1993; Billeter et al. 1992).

The application of the approach based on quantitation of TOCSY peaks to *J*-coupling constant estimation has been hindered by the difficulty in estimating auto-peaks in crowded homonuclear TOCSY spectra.

However, because of the straightforward availability of proton NMR data and their relevance to structural investigation, ^{1}H TOCSY or NOESY crosspeak half-height linewidths have been used to evaluate *J*-couplings (Wang et al. 1997). This method, that bypasses the lack of auto-peak resolution, was proposed for protein spectra, based solely on quantitation of cross-peak linewidths calibrated over typical protein reference data. The experimental acquisition and processing prescriptions to apply this method are rather strict and the calibration fails for statistically disordered residues or regions, or for very large molecules due to substantial deviations of the actual linewidths from the calibrant ranges. Moreover only protein spectra can be treated owing to the specificity of the calibration.

Since the proposals to use TOCSY peaks for *J*-coupling estimation were first advanced, ^{15}N or ^{15}N–^{13}C uniform labeling of protein samples for NMR studies has become nearly routine. Hence sample availability limitations for heteronuclear-resolved ^{1}H TOCSY spectra are no longer a major problem. Indeed, in HSQC-TOCSY spectra of ^{15}N-labeled proteins many spin systems are well resolved and measurement of auto- and cross-peaks is easily achievable. Moreover in amino acid spin systems where proton coherence along the polarization field is transferred from H^N to H^α and to H^β's, the H^N proton is significantly coupled only to H^α. This has the consequences that the general formulae we developed previously (Fogolari et al. 1993) are significantly simplified.

In the following we summarize the theory of coherence transfer under isotropic mixing for the amino acid spin-systems and provide a simple and general recipe that can be used for the derivation of $^{3}J_{HN-H\alpha}$ and $^{3}J_{H\alpha-H\beta}$ coupling constants. The procedure has been implemented in a small C program available from the authors.

Materials and methods

NMR data

The acylphosphatase from the hyperthermophilic archaeon *Sulfolobus solfataricus* (Sso Acp) has been used based on the previous characterization in solution by multidimensional NMR (Corazza et al. 2006). A series of 2D ^{1}H–^{15}N HSQC-TOCSY spectra (Bodenhausen and Ruben 1980) were acquired at 310 K with a Bruker Avance-500 spectrometer on a 0.4 mM U–^{15}N Sso AcP sample dissolved in a H2O/D2O (95/5) containing 50 mM sodium phosphate buffer, pH 5.7, and 50 mM NaCl. The isotropic homonuclear TOCSY mixing process was obtained with WALTZ-16 pulse train (Shaka et al. 1983). The number of WALTZ-16 cycles in the HSQC-TOCSY pulse sequence was varied from 1 to 6, corresponding to mixing times of 2.942, 5.885, 8.827, 11.770, 14.712 and 17.654 ms, respectively. t_1 Quadrature detection was obtained by TPPI or Echo/Antiecho gradient selection, with decoupling during acquisition (Marion and Wuethrich 1983; Keeler et al. 1994). Acquisition parameters were as follows: number of t_1 increments = 300, number of data points in t_2 = 1 K, number of scans = 128, spectral widths: 7002.801 Hz (F2) and 1700.000 Hz (F1). All spectra were acquired and processed with TOPSPIN-1.3 (Bruker Biospin), and analyzed with Felix (Accelrys, San Diego, CA).

Structure based calculation of J-coupling constants

$^{3}J_{HN-H\alpha}$ coupling constants were calculated from the NMR and X-ray structure torsion angles (NMR: pdb id. 1Y9O; X-ray: pdb id. 2BJD (Corazza et al. 2006)) using the Karplus relationship (Karplus 1959), with the parameters reported by Pardi et al. (1984):

$$^{3}J_{HN-H\alpha} = 6.4\cos^2(\phi - 60) - 1.4\cos(\phi - 60) + 1.9 \quad (1)$$

For the NMR ensemble of structures coupling constants were computed for all models and then averaged. $^{3}J_{H\alpha-H\beta}$ coupling constants were calculated from the protein χ_1 torsion angles using the Karplus relationship with the parameters reported by De Marco et al. (1978):

$$^{3}J_{H\alpha-H\beta} = 9.5\cos^2(\theta) - 1.4\cos(\theta) + 1.8 \quad (2)$$

with $\theta = \chi_1 - 120$ for H^{β_2} and $\theta = \chi_1$ for H^{β_3}. H^{β_2} and H^{β_3} are defined according to the IUPAC-IUB conventions (IUPAC-IUB commission on Biochemical Nomenclature 1970).

Coherence transfer in TOCSY experiments

In TOCSY experiments the density matrix (ρ (t)) evolves under the isotropic mixing Hamiltonian:

$$H = \sum_{i=1,\, n;\, j=i+1,\, n} 2\pi J_{ij} \vec{I}_i \cdot \vec{I}_j$$

where J_{ij} is expressed in Hertz and $\hbar$ is set to 1 for simplicity of notation.

We assume that the density matrix at time 0 (ρ_0) is I_{1z}, which means that all the initial magnetization is on spin 1.

The density matrix at time t is the solution of the Liouville–von Neumann equation:

$$\rho(t) = e^{-i\hat{H}t}\rho(0)$$

The equation may be cast as a series expansion in the variable t:

$$\begin{aligned}\rho(t) &= \sum_{n=0,\,\infty} \frac{(-it)^n}{n!}\hat{H}^n\rho(0) \\ &= \sum_{n=0,\,\infty} \frac{(-it)^n}{n!}\underbrace{[H,[H,\ldots,[H,\rho(0)]]]}_{n} \\ &= \rho_0 + \rho_1 + \rho_2 + \rho_3 + \ldots\end{aligned}$$

where

$$\rho_{n+1} = \frac{-it}{n+1}[H,\rho_n]$$

It is possible to show that the expectation value of all I_{iz} operators can be expanded in even powers of time, when the starting density matrix is a linear combination of I_{jz} operators, due to the peculiar symmetry of the Hamiltonian and of the observable operators (Fogolari et al. 1996). For this reason we will be interested in the first even order terms of the expansion. The expansion has been worked out in a previous paper (Fogolari et al. 1993). We realise now that the notation was not free of ambiguities and flawed by missing constant factors in the third and fourth order term. In order to make the expansion useful and unambiguous in notation we recall hereafter the definitions and the commutators which are needed for derivation:

$$\begin{aligned}\Sigma_{ij} &= \frac{I_{iz} + I_{jz}}{2} \\ \Delta_{ij} &= \frac{I_{iz} - I_{jz}}{2} \\ \Pi_{ij} &= I_{ix}I_{jy} - I_{iy}I_{jx} \\ a_{ij} &= 2\pi J_{ij} t\end{aligned}$$

With these notations the following commutation rules hold:

$$\begin{aligned}\left[\vec{I}_i \cdot \vec{I}_j, \Sigma_{ij}\right] &= 0 \\ \left[\vec{I}_i \cdot \vec{I}_j, \Delta_{ij}\right] &= i\Pi_{ij} \\ \left[\vec{I}_i \cdot \vec{I}_j, \Pi_{ij}\right] &= -i\Delta_{ij} \\ \left[\vec{I}_i \cdot \vec{I}_j, \Pi_{ik}\right] &= i\left(I_{jz}\vec{I}_i \cdot \vec{I}_k - I_{iz}\vec{I}_j \cdot \vec{I}_k\right) \\ \left[\vec{I}_i \cdot \vec{I}_j, I_{iz}\vec{I}_j \cdot \vec{I}_k\right] &= i\left(\frac{1}{4}\Pi_{ik} - \frac{1}{4}\Pi_{jk}\right) \\ \left[\vec{I}_i \cdot \vec{I}_j, \Delta_{ik}\right] &= i\frac{1}{2}\Pi_{ij}\end{aligned}$$

The terms of the series expansion can be worked out up to fourth order with some tedious but straightforward work. For instance the zero order term can be written in the following form:

$$\rho_0 = I_{1z} = \Sigma_{1i} + \Delta_{1i}$$

which enables easy computation of the first order term. In the first order term only terms containing $\vec{I}_1 \cdot \vec{I}_i$ in the Hamiltonian do not commute with ρ_0 and therefore:

$$\rho_1 = -i\left[\sum_{i=1,\, n;\, j=i+1,\, n} a_{ij}\vec{I}_i \cdot \vec{I}_j, I_{1z}\right] = \sum_{i=2,\, n} a_{1i}\Pi_{1i}$$

In this and in all other terms the time is included in the coefficients a_{ij}. For the second order term:

$$\rho_2 = \frac{-i}{2}\left[\sum_{i=1,n;j=i+1,n} a_{ij}\vec{I}_i \cdot \vec{I}_j, \sum_{k=2,n} a_{1k}\Pi_{1k}\right]$$

there are three different cases to be considered:

(1) $i = 1, \quad j = k$
(2) $i = 1, \quad j \neq k$
(3) $i = k, \quad j \neq 1$ or $i \neq 1, \quad j = k$

After working out the commutators the result is:

$$\begin{aligned}\rho_2 = &-\frac{1}{2}\sum_{i=2,n} a_{1i}^2\Delta_{1i} \\ &+\frac{1}{2}\sum_{i=2,n;j=2,n;i\neq j} a_{1i}a_{1j}\left(I_{jz}\vec{I}_1 \cdot \vec{I}_i - I_{1z}\vec{I}_i \cdot \vec{I}_j\right) \\ &-\frac{1}{2}\sum_{i=2,n;j=2,n;i\neq j} a_{1i}a_{ij}\left(I_{jz}\vec{I}_1 \cdot \vec{I}_i - I_{iz}\vec{I}_1 \cdot \vec{I}_j\right)\end{aligned}$$

where only the first term contains "observable" coherences of the type I_{kz}. Since we are interested only in such coherences at the fourth order term of the expansion we consider that they can only be obtained from two-spin coherences in the third order term of the expansion. These coherences are obtained from one- or three-spin coherences in the second order term by application of the commutator involving the relevant terms in the Hamiltonian. For this

reason we do not have to deal explicitly with all the terms in the third order term of the expansion, but rather only with those terms that produce single-spin coherences of the type I_{kz} in the fourth order term.

$$\begin{aligned}\rho_3 = &-\frac{1}{3!}\sum_{i=2,n} a_{1i}^3 \Pi_{1i} \\ &+\frac{1}{3!}\sum_{i=2,n;j=2,n;i\neq j} a_{1i}^2 a_{1j}\left(\frac{3}{4}\Pi_{ij} - \frac{5}{4}\Pi_{1j}\right) \\ &+\frac{1}{3!}\sum_{i=2,n;j=2,n;i\neq j} a_{1i}^2 a_{ij}\left(\frac{3}{4}\Pi_{ij} - \frac{1}{4}\Pi_{1j}\right) \\ &+\frac{1}{3!}\sum_{i=2,n;j=2,n;i\neq j} a_{1i} a_{ij}^2\left(\frac{1}{2}\Pi_{i1} - \frac{1}{2}\Pi_{j1}\right) \\ &+\frac{1}{3!}\sum_{i=2,n;j=2,n;i\neq j} a_{1i} a_{ij} a_{1j}\left(\frac{1}{4}\Pi_{1i} - \frac{1}{4}\Pi_{ji}\right)\end{aligned}$$

Each of the terms of the type Π_{mn} will be converted, in the fourth order term of the expansion, into a coherence Δ_{mn} which will be therefore multiplied by a coefficient a_{mn}.

$$\begin{aligned}\rho_4 = &+\frac{1}{4!}\sum_{i=2,n} a_{1i}^4 \Delta_{1i} \\ &-\frac{1}{4!}\sum_{i=2,n;j=2,n;i\neq j} a_{1i}^2 a_{1j} a_{ij}\left(\frac{3}{4}\Delta_{ij}\right) \\ &+\frac{1}{4!}\sum_{i=2,n;j=2,n;i\neq j} a_{1i}^2 a_{1j}^2\left(\frac{5}{4}\Delta_{1j}\right) \\ &-\frac{1}{4!}\sum_{i=2,n;j=2,n;i\neq j} a_{1i}^2 a_{ij}^2\left(\frac{3}{4}\Delta_{ij}\right) \\ &+\frac{1}{4!}\sum_{i=2,n;j=2,n;i\neq j} a_{1i}^2 a_{ij} a_{1j}\left(\frac{1}{4}\Delta_{1j}\right) \\ &-\frac{1}{4!}\sum_{i=2,n;j=2,n;i\neq j} a_{1i}^2 a_{ij}^2\left(\frac{1}{2}\Delta_{i1}\right) \\ &+\frac{1}{4!}\sum_{i=2,n;j=2,n;i\neq j} a_{1i} a_{ij}^2 a_{1j}\left(\frac{1}{2}\Delta_{j1}\right) \\ &-\frac{1}{4!}\sum_{i=2,n;j=2,n;i\neq j} a_{1i}^2 a_{ij} a_{1j}\left(\frac{1}{4}\Delta_{1i}\right) \\ &+\frac{1}{4!}\sum_{i=2,n;j=2,n;i\neq j} a_{1i} a_{ij}^2 a_{1j}\left(\frac{1}{4}\Delta_{ji}\right)\end{aligned}$$

Coherence transfer in ^{1}H–^{15}N HSQC-TOCSY experiments for amino acid spin systems

We will not work out here the last step of the expansion in the general case, but rather we consider how this expansion simplifies for amino acid proton spin systems in ^{1}H–^{15}N HSQC-TOCSY experiments. In such experiments the magnetization at the beginning of the mixing time is on the H^N proton (proton 1 in the above equations), which is significantly coupled only to H^α which is in turn coupled to one or more H^β protons.

All terms containing $a_{1i}a_{1j}$ vanish because $i \neq j$ in the sum and therefore one of the two factors is 0. In addition all terms Π_{1j} do not evolve in Δ_{1j} coherences because the relevant term in the Hamiltonian commutator is multiplied by a_{1j} which is 0 if $a_{1i} \neq 0$ for $i \neq j$. Only few terms must be considered and the final result is a rather simple equation for the fourth order term of the expansion:

$$\begin{aligned}\rho_4 = &+\frac{1}{4!}\sum_{i=2,n} a_{1i}^4 \Delta_{1i} \\ &-\frac{1}{4!}\sum_{i=2,n;j=2,n;i\neq j} a_{1i}^2 a_{ij}^2\left(\frac{3}{4}\Delta_{ij}\right) \\ &+\frac{1}{4!}\sum_{i=2,n;j=2,n;i\neq j} a_{1i}^2 a_{ij}^2\left(\frac{1}{2}\Delta_{1i}\right)\end{aligned}$$

Now if we substitute H^N for 1 and set $a_{1i} \neq 0$ only for i = H^α the above formula reads (we omit H in coefficients' subscripts for simplicity):

$$\begin{aligned}\rho_4 = &+\frac{1}{4!}a_{N\alpha}^4 \Delta_{N\alpha} \\ &+\frac{1}{4!}\sum_{\beta} a_{N\alpha}^2 a_{\alpha\beta}^2\left(\frac{1}{2}\Delta_{N\alpha}\right) \\ &-\frac{1}{4!}\sum_{\beta} a_{N\alpha}^2 a_{\alpha\beta}^2\left(\frac{3}{4}\Delta_{\alpha\beta}\right)\end{aligned}$$

This leads to the following equation for the peak intensities (remember that at time 0 only the H^N has I_z different from 0):

$$\begin{aligned}I_{HN} &= 1 - \frac{1}{2}a_{N\alpha}^2\frac{1}{2} + \frac{1}{4!}a_{N\alpha}^4\frac{1}{2} + \frac{1}{4!}\sum_{\beta} a_{N\alpha}^2 a_{\alpha\beta}^2\left(\frac{1}{2}\frac{1}{2}\right) \\ I_{H\alpha} &= \frac{1}{2}a_{N\alpha}^2\frac{1}{2} - \frac{1}{4!}a_{N\alpha}^4\frac{1}{2} - \frac{1}{4!}\sum_{\beta} a_{N\alpha}^2 a_{\alpha\beta}^2\left(\frac{1}{2}\frac{1}{2}\right) \\ &\quad - \frac{1}{4!}\sum_{\beta} a_{N\alpha}^2 a_{\alpha\beta}^2\left(\frac{3}{4}\frac{1}{2}\right) \\ I_{H\beta} &= \frac{1}{4!}a_{N\alpha}^2 a_{\alpha\beta}^2\left(\frac{3}{4}\frac{1}{2}\right)\end{aligned}$$

Calculation of *J*-coupling constants from ^{1}H–^{15}N HSQC-TOCSY experiments

The simple dependence of the peaks on the coupling constants may be exploited for estimating the latter. We found the following procedure effective. Few HSQC-TOCSY

experiments are recorded at increasing mixing times. The peak intensities normalized by the sum of H^N, H^α and H^β intensities are then considered for the series of experiments. First $^3J_{HN-H\alpha}$ is estimated by fitting the time series of the quantity: $\frac{1-I_{HN}+I_{H\alpha}}{I_{HN}+I_{H\alpha}}$ which is equal, up to $o(t^5)$, to $a_{N\alpha}^2\frac{1}{2}-\frac{1}{4!}a_{N\alpha}^4-\frac{1}{4!}\sum_\beta a_{N\alpha}^2 a_{\alpha\beta}^2\left(\frac{1}{2}\right)\cdot$

The time series of this quantity is fit to $c_2\ t^2$ where c_2 is directly related to $^3J_{HN-H\alpha}$:

$$^3J_{HN-H\alpha}=\frac{\sqrt{2c_2}}{2\pi} \tag{3}$$

Actually it was found convenient not to include in the equation the summation over H^β intensities, proportional to t^4. The rationale for this choice is that the signal to noise ratio is lower for these intensities and therefore they can worsen estimation of $^3J_{HN-H\alpha}$. This approximation is less and less severe as the mixing times become smaller.

The estimated $^3J_{HN-H\alpha}$ is additionally scaled in order not to exceed the maximal value of 9.6 Hz.

Once $^3J_{HN-H\alpha}$ has been estimated the intensities $I_{H\beta}$ are fit to $c_4\ t^4$ and the coupling constants and $^3J_{H\alpha-H\beta}$ are obtained from the coefficient c_4:

$$^3J_{H\alpha-H\beta}=\frac{\sqrt{4!\left(\frac{4}{3}\right)2c_4}}{(2\pi)^2\times{}^3J_{HN-H\alpha}} \tag{4}$$

For glycines a different procedure is adopted because the spin-system connectivity is different from that described above. We consider the TOCSY cross-peak build up quadratic in time. Due to the very strong geminal coupling ($\approx$ –15 Hz) between the two H^α protons only short mixing times (say less or equal to 20 ms) should be considered. An empirical correction derived from simulation results is applied in order to take into account averaging of cross-peak intensities due to efficient magnetization transfer between the two protons.

Simulation of a three-spin system

In order to check the accuracy of the approximation and of the procedure described above we simulated a three spin-system (H^N, H^α and H^β) evolving under isotropic mixing. Coupling constants between H^N and H^α, and between H^α and H^β have been varied between 2.0 and 10.0 Hz and the corresponding magnetization intensities at various mixing times have been computed. *J*-coupling constants have been estimated from time evolution of magnetization intensities, according to the fitting procedure described above, and compared to the true *J*-coupling constants.

The simulation and *J*-coupling estimation from simulated data has been performed also for noisy data. Noise has been added to the computed magnetization intensities. Noise intensities were taken from a zero mean Guassian distribution with standard deviation matching the experimentally estimated value of ca. one thousandth of the overall intensity.

Results

Simulation results

The simulated evolution of peak intensities for a representative three-spin system with $^3J_{HN-H\alpha}$ and $^3J_{H\alpha-H\beta}$ equal to 4.0 and 8.0 Hz, respectively, is shown in Fig. 1. Up to approximately 30 ms mixing time the Taylor expansion provides a very good approximation of the true time evolution. In general the error is fitted excellently by a single coefficient multiplied by the sixth power of time. The agreement between time evolution of the peaks and the approximation provided by the time series approximation supports the idea of inverting the time series expansion in order to compute coupling constants. *J*-coupling constants were estimated from simulated data at 5 mixing times ranging from 4 to 20 ms for different *J*-coupling constants according to the equations reported in the materials and methods section. In the absence of noise the errors in the estimated $^3J_{HN-H\alpha}$ and $^3J_{H\alpha-H\beta}$ are less than 0.2% and 7%, respectively.

The agreement between true and calculated $^3J_{HN-H\alpha}$ and $^3J_{H\alpha-H\beta}$ coupling constants gets worse with increasing isotropic mixing times, but the discrepancies remain lower than 1% and 15% of the real values, respectively, up to 30 ms mixing times for realistic *J*-coupling constants in the absence of noise. When noise is added the agreement is deteriorated, in particular because, when $^3J_{HN-H\alpha}$ is small, the noise is comparable to the intensity of the transfer from

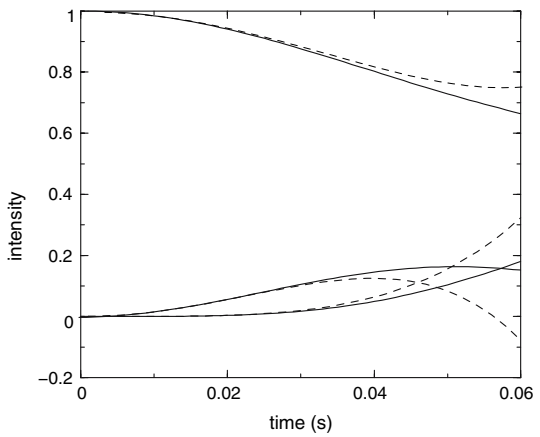

Fig. 1 Time evolution of peak intensities from numerical density matrix calculations (continuous line) and from Taylor series expansion (dotted line). Curves refer to H^N (upper), H^α (middle), H^β (lower)

H^N to H^β, introducing large errors in $^3J_{H\alpha-H\beta}$ which is estimated from the $I_{H\beta}$ intensity.

Transfer data for five mixing times (0.004, 0.008, 0.012, 0.016 and 0.020) have been simulated in the presence of noise for coupling constants $^3J_{HN-H\alpha}$ and $^3J_{H\alpha-H\beta}$ equal to 2, 4, 6, 8, 10 Hz.

A similar test with due corrections has been applied to glycine spin system. The plot of the reconstructed and true $^3J_{HN-H\alpha}$ and $^3J_{H\alpha-H\beta}$ and $^3J_{HN-H\alpha1}$ and $^3J_{HN-H\alpha2}$ for glycines is reported in Fig. 2. For such short mixing times the agreement between true and calculated coupling constants is in general very good for $^3J_{HN-H\alpha}$, while for $^3J_{H\alpha-H\beta}$ it crucially depends on the signal to noise ratio for $I_{H\beta}$. In the present simulation, when $^3J_{HN-H\alpha}$ is smaller than 4.0 Hz, estimation of $^3J_{H\alpha-H\beta}$ is very imprecise.

Experimental results

$^3J_{HN-H\alpha}$ determination

The quality of the spectra used for the present study is illustrated by Figs. 3 and 4. In the latter figure the progressive build up of TOCSY peaks for H^βs is apparent. The six spectra shown in this figure have been used for the analysis.

Due to progressive deterioration of the statistically flexible N-terminal tail of Sso AcP (Corazza et al. 2006), the coupling constants have been estimated and compared with experimental data starting from residue Leu 13. Similarly the C-terminal Tyr 101 residue was not considered because the $^3J_{HN-H\alpha}$ coupling constants computed on the two monomers in the crystal structure differ by 2.8 Hz making comparison invalid. Moreover 3 out of the 88 residues are prolines, and 15 residues were excluded because of severe overlap of the auto-peak and/or the cross peak. For the remaining 70 residues $^3J_{HN-H\alpha}$ coupling constants were estimated, including glycine residues. For the latter the strong geminal coupling causes an efficient magnetization transfer between the two H^α protons, so that the intensities of the two estimated $^3J_{HN-H\alpha}$ are very close. Application of the empirical correction derived from simulation data analyses greatly improves the performance of the method. At the end of the filtering process the analysis concerns 62 residues with sidechains and 8 glycines. 19 of the 62 residues with sidechain analyzed present a partial overlap of auto-peak and/or cross peak. In the following results obtained for residues with sidechain and glycines are discussed separately.

Comparison with NMR structural data was also performed, but in general the agreement was worse because of artifacts due to poorly defined structural regions. We discuss in the following only the comparison with $^3J_{HN-H\alpha}$ values computed on the X-ray structure.

The results are shown in Fig. 5. The correlation coefficient between calculated and structurally derived $^3J_{HN-H\alpha}$ coupling constants is as high as 0.93 both for all or just the best experimental data and the RMSD is remarkably low (Table 1). These results have been compared with the results obtained by using the method of Wishart and coworkers (Wang et al. 1997), which is a widely accepted method for computing *J*-couplings from TOCSY and NOESY spectra. The accuracy of Wishart's method is comparable to that obtainable with other methods proposed so far (Wang et al. 1997) with the exception of the method of Vuister and Bax which appears to be the most accurate (Vuister and Bax, 1993). It should be noted however that the latter method requires recording a 3D-NMR experiment whereas Wishart's method and the one presented here require just 2D-NMR experiments. Correlation coefficients and RMSDs between computed and structurally derived $^3J_{HN-H\alpha}s$ are reported in Table 1. The correlation coefficient is rather high for both the TIE (Tocsy Intensity Evolution) method presented here and Wishart's method, but the accuracy, assessed using the RMSD, is definitely higher for the TIE method.

Since the estimation of $^3J_{HN-H\alpha}$ based on simulation for short mixing times, as the ones used here, should be quite accurate, discrepancies with the values computed for the X-ray experimental structures were further analyzed. There are six $^3J_{HN-H\alpha}$ deviating for more than 1.5 Hz (Fig. 6). Four of these, namely Leu 23, Ala 78, Ala 79 and Asp 51, are

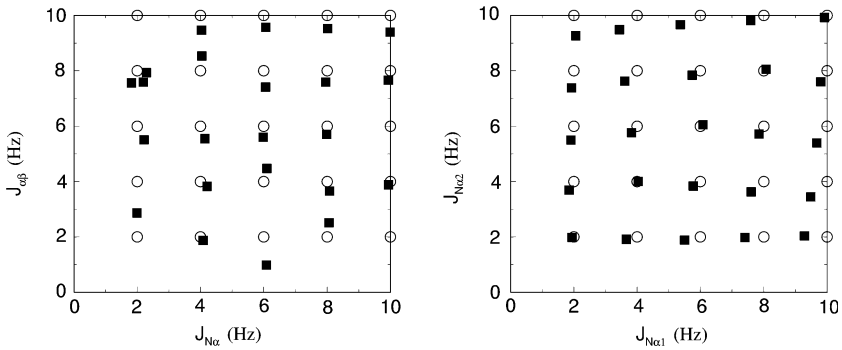

Fig. 2 Estimated (squares) and true (filled circles) *J*-coupling constants for three-spin general amino acid-like systems (left panel) and glycine-like spin-system (right panel). Estimated and true coupling *J*-coupling constants are displayed, based on intensity time evolution at 5 mixing times ranging from 4 to 20 ms. Noise has been added to the simulated intensities

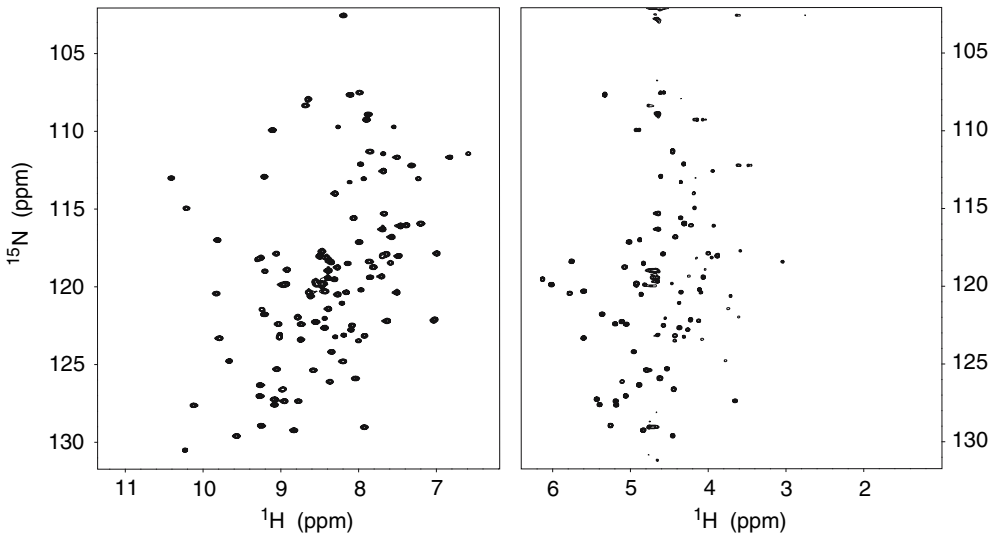

Fig. 3 The 500 MHz ^{1}H–^{15}N 2D HSQC-TOCSY spectrum with mixing time of 11.770 ms. All spectra were acquired at 310 K on a 0.4 mM Sso AcP sample in 50 mM phosphate buffer, 50 mM NaCl, at pH 5.7

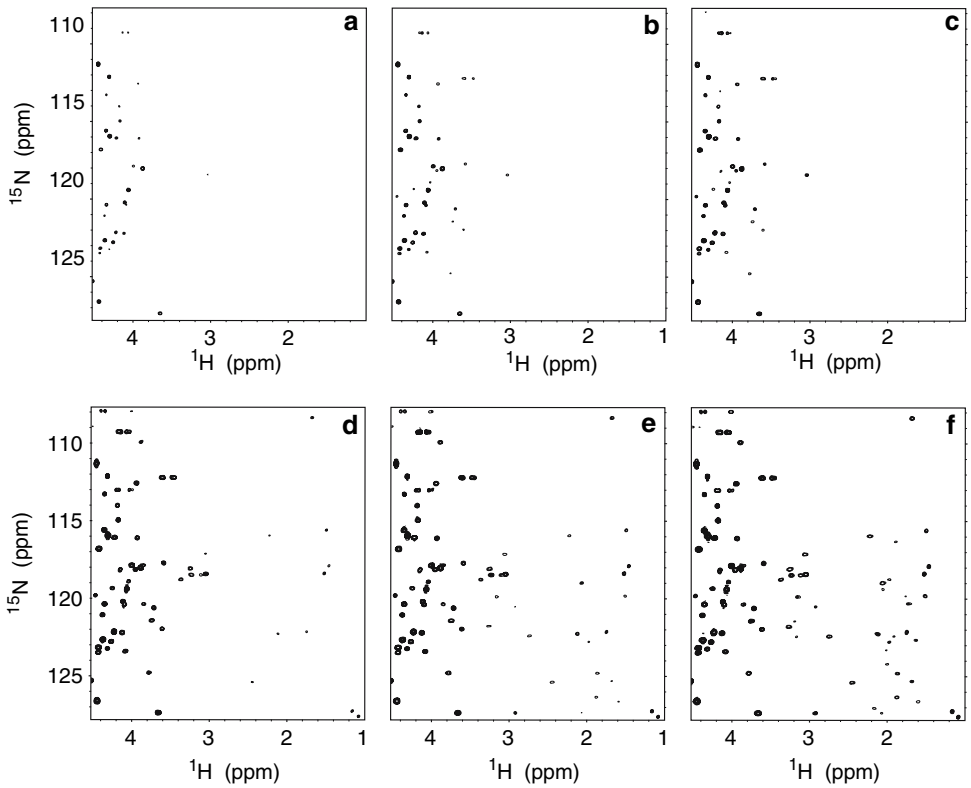

Fig. 4 Representative ^{1}H–^{15}N 2D HSQC-TOCSY spectra: from panel (**a**) to (**f**) the number of WALTZ-16 cycles varies from 1 to 6, corresponding to mixing times of 2.942, 5.885, 8.827, 11.770, 14.712 and 17.654 ms, respectively

found in loops at the same end of the molecule. Asp 51 is further involved in intermolecular contacts in the crystal. The proximity of these residues suggests that this region could be flexible in solution. The RMSDs of residues Leu 23 and Asp 51 are indeed two of the largest local RMSDs upon superposition of the N atoms of the two crystallographic monomers, showing some degree of conformational freedom. Moreover, the temperature factors of Ala 78 and Ala 79 are among the highest of the crystal structure. The other two residues showing a large discrepancy are Ala 64 and Arg 39 which are in the first and last turn of the two anti-parallel helices, respectively, and are close to each other. The position of the two residues is well defined in the crystal structure, however, the angle of Ala 64 is one of the most poorly defined in the 20 deposited NMR structures resulting in a computed $^{3}J_{HN-H\alpha}$ coupling constant of 7.2 ± 1.9 Hz, in

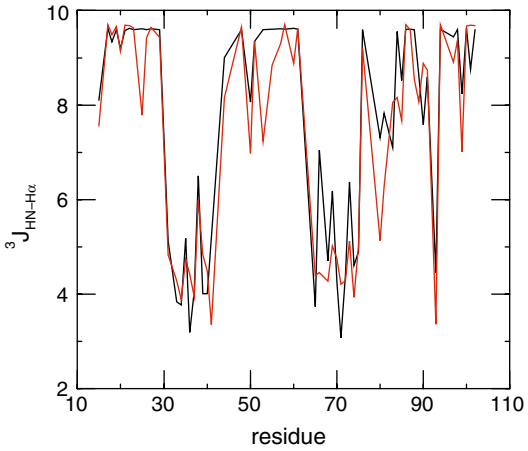

Fig. 5 $^3J_{HN-H\alpha}$ coupling constants computed from TOCSY experiments (black line) and calculated according to Karplus equation from the crystallographic structure (red line)

Table 1 Performance of the method—the correlation coefficients and RMSDs between computed and structurally derived $^3J_{HN-H\alpha}$s

Method	corr. coef.	RMSD
TIE[a]	0.93 (0.93)	0.89 (0.85)
Wishart[b]	0.85 (0.81)	1.29 (1.31)

[a] The row TIE refers to the method presented here (Tocsy intensity evolution)

[b] The row Wishart refers to Wishart's and coworkers' method. In parenthesis the corresponding values obtained for the dataset free of overlap are given

agreement with the estimated coupling constant of 7.0 Hz, suggesting that also for these two residues the discrepancy could be due to flexibility. As a possible confirmation to this explanation it should be noted that the $^3J_{HN-H\alpha}$ coupling constants computed for Arg 39 differ by 0.6 Hz in the two crystallographic monomers. The value for monomer B (4.0 Hz) would not be inconsistent with the computed value of 5.1 Hz, because the peaks of Arg 39 are among those affected by overlap. Arg 39 is also present in a double form in the crystallographic monomer A.

$^3J_{HN-H\alpha1}$ and $^3J_{HN-H\alpha2}$ determination for glycines

No stereo specific assignment was available for glycines. Based on simulation results for mixing times like those used here, the relative magnitude of the coupling constants can be assessed. It should be noted that for all mixing times one of the two crosspeaks is larger than the other, thus making the relative magnitude of the coupling constants out of doubt.

The results are reported in Table 2. Error bars have been estimated by randomly adding noise to peak volumes in such a way that the standard deviation of the noise is 0.0025 times the intensity of the starting auto-peak. This figure is consistent with experimental estimation and with the range of estimated values for $^3J_{H\alpha-H\beta}$ for alanines.

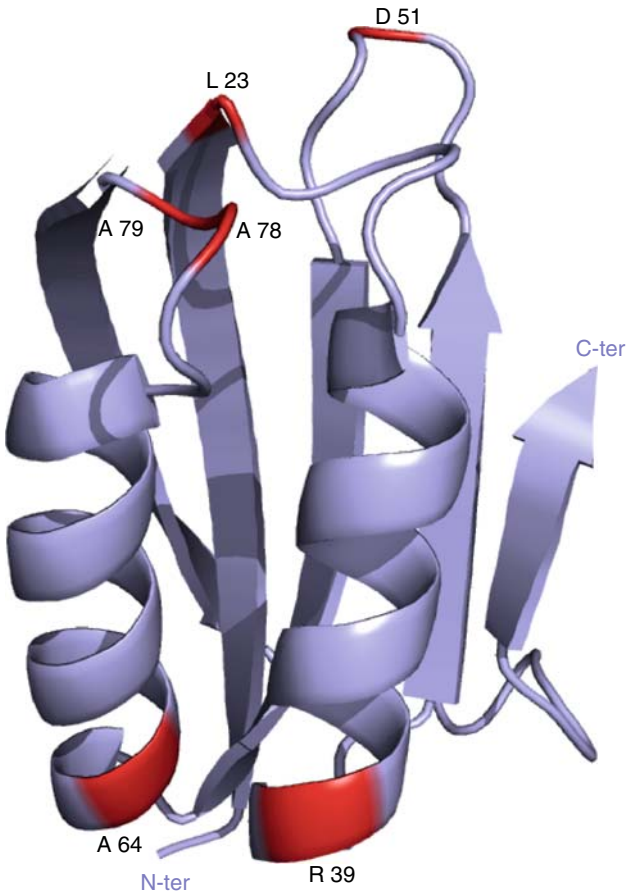

Fig. 6 Ribbon diagram of Sso AcP showing the β-sandwich domain composed of four antiparallel and one parallel β-strands, assembled in a five-stranded, twisted β-sheet facing two antiparallel α-helices. Residues that show a deviation larger than 1.5 Hz, between computed (TIE) and structurally derived (X-ray) coupling constants, are highlighted in red

Table 2 Performance of the TIE method—computed and structurally derived coupling constants for glycines[a]

Residue	TIE	X-ray
GLY 22	8.2 (0.04)/2.4 (0.11)	9.0/1.9
GLY 26	6.2 (0.05)/5.3 (0.08)	6.7/5.4
GLY 41	6.5 (0.05)/5.9 (0.08)	6.4/6.3
GLY 44	5.6 (0.06)/3.3 (0.11)	9.0/1.9
GLY 52	8.9 (0.04)/4.0 (0.09)	7.6/5.7
GLY 60	4.2 (0.08)/3.0 (0.13)	5.5/3.1
GLY 75	6.2 (0.05)/4.7 (0.09)	6.5/6.2
GLY 93	6.3 (0.05)/6.0 (0.08)	6.9/4.0

[a] Coupling constants are listed in progressive order because no stereospecific assignment was available. Estimated error bars (see text) are reported in parentheses

The correlation coefficient between computed and structurally derived coupling constants is in this case 0.79 and the RMSD is 1.3 Hz. Since these figures are computed under the assumption that we are able to recover correctly at least the relative magnitude of the coupling constants, some correlation between computed and structurally derived coupling constants is expected. In order to provide an estimate of the expected correlation coefficient and RMSD due to ordering the pair of coupling constants to be compared, a null hypothesis test was performed where all computed values were swapped randomly among residues, while maintaining the ordering of the pair. For this randomized set the correlation coefficient drops to 0.44 and the RMSD is 2.0 Hz.

$^3J_{H\alpha-H\beta}$ determination

For the analysis of $^3J_{H\alpha-H\beta}$ it is necessary to detect the transfer of magnetization from H^N to H^β nuclei. Since the signal builds up with the fourth power of time, the intensity is weak (two to three orders of magnitude smaller than the auto-peak intensity) and often it cannot be safely separated from background noise. In practice reliable quantification of H^β peaks requires that the transfer from H^N to H^α be efficient for transfer to proceed from H^α to H^β. These considerations impose a threshold value of 6.0 Hz for the intensity of $^3J_{HN-H\alpha}$ with the present noise conditions. For values below this threshold the H^β crosspeak is either undetectable or inaccurately measured. This rules out the possibility of using the method, in the present conditions, for residues in α-helices. When no H^β signal is observed, despite the estimated $^3J_{HN-H\alpha}$ is larger than the 6.0 Hz threshold, we set the relative $^3J_{H\alpha-H\beta}$ to the minimum possible value of 1.7 Hz. The comparison of computed and structurally derived $^3J_{H\alpha-H\beta}$ coupling constants could be afforded for 30 residues, including six alanines whose fixed $^3J_{H\alpha-H\beta}$ (7.4 Hz) was considered only for the sake of testing the reliability of the TIE approach.

As expected the agreement between computed and structurally derived values gets worse. For small coupling constants the signal is not safely detected from noise and this results in assigning the minimum possible value (1.7 Hz) to such constants. On the other hand larger coupling constants seem to be mostly underestimated. Due to the approximations involved it is more plausible ascribing such effect to the method itself rather than to the mobility of the sidechains. It is worth noting, however, that a clear indication of the type of conformation (gauche or trans) and χ_1 torsion angle could be gained by the analysis, provided that stereospecific assignment of H^β protons is available. Consistent underestimation of larger coupling constants results in a RMSD of 1.9 Hz while the correlation coefficient is 0.72 for the data of Table 3. As for glycines, error bars have been estimated by randomly adding noise to peak volumes in such away that the standard deviation of the noise is 0.0025 times the intensity of the starting auto-peak. It should be clear that the error refers only to the effect of noise on peak integration. Other sources of systematic error like overlap of peaks or the effect of the approximations involved in the method are not taken into account.

Table 3 Performance of the method—computed (column TIE) and structurally derived (column X-ray) coupling constants

Residue	TIE[a]	X-ray[a]
ALA 18	7.4 (0.94)	7.4
ALA 46	7.8 (0.70)	7.4
ALA 58	7.3 (0.82)	7.4
ALA 64	9.3 (1.33)	7.4
ALA 78	9.3 (1.25)	7.4
ALA 79	8.2 (1.15)	7.4
VAL 20	9.2 (0.67)	12.8
VAL 24	1.7 (n.a.)	2.9
VAL 27	1.7 (n.a.)	2.5
VAL 56	9.0 (0.65)	12.9
VAL 81	8.3 (1.13)	12.8
VAL 84	9.6 (0.56)	12.9
ILE 42	7.6 (0.94)	12.7
THR 100	1.7 (n.a.)	3.6
TYR 21	8.9 (0.46)/3.9 (0.91)	12.9/3.5
LEU 23	4.1 (0.61)/9.1 (0.87)	2.6/12.3
ASP 85	7.1 (0.75)/9.6 (0.51)	7.1/7.2
PHE 88	9.5 (1.02)/1.7 (1.47)	12.7/2.7
PHE 95	10.5 (0.59)/4.6 (0.93)	12.8/4.1
PHE 98	1.7 (1.12)/10.2 (0.57)	1.8/11.8
GLU 99	8.3 (0.88)/1.7 (n.a.)	12.8/3.6
ARG 19	6.6 (0.87)/3.6 (1.00)	8.0/7.5
GLN 25	7.2 (0.61)/1.7 (n.a.)	12.8/2.8
LEU 49	8.6 (0.67)/1.7 (n.a.)	12.8/2.7
ARG 71	8.7 (1.32)/1.7 (n.a.)	12.1/2.0
LYS 83	1.7 (n.a.)/5.0 (1.03)	3.9/12.9
LYS 92	9.3 (0.61)/1.7 (n.a.)	12.4/4.9

[a] When two values are listed they refer to H^{β_2} and H^{β_3}, respectively. The lower part of the table lists coupling constants for residues where no stereospecific assignment was available. Estimated error bars (see text) are reported in parentheses, n.a. stands for not applicable because the coupling constant has been assigned the lowest possible value of 1.7 Hz due to absence of detectable signal in well resolved regions

Conclusions

TOCSY experiments convey more information than the bare topology of the studied spin-systems. The time evolution of TOCSY peaks depends on *J*-couplings and can be computed. The series expansion of the density matrix during a TOCSY experiment shows that for short mixing times, and for typical coupling constants found in amino acids the crosspeaks may be described by quadratic and quartic terms. The relationship between time series coefficients and *J*-couplings is in general complex, but simplifies for the topology of amino acid, where the amide proton is coupled only to the H^{α} proton which is in turn coupled only to the H^{β} protons. In the presented approach we exploit the resolution of auto- and cross-peaks in 2D ^{1}H–^{15}N HSQC-TOCSY experiments to estimate $^{3}J_{HN-H\alpha}$ and $^{3}J_{H\alpha-H\beta}$ coupling constants from the time evolution of the peaks. Based on the results we conclude that the method performs comparably or better than the few other methods available for estimating $^{3}J_{HN-H\alpha}$ coupling constants, and provides useful estimates of $^{3}J_{H\alpha-H\beta}$ coupling constants.

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