Journal of Biomolecular NMR, 4 (1994) 587–593
ESCOM

J-Bio NMR 203

HNCCH-TOCSY, a triple resonance experiment for the correlation of backbone $^{13}C^{\alpha}$ and ^{15}N resonances with aliphatic side-chain proton resonances and for measuring vicinal $^{13}CO,^{1}H^{\beta}$ coupling constants in proteins

Rüdiger Weisemann*, Frank Löhr and Heinz Rüterjans**

Institut für Biophysikalische Chemie der Johann Wolfgang Goethe Universität Frankfurt am Main, Biozentrum, Marie Curie Strasse, D-60439 Frankfurt am Main, Germany

Received 10 March 1994
Accepted 13 April 1994

Keywords: 3D triple resonance NMR spectroscopy; Backbone/side-chain resonance assignments; $^{13}CO,^{1}H^{\beta}$ coupling constants; *Desulfovibrio vulgaris* flavodoxin

SUMMARY

A 3D triple resonance experiment has been designed to provide intraresidual and sequential correlations between amide nitrogens and α-carbons in uniformly $^{13}C/^{15}N$-labeled proteins. In-phase $^{13}C^{\alpha}$ magnetization is transferred to the aliphatic side-chain protons via the side-chain carbons using a CC-TOCSY mixing sequence. Thus, the experiment alleviates the resonance assignment process by providing information about the amino acid type as well as establishing sequential connectivities. Leaving the carbonyl spins untouched throughout the transfer from $^{13}C^{\alpha}$ to $^{1}H^{\beta}$ leads to E.COSY-type cross peaks, from which the $^{3}J_{H^{\beta}CO}$ coupling constants can be evaluated. The pulse sequence is applied to oxidized *Desulfovibrio vulgaris* flavodoxin.

The resonance assignment of a protein provides the basis for the interpretation of structural data, derived from multidimensional NMR experiments. As a first step, the approach generally applied for $^{13}C/^{15}N$-labeled proteins leads to the assignment of all backbone resonances. The identification of spin systems is mainly performed in subsequent experiments, without direct correlation between backbone and side-chain resonances. In this communication we present a triple resonance 3D NMR technique which correlates the backbone ^{15}N and $^{13}C^{\alpha}$ resonances with the aliphatic side-chain ^{1}H resonances in $^{15}N/^{13}C$-labeled proteins. In contrast to the previously published HCC(CO)NH-type experiments (Montelione et al., 1992; Clowes et al., 1993; Grzesiek et al., 1993a; Logan et al., 1993), this technique does not include a relay transfer via the carbonyl spin. Correlating the amide nitrogen directly with the α-carbon leads to intra- and interresidual

*Present address: Bruker Analytische Messtechnik GmbH, Silberstreifen, D-76287 Rheinstetten, Germany.
**To whom correspondence should be addressed.

magnetization transfers due to the $^1J_{NC\alpha}$ and $^2J_{NC\alpha}$ scalar couplings, as in previously described triple resonance techniques (Montelione and Wagner, 1990; Kay et al., 1990,1991; Boucher et al., 1992; Clubb et al., 1992). The HNCCH-TOCSY approach proposed by us inevitably suffers from relatively long delay durations, necessary to achieve a polarization transfer from nitrogen to carbon. This drawback can be partly overcome by a careful design of the experiment. The evolutions of chemical shift and scalar couplings are allowed to occur simultaneously during the experiment in a constant-time manner. In particular, no loss of magnetization due to the C^α-C^β coupling during the C^α evolution period is observed if the constant-time delay is tuned to $1/^1J_{C\alpha C\beta}$. Moreover, this method offers a potentially maximal resolution in the C^α dimension when compared to an HNCA experiment. Mixing of ^{13}C magnetization is achieved with a ^{13}C-^{13}C TOCSY sequence, finally leading to the detection of all aliphatic proton resonances. The combination of the HNCH-type experiment and the CCH-TOCSY (Bax et al., 1990; Fesik et al., 1990) technique thus allows the identification of the amino acid type of sequentially connected residues.

As the HNCCH-TOCSY technique may be viewed as a combination of well-known experimental schemes, only a brief description of the pulse sequence will be given. In the initial step, proton magnetization is transferred to the amide nitrogen via a refocused INEPT (Morris and Freeman, 1979; Burum and Ernst, 1980) scheme. Evolution of the nitrogen chemical shift is monitored during the constant-time period T_N. Simultaneously, antiphase magnetization builds up with respect to the $^{13}C^\alpha$ of the same amino acid residue and that of the preceding residue due to the $^1J_{NC\alpha}$ and $^2J_{NC\alpha}$ coupling, respectively. To allow for maximal resolution in the F1 domain, the constant-time period is split into four parts with separate refocusing of the $^1J_{NCO}$ coupling (Grzesiek et al., 1993b). Alternatively, the experiment can be recorded with only one refocusing pulse, moving across the beginning of the ^1H composite pulse decoupling sequence. Following the pair of 90° pulses applied to ^{15}N and $^{13}C^\alpha$, the antiphase carbon magnetization refocuses with respect to the coupled ^{15}N nuclei and evolves according to the $^{13}C^\alpha$ chemical shift during t2. α-Carbon chemical shift evolution is executed in a constant-time manner, with the delay T_C set to the inverse of the $^1J_{C\alpha C\beta}$ coupling constant. Refocusing of the $^1J_{C\alpha CO}$ coupling is achieved by applying a 180° pulse on ^{13}CO, as indicated in Fig. 1. Note that, in order to observe an E.COSY pattern in the final spectrum (i.e., a splitting of the multiplets along F2 due to the $^1J_{C\alpha CO}$ coupling), this pulse must be omitted. In-phase magnetization of the $^{13}C^\alpha$ nuclei is transferred along the side chain in the following ^{13}C,^{13}C TOCSY step. This can be carried out as a z-filtered DIPSI-3 isotropic mixing sequence (Shaka et al., 1988), or by using a G3-MLEV16 expansion (Eggenberger et al., 1992a). The former is applied in the ^{13}CO-decoupled HNCCH-TOCSY experiment, whereas the latter must be applied in the coupled version of the experiment, in order not to disturb the ^{13}CO spin state during the mixing period. In the z-filtered version, the ^{13}C offset frequency is shifted upfield from the $^{13}C^\alpha$ spectral region to the center of the aliphatic ^{13}C resonances after the first hatched 90° pulse. Power levels on the ^{13}C channel are switched after the first and before the second hatched 90° pulse. After the mixing period, the magnetization at the aliphatic carbons is transferred to their covalently linked protons in a reverse refocused INEPT step for detection during t3. ^{13}C decoupling during acquisition is achieved with a GARP (Shaka et al., 1985) modulation in the case of the ^{13}CO-decoupled experiment and with a G3-MLEV16 expansion in the case of the ^{13}CO-coupled version. Nitrogen decoupling during t3 is optional in both cases, however, it is desirable to remove the effect of the $^3J_{H\beta N}$ coupling on the multiplet structure of the H^β,C^α cross peaks if the $^3J_{H\beta CO}$ coupling is to be determined.

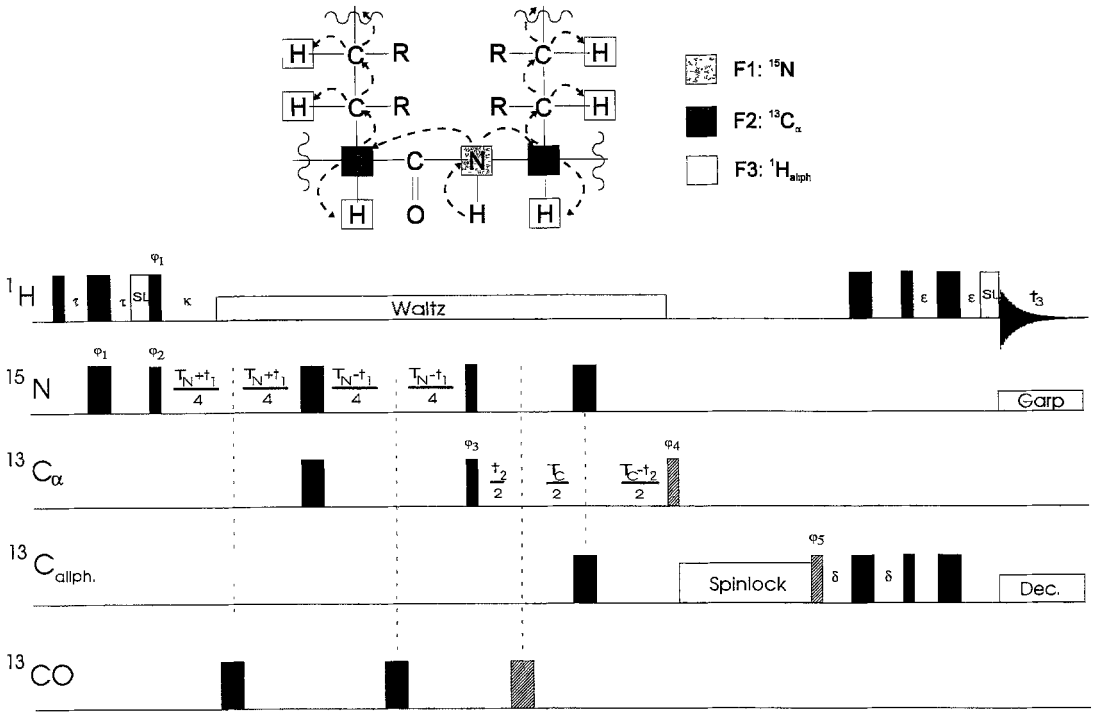

Fig. 1. Combined pulse scheme of the HNCCH-TOCSY experiment in the ^{13}CO-coupled and -decoupled mode. The inset shows the magnetization transfer pathway. Narrow and wide bars represent 90° and 180° pulses, respectively. Unless otherwise indicated, pulses are applied along the x-axis. Hatched pulses are applied in the ^{13}CO-decoupled HNCCH-TOCSY experiment only. In the ^{13}CO-coupled version of the experiment, ^{15}N decoupling during acquisition is achieved using GARP modulation with a field strength of 1.0 kHz. Proton decoupling is accomplished by a synchronous WALTZ-16 scheme, with an RF field of 5.0 kHz. Proton spin-lock pulses (SL) are applied for durations of 2.0 and 1.0 ms, respectively. Carbonyl pulses are implemented as phase-modulated shaped pulses with a profile corresponding to the central lobe of a 'sinc' function (sin(x)/x) and a duration of 108 μs. The carrier for the C^{α} pulses is positioned at 60.0 ppm, while the pulses on the aliphatic carbons are applied at 45.0 ppm. In the carbonyl-decoupled version, the ^{13}C pulses are implemented as low-power square pulses with an RF field of 4.6 kHz for C^{α}, and with field strengths of 5.3 (90°) and 11.8 kHz (180°) for the aliphatic carbons. A 7 kHz DIPSI-3 mixing period of 22.8 ms duration is employed. ^{13}C decoupling during acquisition is accomplished by a GARP scheme, with an RF field of 2.5 kHz. In the carbonyl-coupled version, all carbon pulses (except for ^{13}CO) are implemented as Gaussian cascades G4 and G3 (Emsley and Bodenhausen, 1990) of 400 and 256 μs duration, respectively. Mixing of ^{13}C magnetization is achieved with an MLEV-16 expansion of G3 pulses. The mixing time is set to 12 ms, corresponding to three MLEV-16 cycles. Band-selective decoupling of the aliphatic carbons during t_3 is achieved using an MLEV-16 expansion of a 614 μs G3 pulse cascade. Delay durations are: τ = 2.3 ms; κ = 5.4 ms, T_N = 22.0 ms, T_C = 27.0 ms; δ = 1.0 ms; ε = 1.7 ms. Phase cycling is as follows: ϕ_1 = y,−y; ϕ_2 = 4(x),4(−x); ϕ_3 = 2(x),2(−x); ϕ_4 = 8(y),8(−y); ϕ_5 = 16(y),16(−y); acq. = x,2(−x),x,−x,2(x),−x (coupled version); acq. = x,2(−x),x,2(−x,2(x),−x),x,2(−x),x,−x,2(x),−x,2(x,2(−x),x),−x,2(x),−x (decoupled version). Quadrature in the t_2 domain is obtained using the States–TPPI method (Marion et al., 1989) by incrementing phase ϕ_3, while in the t_1 domain the phase ϕ_2 is changed in the TPPI manner (Marion and Wüthrich, 1983).

The HNCCH-TOCSY pulse sequence was applied to flavodoxin, a 16.3 kDa protein from the sulfate-reducing organism *Desulfovibrio vulgaris* (Mayhew and Tollin, 1992; Knauf et al., 1993). In Fig. 2, two F2,F3 planes from a ^{13}CO-decoupled HNCCH-TOCSY spectrum of flavodoxin in its oxidized state are shown. The assignments derived from intra- and interresidual connectivities

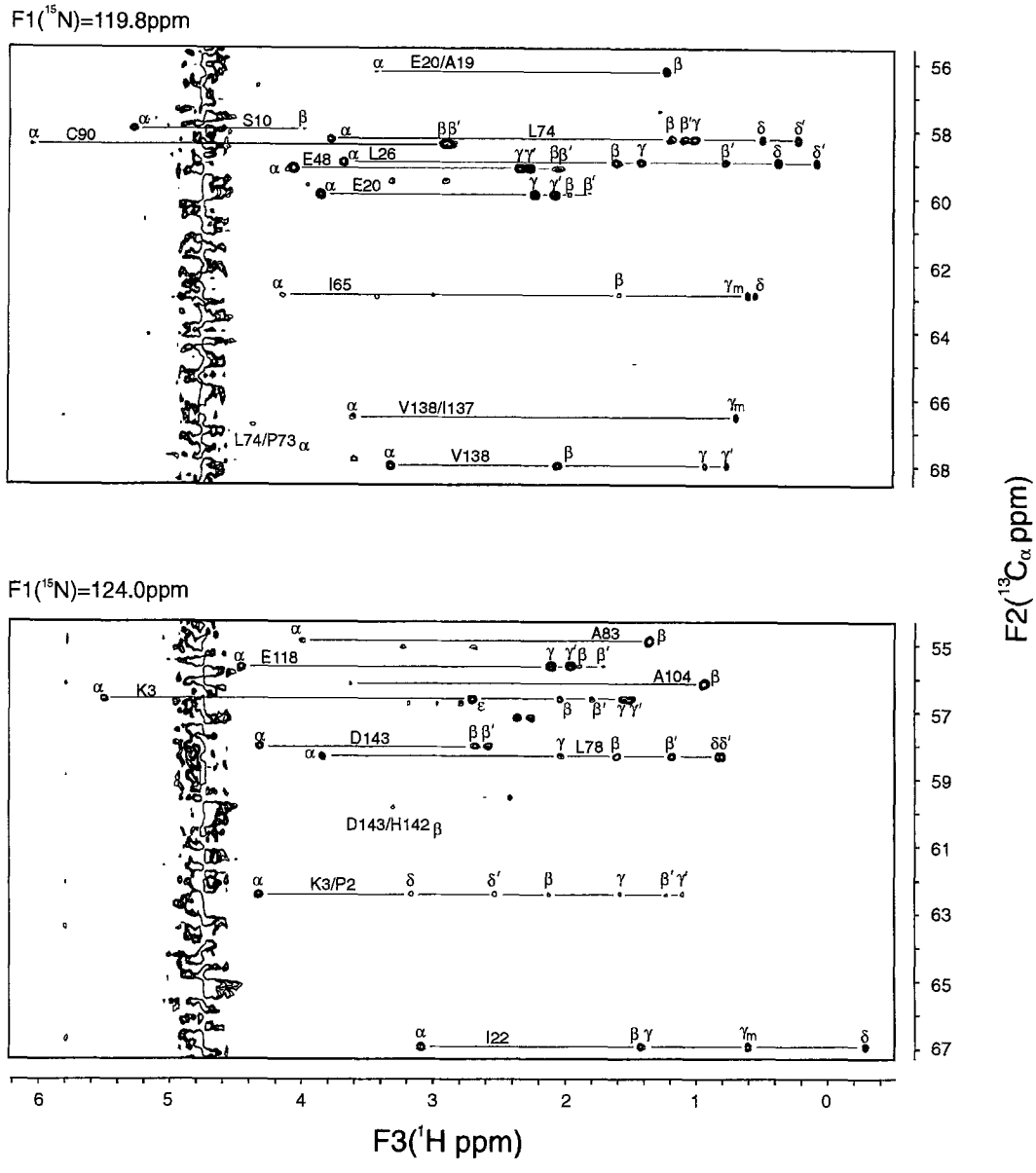

Fig. 2. Representative F2,F3 slices from a decoupled HNCCH-TOCSY spectrum, obtained from a 2.2 mM sample of uniformly $^{13}C/^{15}N$-labeled oxidized *Desulfovibrio vulgaris* flavodoxin in 95% H_2O/5% D_2O, pH 7.0, 27 °C. The experiment was performed on a Bruker AMX 600 spectrometer, equipped with a three-channel interface and a triple resonance $^1H/^{13}C/^{15}N$ probe. Cross peaks belonging to the same spin system are connected by horizontal lines, with assignments indicated for the side-chain proton resonances. Nonlabeled signals arise from correlations exhibiting maximum intensity in neighbouring planes. Sequential cross peaks for the pairs E48/F47 and C90/A89 (top), and I22/T21, A83/G82, A104/G103 and E118/A117 (bottom) are located outside the plotted regions. The 3D spectrum results from a (64 real) × (80 complex) × (1024 real) data matrix, recorded in 4.3 days with 32 scans per increment and acquisition times of 17.3, 26.2 and 68.6 ms in the t1, t2 and t3 dimensions, respectively. Spectral widths were 1825 Hz in the F1(^{15}N) domain and 3012 Hz in the F2($^{13}C^{\alpha}$) domain. Time-domain data were extendend by linear prediction and zero-filling, prior to Fourier transformation. The real part of the 3D spectrum consists of 64 × 256 × 1024 real data points.

are indicated. In many cases it is possible to identify the complete aliphatic side-chain proton spin system, and information about neighbouring spin systems can be obtained in the same plane if a sequential set of signals exists. For flavodoxin this is true for 82% of the amino acid residues, whereas intraresidual cross peaks can be observed for all non-proline residues. Thus, data from HNCA spectra can be supplemented with aliphatic proton chemical shifts, leading to the amino acid identification. In contrast to the HC(C)NH-TOCSY experiment proposed by Lyons and Montelione (1993), the intraresidual and sequential cross peaks can be discriminated due to their different $^{13}C^{\alpha}$ chemical shifts. The slices shown in Fig. 2 clearly demonstrate the improved resolution obtainable in the C^{α} domain when compared to the HNCA experiment.

Minor modifications of the HNCCH-TOCSY scheme, i.e., the application of band-selective ^{13}C pulses and the omission of a pulse on ^{13}CO during the C^{α} constant-time evolution period, permit the measurement of the $^{3}J_{H\beta CO}$ coupling from H^{β},C^{α} cross peaks in an E.COSY-type fashion (Griesinger et al., 1985,1986). Several other methods have been reported for the determination of this coupling constant. A qualitative analysis can be achieved by the HN(CO)HB experiment through evaluation of cross-peak intensities (Grzesiek et al., 1992). For the quantitative measurement of coupling constants to an unprotonated heteronucleus, methods can be employed for the deconvolution of the cross-peak shape in HMBC spectra (Bax and Summers, 1986; Bermel et al., 1989; Titman et al., 1989; Richardson et al., 1991). This approach is limited by large ^{1}H linewidths and resonance overlap, typically observed for biomolecules. To overcome these limitations, 3D NMR experiments can be devised based on a magnetization transfer through large one-bond couplings, yielding superior transfer efficiency and signal dispersion. In the soft-HCCH-COSY experiment (Eggenberger et al., 1992b), the carbonyl ^{13}C remains undisturbed during the whole sequence, leading to an E.COSY-type pattern using the $^{1}J_{C\alpha CO}$ coupling to separate the multiplet components in the C^{α} dimension. As the constant-time evolution (T_C) in C,C-COSY-type experiments must be set to $(2n-1)/2(^{1}J_{C\alpha C\beta})$, where n is often chosen equal to 1 for reasons of sensitivity, extensive linear prediction of the time-domain data is required to resolve the passive splitting. When a bandwidth-limited C,C-TOCSY transfer is used in this kind of experiment, sufficient resolution can be obtained by setting the constant-time C^{α} evolution period equal to $1/^{1}J_{C\alpha C\beta}$. This was demonstrated for a new band-selective mixing scheme applied to ^{13}C-labeled alanine (Schmidt et al., 1993). In the HNCCH-TOCSY experiment presented in this communication, the constant-time period T_C is used for the evolution of C^{α} chemical shifts, as well as for the refocusing of the $^{1}J_{NC\alpha}$ and $^{2}J_{NC\alpha}$ couplings. By allowing these processes to occur simultaneously, it is possible to introduce a ^{15}N evolution period without major sensitivity losses owing to a transverse relaxation of the C^{α} spin. Moreover, using a second heteronucleus in the third frequency domain reduces resonance overlap considerably. Figure 3 shows representative cross peaks from a ^{13}CO-coupled HNCCH-TOCSY experiment recorded on *D. vulgaris* flavodoxin. The ^{13}C,^{13}C TOCSY mixing time in this experiment was set to 12 ms to optimize magnetization transfer from C^{α} to C^{β}.

The information obtained from this experiment, together with a determination of the $^{3}J_{H\alpha H\beta}$ and $^{3}J_{H\beta N}$ coupling constants, allows an unambiguous stereospecific assignment of the β-methylene proton resonances and the determination of the χ^{1} dihedral angle.

In conclusion, the 3D HNCCH-TOCSY experiment in the ^{13}CO-decoupled mode, like the HNCA experiment, provides sequential and intraresidual connectivities between the amide nitrogen and the α-carbons, as well as intraresidual connectivities between side-chain protons. The

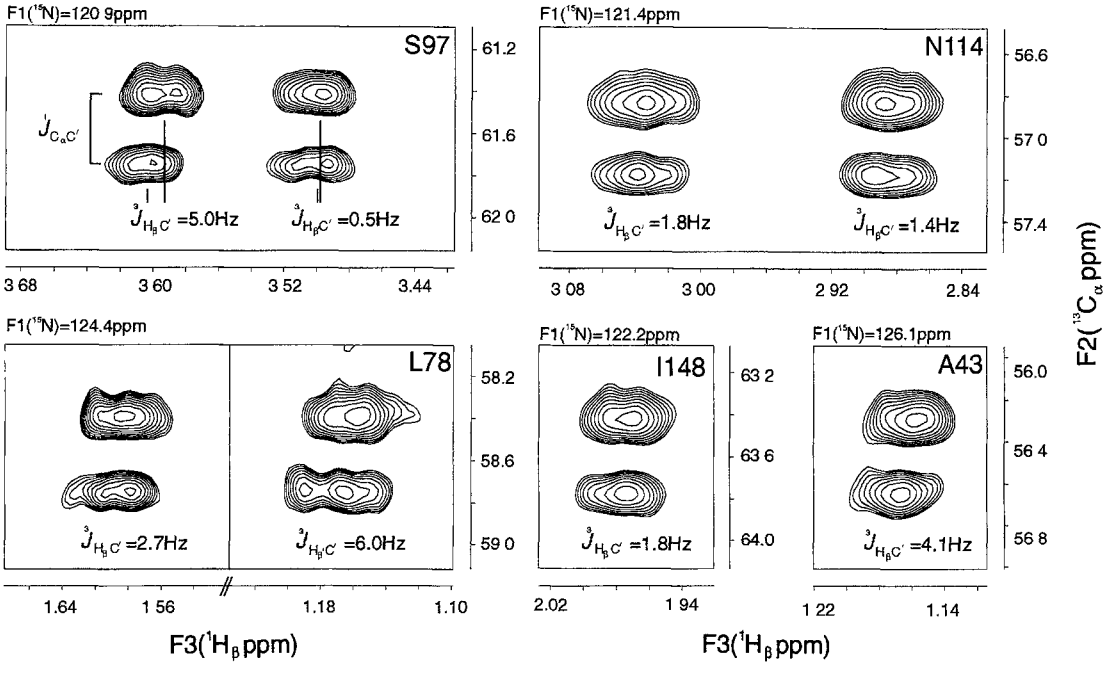

Fig. 3. Expansions of F2,F3 slices from a coupled HNCCH-TOCSY spectrum of oxidized *D. vulgaris* flavodoxin recorded at 27 °C on a Bruker AMX 600 spectrometer. The H^{β},C^{α} cross peaks exhibit an E.COSY-type multiplet pattern, with a large splitting along F2 due to the $^{1}J_{C^{\alpha}CO}$ coupling and a small displacement along F3 due to the $^{3}J_{H^{\beta}CO}$ coupling. Assignments, as well as the measured ^{3}J coupling constants are indicated. The spectrum was recorded with 32 scans per increment and spectral widths of 1678 Hz in F1, 3906 Hz in F2 and 7463 Hz in F3. The 3D data set consisted of (40 real) × (104 complex) × (2048 real) points, corresponding to acquisition times of 11.6, 26.5 and 137.2 ms (t1,t2,t3) and a total measuring time of 4.4 days. Linear prediction was employed in the t1 and t2 domains, extending the raw data to 64 (real) and 152 (complex) points. A squared sine-bell function shifted by 70° was applied to all dimensions prior to zero-filling. The absorptive part of the final 3D spectrum consisted of 64 × 512 × 2048 data points. The coupling constants were extracted from appropriate F3 traces, as described by Schwalbe et al. (1993). The error of determination is assumed to be at least equal to the digital resolution of the selected traces after zero-filling to 16K data points (0.46 Hz/point).

information obtained from this experiment is thus complementary to that from a 3D C(CO)NH experiment (Grzesiek et al., 1993a). The ^{13}CO-coupled 3D HNCCH-TOCSY was derived from the former experiment by minor modifications of the pulse scheme. We have shown that with this experiment the $^{3}J_{H^{\beta}CO}$ coupling constant is accessible from E.COSY-type multiplets if band-selective pulses are chosen for excitation and mixing of ^{13}C magnetization.

ACKNOWLEDGEMENTS

A grant from the Deutsche Forschungsgemeinschaft (Ru145/11-1) is gratefully acknowledged. The preparation of the protein sample was carried out in the Department of Biochemistry, University College, Dublin, Ireland. We thank Prof. S. Mayhew and M. Knauf for support in labeling and isolating the flavodoxin of *D. vulgaris*.

REFERENCES

Bax, A. and Summers, M.F. (1986) *J. Am. Chem. Soc.*, **108**, 2093–2094.
Bax, A., Clore, G.M. and Gronenborn, A.M. (1990) *J. Magn. Reson.*, **88**, 425–431.
Bermel, W., Wagner, K. and Griesinger, C. (1989) *J. Magn. Reson.*, **83**, 223–232.
Boucher, W., Laue, E.D., Campbell-Burk, S. and Domaille, P.J. (1992) *J. Am. Chem. Soc.*, **114**, 2262–2264.
Burum, D.P. and Ernst, R.R. (1980) *J. Magn. Reson.*, **39**, 163–168.
Clowes, R.T., Boucher, W., Hardman, C.H., Domaille, P.J. and Laue, E.D. (1993) *J. Biomol. NMR*, **3**, 349–354.
Clubb, R.T., Thanabal, V. and Wagner, G. (1992) *J. Biomol. NMR*, **2**, 203–210.
Eggenberger, U., Schmidt, U.P., Sattler, M., Glaser, S.J. and Griesinger, C. (1992a) *J Magn. Reson.*, **100**, 604–610.
Eggenberger, U., Karimi-Nejad, Y., Thüring, H., Rüterjans, H. and Griesinger, C. (1992b) *J. Biomol. NMR*, **2**, 583–590.
Emsley, L. and Bodenhausen, G. (1990) *Chem. Phys. Lett.*, **165**, 469–476.
Fesik, S.W., Eaton, H.L., Olejniczak, E.T. and Zuiderweg, E.R.P. (1990) *J. Am. Chem. Soc.*, **112**, 886–888.
Griesinger, C., Sørensen, O.W. and Ernst, R.R. (1985) *J. Am. Chem. Soc.*, **107**, 6394–6396.
Griesinger, C., Sørensen, O.W. and Ernst, R.R. (1986) *J. Chem. Phys.*, **85**, 6837–6852.
Grzesiek, S., Ikura, M., Clore, G.M., Gronenborn, A.M. and Bax, A. (1992) *J. Magn. Reson.*, **96**, 215–221.
Grzesiek, S., Anglister, J. and Bax, A. (1993a) *J. Magn. Reson. Ser. B*, **101**, 114–119.
Grzesiek, S., Anglister, J., Ren, H. and Bax, A. (1993b) *J. Am. Chem. Soc.*, **115**, 4369–4370.
Kay, L.E., Ikura, M., Tschudin, R. and Bax, A. (1990) *J. Magn. Reson.*, **89**, 496–514.
Kay, L.E., Ikura, M. and Bax, A. (1991) *J. Magn. Reson.*, **91**, 84–92.
Knauf, M., Löhr, F., Curley, G.P., O'Farrell, P., Mayhew, S.G., Müller, F. and Rüterjans, H. (1993) *Eur. J. Biochem.*, **213**, 167–184.
Logan, F.M., Olejniczak, E.T., Xu, R.X. and Fesik, S.W. (1993) *J. Biomol. NMR*, **3**, 225–231.
Lyons, B.A. and Montelione, G.T. (1993) *J. Magn Reson. Ser. B*, **101**, 206–209.
Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989) *J. Magn. Reson.*, **85**, 393–399.
Marion, D. and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.*, **113**, 967–974.
Mayhew, S.G. and Tollin, G. (1992) In *Chemistry and Biochemistry of Flavoenzymes*, Vol. 3 (Ed, Müller, F.) CRC Press, Boca Raton, FL, pp. 389–426.
Montelione, G.T., Lyons, B.A., Emerson, S.D. and Tashiro, M. (1992) *J Am. Chem. Soc.*, **114**, 10974–10975.
Montelione, G.T. and Wagner, G. (1990) *J. Magn. Reson.*, **87**, 183–188.
Morris, G.A. and Freeman, R. (1979) *J. Am. Chem. Soc.*, **101**, 760–762.
Richardson, J.M., Titman, J.J., Keeler, J. and Neuhaus, D. (1991) *J. Magn. Reson.*, **93**, 533–553.
Schmidt, P., Schwalbe, H., Glaser, S.J. and Griesinger, C. (1993) *J. Magn. Reson. Ser. B*, **101**, 328–332.
Schwalbe, H., Rexroth, A., Eggenberger, U., Geppert, T. and Griesinger, C. (1993) *J. Am. Chem. Soc.*, **115**, 7878–7879.
Shaka, A.J., Barker, P.B. and Freeman, R. (1985) *J. Magn. Reson.*, **64**, 547–552.
Shaka, A.J., Lee, C.J. and Pines, A. (1988) *J Magn. Reson.*, **77**, 274–293.
Titman, J.J., Neuhaus, D. and Keeler, J. (1989) *J. Magn. Reson.*, **85**, 111–131.