JOURNAL OF MAGNETIC RESONANCE **124,** 255–258 (1997)
ARTICLE NO. MN961050

H_2NCO–E.COSY, a Simple Method for the Stereospecific Assignment of Side-Chain Amide Protons in Proteins

FRANK LÖHR AND HEINZ RÜTERJANS*

Institut für Biophysikalische Chemie, Johann Wolfgang Goethe-Universität, Biozentrum N230, Marie Curie-Straße 9, D-60439 Frankfurt am Main, Germany

Received October 28, 1996

It has long been recognized that stereospecific resonance assignments significantly improve the quality of protein structures determined by NMR (*1–8*). Both the number and the precision of distance constraints involving diastereotopic methylene protons and isopropyl methyl protons is enhanced when this kind of information is available. Without taking recourse to long-range dipolar interactions in known tertiary structures, these assignments can be obtained from a combination of vicinal coupling constants and intraresidual NOEs. The most reliable strategy makes use of the incorporation of stereoselectively deuterated amino acids (*9–14*) or biosynthetically directed fractional ^{13}C labeling (*15–18*). A similar approach is, of course, not applicable for the individual assignment of exchanging nuclei such as side-chain amide protons of asparagine and glutamine residues. Although methods have been introduced for the selective detection of NH_2 groups (*19, 20*), no distinction can be made for signals of protons in the trans ($H^{\delta 21}$ and $H^{\epsilon 21}$ for Asn and Gln, respectively) and cis ($H^{\delta 22}$ and $H^{\epsilon 22}$) configuration with respect to the carbonyl oxygen.

In random conformations, a chemical-shift difference of approximately 0.7 ppm was reported for the two geminal protons (*21*). The resonance frequencies for the trans protons of primary amide groups in small organic compounds (*22*) and Asn and Gln derivatives (*23*), as well as in Asn-containing peptides (*24*), were correspondingly found to appear to low field from those of the cis protons, but in proteins this order can be reversed by the influence of aromatic rings in the vicinity of the NH_2 groups or by hydrogen-bond formation. In some cases, the individual assignment can be obtained on the basis of intraresidual (i.e., H^{β}–H^{δ}, H^{γ}–H^{ϵ}) NOE contacts (*25–27*) as suggested by Montelione *et al.* (*24*). The intensity ratio between the intraresidual NOE cross peaks of the trans and cis protons, however, depends on the χ^2 and χ^3 torsion angles in asparagine and glutamine side chains, respectively, and it may not always be obvious which resonance belongs to which position. Especially in larger proteins, the analysis of intraresidual NOE intensities can be rendered difficult by spin diffusion and spectral overlap. In this Communication, a sensitive two-dimensional method is described which exploits vicinal ^1H, ^{13}C couplings in order to differentiate between the two positions.

The pulse scheme depicted in Fig. 1 is based on the HNCO experiment (*28*), employing an HSQC- rather than an HMQC-type transfer between ^{15}N and ^{13}CO spins. Since ^{15}N antiphase magnetization with respect to the attached protons is not refocused, correlations of the side-chain amide groups can be detected with the same intensity as those of the polypeptide backbone. In uniformly ^{13}C-labeled proteins, the evolution of the one-bond coupling between the carbonyls and the directly bound aliphatic carbons leads to a splitting of the cross peaks in the F_1 dimension. Pulses on ^{13}CO are applied band-selectively in order not to disturb the spin states of the aliphatic carbons. As a result, E.COSY-like (*29*) multiplets are obtained from which $^3J(H^{\delta}, C^{\beta})$ and $^3J(H^{\epsilon}, C^{\gamma})$ coupling constants can be determined for asparagine and glutamine side chains, respectively. Solvent suppression is achieved with the WATERGATE (*30*) technique, while the saturation of the water magnetization is avoided by keeping it along the z axis during the application of B_0-gradient pulses with the use of water-selective 90° pulses (*31, 32*). The final ^{15}N pulse is displaced from the center of the reverse INEPT delay (*33*), allowing the use of longer and thus more selective pulses on the H_2O resonance, while fixing the duration for refocusing of proton antiphase magnetization at $(2^1J_{NH})^{-1} = 2\tau'$.

The $H_2(N)CO$–E.COSY sequence was applied to the individual assignment of side-chain amide resonances of $^{13}C/^{15}N$-labeled flavodoxin from the sulfate-reducing organism *Desulfovibrio vulgaris.* The protein, which consists of 147 amino acids and a noncovalently bound FMN molecule, was dissolved in 10 m*M* potassium phosphate

* To whom correspondence should be addressed.

1090-7807/97 $25.00

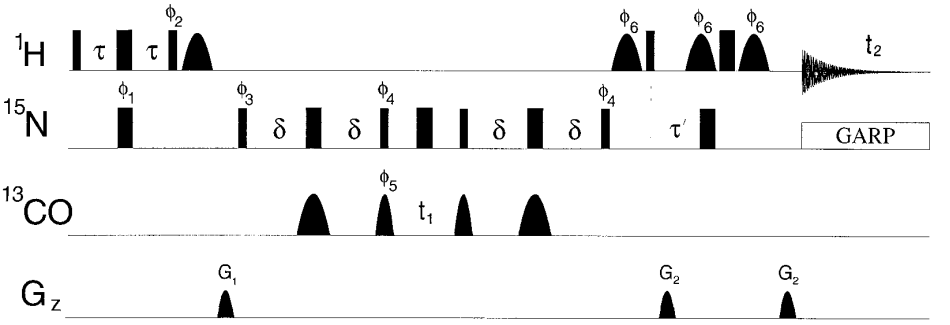

FIG. 1. Pulse scheme for the $H_2(N)CO$–E.COSY experiment. Narrow and wide bars denote hard 90° and 180° rectangular pulses, respectively. Band-selective excitation and inversion of the carbonyl spins is achieved by using G4 and G3 Gaussian cascades (*35*) with a duration of 0.5 ms, applied at an offset of 175.7 ppm. The second G4 pulse has a time-reversed amplitude profile. GARP-1 modulation (*36*) with an RF field strength of 0.75 kHz is used for ^{15}N decoupling during acquisition. The proton carrier is placed at the position of the H_2O resonance throughout the sequence. Gaussian-shaped pulses with a duration of 2.5 ms are employed for selective excitation of the water signal. The durations and strengths of the sine-bell shaped *z*-gradient pulses are G_1, 1 ms, 10 G cm^{-1}; and G_2, 0.8 ms, 35 G cm^{-1}. Phase cycling is as follows: $\phi_1 = x, -x$; $\phi_2 = y, -y$; $\phi_3 = 2(x), 2(-x)$, $\phi_4 = y$; $\phi_5 = 4(x), 4(-x)$; $\phi_6 = -x$; rec. $= x, 2(-x), x, -x, 2(x), -x$. The phase of the selective proton pulses is adjusted to compensate for a small phase difference with respect to hard pulses caused by the change of power levels. Nonlabeled pulses are applied along the *x* axis. Quadrature in the ^{13}CO dimension is achieved by TPPI (*37*) applied to ϕ_5. Delay durations are $\tau = 2.3$ ms, $\delta = 12$ ms, $\tau' = 2.7$ ms.

buffer, pH 7, containing 5% D_2O (sample concentration: 1.4 m*M*). A trace amount of NaN_3 was added to prevent bacterial growth. The experiment was performed at a temperature of 27°C on a Bruker DMX 600 spectrometer, equipped with an actively shielded *z*-gradient triple-resonance probe and a gradient amplifier unit. Spectral widths were 3125 Hz in F_1 and 9058 Hz in F_2. For each of 352 t_1 values, 2048 complex data points were recorded, corresponding to acquisition times of 56.3 ms in t_1 and 226.1 ms in t_2. Accumulation of 16 scans per increment resulted in a measuring time of 1.75 h. Time-domain data were multiplied by squared sine-bell functions shifted by 70° and 90° in t_1 and t_2, respectively. Zero-filling was applied prior to Fourier transformation to yield a digital resolution of 6.1 Hz/point in the ^{13}CO domain and 0.55 Hz/point in the ^1H_N domain.

Due to the partial-double-bond character of the CO–NH_2 linkage, the two amide protons adopt a syn- and an antiperiplanar conformation with respect to the carbon atom directly bound to the carbonyl group. Consequently, one large and one smaller $^3J(H^{\delta(\epsilon)}, C^{\beta(\gamma)})$ coupling constant can be expected for asparagine (glutamine) residues. For acetamide, a value of 7.1 Hz for the coupling between the methyl carbon and one of the amide protons and a vanishingly small coupling for the second proton was reported (*34*). With the use of the $H_2(N)CO$–E.COSY experiment, $^3J(H^{\delta(\epsilon)22}, C^{\beta(\gamma)})$ coupling constants ranging from 5.5 to 7.2 Hz and $^3J(H^{\delta(\epsilon)21}, C^{\beta(\gamma)})$ coupling constants between 0.5 and 1.0 Hz were determined for the two asparagine and the three glutamine residues of flavodoxin. Thus, stereospecific assignments could be obtained in each case. Three examples are shown in Fig. 2. Due to the large difference of the vicinal coupling constants within one pair of amide protons, a distinction of the two positions can be achieved merely by visual inspection, rendering a quantitative evaluation of the E.COSY multiplet patterns unnecessary. As can be seen in Fig. 2 for Asn 114, this holds true even in the presence of partial overlap. Note that in the case of Gln 121 the order of chemical shifts is reversed when compared to the random-coil values.

Since the $H_2(N)CO$–E.COSY spectrum recorded with the pulse sequence in Fig. 1 also contains signals of the backbone amide groups, additional information about $^3J(H^{N'}_{(i)}, C^{\alpha}_{(i-1)})$ couplings, which depend on the ω torsion angle, is obtained. Therefore, the method is suitable for the detection of cis peptide bonds for all but proline residues. As judged from the small $^3J(H^{N'}_{(i)}, C^{\alpha}_{(i-1)})$ coupling constants, all peptide bonds in flavodoxin were found in the trans configuration, with the exception of a few cases where overlap obscured the E.COSY patterns.

In conclusion, a pulse sequence has been proposed which provides unambiguous stereospecific resonance assignments for side-chain amide protons in doubly labeled polypeptides in a relatively short measuring time. The distinction relies on the different magnitudes of the scalar couplings involving the vicinal carbon spins. In larger proteins, where spectral overlap may prevent the evaluation of the E.COSY-type multiplets, the resolution can easily be improved by exploiting the dispersion of ^{15}N chemical shifts in a third dimension. Alternatively, the experiment could be modified to an NH_2-filtered version (*20*) in order to eliminate cross peaks from backbone H(N)CO-correlations.

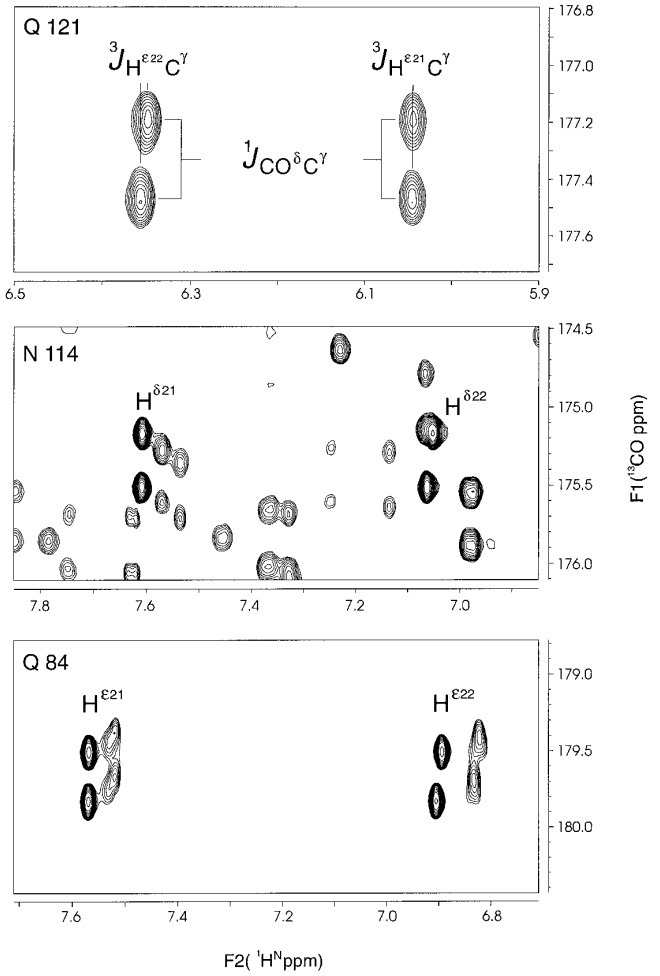

FIG. 2. Expanded region from the 2D H_2(N)CO-E.COSY spectrum of oxidized *Desulfovibrio vulgaris* flavodoxin, illustrating the stereospecific assignment of the side-chain amide protons of residues Gln 121, Asn 114, and Gln 84. The multiplets exhibit a large splitting along F_1 due to CO^γ, C^β or CO^δ, C^γ one-bond couplings. The displacement of the two components along F_2 serves to differentiate the $^1H^{\delta 21}$ or $^1H^{\epsilon 21}$ from $^1H^{\delta 22}$ or $^1H^{\epsilon 22}$ resonances.

ACKNOWLEDGMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Ru 145/11-2). We thank Dr. István Pelczer (Princeton University) for pointing out to us the potential usefulness of the vicinal H, C couplings involving side-chain amide protons and for giving insight into his work on this subject. The aid of Professor Stephen G. Mayhew (University College Dublin) in labeling and isolating the *D. vulgaris* flavodoxin is gratefully acknowledged.

REFERENCES

1. E. R. P. Zuiderweg, R. Boelens, and R. Kaptein, *Biopolymers* **24,** 601–611 (1985).
2. S. G. Hyberts, W. Märki, and G. Wagner, *Eur. J. Biochem.* **164,** 625–635 (1987).
3. G. Wagner, W. Braun, T. F. Havel, T. Schaumann, N. Go, and K. Wüthrich, *J. Mol. Biol.* **196,** 611–639 (1987).
4. P. L. Weber, R. Morrison, and D. Hare, *J. Mol. Biol.* **204,** 483–487 (1988).
5. A. Arseniev, P. Schultze, E. Wörgötter, W. Braun, G. Wagner, M. Vašák, J. H. R. Kägi, and K. Wüthrich, *J. Mol. Biol.* **210,** 637–657 (1988).
6. P. C. Driscoll, A. M. Gronenborn, and G. M. Clore, *FEBS Lett.* **243,** 223–233 (1989).
7. P. Güntert, W. Braun, M. Billeter, and K. Wüthrich, *J. Am. Chem. Soc.* **111,** 3997–4004 (1989).
8. R. X. Xu, E. T. Olejniczak, and S. W. Fesik, *FEBS Lett.* **305,** 137–143 (1992).
9. M. Kainosho and K. Ajisaka, *J. Am. Chem. Soc.* **97,** 5630–5631 (1975).
10. J. Kobayashi, U. Nagai, T. Higashijima, and T. Miyazawa, *Biochim. Biophys. Acta* **577,** 195–206 (1979).
11. A. J. Fischman, D. H. Live, H. R. Wyssbrod, W. C. Agosta, and D. Cowburn, *J. Am. Chem. Soc.* **102,** 2533–2539 (1980).
12. D. M. LeMaster, *FEBS Lett.* **223,** 191–196 (1987).
13. G. Ostler, A. Soteriou, C. M. Moody, J. A. Khan, B. Birdsall, M. D. Carr, D. W. Young, and J. Feeney, *FEBS Lett.* **318,** 177–180 (1993).
14. R. W. Curley Jr., M. J. Panigot, A. P. Hansen, and S. W. Fesik, *J. Biomol. NMR* **4,** 335–340 (1994).
15. H. Senn, B. Werner, B. A. Messerle, C. Weber, R. Traber, and K. Wüthrich, *FEBS Lett.* **249,** 113–118 (1989).
16. D. Neri, T. Szyperski, G. Otting, H. Senn, and K. Wüthrich, *Biochemistry* **28,** 7510–7516 (1989).
17. D. Neri, G. Otting, and K. Wüthrich, *Tetrahedron* **46,** 3287–3296 (1990).
18. T. Szyperski, D. Neri, B. Leiting, G. Otting, and K. Wüthrich, *J. Biomol. NMR* **2,** 323–334 (1992).
19. J. M. Schmidt and H. Rüterjans, *J. Am. Chem. Soc.* **112,** 1279–1280 (1990).
20. B. T. Farmer II and R. A. Venters, *J. Biomol. NMR* **7,** 59–71 (1996).
21. A. Bundi and K. Wüthrich, *Biopolymers* **18,** 285–297 (1979).
22. A. G. Redfield and S. Waelder, *J. Am. Chem. Soc.* **101,** 6151–6162.
23. N. R. Krishna, K. P. Sarathy, D.-H. Huang, R. L. Stephens, J. D. Glickson, C. W. Smith, and R. Walter, *J. Am. Chem. Soc.* **104,** 5051–5053.
24. G. T. Montelione, E. Arnold, Y. C. Meinwald, E. R. Stimson, J. B. Denton, S.-G. Huang, J. Clardy, and H. A. Scheraga, *J. Am. Chem. Soc.* **106,** 7946–7958 (1984).
25. A. D. Kline, W. Braun, and K. Wüthrich, *J. Mol. Biol.* **204,** 675–724 (1988).
26. G. T. Montelione, K. Wüthrich, A. W. Burgess, E. C. Nice, G. Wagner, K. D. Gibson, and H. A. Scheraga, *Biochemistry* **31,** 236–249 (1992).
27. F. J. Moy, Y.-C. Li, P. Rauenbuehler, M. E. Winkler, H. A. Scheraga, and G. T. Montelione, *Biochemistry* **32,** 7334–7353.
28. L. E. Kay, M. Ikura, R. Tschudin, and A. Bax, *J. Magn. Reson.* **89,** 496–514 (1990).
29. C. Griesinger, O. W. Sørensen, and R. R. Ernst, *J. Am. Chem. Soc.* **107,** 6394–6396 (1985).

30. M. Piotto, V. Saudek, and V. Sklenář, *J. Biomol. NMR* **2,** 661–665 (1992).

31. S. Grzesiek and A. Bax, *J. Am. Chem. Soc.* **115,** 12593–12594 (1993).

32. L. E. Kay, G. Y. Xu, and T. Yamazaki, *J. Magn. Reson. A* **109,** 129–133 (1994).

33. J.-P-Simorre, G. R. Zimmermann, A. Pardi, B. T. Farmer II, and L. Mueller, *J. Biomol. NMR* **6,** 427–432 (1995).

34. D. E. Dorman and F. A. Bovey, *J. Org. Chem.* **38,** 1719–1722 (1973).

35. L. Emsley and G. Bodenhausen, *Chem. Phys. Lett.* **165,** 469–476 (1990).

36. A. J. Shaka, P. B. Barker, and R. Freeman, *J. Magn. Reson.* **64,** 547–552 (1985).

37. D. Marion and K. Wüthrich, *Biochem. Biophys. Res. Commun.* **113,** 967–974 (1983).