Journal of Biomolecular NMR **30:** 423–429, 2004.

Side-chain assignments of methyl-containing residues in a uniformly ^{13}C-labeled hemoglobin in the carbonmonoxy form

Yu Zheng[a], Janel L. Giovannelli[b], Nancy T. Ho[b], Chien Ho[b] & Daiwen Yang[a,*]
[a]*Department of Biological Sciences and Department of Chemistry, 14 Science Drive 4, Singapore 117543;*
[b]*Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA 15213, U.S.A.*

Received 20 July 2004; Accepted 28 September 2004

Key words: hemoglobin, methyl group, protein structure, side-chain assignment

Abstract

Sequence-specific assignment of the methyl groups in large proteins can be obtained from an MQ-(H)CC$_m$H$_m$-TOCSY experiment on uniformly ^{13}C-labeled proteins without deuteration (Yang et al., 2004). Here the procedure is further demonstrated on a uniformly ^{13}C-labeled α-chain or β-chain of human normal adult hemoglobin (65 kDa) in the carbonmonoxy form. In addition, a strategy is presented for assigning protons of methyl-containing residues of uniformly ^{13}C-labeled large proteins, on the basis of prior methyl assignments based on MQ-(H)CCH-TOCSY and H(C)C$_m$H$_m$-TOCSY experiments. Assignment of about 80% of the side-chain resonances of methyl-containing residues of carbonmonoxyhemoglobin has been obtained.

Introduction

Hemoglobin (Hb) is the iron-containing oxygen-transport metalloprotein inside the red cells of mammals and other animals. For reviews on Hb, see Dickerson and Geis (1983), Lukin and Ho (2004), and Barrick et al. (2004). Human normal adult hemoglobin (Hb A) is a tetramer with a molecular weight of about 65 kDa, consisting of two α-chains and two β-chains. Each α-chain contains 141 amino acids that coil into seven α-helical regions and each β-chain contains 146 amino acids that form eight α-helical regions. The physiological function of Hb is to transport oxygen from the lungs to the tissues. Hb binds O_2, CO, and NO reversibly and cooperatively, i.e., the binding of the first ligand enhances the binding of subsequent ligands. The ligand-binding affinity of Hb A is regulated by pH (the Bohr effect) and allosteric effectors, such as 2,3-bisphosphoglycerate (2,3-BPG). The four subunits pack together through hydrophobic and hydrogen-bond interactions to form a quaternary structure. The arrangement of the subunits of Hb depends on the ligation state of the protein, and is related to the physiological function of Hb. X-ray crystallographic studies of deoxy- and liganded Hb A have found that there are at least three different quaternary structural forms (T, R, and R2), in which the structures of each subunit are very similar; however, there are differences in the arrangement between $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces (Silva et al., 1992; Mueser et al., 2000). T symbolizes the deoxy quaternary structure of crystalline deoxy-Hb A, and R and R2 represent the quaternary structures of crystalline liganded carbonmonoxy-Hb A (HbCO A) in high salt and low salt conditions, respectively. More recently, by using NMR residual dipolar measurements on ^{15}N-labeled recombinant HbCO A (rHbCO A), we have found that the solution structure of

*To whom correspondence should be addressed. E-mail: dbsydw@nus.edu.sg

HbCO A is distinctly different from the previously determined R and R2 crystal structures and that the solution structure of HbCO A is a dynamic intermediate between R and R2 structures (Lukin et al., 2003). In spite of extensive studies on the Hb molecule, the detailed structure-function relationship in Hb is not fully understood and many aspects remain controversial. This may arise from a lack of information on the structure and dynamics of Hb under physiological conditions.

As a first step toward a detailed investigation of the structure and dynamics of Hb A in solution, side-chain resonances need to be assigned. For small and medium-sized proteins, side-chain assignments are made using a set of TOCSY-based experiments (e.g., H(CCO)NH-TOCSY, C(CO)NH-TOCSY and HCCH-TOCSY) (Fesik et al., 1990; Montelione et al., 1992; Gardner et al., 1996, 1998). For proteins larger than 30 kDa, this set of experiments may not work because of a severe overlap of ^{1}H-^{13}C correlations in the HCCH spectrum and an inefficiency of magnetization transfer from the side-chain spins to the backbone amide protons in the ^{1}HN-observed spectra. Even for sequential assignments of a large protein, it is necessary to enhance the efficiency of magnetization transfer through an increase of the transverse relaxation time (T_2) by deuteration (LeMaster, 1990; Yamazaki et al., 1994). Deuteration can also facilitate the assignments of side-chain ^{13}C resonances in large multimeric proteins using a 2D ^{13}C-observed CCC-TOCSY experiment (Eletsky et al., 2003). However, the assignments of ^{1}H resonances still require a partial or complete protonation of the side chains on the basis of ^{13}C- and ^{15}N-resolved ^{1}H-^{1}H NOESY spectra (Eletsky et al., 2003). In this case, the assignments are hindered by the poor dispersion of (^{1}H,^{13}C) spin pairs for CH and CH_2 groups. Using specific isotopic labeling techniques, methyl groups in very large proteins can be assigned using COSY-based experiments recently developed by the Kay group (Tugarinov and Kay, 2003a, b). Very recently, we have developed an MQ-(H)CC$_m$H$_m$-TOCSY (where MQ represents multiple-quantum and the subscript m denotes methyl groups that are selected for observation) experiment for the assignment of methyl groups in uniformly ^{13}C-labeled proteins, one which has been successfully applied to a 42 kDa AcpS trimer (Yang et al., 2004). In this study, we have applied our newly developed pulse sequence to assign the methyl resonances in the α- and β-chains of rHbCO A, using only uniformly ^{13}C-labeled protein. Using the same procedure, other side-chain ^{13}C resonances in methyl-containing residues can also be assigned. A non-constant-time MQ-(H)CCH-TOCSY experiment is proposed to assign some Leu methyl groups that display very weak signals in the MQ-(H)CC$_m$H$_m$-TOCSY experiment due to the strong coupling effect. In addition, we propose a strategy to assign side-chain ^{1}H resonances of methyl-containing residues with H(C)C$_m$H$_m$-TOCSY and MQ-(H)CCH-TOCSY experiments.

Materials and methods

NMR spectroscopy

Chain-specifically ^{13}C-labeled rHbCO A samples were prepared as described previously (Simplaceanu et al., 2000). NMR experiments were performed on samples of ~1.0 mM protein (in the tetramer), 20 mM sodium phosphate, pH 7.0, and 100% D_2O at 34 °C. All spectra were recorded on a Bruker Avance 500 MHz NMR spectrometer equipped with pulse gradient units and an actively shielded cryoprobe. Methyl resonances were assigned using the MQ-(H)CC$_m$H$_m$-TOCSY experiments as described previously (Yang et al., 2004). Several Leu methyl groups were assigned with 3D MQ-(H)CCH-TOCSY experiments using the pulse sequences illustrated in Figure 1a. Protons at methylene and methine positions in methyl-containing residues were obtained with 3D H(C)C$_m$H$_m$-TOCSY experiments using the pulse scheme shown in Figure 1b. The MQ-(H)CCH-TOCSY experiments were also used to confirm the assignments of non-methyl protons. The 3D MQ-(H)CC$_m$H$_m$-TOCSY data comprising $64 \times 70 \times 512$ complex points with spectral widths of 8000, 2516, and 8000 Hz in F1, F2, and F3 dimensions (corresponding to acquisition times of 7.7, 27.4, and 64 ms, respectively) were collected with 4 scans and an inter-scan delay of 0.95 s for each FID, giving rise to a net experimental time of 24.5 h. The 3D H(C)C$_m$H$_m$-TOCSY data set consisting of $32 \times 70 \times 512$ complex points with spectral widths of 3500, 2516, and 8000 Hz in F1, F2,

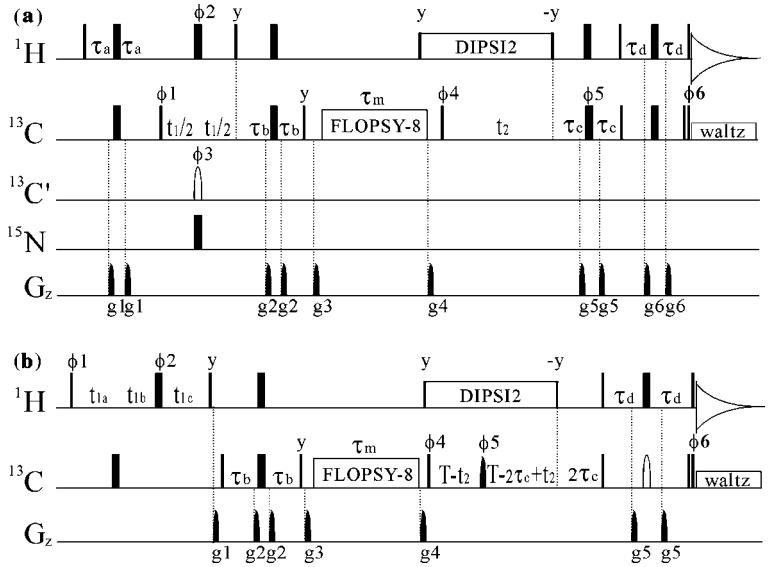

Figure 1. Pulse sequences for the MQ-(H)CCH-TOCSY (a) and H(C)C$_m$H$_m$-TOCSY (b) experiments. All narrow (wide) rectangular pulses have flip angles of 90° (180°). The ^{1}H carrier is at 4.7 ppm while the ^{13}C carrier is centered at 41 ppm. For scheme B, the ^{13}C carrier is jumped to 17 ppm immediately prior to the g4 gradient pulse. All ^{1}H pulses are applied with a 23 kHz field; ^{1}H DIPSI2-decoupling elements make use of a 6.25 kHz field. All ^{13}C rectangular pulses employ a 16.8 kHz field and the ^{13}C shaped pulses have REBURP profiles. The 180° shaped ^{13}C pulse (filled) has a duration of 400 μs and is phase-modulated by 24 ppm while the second one (empty) has a duration of 1.5 ms. The ^{13}C spin-lock field strength for FLOPSY is 7 kHz. A decoupling power of 1.25 kHz is used during acquisition. The 180° pulse on C′ has a SEDUCE profile with a duration of 250 μs (center of excitation 176 ppm). The pulse on ^{15}N is omitted for ^{13}C-labeled samples. The delays used are: $\tau_a = 1.4$ ms; $\tau_b = 1.1$ ms; $\tau_c = 1$ ms for scheme A and 0.75 ms for scheme B; $\tau_d = 1.6$ ms; $\tau_m = 17$ ms; $T = 14$ ms; $t_{1a} = 1.4$ ms + t_1; $t_{1b} = t_1 - t_1'$; $t_{1c} = 1.4$ ms − t_1'; $t_1' = 1.4$ ms/(ni-1) where ni is the total complex points in the t_1 dimension. The phase cyclings employed are: $\phi_1 = 4(x), 4(-x)$; $\phi_2 = x, y, -x, -y$; $\phi_3 = 2(x), 2(-x)$; $\phi_4 = y$; $\phi_5 = 2(x), 2(y), 2(-x), 2(-y)$; $\phi_6 = 4(x), 4(-x)$; rec = x, -x, -x, x, -x, x, x, -x. The duration and strengths of the sine-shaped gradients are: g1 = (0.5 ms, 20 G/cm); g2 = (0.3 ms, 25 G/cm); g3 = (1 ms, 25 G/cm); g4 = (1 ms, 20 G/cm); g5 = (0.5 ms, 20 G/cm); g6 = (1 ms, 10 G/cm). Quadrature detection in F1 and F2 is achieved by State-TPPI of ϕ_1 and ϕ_4, respectively.

and F3 dimensions was acquired using 8 scans and a relaxation delay of 0.95 s for each increment, resulting in a total experimental time of 24.5 h. The MQ-(H)CCH-TOCSY data comprising $64 \times 30 \times 512$ complex points with spectral widths of 8000, 3774, and 8000 Hz in F1, F2, and F3 dimensions were collected with an inter-scan delay of 0.95 s and 8 scans per increment, resulting in a total experimental time of 20.5 h. All data sets were apodized with a sine weighting function shifted by 72° in the direct proton dimension. The t_1 and t_2 domains were doubled by linear prediction prior to the application of a cosine-squared window function. After zero filling and Fourier transformation, all the final data sets comprised $256 \times 256 \times 1024$ points along the F1, F2, and F3 dimensions, respectively. Processing of the spectra was carried out using NMRPipe and analyzed with NMRView.

MQ-(H)CCH-TOCSY experiment

Figure 1a shows the pulse sequence for establishing (^{13}C, ^{13}C, ^{1}H) correlations through a TOCSY scheme, which is similar to the original HCCH-TOCSY (Bax et al., 1990; Fesik et al., 1990) and MQ-(H)CC$_m$H$_m$-TOCSY (Yang et al., 2004) experiments. A non-constant-time acquisition mode in the t_2 period is used to replace the constant-time (CT) t_2 period in the MQ-(H)CC$_m$H$_m$-TOCSY experiment. In addition, a non-selective ^{13}C 180° pulse is used in the last INEPT period to allow the detection of all aliphatic ^{1}H spins. The non-CT MQ-(H)CCH-TOCSY experiment proposed here has lower resolution in the F2 dimension than the CT MQ-(H)CC$_m$H$_m$-TOCSY experiment due to J_{CC} couplings and a short acquisition time in this dimension. However, the former is significantly more sensitive, especially for Leu residues with strong scalar coupling interactions, because ^{13}C

magnetization cannot be refocused completely for strongly coupled spin systems during the CT period.

H(C)C_mH_m-TOCSY experiment

In principle, one can assign the resonances of most side-chain ^{1}H and ^{13}C spins using the 3D HCCH-TOCSY or MQ-(H)CCH-TOCSY experiments. In practice, it is very difficult to do so because of poor resolutions in both indirect dimensions and poor dispersion of most ^{1}H-^{13}C correlations for large proteins. To assign side-chain protons in methyl-containing residues, an H(C)C_mH_m-TOCSY experiment is proposed here (Figure 1b), which is similar to the HCCH-TOCSY experiment (Bax et al., 1990; Uhrin et al., 2000). This procedure correlates methyl ^{1}H with all aliphatic ^{1}H spins in the same residue through a ^{13}C TOCSY mixing scheme. The relatively good dispersion of methyl ^{1}H-^{13}C correlations and the slow decay of the methyl spins in uniformly ^{13}C-labeled protein make ^{1}H assignment possible (Liu et al., 2003). The pulse sequence is similar to that of the MQ-(H)C_mH_m-TOCSY experiment, but ^{1}H chemical shifts instead of ^{13}C shifts are recorded in the t_1 period in a single-quantum (SQ) mode. Although multiple-quantum (MQ) coherences (H_xC_y) have longer relaxation times than SQ coherences (H_x), the MQ mode involves additional signal loss from ^{13}C-^{13}C couplings in the t_1 period. The SQ mode is more sensitive than the MQ mode without selective ^{13}C decoupling in the t_1 period and is thus used in this experiment.

Correction of ^{13}C chemical shifts

Assignment of CH_3 groups relies on prior assignments of $^{13}C_\alpha$ and $^{13}C_\beta$ chemical shifts. For rHbCO A, sequential assignments were obtained from perdeuterated samples (Lukin et al., 2004). Due to ^{2}H isotope effects, ^{13}C chemical shifts observed in a perdeuterated sample are smaller than those observed in a protonated sample. To make ^{13}C chemical shifts consistent for both samples, ^{2}H isotope effects are corrected according to the following equation (Venters et al., 1996):

$$\delta C(H) = \delta C(D) - ({}^1\Delta C(D)*d_{1b} + {}^2\Delta C(D)*d_{2b} + {}^3\Delta C(D)*d_{3b}), \quad (1)$$

where δC(H) and δC(D) are the chemical shifts of a ^{13}C spin in protonated and perdeuterated samples, respectively; $^n\Delta$C(D) represents the n-bond isotope effect per deuteron; and d_{nb} is the number of deuterons n bonds removed from the ^{13}C nucleus. Due to the negligible magnitude of $^4\Delta$C(D) in saturated alkanes, Equation 1 has been restricted to isotope shifts over three bonds or fewer. The three $^n\Delta$C(D) constants used are: $^1\Delta C(D) = -0.29$; $^2\Delta C(D) = -0.13$; $^3\Delta C(D) = -0.07$ (Venters et al., 1996).

Results and discussion

Methyl assignments

Figure 2 shows representative F1–F3 slices from the MQ-(H)CC_mH_m-TOCSY spectrum of ^{13}C-labeled α-chain of rHbCO A. Note that there is no Ile in HbCO A (Dickerson and Geis, 1983). For a given set of signals on one slice, firstly, the amino acid type of the residue contributing to these signals is determined from the spectral pattern, since each type of amino acid displays a characteristic pattern, as shown in Figure 2. Secondly, the chemical shifts of ($^{13}C_\alpha$, $^{13}C_\beta$) are

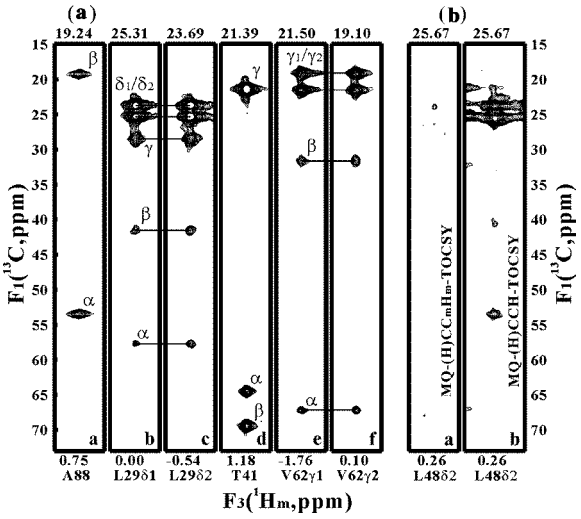

Figure 2. Representative F1–F3 slices from the MQ-(H)CC_mH_m-TOCSY (a) and MQ-(H)CCH-TOCSY (b) spectra of ^{13}C-labeled α-chain of rHbCO A. Each slice is labeled with the identity of the methyl-containing residue, and the F2 (^{13}C) frequency in ppm is indicated at the top of each slice.

measured from the same slice and compared with the corrected ($^{13}C_\alpha$, $^{13}C_\beta$) shifts from prior sequential assignments. Lastly, if the ($^{13}C_\alpha$, $^{13}C_\beta$) shifts uniquely match the shifts of residue N, the set of signals is assigned to residue N. For example, slice *b* with 5 peaks at positions ($^{13}C_i$, $^{13}C_m$, $^{1}H_m$) in Figure 2a, where i = α, β, γ, δ_1 and δ_2, corresponds obviously to a Leu. The ($^{13}C_\alpha$, $^{13}C_\beta$) shifts uniquely match the shifts of Leu29, within a threshold of 0.3 ppm. All aliphatic carbons of Leu29 are subsequently obtained from this slice and the chemical shift of $^{1}H_{\delta 1}$ for Leu29 is also measured from the F3 dimension. With this procedure, we assigned 72 out of 92 methyl groups (excluding Met) for the α-chain and 76 out of 94 for the β-chain. Due to the degeneracy of ($^{13}C_\alpha$, $^{13}C_\beta$) shifts within a threshold of 0.15 ppm, 14 and 16 methyl groups in the α-chain and β-chain, respectively, cannot be uniquely assigned. Respectively, 6 and 2 Leu methyls in the α-chain and β-chain cannot be assigned because of the absence of $^{13}C_\alpha$ and $^{13}C_\beta$ peaks in the MQ-(H)CC$_m$H$_m$-TOCSY spectra. Slice *a* in Figure 2b shows one such example. It occurs when the chemical shift difference between $^{13}C_{\delta 1}$/$^{13}C_{\delta 2}$ and $^{13}C_\gamma$ is smaller than or close to the $^{1}J_{CC}$ value due to the strong coupling effect. To assign these methyl groups, a non-CT MQ-(H)CCH-TOCSY experiment can be used.

Slice *b* in Figure 2b taken from the non-CT MQ-(H)CCH-TOCSY spectrum shows the correlations between $^{13}C_\delta$ and all aliphatic carbons in the same Leu residue as shown in slice *a*. Using the ($^{13}C_\alpha$, $^{13}C_\beta$) chemical shifts obtained from this slice, the signals were assigned to Leu48δ_2. The 6 and 2 Leu methyl groups in the α- and β-chains respectively, which give rise to very weak signals in the MQ-(H)CC$_m$H$_m$-TOCSY experiment, were assigned with the MQ-(H)CCH experiments. Figure 3 shows the ^{1}H-^{13}C HSQC spectra of α- and β-chains with assignments.

Assignment of side-chain protons in methyl-containing residues

Figure 4 shows a number of slices taken from the H(C)C$_m$H$_m$-TOCSY experiment. Each methyl ^{1}H correlates with all aliphatic protons in the same residue. However, this experiment does not provide direct ^{1}H-^{13}C correlations of pairs of covalently bonded atoms. Assignment of ^{1}H

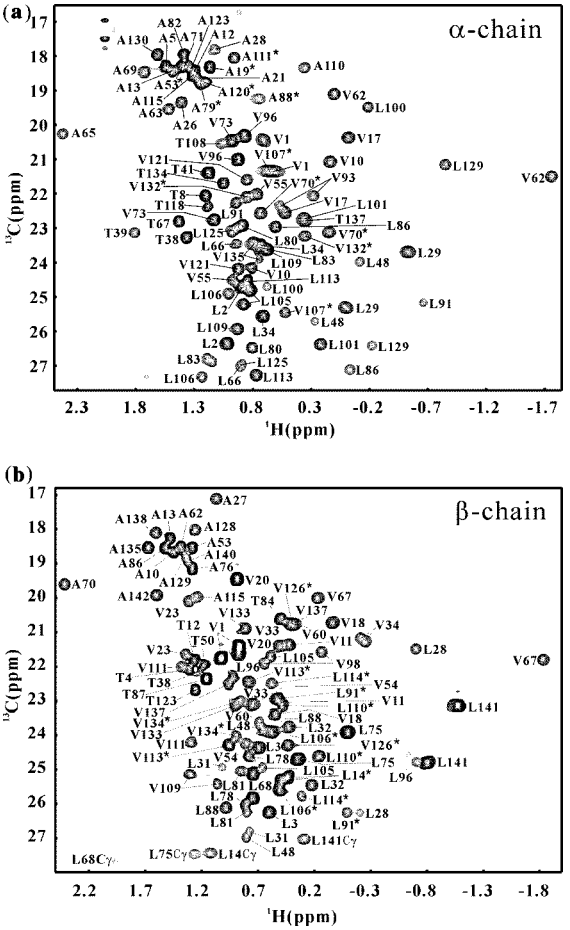

Figure 3. CT ^{13}C-^{1}H HSQC of the ^{13}C-labeled α-chain and β-chain of rHbCO A. Cross-peaks are labeled with their assignments. Ambiguous assignments are indicated with asterisks (*).

chemical shifts is based on empirical ^{1}H chemical shift ranges of different protons and the assignment of methyl groups. In many cases, the assignment is straightforward. However, discrimination of $^{1}H_\alpha$ and $^{1}H_\beta$ spins for Thr and $^{1}H_\beta$ and $^{1}H_\gamma$ spins for Leu also needs spectral information from the MQ-(H)CCH-TOCSY experiment which provides direct ^{1}H-^{13}C correlations of pairs of covalently bonded atoms. For example, the assignment of T134H$_\alpha$ of the α-chain can be confirmed from the correlations [$^{13}C_i$, $^{13}C_\alpha$, $^{1}H_\alpha$], where i = α, β, and γ, positioned at T134C$_\alpha$ in the MQ-(H)CCH-TOCSY spectrum (slice *c*, Figure 5). On the basis of slices *b* and *c* in Figure 5, one can also assign T134H$_\alpha$ and it seems that the H(C)C$_m$H$_m$-TOCSY experiment is not necessary in this particular case. According to slices *e* and *f* in Figure 5, however, one cannot

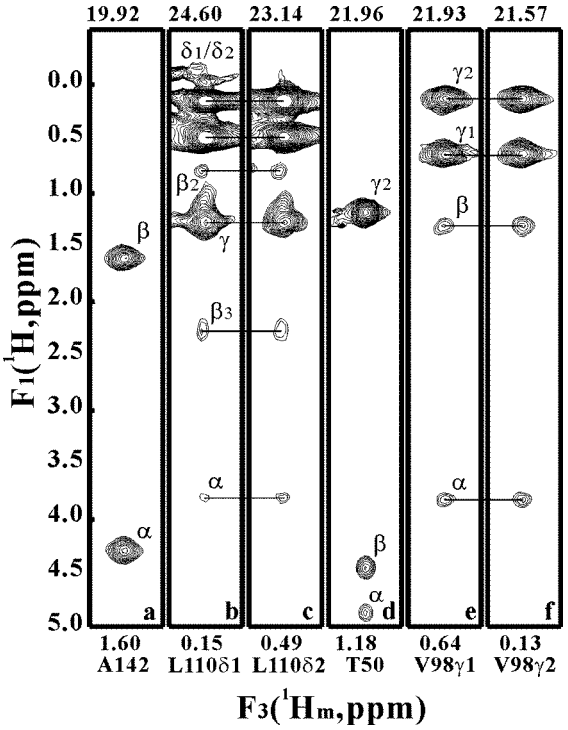

Figure 4. Representative F1–F3 slices from the H(C)C_mH_m-TOCSY spectrum of ^{13}C-labeled β-chain of rHbCOA. Each F1(^{1}H)–F3(^1H_m) slice is labeled with the identity of the methyl-containing residue, and the F2 ($^{13}C_m$) frequency in ppm is indicated at the top of each slice.

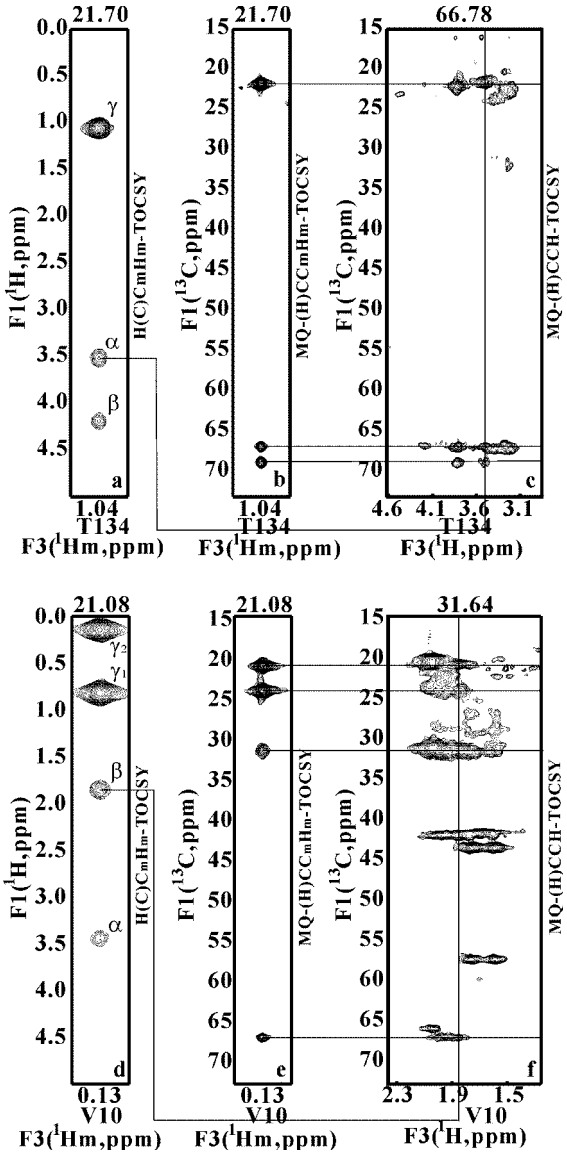

Figure 5. F1-F3 slices taken from the spectra of H(C)C_mH_m-TOCSY, MQ-(H)CC_mH_m-TOCSY and MQ-(H)CCH-TOCSY experiments. The corresponding experiment for each slice is labeled beside the slice. The chemical shift of the F2 dimension is labeled on the top of each slice.

determine the chemical shift of V10H_β because two or more protons in the range of 1.85–2.1 ppm correlate with sets of ^{13}C resonances with very similar chemical shifts to ($^{13}C_\alpha$, $^{13}C_\beta$, $^{13}C_\gamma$) of V10 as shown on slice *f*. On the other hand, V10H_β can be easily assigned from slice *d*. Actually, most ^{1}H resonances cannot be assigned without H(C)C_mH_m-TOCSY data because the MQ-(H)CCH-TOCSY spectrum had poor resolutions in both indirect dimensions and poor dispersion of most ^{1}H-^{13}C correlations. We have unambiguously assigned 90 out of 137 non-methyl protons in methyl-containing residues of the α-chain and 89 out of 137 non-methyl protons of the β-chain. Nearly all unassigned protons were $^1H_\beta$ in Leu residues because the experimental sensitivity for CH_2 groups in which the two protons are magnetically different and two sets of correlations exist is lower than that for CH and CH_3 groups. The results are summarized in Table 1.

Conclusion

In summary, methyl resonances of large proteins, e.g., rHbCO A, can be assigned using uniformly ^{13}C-labeled proteins with the MQ-(H)CC_mH_m-TOCSY experiment. In addition, most side-chain ^{1}H and ^{13}C resonances of methyl-containing residues can be assigned with the H(C)C_mH_m-TOCSY

Table 1. Summary of assignment of non-methyl protons in methyl-containing residues of both α- and β-chains of rHbCOA

rHbCOA α-chain (number of assigned/total protons)			
Ala	H_α 13/21		
Thr	H_α 9/9	H_β 9/9	
Val	H_α 10/13	H_β 10/13	
Leu	H_α 15/18	H_β 8/36	H_γ 16/18
rHbCOA β-chain (number of assigned /total protons)			
Ala	H_α 14/15		
Thr	H_α 6/7	H_β 6/7	
Val	H_α 15/18	H_β 15/18	
Leu	H_α 13/18	H_β 8/36	H_γ 12/18

and MQ-(H)CCH-TOCSY experiments. The non-CT MQ-(H)CCH-TOCSY experiment is also complementary to the MQ-(H)CC_mH_m-TOCSY experiment for the assignment of methyl groups in Leu residues. The experiments proposed here, together with the MQ-(H)CC_mH_m-TOCSY procedure, facilitate the study by NMR of structure, dynamics and structure-activity-relationship of large proteins, especially multimeric proteins, without the use of expensive specific isotope labeling.

Acknowledgements

This research is supported by a Young Investigator Award from the Biomedical Research Council (BMRC) of the Agency for Science, Technology and Research (A*STAR) of Singapore to D.W.Y. This research is also supported by research grants from the U.S. National Institutes of Health (R01HL-24525 and P01HL-71064) to C.H.

References

Barrick, D., Lukin, J.A., Simplaceanu, V. and Ho, C. (2004) *Meth. Enzymol,* **378**, 28–54.

Bax, A., Clore, G.M. and Gronenborn, A.M. (1990) *J. Magn. Reson.,* **88**, 425–431.

Dickerson, R.E. and Geis, I. (1983) *Hemoglobin: Structure, Function, Evolution, and Pathology*, Benjamin/Cummings, Menlo Park, CA.

Eletsky, A., Moreira, O., Kovacs, H. and Pervushin, K. (2003) *J. Biomol. NMR,* **26**, 167–179.

Fesik, S.W., Eaton, H.L., Olejniczak, E.T., Zuiderweg, E.R.P., McIntosh, L.P. and Dahlquist, F.W. (1990) *J. Am. Chem. Soc.,* **112**, 886.

Gardner, K.H., Konrat, R. and Kay, L.E. (1996) *J. Biomol. NMR,* **8**, 351–356.

Gardner, K.H., Zhang, X.C., Gehring, K. and Kay, L.E. (1998) *J. Am. Chem. Soc.,* **120**, 11738–11748.

LeMaster, D.M. (1990) *Quart. Rev. Biophys.,* **23**, 133–174.

Liu, W., Zheng, Y., Cistola, D.P. and Yang, D.W. (2003) *J. Biomol. NMR,* **27**, 351–364.

Lukin, J.A. and Ho, C. (2004) *Chem. Rev.,* **104**, 1219–1230.

Lukin, J.A., Kontaxis, G., Simplaceanu, V., Yuan, Y., Bax, A. and Ho, C. (2003) *Proc. Natl. Acad. Sci. USA,* **91**, 11113–11117.

Lukin, J.A., Kontaxis, G., Simplaceanu, V., Yuan, Y., Bax, A. and Ho, C. (2004) *J. Biomol. NMR,* **28**, 203–204.

Montelione, G.T., Lyons, B.A., Emerson, S.D. and Tashiro, M. (1992) *J. Am. Chem. Soc.,* **114**, 10974–10975.

Mueser, T.C., Rogers, P.H. and Arnone, A. (2000) *Biochemistry,* **39**, 15353–15364.

Silva, M.M., Rogers, P.H. and Arnone, A. (1992) *J. Biol. Chem,* **267**, 17248–17256.

Simplaceanu, V., Lukin, J.A., Fang, T.Y., Zou, M., Ho, N.T. and Ho, C. (2000) *Biophys. J.,* **79**, 1146–1154.

Tugarinov, V. and Kay, L.E. (2003a) *J. Am. Chem. Soc.,* **125**, 5701–5706.

Tugarinov, V. and Kay, L.E. (2003b) *J. Am. Chem. Soc.,* **125**, 13868–13878.

Uhrin, D., Uhrinova, S., Leadbeater, C., Nairn, J., Price, N.C. and Barlow, P.N. (2000) *J. Magn. Reson.,* **142**, 288–293.

Venters, R.A., Farmer, B.T., Fierke, C.A. and Spicer, L.D. (1996) *J. Mol. Biol.,* **264**, 1101–1116.

Yamazaki, T., Lee, W., Arrowsmith, C.H., Muhandiram, D.R. and Kay, L.E. (1994) *J. Am. Chem. Soc.,* **116**, 11655–11666.

Yang, D.W., Zheng, Y., Liu, D.J. and Wyss, D.F. (2004) *J. Am. Chem. Soc.,* **126**, 3710–3711.