Journal of Biomolecular NMR (2006) 34: 167–177
DOI 10.1007/s10858-006-0008-8

Article

Utilization of methyl proton resonances in cross-saturation measurement for determining the interfaces of large protein–protein complexes

Hideo Takahashi[a,*], Mayumi Miyazawa[b], Yasuo Ina[c], Yoshifumi Fukunishi[a], Yumiko Mizukoshi[c], Haruki Nakamura[a,d] & Ichio Shimada[a,b,*]

[a]*Biological Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), Aomi 2-41-6, Koto-ku, Tokyo 135-0064, Japan;* [b]*Graduate School of Pharmaceutical Sciences, The University of Tokyo Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan;* [c]*Japan Biological Information Research Center (JBIRC), Japan Biological Informatics Consortium (JBIC), Hachobori, Chuo-ku, Tokyo, 104-0032, Japan;* [d]*Institute for Protein Research, Osaka University, Yamadaoka, Suita, Osaka, 565-0871, Japan*

Received 13 October 2005; Accepted 17 January 2006

Key words: cross-saturation, deuteration, methyl protons, methyl-TROSY, protein–protein complex

Abstract

Cross-saturation experiments allow the identification of the contact residues of large protein complexes (MW > 50 K) more rigorously than conventional NMR approaches which involve chemical shift perturbations and hydrogen-deuterium exchange experiments [Takahashi et al. (2000) *Nat. Struct. Biol.*, **7**, 220–223]. In the amide proton-based cross-saturation experiment, the combined use of high deuteration levels for non-exchangeable protons of the ligand protein and a solvent with a low concentration of ^1H_2O greatly enhanced the selectivity of the intermolecular cross-saturation phenomenon. Unfortunately, experimental limitations caused losses in sensitivity. Furthermore, since main chain amide protons are not generally exposed to solvent, the efficiency of the saturation transfer directed to the main chain amide protons is not very high. Here we propose an alternative cross-saturation experiment which utilizes the methyl protons of the side chains of the ligand protein. Owing to the fast internal rotation along the methyl axis, we theoretically and experimentally demonstrated the enhanced efficiency of this approach. The methyl-utilizing cross-saturation experiment has clear advantages in sensitivity and saturation transfer efficiency over the amide proton-based approach.

Introduction

Protein–protein interactions play a critical role in various aspects of biological systems and the identification of the molecular interface of a protein complex can provide valuable information for the rational design of functional molecules. Many attempts to identify the interaction sites of proteins have been made by using NMR spectroscopy. Recently, we developed a novel NMR method, termed the cross-saturation experiment, which enables us to identify the contact residues of a large protein complex (MW > 50 K) more rigorously than conventional NMR approaches which involve chemical shift perturbation and hydrogen-deuterium exchange experiments (Takahashi et al., 2000). This method was also applied to higher molecular weight protein complexes by utilizing the fast exchange process between free and bound states of the ligand protein (transferred cross-saturation experiment; Nakanishi et al., 2002).

*To whom correspondence should be addressed. E-mails: hid@jbirc.aist.go.jp, shimada@iw-nmr.f.u-tokyo.ac.jp

For the successful application of cross-saturation experiments to large molecular weight complexes, a high level of deuteration for ligand proteins is required, resulting in the effective suppression of spin diffusion in the ligand proteins. To satisfy this requirement, a solvent with a low concentration of $^{1}H_2O$ is used in the cross-saturation experiment. However, since the cross-saturation effect is detected by the labile main chain amide protons, this experimental limitation causes a loss in sensitivity. Furthermore, because the main chain amide protons generally form the framework structure of the protein and are less exposed on the molecular surface, they are generally less susceptible to saturation transfer from the target molecule to the ligand molecule.

Here we report an alternative cross-saturation method, which utilizes the side chain methyl protons of the ligand protein. The side chains of a protein usually have direct contacts with other molecules; therefore, the efficiency of the saturation transfer for side chain protons is expected to be higher than that for main chain amide protons. Furthermore, the $^{1}H-^{13}C$ shift correlation spectra of the methyl groups are generally well-resolved, and because of the fast rotation about the methyl symmetry axis, the methyl group exhibits narrow line widths which in turn leads to excellent sensitivity. Additionally, the dynamic properties of the methyl groups cause efficient longitudinal relaxation of the methyl protons (Ishima et al., 1991), which is expected to affect the saturation transfer efficiency in the cross-saturation experiment.

This new experiment was applied to the B-domain of Protein A (FB) in which the methyl groups were selectively protonated and complexed with the Fc fragment of human IgG1 (MW~64 K). Efficient cross-saturation phenomena were observed for the methyl-containing residues that were located at the interface of the complex, and the sensitivity of this experiment was exceedingly high, as compared with the previous experiments.

Materials and methods

DB analysis for the distribution of methyl protons at the molecular interface

Sixty-nine non-redundant protein–protein complexes were selected from the PDB. Six complexes [4cpa, 3hfl, 1brs, and 2btf; (*2) and (*3)] were not used from the original 75 complexes in Table 1 of Lo Conte et al. (1999) because of their ambiguous

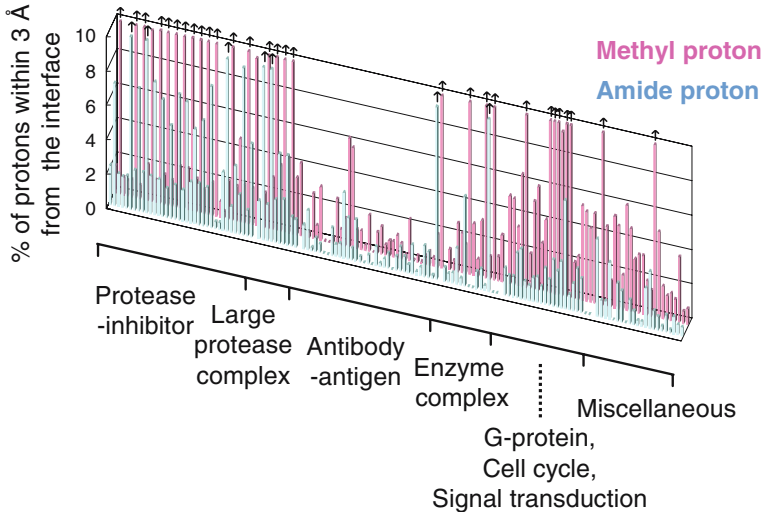

Figure 1. Histogram representation of the probability of methyl and amide protons to be located within 3.0 Å from the molecular interface of the protein–protein complex. The analysis procedures are described in Materials and Methods. The classification of protein complexes (69 PDB files) was according to Lo Conte et al. (1999). Protease-inhibitor: 2ptc, 1avw, 1mct, 3tpi, 1tgs, 1cho, 1acb, 1cbw, 1ppf, 1fle, 2kai, 1hia, 3sgb, 1mkw, 1cse, 2sic, 2sni, and 1stf; Large protease complexes: 1bth, 4htc, 1tbq, 1toc, and 1dan; Antibody-antigen: 1vfb, 1mlc, 1jhl, 3hfm, 1fbi, 1mel, 1jel, 1nsn, 1osp, 1nca, 1nmb, 1eo8, 1dvf, 1iai, 1nfd, 1kb5, and 1ao7; Enzyme complexes: 1dfj, 1dhk, 1fss, 1gla, 1udi, 1ydr, and 2pcc; G-protein, cell cycle, signal transduction: 1tx4, 1gua, 1a2k, 1efu, 1aip, 1gg2, 1got, 2trc, 1agr, 1fin, and 1a0b; Miscellaneous: 1fc2, 1igc, 1ak4, 1efn, 1atn, 1dkg, 1ebp, 1hwg, 1seb, 1tco, and 1ycs. Bar graphs that show a 10% ratio in the figure actually indicate that the ratio is larger than 10% (indicated by small arrows).

representation of amino acids (GLX) or the absence of the file in the PDB. Hydrogen atoms were added and energy minimizations were performed for each complex structure to optimize the geometry of the hydrogen atoms by using in-house software prestoX (Fukunishi et al., 2003). One of the components is defined as "Ligand Protein (LP)" and the other(s) are defined as "Target Protein(s) (TP)". The distances between each methyl proton (Ala-β, Ile-γ2 and δ1, Leu-δ1 and δ2, Met-ε, Thr-γ, Val-γ1 and γ2) of LP and all protons of TP were calculated. The number of methyl protons with distances within 3.0 Å of the protons of TP was counted as the protons located at the interface of the complex, and the ratio of this number to the total number of methyl protons was calculated. The same calculations were also performed with the interchange between LP and TP. The distribution of the amide protons at the molecular interface was estimated in a similar way, by using the amide protons instead of the methyl protons.

Sample preparation

The ^{2}H (^{1}H, ^{13}C-methyl)-labeled sample of FB was prepared by growing bacteria, according to the protocol described by Goto et al. (Goto et al., 1999), but [$^{2}H_7$]-glucose was used, instead of [$^{2}H_7$, $^{13}C_6$]-glucose. In order to assign the two diastereotopic methyl groups of the seven leucyl residues in FB, a mixture of 10% [$^{13}C_6$]-glucose and 90% [$^{12}C_6$]-glucose was used (Senn et al., 1989).

The isolation and purification procedures of the FB fragments were previously described (Torigoe et al., 1990). The purified protein sample was dissolved in a solution of $^{2}H_2O$, 50 mM potassium phosphate, pH 6.0, with 150 mM NaCl, and was incubated overnight at 37 °C. After this incubation procedure, no residual amide proton resonances of FB were observed in the ^{1}H-NMR spectrum (Figure 2(a)).

The Fc fragments of human myeloma protein IgG(k) Ike-N were also prepared as previously reported (Gouda et al., 1992).

Simulation study

The cross-saturation experiment was theoretically treated by using modified Bloch equations. When the protons of protein II (target protein for saturation: $m+1 \leq j \leq n$) are irradiated instantaneously, the modified Bloch equations for the

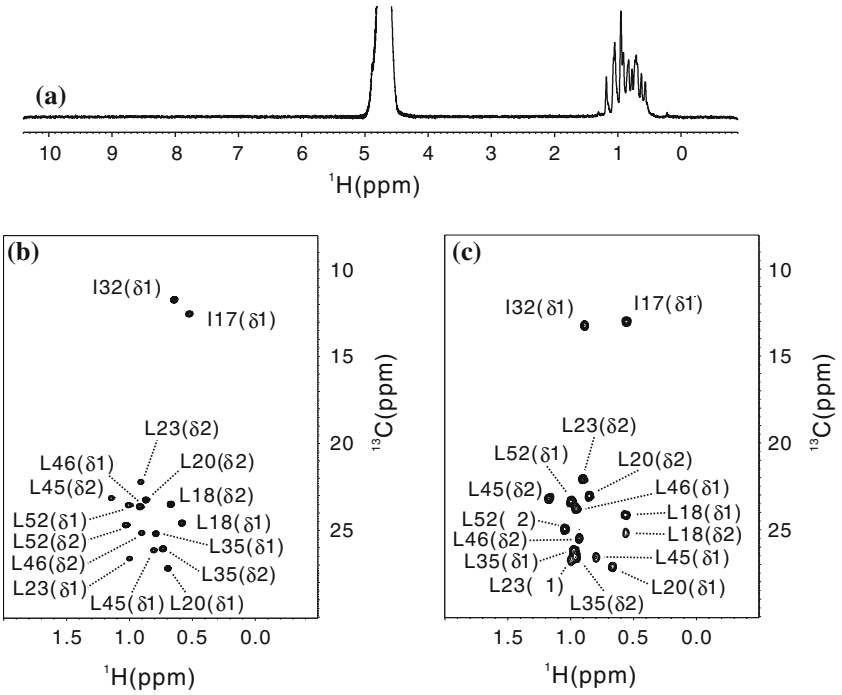

Figure 2. (a) ^{13}C-decoupled ^{1}H-NMR spectra of ^{2}H (^{1}H, ^{13}C-methyl)-labeled FB in $^{2}H_2O$, 50 mM potassium phosphate, pH 6.0 with 150 mM NaCl at 25 °C. Methyl regions of the ^{13}C–^{1}H shift correlation spectra of ^{2}H (^{1}H, ^{13}C-methyl)-labeled FB (b) and the FB–Fc complex (c). Spectra were recorded with ^{2}H decoupling during the ^{13}C evolution periods.

protons of protein I (protein for which the molecular interface is determined:$1 \leq i \leq m$) could be written as:

$$\frac{dM_z^i}{dt} = -\rho_i(M_z^i - M_0^i) - \sum_{k=1,k\neq i}^{m} \sigma_{ik}(M_z^k - M_0^k) + \sum_{j=m+1}^{n} \sigma_{ij} M_0^j \qquad [1]$$

where M_z represents the instantaneous longitudinal magnetization, M_0 represents the thermal equilibrium values of magnetization, and ρ_i and σ_{ij} represent the auto-relaxation rate constant of proton i and the cross-relaxation rate constant between protons i and j, respectively. By using matrix notation, equation [1] can be rewritten as follows:

$$\frac{d\boldsymbol{\eta}}{dt} = -\mathbf{R}\cdot\boldsymbol{\eta} + \Sigma$$

where

$$\eta = \begin{pmatrix} \frac{(M_z^1 - M_0)}{M_0} \\ \cdot \\ \cdot \\ \cdot \\ \frac{(M_z^m - M_0)}{M_0} \end{pmatrix}$$

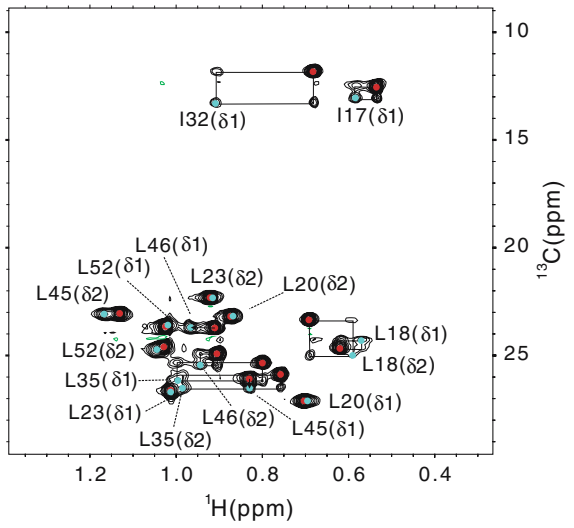

Figure 3. The assignment of the resonances originating from the Fc-bound form of ^{2}H (^{1}H, ^{13}C-methyl)-labeled FB. A longitudinal two-spin-order exchange experiment for the ^{2}H (^{1}H, ^{13}C-methyl)-labeled FB–Fc complex was performed in ^2H_2O, 50 mM potassium phosphate, pH 5.0 with 150 mM NaCl at 37 °C. The cross-peaks originating from the free FB and the Fc-bound form of FB are marked with light green and red, respectively.

$$\mathbf{R} = \begin{pmatrix} \rho_1 & \sigma_{\cdot 12} & \cdot & \sigma_{1\cdot(m-1)} & \sigma_{1\cdot m} \\ \sigma_{2\cdot 1} & \rho_2 & \cdot & \sigma_{2\cdot(m-1)} & \sigma_{2\cdot m} \\ \cdot & \cdot & \cdot & \cdot & \cdot \\ \sigma_{(m-1)\cdot 1} & \sigma_{(m-1)\cdot 2} & \cdot & \rho_{m-1} & \sigma_{(m-1)\cdot m} \\ \sigma_{m\cdot 1} & \sigma_{m\cdot 2} & \cdot & \sigma_{m\cdot(m-1)} & \rho_m \end{pmatrix}$$

$$\Sigma = \begin{pmatrix} \sum_{j=m+1}^{n} \sigma_{1\cdot j} \\ \cdot \\ \cdot \\ \cdot \\ \sum_{j=m+1}^{n} \sigma_{m\cdot j} \end{pmatrix}$$

The above equation can be solved to give,

$$\boldsymbol{\eta} = \{\mathbf{E} - \mathbf{T}\cdot\exp(-\Lambda\cdot t)\cdot\mathbf{T}^{-1}\}\cdot\mathbf{R}^{-1}\cdot\Sigma \qquad \Lambda = \mathbf{T}\cdot\mathbf{R}\cdot\mathbf{T}^{-1} \qquad [2]$$

where Λ is the diagonalization form of the relaxation matrix $\mathbf{R}$, and $\mathbf{T}$ is the matrix that diagonalizes the matrix $\mathbf{R}$.

Simulation of the cross-saturation experiment was performed with a modified CORONA program (Eisenmesser et al., 2000). The modification was made to solve equation [2], although the original input/output interface was utilized.

The proton arrangement for the simulation is shown in Figure 5(a). The fast methyl group rotation with local correlation times of 50 ps or 100 ps was assumed. For this simulation, the protons within the protein are assumed to experience instantaneous saturation. The dependence of

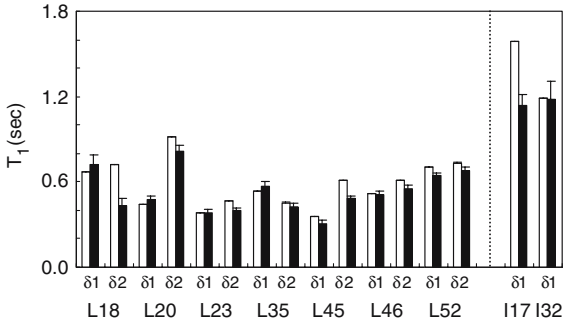

Figure 4. Longitudinal relaxation times for the methyl protons of ^{2}H (^{1}H, ^{13}C-methyl)-labeled FB in the absence (open bar) and presence (filled bar) of FB. Experimental conditions are described in Materials and Methods.

the fractional intensity change $[(I - I_0)/I_0]$ of each proton of the methyl groups on the irradiation times was plotted. The simulations were carried out for a spectrometer frequency of 700.13 MHz.

NMR measurements

All measurements were performed on a Bruker Avance 700 MHz spectrometer. Unless otherwise noted, experiments were conducted using samples in 50 mM potassium phosphate, pH 6.0, with 150 mM NaCl in 99.9% ^2H_2O. Spectra were processed and analyzed using NMRPipe (Delaglio et al., 1995).

The $^1H-^{13}C$ CT-HSQC experiments (Vuister and Bax, 1992) for ^2H (^1H, ^{13}C-methyl)-labeled FB were performed with ^2H broadband decoupling during the ^{13}C constant time evolution period ($1/J_{CC} \sim 26.6$ ms). Spectra were recorded with 80 complex $t_1(^{13}C)$ points and 512 complex $t_2(^1H)$ points, respectively. The ^1H spectral width was 9328.4 Hz and the ^{13}C spectral width was 4225.2 Hz. In the case of the Fc-bound form of FB, the $^1H-^{13}C$ shift correlation spectra were performed with the $^1H-^{13}C$ HMQC pulse sequence (Ollerenshaw et al., 2003; Tugarinov et al., 2003; Müller, 1979) with ^2H broadband decoupling during the ^{13}C evolution period.

On the basis of the assignments of the methyl resonances of FB alone, the assignments for the methyl resonances originating from the Fc-bound state of FB were made by using a longitudinal two-spin-order exchange experiment (Wider et al., 1991). Six molar equivalents (0.75 mM) of ^2H (^1H, ^{13}C-methyl)-labeled FB were added to Fc (0.125 mM) dissolved in^2H_2O, 50 mM potassium phosphate, pH 5.0 with 150 mM NaCl at 37 °C. The mixing time for two-spin-order exchange was set to 50 ms.

The longitudinal relaxation times (T_1's) of the leucine and isoleucine ($\delta 1$) methyl protons of ^{13}C, ^2H (^1H-methyl)-labeled FB, at a field strength corresponding to the ^1H frequency of 700.13 MHz at 37 °C, were determined by an inversion-recovery scheme followed by a $^1H-^{13}C$ shift correlation pulse sequence ($^1H-^{13}C$ CT-HSQC or $^1H-^{13}C$ HMQC). The following relaxation delay times were used: 0.001, 0.01, 0.05, 0.1, 0.2, 0.4, 0.7, 1.0, 1.3, 1.6, and 2.0 s. The data thus obtained were processed, and each resonance was fitted to a single exponential decay curve, in order to derive the longitudinal relaxation time of each methyl proton. Although the longitudinal relaxation of proton magnetization is generally expected to be a non-exponential (or multi-exponential) decay, the obtained data fit nicely to a single exponential decay for this fairly deuterated sample.

Cross-saturation measurements were conducted on a 0.8 mM sample of ^2H (^1H, ^{13}C-methyl)-labeled FB combined with 0.4 mM of the Fc fragment. Saturation of the protons of the Fc fragment was done using the WURST-2 decoupling scheme (Kupce and Wagner, 1995). The maximum RF amplitude was 0.17 kHz for WURST-2 (the adiabatic factor $Q_0 = 1$). The saturation frequency was set to 6.0 ppm. The experiments were performed at 37 °C. The pulse sequence with all appropriate parameters is provided as supporting information.

Results and discussion

Distribution of methyl protons at the molecular interface

The propensity of methyl protons to be located at the molecular interface of protein complexes was estimated. Protein–protein complexes were selected from 69 non-redundant files from the PDB (Lo Conte et al., 1999) and calculations were performed with the procedure described in the Materials and Methods section. Figure 1 shows a histogram representation of the probability of methyl and amide protons to be located within 3.0 Å from the molecular interface of a protein–protein complex. This probability for methyl protons is comparable to or even larger than that of amide protons. As for the antibody-antigen complexes, the probability for the methyl groups to be at the interface is lower than those of the other complexes (Figure 1). This is probably due to their being composed of less hydrophobic and more polar amino acid residues than other protein–protein complexes (Lo Conte et al., 1999). The present analysis suggests that the methyl protons are suitable as a probe in the cross-saturation experiment for identifying molecular interfaces, even though it has been suggested that the propensities of methyl containing amino acids to be at the interface are not as high compared to other amino acids (Hu et al., 2000; Ma et al., 2003).

Selective methyl group protonation of a perdeuterated protein and resonance assignment of the methyl groups

Goto et al. developed an efficient protocol for the production of highly deuterated proteins with protons selectively incorporated into the valine, leucine, and isoleucine (δ1) methyl groups (Goto et al., 1999). By using this protocol, high levels of protonation were attained for the methyl groups of the leucine and isoleucine (δ1 only) residues of FB (there are no valine residues in FB), while the $^{13}CH_2D$ and $^{13}CHD_2$ isotopomers were not produced, as judged by the ^{2}H-decoupled $^1H-^{13}C$ constant time HSQC spectrum (Figure 2(b)). Figure 2(c) shows the $^1H-^{13}C$ HMQC spectrum of ^2H (^{1}H, ^{13}C-methyl)-labeled FB complexed with the Fc of human IgG1. Although the molecular weight of this complex was more than 60 K, the methyl signals were well-resolved and the sensitivity of the signals was high. In this case, the CT-HSQC scheme was not adequate because the constant time evolution ($\sim$26.6 ms) for the ^{13}C evolution reduced the sensitivity. Furthermore, as pointed out by Kay's group (Ollerenshaw et al., 2003; Tugarinov et al., 2003), the HMQC scheme has an advantage over that of HSQC for the FB–Fc complex due to its large molecular weight and relaxation properties.

In order to assign the resonances originating from the side chain methyl groups of a large protein (MW > 50 K), one can utilize the magnetization transfer from the methyl protons to the assigned main chain amide groups or the assigned carbons (C_α and/or C_β) (Hilty et al., 2002; Lohr and Ruterjans, 2002; Tugarinov and Kay, 2003a; Tugarinov and Kay, 2003b; Yang et al., 2004). However, in our case, it was feasible to assign the methyl resonances originating from the Fc-bound form of FB by using longitudinal two-spin-order exchange between the free and bound forms of FB (Figure 3) (Wider et al., 1991).

Longitudinal relaxation time of methyl protons

It is well known that the relaxation mechanism of methyl protons is dominated by the fast methyl group rotation (Ishima et al., 1991). Therefore, the longitudinal relaxation time (T_1) of the methyl protons is not strongly dependent on the rotational correlation time of the molecule. Figure 4 shows the T_1 values of the methyl protons of FB, in the absence and presence of the human IgG1 Fc fragment. Although the other protons of FB were well deuterated (Figure 2(a)), the T_1 values of the methyl protons were relatively short. The average T_1 value for the methyl protons of the leucine residues of ^{2}H (^{1}H, ^{13}C-methyl)-labeled FB was 0.58 s in the absence of Fc and 0.53 s in the presence of Fc. In the case of the δ1 methyl protons for the isoleucine residues, the T_1 values were larger than 1.0 s. The relatively long T_1 values for the isoleucine residues, as compared to those for the leucine residues, would be explained by the deuteration of the neighboring γ2 methyl protons of its own residues and/or the different dynamic properties between the δ methyl groups of the leucines and the δ1 methyl groups of the isoleucines.

Methyl-utilizing cross-saturation – simulation

Since the cross-saturation experiment is easily formulized with a complete relaxation matrix analysis (Lane et al., 2001; Jayalakshmi and Krishna, 2002), we can assess the feasibility of the methyl-utilized cross-saturation experiment with a simulation study.

The most important consideration for the successful application of the cross-saturation experiment is the suppression of spin diffusion in a large molecular weight protein complex; otherwise, the accurate identification of the interaction surface can no longer be achieved. In our previous version of the cross-saturation experiment, this requirement was satisfied by combining high level deuteration for the carbon-bound protons of the ligand protein and a solvent with a high deuterium content (Takahashi et al., 2000). In the case of the methyl-utilizing cross-saturation experiment, the short T_1 values for the methyl protons are expected to suppress the spin diffusion effect as occurs in small molecules. In order to confirm this, a simulation of the cross-saturation experiment was performed with a hypothetical model of a ligand–protein complex for which rotational correlation times of 30 ns and 60 ns were assumed. Figure 5(a) shows the spatial arrangement of protons for the ligand–protein complex model. In this system, the saturation of the "Methyl (2)" protons is mainly mediated by the spin diffusion of the saturated "Methyl (1)" protons.

First, each methyl group is assumed to be virtually fixed, and the protons of the target proteins are saturated. In the case of a large protein complex, which has a rotational correlation time of 60 ns corresponding to a molecular weight of more than 100 K, rapid saturation transfers would occur for both methyl protons (Figure 5(b)). Since the spin diffusion phenomena of large molecular weight protein complexes are so efficient, the saturation profiles of both methyl group protons show similar time dependencies. The saturation factors of both methyl group protons effectively show the same values for more than a 1.0 s saturation time. However, in a realistic case, if methyl

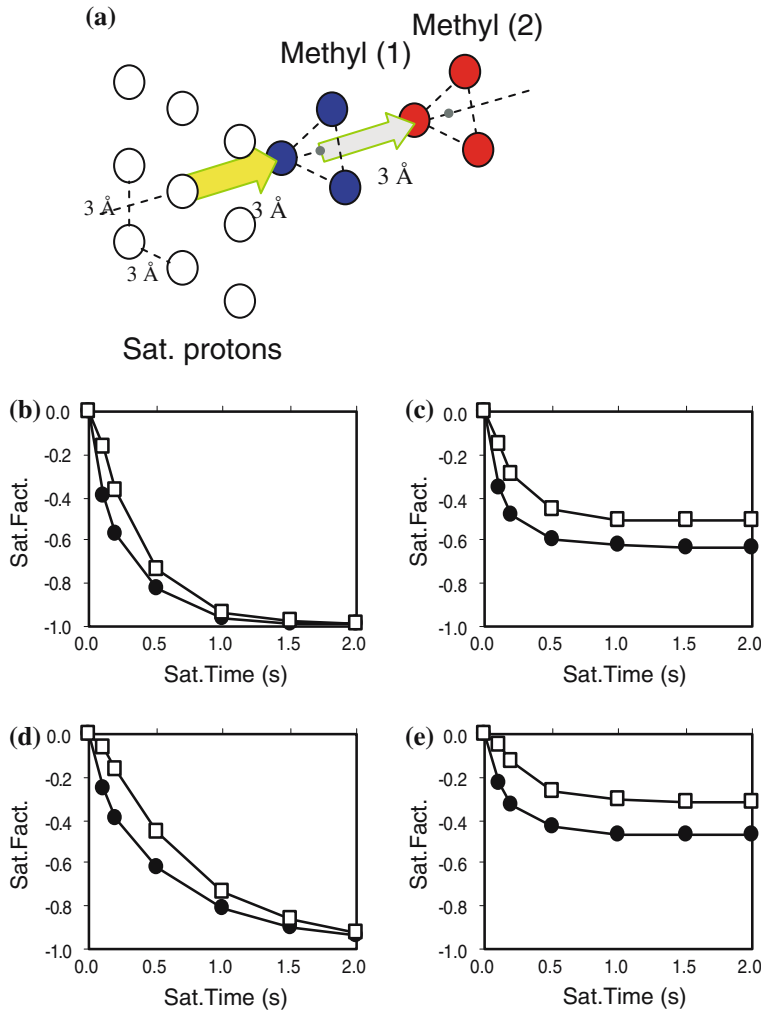

Figure 5. (a) Proton arrangement for the simulation study of the methyl-utilizing cross-saturation experiment. The filled circles (blue and red) represent the methyl protons of the ligand protein ["Methyl (1)" and "Methyl (2)," respectively], while the nine open circles represent the protons of the target protein. The nine protein protons were placed on a grid with 3 Å separation. The distance between the center proton of the protein protons and the pseudo center proton of "Methyl (1)" is assumed to be 3 Å. The distance between the two pseudo center protons of the methyl groups is 3 Å. (b)–(e) The dependencies of the fractional intensity change [Saturation factor $(I-I_0)/I_0$] of each proton of the methyl groups on the irradiation time are plotted. Filled circles and open squares represent "Methyl (1)" and "Methyl (2)," respectively. (b) Simulation 1: Methyl group is assumed to be virtually fixed. Overall correlation time of the system is assumed to be 60 ns. (c) Simulation 2: Methyl groups are assumed to be rotated along their methyl axes, respectively. Overall correlation time of the system is assumed to be 60 ns, while the local correlation times for the methyl groups are assumed to be 50 ps. (d) Simulation 3: Methyl group is assumed to be virtually fixed. Overall correlation time of the system is assumed to be 30 ns. (e) Simulation 4: Methyl groups are assumed to be rotated along their methyl axes, respectively. Overall correlation time of the system is assumed to be 30 ns, while the local correlation times for the methyl groups are assumed to be 50 ps.

protons undergo rapid rotational motion with a local correlation time of 50 ps, then the time dependencies of the saturation for the two methyl group protons are apparently different (Figure 5(c)). Both methyl group protons rapidly reach a different steady state value (−0.63 and −0.50 for "Methyl (1)" and "Methyl (2)," respectively) after approximately 0.5 s of irradiation time. Considering the fact that the saturation factor of "Methyl (2)" is larger than −0.1 in the absence of "Methyl (1)," the spin diffusion has not completely vanished. However, the observed difference in the saturation factors for "Methyl (1)" and "Methyl (2)" indicates that the spin diffusion effect has been partially suppressed in the methyl-utilizing cross-saturation experiment. The simulations with a methyl local correlation time of 100 ps showed similar tendencies, but the final steady state values were slightly lower (−0.55 and −0.40 for "Methyl (1)" and "Methyl (2)," respectively; data not shown) than those of 50 ps. In the case of a "medium-sized" protein complex with a 30 ns rotational correlation time, similar saturation profiles were obtained (Figure 5(d) and (e)).

The rapid rotational motion of the methyl groups enhances the fast longitudinal relaxation, and the spin diffusion effect is partially suppressed in a large molecular weight protein complex. This result indicates that the cross-saturation experiment using methyl protons can be effectively applied to large molecular weight protein complexes.

Methyl-utilizing cross-saturation – experiment

The methyl-utilizing cross-saturation experiment was performed in a similar manner as the amide proton-based cross-saturation experiment (Takahashi et al., 2000; see Materials and Methods). As shown in Figure 2(a), there were no proton resonances from ^{2}H (^{1}H, ^{13}C-methyl)-labeled FB (PBS 99.8% D_2O) at shifts higher than 1.3 ppm. The residual HDO signal can be seen at ~4.7 ppm in Figure 2(a). Therefore, irradiation using an RF (radio frequency) field was applied at approximately 3.5 ppm ~8.5 ppm, a region corresponding to the residual amide protons, aromatic protons, most of the alpha protons, and some of the other aliphatic protons of the Fc fragment, with a band-selective adiabatic saturation scheme (Takahashi et al., 2000; Kupce and Wagner, 1995). The $^{1}H-^{13}C$ HMQC spectra observed for the complex between the ^{2}H (^{1}H, ^{13}C-methyl)-labeled FB and the Fc fragment, with and without irradiation, are shown in Figure 6(a). Based on the spectra with and without the irradiation, the intensity ratios for each methyl proton were calculated, and are summarized in Figure 6(b). The experiment was carried out with 0.8 mM labeled FB with 0.5 molar equivalent of the Fc fragment, and the total measuring time was 3 h, which was much shorter than that of the original amide proton-based cross-saturation experiment (~1 day) with a highly deuterated solvent (90% $^{2}H_2O$) (Supplementary material for the spectral comparison between methyl-utilizing and amide proton-based cross-saturation experiments are provided.). Although the irradiation time was relatively short (0.3 s) compared with that of the original amide proton-based cross-saturation experiment (usually longer than 1 s), the signal intensities for some methyl protons were moderately decreased (intensity ratio was less than 0.8) with the irradiation. This result indicates that the saturation transfer from the Fc protons to the methyl protons of FB, which are located at the molecular interface, was highly efficient.

In the case of the amide proton-based cross-saturation experiment, in order to suppress the spin diffusion effect of the large protein complex and to detect the molecular interface selectively, it is necessary to use a solvent with a high (80~90%) $^{2}H_2O$ content (Takahashi et al., 2000), which leads to sensitivity losses in the experiment. On the other hand, since the spin diffusion effect of the methyl protons is intrinsically suppressed, as shown in the simulation study, no sacrifices need to be made for the sensitivity in the methyl-utilizing cross-saturation experiment.

Figure 6(c) shows the irradiation time dependency for the selected methyl groups. The saturation of the methyl protons reached equilibrium within a relatively short irradiation time (less than 1 s), as shown in the simulation experiments, which indicates a fast relaxation of the methyl protons.

Among the 16 methyl proton resonances originating from the Ile and Leu residues of FB, the intensity ratios of Leu18 ($\delta 1$ and $\delta 2$) and Ile32 ($\delta 1$) in the cross-saturation experiment exhibited the lowest values (less than 0.7 with more than 0.5 s irradiation), which indicated that these residues

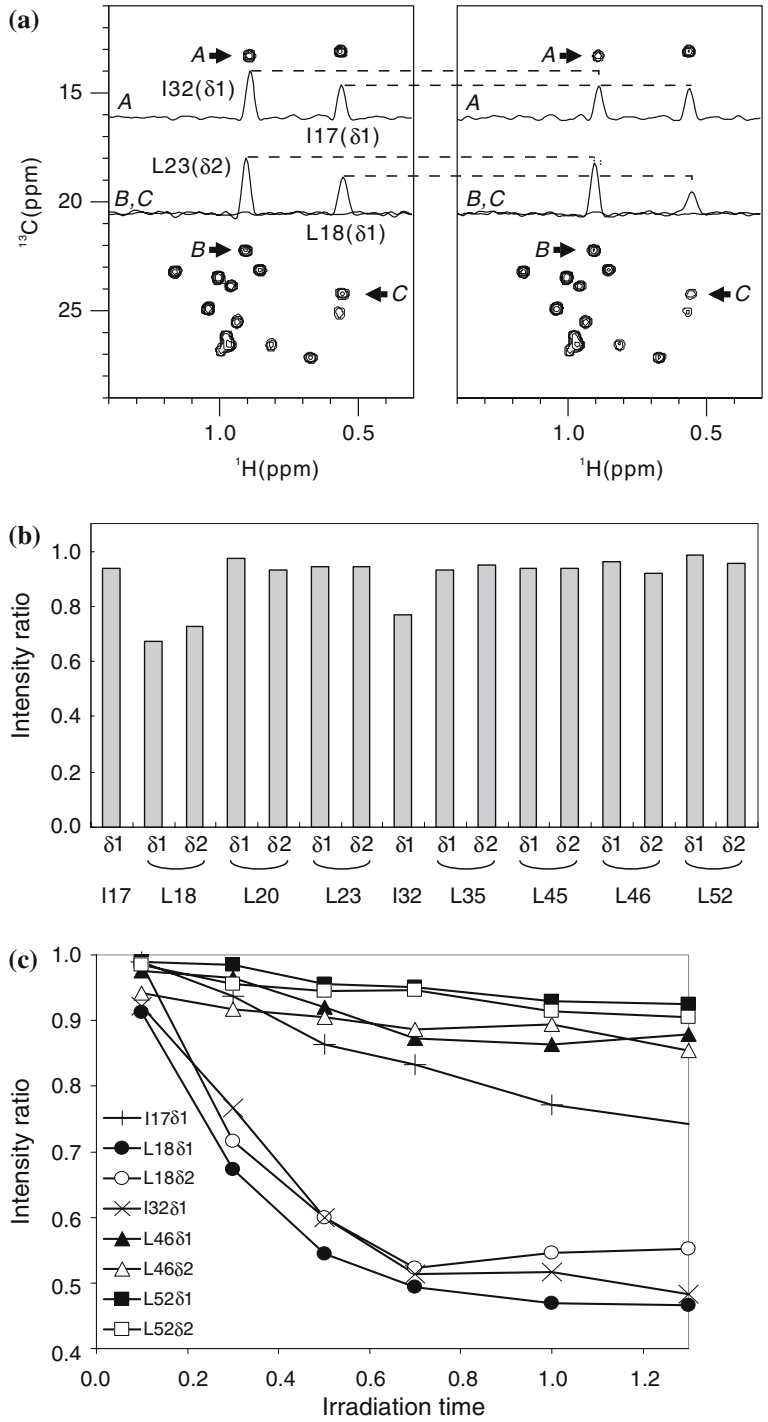

Figure 6. Methyl-utilizing cross-saturation experiment. (a) $^{1}H-^{13}C$ HMQC spectra observed for ^{2}H (^{1}H, ^{13}C-methyl)-labeled FB in complex with the Fc fragment, without (left) and with (right) irradiation. F_2 cross-sections through Ile17(δ1) and Ile32(δ1); Leu23(δ2); and Leu18(δ1) of the FB are also shown in the figure. The corresponding F_1 frequencies (A–C) are indicated with arrows in the spectra. The experimental conditions are described in Materials and Methods and the irradiation time was set to 0.3 s. (b) Ratio of signal intensities originating from the Leu and Ile methyl groups, with and without irradiation, in the cross-saturation experiments. (c) Irradiation time dependency on the intensity ratios of the selected methyl resonances. The irradiation times used were 0.1, 0.3, 0.5, 0.7, 1.0 and 1.5 s.

are located in the molecular interface with human Fc. These residues were also included in the contact residues which were determined by the previous amide proton-based experiment (Takahashi et al., 2000). This result was confirmed by the X-ray crystallographic structure of the FB–Fc complex (Figure 7).

Since the methyl-utilizing cross-saturation experiment can be extended to a transferred type experiment (Nakanishi et al., 2002), it would be easy to apply it to larger protein complexes with high sensitivity. We have already applied the transferred type methyl-utilizing cross-saturation experiment to the molecular interaction between ^{2}H (^{1}H, ^{13}C-methyl)-labeled FB and intact mouse IgG1, with a molecular mass beyond 150 kDa (Nakanishi et al., 2002). Essentially the same result was obtained with that of the FB–Fc complex in a 3 h experiment with reasonable sample conditions [0.8 mM ^{2}H (^{1}H, ^{13}C-methyl)-labeled FB with 0.16 mM mouse IgG1, pH 6.0, 25 °C; experimental data are provided as supplementary files].

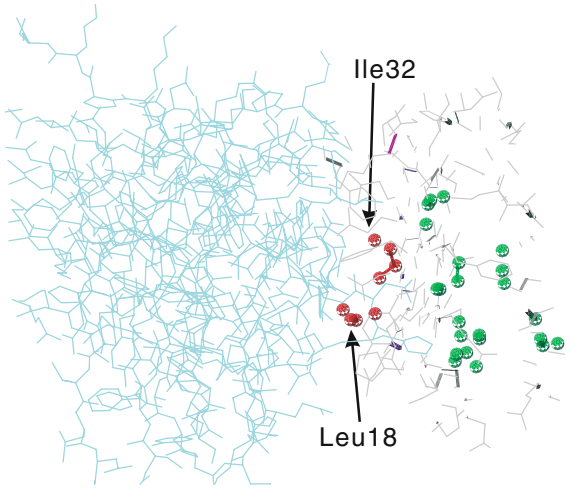

Figure 7. Three-dimensional structure of the FB–Fc complex (PDB code 1FC2). Molecular graphics images were produced using MOLMOL (Koradi et al., 1996). The Fc molecule is colored in cyan. The backbone of FB is rendered with a "neon" representation. The side chains of Ile and Leu of FB are rendered with a "ball-and-stick" representation and are colored in green, and those of Leu18 and Ile32 are colored in red. The contact residues which were determined by the previous amide-based cross-saturation experiments are colored in magenta in the backbone of FB.

Concluding remarks

In the present study, we have developed a methyl-utilizing cross-saturation experiment. Methyl groups are useful as NMR probes because their resonances are intense and well-dispersed (Hajduk et al., 2000). Furthermore, the recently developed methyl-TROSY principle ensures the advantage of sensitivity for the methyl probes of large molecules (Ollerenshaw et al., 2003; Tugarinov et al., 2003; Hamel and Dahlquist, 2005). Therefore, the methyl-utilizing cross-saturation experiment is clearly superior to an amide proton-based cross-saturation experiment from a sensitivity standpoint (the comparison of both experiments is presented in Figure S3 of the supplementary materials).

Since the suppression of the spin diffusion effect originating from the fast longitudinal relaxation of the methyl protons is assured, a highly accurate determination of the binding site can be made. Additionally, the relatively short longitudinal relaxation time of the methyl protons ensures the short relaxation delay in the experiment, which decreases the total measuring time. Therefore, the utilization of an Ernst angle pulse and the optimization of the relaxation delay further enhance the sensitivity of the experiments (Ross et al., 1997). Additionally, the sensitivity gain would be more pronounced with the use of the selective methyl label of Ile, Leu and Val residues proposed by Hajduk et al. (2000) for the suppression of J_{CC} spin coupling.

In this study, we performed the methyl-utilizing cross-saturation experiment with Ile and Leu residues in which the methyl protons were selectively protonated. The methyl-utilizing cross-saturation experiment can in principle be performed with other methyl-containing amino acid residues, e.g., Ala, Met, and Thr, since fast longitudinal relaxation properties for the methyl protons are also ensured (Ishima et al., 1991). If deuterated Ala, Met, and Thr residues in which the methyl groups are selectively protonated become available in the near future, we will be able to obtain more probes and take full advantage of the methyl-utilizing cross-saturation experiment.

In our previous experiment (Takahashi et al., 2000), the amide proton-based cross-saturation experiment was successfully applied for determining the molecular interface of the FB–Fc complex with a sample of ~1 mM concentration. However,

solubility issues or problems in obtaining samples in sufficient quantitites are often encountered. Thus, the amide proton-based cross-saturation experiment frequently suffers from its low sensitivity and the outcome of the experiment is uncertain. Therefore, the methyl-utilizing cross-saturation experiment offers an undisputed enhancement over the amide proton-based approach, especially in the case of low solubility and large molecular weight complex samples. Unfortunately, although a 3D-structural DB search of protein complexes indicated that methyl groups would be applicable to interaction analysis, the absolute number of methyl probes is apparently smaller than that of the amide groups. Therefore, the combined use of the methyl-utilizing cross-saturation experiment and the amide proton-based cross-saturation experiment would be recommended for highly accurate determinations of the molecular interfaces of large and intractable protein complexes.

Electronic supplementary material is avlailable at http://dx.doi.org/10.1007/s10852-006-0008-8

Acknowledgements

The authors are grateful to Professor Gerhard Wagner (Harvard Medical School) for useful discussions. This work was supported by grants from the New Energy and Industrial Technology Development Organization.

References

Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J. and Bax, A. (1995) *J. Biomol. NMR* **6**, 277–293.

Eisenmesser, E.Z., Zabell, A.P.R. and Post, C.B. (2000) *J. Biomol. NMR* **17**, 17–32.

Fukunishi, Y., Mikami, Y. and Nakamura, H. (2003) *J. Phys. Chem. B.* **103**, 13201–13210.

Goto, N.K., Gardner, K.H., Mueller, G.A., Willis, R.C. and Kay, L.E. (1999) *J. Biomol. NMR* **13**, 369–374.

Gouda, H., Torigoe, H., Saito, A., Sato, M., Arata, Y. and Shimada, I. (1992) *Biochemistry* **31**, 9665–9672.

Hajduk, P.J., Augeri, D.J., Mack, J., Mendoza, R., Yang, J.G., Betz, S.F. and Fesik, S.W. (2000) *J. Am. Chem. Soc.* **122**, 7898–7904.

Hamel, D.J. and Dahlquist, F.W. (2005) *J. Am. Chem. Soc.* **127**, 9676–9677.

Hilty, C., Fernandez, C., Wider, G. and Wüthrich, K. (2002) *J. Biomol. NMR* **23**, 289–301.

Hu, Z., Ma, B., Wolfson, H. and Nussinov, R. (2000) *Proteins* **39**, 331–342.

Ishima, R., Shibata, S. and Akasaka, K. (1991) *J. Magn. Reson.* **91**, 455–465.

Jayalakshmi, V. and Krishna, N.R. (2002) *J. Magn. Reson.* **155**, 106–118.

Koradi, R., Billeter, M. and Wüthrich, K. (1996) *J. Mol. Graphics* **14**, 29–32.

Kupce, E. and Wagner, G. (1995) *J. Magn. Reson. B* **109**, 329–333.

Lane, A.N., Kelly, G., Ramos, A. and Frenkiel, T.A. (2001) *J. Biomol. NMR* **21**, 127–139.

Lo Conte, L., Chothia, C. and Janin, J. (1999) *J. Mol. Biol.* **285**, 2177–2198.

Lohr, F. and Ruterjans, H. (2002) *J. Magn. Reson.* **156**, 10–18.

Ma, B., Elkayam, T., Wolfson, H. and Nussinov, R. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 5772–5777.

Müller, L. (1979) *J. Am. Chem. Soc.* **101**, 4481–4484.

Nakanishi, T., Miyazawa, M., Sakakura, M., Terasawa, H., Takahashi, H. and Shimada, I. (2002) *J. Mol. Biol.* **318**, 245–249.

Ollerenshaw, J.E., Tugarinov, V. and Kay, L.E. (2003) *Magn. Reson. Chem.* **41**, 843–852.

Ross, A., Salzmann, M. and Senn, H. (1997) *J. Biomol. NMR* **10**, 389–396.

Senn, H., Werner, B., Messerle, B.A., Weber, C., Traber, R. and Wüthrich, K. (1989) *FEBS Lett.* **249**, 113–118.

Takahashi, H., Nakanishi, T., Kami, K., Arata, Y. and Shimada, I. (2000) *Nat. Struct. Biol.* **7**, 220–223.

Torigoe, H., Shimada, I., Saito, A., Sato, M. and Arata, Y. (1990) *Biochemistry* **29**, 8787–8793.

Tugarinov, V., Hwang, P.M., Ollerenshaw, J.E. and Kay, L.E. (2003) *J. Am. Chem. Soc.* **125**, 10420–10428.

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.

Vuister, G.W. and Bax, A. (1992) *J. Magn. Reson.* **98**, 428–435.

Wider, G., Neri, D. and Wüthrich, K. (1991) *J. Biomol. NMR* **1**, 93–98.

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