Overcoming the Ambiguity Problem Encountered in the Analysis of Nuclear Overhauser Magnetic Resonance Spectra of Symmetric Dimer Proteins

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NMR[1] spectroscopy has developed into a generally accepted method for the determination of the three-dimensional solution structure of proteins with molecular weights up to ≈20 kDa. In many instances, proteins are only biologically active when they are combined into symmetrical dimers. The structure determination of such dimers by means of NMR is obstructed because it is intrinsically impossible to distinguish between their intra- and intersubunit NOEs in regular NOESY spectra. Yet, NMR structures of some dimeric proteins have been obtained.[2-4] In most of these cases, a comparison of the NMR data with the coordinates of the available crystal structure of the same[3] or an homologous protein[4] made it possible to sort out the ambiguities.

It is, however, possible to resolve the ambiguity problem with NMR by using asymmetric isotope labeling.[5,6] So far, this has been achieved by using heterodimers composed of different selectively deuterated proteins. The method necessitates at least two protein preparations, each containing a different deuteration level of their corresponding amino acids. The intersubunit NOEs can be identified in an indirect manner through comparison of the NOESY spectra of the heterodimers with that of each selectively deuterated homodimer.

However, a more practical approach for overcoming the ambiguity problem with NMR is feasible. After formation of heterodimers between uniformly ^{13}C-labeled and unlabeled (^{12}C) protein, intersubunit NOEs can be identified directly using an appropriate NMR method. In this paper, we demonstrate this approach for the dimeric gene V protein (GVP) encoded by the filamentous bacteriophage M13.

Intra- and intersubunit NOEs in symmetric dimer proteins can be distinguished in a very convenient manner provided that their subunits can be made magnetically nonequivalent. By mixing ^{13}C-labeled and unlabeled dimer proteins under the appropriate conditions, a mixture can be generated of doubly unlabeled homodimers, doubly ^{13}C-labeled homodimers, and heterodimers consisting of both a ^{13}C-labeled and an unlabeled monomer. The pulse sequence used to study the asymmetrically labeled symmetric dimer complex is depicted in Figure 1. It is a variant of the 2D heteronuclear double-half-filter NOESY experiment described previously.[7] Two data sets are recorded, one in which both ^{13}C 180° editing pulses are applied and one in which both ^{13}C 180° editing pulses are effectively omitted. Addition of the two recordings results in a spectrum in which NOE cross-peaks are observed among ^{13}C-bound protons as well as among ^{12}C-bound protons. Subtraction of these recordings yields a spectrum in which only NOE cross-peaks are observed between on one hand ^{13}C-bound protons and on the other hand ^{12}C-bound protons, thus yielding a subspectrum containing only the desired intersubunit NOEs.

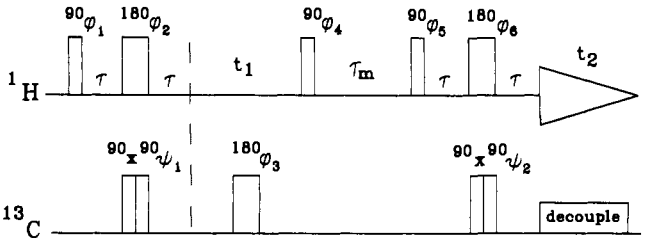

Figure 1. Pulse scheme of the 2D $^{13}C(\omega1,\omega2)$ double-half-filter NOESY experiment to study symmetric dimers. The delay τ is chosen as $\tau = 1/[2^1J(^{13}C,^1H)]$. Low-power GARP decoupling[14] is used during the detection period on both the aromatic and aliphatic ^{13}C resonances utilizing an external CW amplifier driven by two synthesizers and interfaced to a GARP box (Tschudin Associates). The phases $\phi1$–$\phi6$ are independently alternated between x and $-x$, which results in a phase cycle of 64 steps. The receiver phase is inverted whenever the phase of a 90° ^1H pulse is alternated. Data are acquired with the TPPI[15] method for obtaining quadrature detection in the $t1$ dimension by alternating the phase of the first ^1H pulse. The basic phase cycle is repeated two times with the phase $\Psi_1 = \Psi_2 = x$ and $\Psi_1 = \Psi_2 = -x$, respectively. The two data sets are recorded in an interleaved manner and stored separately.

Compared to the protocol in earlier experiments[7] in which the contents of the above mentioned subspectra are further separated in $^{13}C(\omega1)$–$^{13}C(\omega2)$ doubly filtered and $^{13}C(\omega1)$–$^{13}C(\omega2)$ doubly selected subspectra, as well as $^{13}C(\omega1)$-filtered/$^{13}C(\omega2)$-selected and $^{13}C(\omega1)$-selected/$^{13}C(\omega2)$-filtered subspectra, respectively, the present approach offers a reduction of the measuring time by a factor of 2. The original protocol would, in principle, be applicable to symmetric dimers, but contrary to the case of asymmetric complexes,[8] the four subspectra mentioned above would be pairwise identical to one another. Therefore, the two-step phase cycle (with $\Psi_1 = \Psi_2 = x$ or $-x$) is the most efficient way to extract all the relevant information of the asymmetrically labeled dimers.

The proposed experiment was applied to mutant Tyr41 → His (Y41H) of the single-stranded DNA binding protein, M13 gene V protein (GVP), which in solution occurs as a symmetric dimer.[9,10] In order to be able to perform the experiment, conditions at which GVP ^{13}C–^{12}C heterodimers are formed were sorted out. The procedure was tested with a mixture of GVP mutant Y41H and M13 wild-type GVP of which hybrids could be distinguished from the respective homodimers on a monoS cation-exchange FPLC-column. It turned out that formaton of heterodimers can be achieved under mild conditions (overnight incubation at 20 °C) by mixing ^{13}C-labeled and unlabeled GVP Y41H at a protein concentration of ≈1 μM, at which half of the protein molecules occur as monomers ($K_d \approx 10^{-6}$ M for the dimer complex). Subsequent concentration of the diluted protein solution generates a mixture of three species: the originally prepared homodimers and the wanted, novel, magnetic heterodimers.

Figure 2 shows parts of the two subspectra which were obtained after addition and subtraction of the recorded data sets. Subspectrum B displays various intermonomeric NOEs, some of which are indicated. It is noted that NOEs originating from the homodimers, either doubly unlabeled or doubly ^{13}C-labeled

(1) Abbreviations and symbols used: NMR, nuclear magnetic resonance; NOE, Nuclear Overhauser Enhancement; NOESY, two-dimensional NOE spectroscopy; FPLC, fast protein liquid chromatography; TPPI, time-proportional phase incremention; GARP, globally optimized alternating-phase rectangular pulses; GVP, gene V protein; Y41H tyrosine substituted by histidine.

(2) Clore, G. M.; Apella, E.; Yamada, M.; Matsushima, H.; Gronenborn, A. M. *Biochemistry* **1989**, *29*, 1689–1701.

(3) Kay, L. E.; Forman-Kay, J.; McCubbin, W.; Kay, C. *Biochemistry* **1991**, *30*, 4323–4333.

(4) Breg, J. N.; van Opheusden, J. H. J.; Burgering, M. J. M.; Boelens, R.; Kaptein, R. *Nature* **1990**, *346*, 586–589.

(5) Weiss, M. *J. Magn. Reson.* **1990**, *86*, 626–632.

(6) Arrowsmith, C. H.; Pachter, R.; Altman, R. B.; Iyer, S. B.; Jardetzky, O. *Biochemistry* **1990**, *29*, 6332–6341.

(7) Otting, G.; Wüthrich, K. *J. Magn. Reson.* **1989**, *85*, 586–594.

(8) Wider, G.; Weber, C.; Traber, R.; Widmer, H.; Wüthrich, K. *J. Am. Chem. Soc.* **1990**, *112*, 9015–9016.

(9) Folkers, P. J. M.; Stassen, A. P. M.; van Duynhoven, J. P. M.; Harmsen, B. J. M.; Konings, R. N. H.; Hilbers, C. W. *Eur. J. Biochem.* **1991**, *200*, 139–148.

(10) Folkers, P. J. M.; van Duynhoven, J. P. M.; Jonker, A. J.; Harmsen, B. J. M.; Konings, R. N. H.; Hilbers, C. W. *Eur. J. Biochem.* **1991**, *202*, 349–360.

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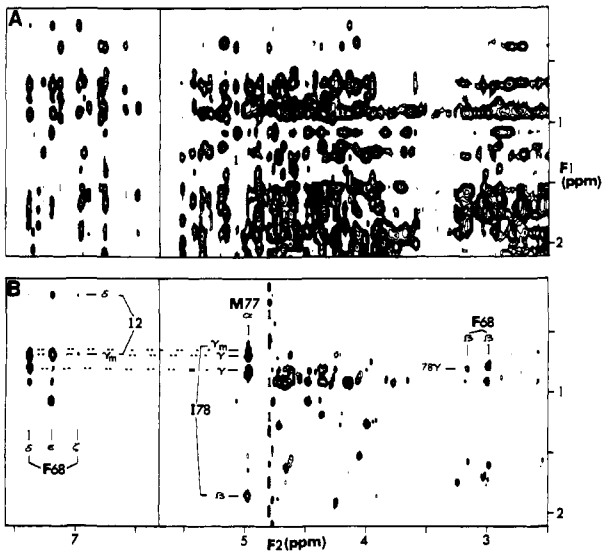

Figure 2. Two spectral regions from the 1H NOESY(D_2O) spectrum of the mutant Y41H gene V protein dimer complex (19.4 kDa) recorded at 600 MHz using the double-half-filter experiment of Figure 1 (τ = 3.5 ms, τ_{mix} = 120 ms, $t1$ and $t2$ aquisition times 24 and 123 ms, total recording time 56 h, concentration of GVP heterodimers present ≈ 0.7 mM, pD = 5.2, t = 27 °C). (A) Subspectrum obtained after addition of the two recorded data sets. In the spectrum, the cross-peaks between ^{12}C-bound protons in both $\omega1$ and $\omega2$ as well as cross-peaks between ^{13}C-bound protons in both dimensions are superimposed. (B) Subspectrum obtained after subtraction of the two recorded data sets. The spectrum contains cross-peaks between ^{12}C-bound protons in $\omega1$ and ^{13}C-bound protons in $\omega2$ plus cross-peaks between ^{13}C-bound protons in $\omega1$ and ^{12}C-bound protons in $\omega2$. Some of the intermolecular identified NOEs are indicated.

material, are canceled in this spectrum. Possible artifacts which may occur due to insufficiently suppressed ^{13}C-bound proton signals pose no particular problem as these are largely attenuated due to efficient proton relaxation in such large uniformly ^{13}C-labeled systems. For smaller molecules, however, it may be useful to employ the recently described double filters which are tunable to different $^1J_{H,X}$ values[11] but exhibit longer delay times.

Inspection of spectrum B reveals that of all aromatic residues of GVP, only NOE cross-peaks stemming from Phe68 emerge in the spectrum. The resonances of this aromatic residue particularly connect to those of Ile78. Thus, the Phe68 spin system of one monomer is in close proximity of that of Ile78 of the symmetry-related monomer. Interestingly, the regular NOESY spectrum also displays a $d_{\alpha\alpha}$ contact between residues 68 and 78 (shown in ref 10) which does not appear in spectrum B, demonstrating that this is an intrasubunit connectivity. Both residues are therefore also in close proximity of one another within each monomer. The existence of both intra- and intersubunit NOEs between the Phe68 and Ile78 spin systems demonstrates that these two amino acids are close to the dyad axis of the GVP dimer. This observation does not conform to the X-ray data obtained for wild-type GVP[12] which indicated that residues Phe68 and Met77 are positioned in the center of the molecule.

In conclusion, it is shown that intersubunit NOEs can be unambiguously identified in symmetric dimer proteins using heterodimers consisting of both a ^{13}C-labeled and an unlabeled monomer. The NMR method is applicable to dimeric proteins with molecular weights of at least 20 kDa. The NMR experiment, of which the dimensionality can easily be extended,[13] provides data which are very valuable for the determination of the three-dimensional structure of symmetric dimer proteins.

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(11) Gemmecker, G.; Olejniczak, E. T.; Fesik, S. W. *J. Magn. Reson.* **1992**, *96*, 199–204.
(12) Brayer, G. D.; Mc Pherson, A. *J. Mol. Biol.* **1983**, *169*, 565–596.
(13) Wider, G.; Weber, C.; Wüthrich, K. *J. Am. Chem. Soc.* **1991**, *113*, 4676–4678.
(14) Shaka, A. J.; Barker, P. B.; Freeman, R. *J. Magn. Reson.* **1985**, *64*, 547–552.
(15) Marion, D.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967–974.