JOURNAL OF MAGNETIC RESONANCE, Series B 106, 195–198 (1995)

Identification of Protein-Mediated Indirect NOE Effects in a Disaccharide–Fab′ Complex by Transferred ROESY

S. RAO AREPALLI,*[1] CORNELIS P. J. GLAUDEMANS,* G. DOYLE DAVES, JR.,*[2] PAVOL KOVAC,* AND AD BAX †[3]

* *Laboratory of Medicinal Chemistry and* † *Laboratory of Chemical Physics, National Institutes of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892-0520*

Received November 7, 1994

The conformation of a ligand bound to a macromolecule can be studied by transferred NOE (TRNOE) experiments (*1–7*). Recent reports question the validity of the commonly used assumption that macromolecule-mediated indirect NOE effects are negligible in TRNOE spectra (*8, 9*). It also was clear from these studies that such indirect effects in practice will be difficult to identify because, to a good approximation, the cross-peak-buildup curve for such an indirect interaction is nearly linear when using an excess of ligand. It shows only a very short lag time, on the order of the inverse of the intermolecular cross-relaxation rate in the bound state, σ_b^{-1}. Even if signal-to-noise permits such a lag period to be detected, it may be difficult to distinguish this from the intrinsic lag time occurring when the off rate is not much higher than the difference in chemical shifts for ligand protons in the free and bound states (*8*). Here we demonstrate that indirect, protein-mediated, TRNOE effects may easily be recognized by repeating the NOE measurement in the rotating frame (*10*), i.e., by conducting a transferred ROESY experiment. The method will be demonstrated for a disaccharide ligated with its cognate Fab′ fragment. In a previous study, we concluded that this disaccharide undergoes a significant conformational change when binding to its specific antibody (*11*). This conclusion was based primarily upon the observation of a cross peak between two protons located on the two different sugar rings. Here we show that this cross peak is dominated by an indirect effect, mediated by a protein proton, and that the recorded NMR data contain insufficient structural information for determining the conformation of the carbohydrate in the Fab′-bound state.

The theory underlying transferred NOE experiments has been described in detail by Clore and Gronenborn for the case where the ligand off rate, k_{off}, is much larger than the difference, δ, in ^{1}H chemical shift for molecules in the free and bound states, and also much larger than the applicable intraligand cross-relaxation rate in the bound state, σ_b (*2, 3*). A relaxation-matrix analysis of the transferred NOE outside of these limits has been presented by London *et al.* (*8*). This study, and an analogous relaxation-matrix analysis by Zheng and Post (*9*), also underscored the potential importance of indirect transferred NOE effects that are mediated via a protein proton. The buildup of such an indirect effect is essentially indistinguishable from a direct intraligand NOE. Quantitative analysis of the indirectly transferred NOE effect requires the use of a relaxation-rate matrix, which is only possible if a detailed structure of both the bound ligand and all protein residues in the vicinity of the ligand is known.

However, qualitatively it is easily seen that an indirect protein-mediated NOE effect can be far larger compared to the situation where the relay occurs via a ligand proton in the same position. Consider, for example, two ligand protons, A and B, that are 5 Å apart, and a third proton C (either ligand or protein), equidistant from A and B, with $r_{AC} = r_{BC} = 3$ Å. If the z magnetization of spin A is inverted by a selective 180° pulse, this will decrease the z magnetization of C at a rate $\sigma_{b(3\text{ Å})}$. In the limit of fast exchange, if C is a ligand proton, the ligand will leave its binding site before any significant indirect transfer from C to B has occurred. Thus, neglecting cross relaxation in the free state, transfer from C to B can reoccur only once the ligand rebinds and requires a total bound time (after multiple rebinding) which is not much smaller than $1/\sigma_{b(3\text{ Å})}$. Thus, in the limit of fast exchange, it appears that all cross-relaxation rates are attenuated by p_b, where p_b is the fraction of bound ligand ($p_b + p_f = 1$, where p_f is the fraction of free ligand), and the buildup of indirect transfer via C is attenuated by p_b^2. This quadratic attenuation does not occur if C is a protein proton: Magnetization transferred from A to C is efficiently relayed to proton B because C is always exchanging magnetization with the pools of A and B protons at a rate $\sigma_{b(3\text{ Å})}$. Consequently, if C is a protein proton, the rate of indirect magnetization transfer is proportional to p_b, and direct and indirect NOE cross peaks show the same dependence on p_b. If C is in thermal contact with other protein protons, these will compete with the transfer from C to B, thereby atten-

[1] Current address: Food and Drug Administration, 9200 Corporate Blvd., Rockville, MD 20850.

[2] Current address: School of Science, Rensselaer Polytechnic Institute, Troy, NY 12180-3590.

[3] To whom reprint requests should be addressed.

1064-1866/95 $6.00

uating the amount of indirect magnetization transfer between A and B. For example, if C were 2.5 Å from three other protein protons, this would attenuate the relay to B approximately 10-fold.

When the NOE is measured in the rotating frame, indirect effects are of a sign opposite to that of direct interactions and the effects destructively interfere. Consequently, cross peaks resulting from indirect ROE interactions generally are much weaker than the corresponding indirect NOE cross peaks, and they can be identified by their sign (*10, 12*). To date, the ROESY experiment has been used primarily to study the conformation of intermediate-sized molecules for which $\omega\tau_c \sim 1$, where ω is the angular ^{1}H Larmor frequency and τ_c is the rotational correlation time. In this τ_c range, the NOE buildup rates are near zero and the ROESY experiment offers much better sensitivity than NOESY. However, even for macromolecules, ROESY experiments can give high-quality spectra (*13–15*), although the maximum obtainable cross-peak intensity tends to be lower compared to NOESY by ~30% (*14*). Here we propose to use transferred ROESY for studying the conformation of a ligand bound to a macromolecule as this method permits unambiguous identification of indirect effects. The pulse sequence can be used in an unmodified form (*10, 16*) and, in contrast to transferred NOESY, no special filter (*17*) is needed to attenuate the intensity of the macromolecule resonances because the ROESY mixing time generally is much longer than the $T_{1\rho}$ of the macromolecule protons.

Experiments are demonstrated for a sample containing 5 m*M* methyl *O*-β-D-galactopyranosyl-(1,6)-4-deoxy-2-deuterio-4-fluoro-β-D-galactopyranoside (*18*) and 130 μ*M* Fab′, derived from the monoclonal antibody IgA X24, which is a prototype of the anti-(1,6)-β-D-galactopyranan antibody family (*19*). The above ligand was previously shown to bind IgA X24 with an affinity identical to that of the (nondeuterated, 4-OH) disaccharide. The sample contained phosphate-buffered saline (150 m*M*), pH 7.4, and all experiments were carried out on a Bruker AMX-600 spectrometer, at a temperature of 45°C. The delay time between scans was 2.5 s in all experiments. A ROESY reference spectrum has been recorded for a sample containing 12 m*M* of the disaccharide, in the absence of Fab′. Except for a NOESY spectrum optimized for detecting Fab′–sugar NOE interactions, all 2D spectra result from 300* × 1024* data matrices (*n** refers to *n* complex points), with acquisition times of 90 ms (t_1) and 106 ms (t_2). The carrier was positioned at 6 ppm, and for the ROESY experiments a spin-lock field strength of 4 kHz was used, with both parameters chosen such that Hartmann–Hahn effects are minimized (*16*). Signal averaging and phase cycling were executed after completing a full cycle (using two scans per complex t_1 increment) for the entire 2D matrix. This method of averaging reduces diagonal-peak distortion which otherwise results from very small instabilities in sample temperature and RF electronics that occur on a time scale of hours or longer.

The TRNOE spectrum was recorded by coadding the results of four mixing times of 300, 301, 305.2, and 306.2 ms (in order to minimize zero-quantum artifacts (*20*)). In order to attenuate the background of antibody resonances in the TRNOE spectrum, the pulse scheme includes a short (25 ms) $T_{1\rho}$ filter (γB_1 = 10 kHz) immediately prior to the t_1 evolution period (*17*). The ROESY spectra have been recorded using a single mixing time of 150 ms.

Figure 1 shows the TRNOE spectrum obtained for the disaccharide in the presence of Fab′. The spectrum is very similar to that shown previously (*11*) for a sample containing the disaccharide with the intact antibody, X24. In that study,

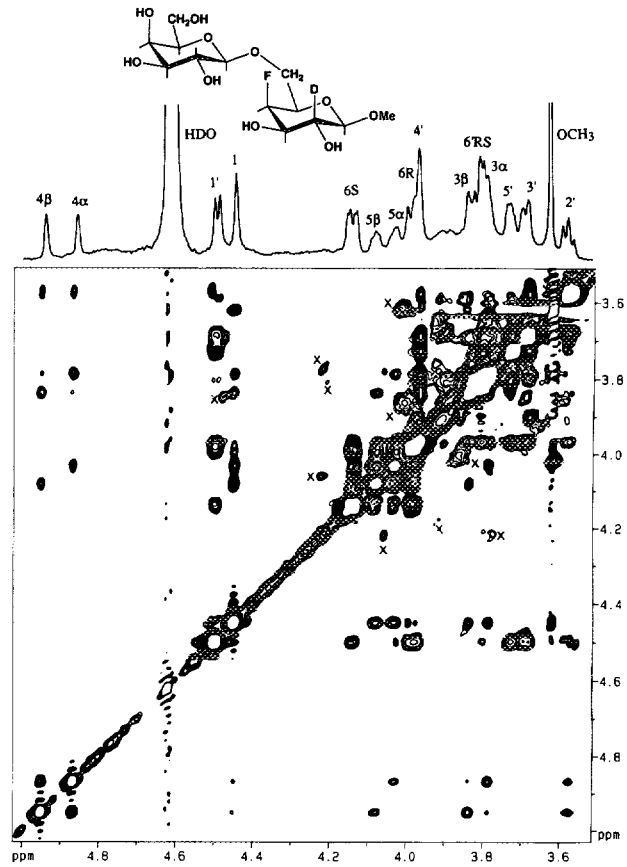

FIG. 1. Transferred NOE spectrum of 5 m*M* of the disaccharide, shown in the inset, in the presence of IgA X24 Fab′ (0.129 m*M*), recorded at 600 MHz. The average mixing-time duration was 303 ms, and a 25 ms spin-lock filter (*17*) was used for minimizing Fab′ signals. Along the top is shown the 1D spectrum corresponding to the first t_1 increment in the TRNOE spectrum, but in the absence of HDO presaturation. The labels α and β refer to the spin state of the fluorine which has a large *J* coupling to H3, H4, and H5. Several cross peaks that represent intramolecular Fab′ NOE interactions which have not completely been removed by the spin-lock filter are marked with a ×. Total measuring time, 22 h. Protons on the O-methylated galactose are H1–H6; protons on the other galactose residues are H1′–H6′.

some intraresidue spin diffusion in the TRNOE had been noted which, owing to the slow dissociation rate (approximately 100 s^{-1}), occurred during a single bound cycle. Due to the ~3 times smaller size of the Fab' relative to the intact antibody, these indirect effects (for example, between H1 and H4) are much weaker in the present case. The interresidue NOE between H4 and H2' has an intensity similar to what was observed in the earlier study, however. This cross peak had been interpreted as an indication of a relatively short distance between these two protons, requiring an unusual conformation of the glycosidic linkage (*11*). Intramolecular spin diffusion can safely be excluded as H4 and H2' do not share a third proton to which both exhibit NOEs, and the similar relative intensity of this cross peak in the case of the Fab' and the antibody confirms this conclusion.

Figure 2A shows the 150 ms ROESY spectrum of the free disaccharide and Fig. 2B is the spectrum recorded in the presence of Fab'. Except for a few additional peaks in the transferred ROESY spectrum, which correspond to the few highly mobile sites on the Fab' with long $T_{1\rho}$ values, the two ROESY spectra qualitatively are very similar. Quantitative analysis shows, however, that the cross peak to diagonal peak ratio is, on average, approximately 3 times higher in the spectra recorded in the presence of Fab'. The most important difference between the transferred ROESY spectrum of Fig. 2B and the TRNOE spectrum of Fig. 1 is found for the interresidue cross peak between H4 and H2'. In both spectra, the resonance of H4 shows a 50 Hz splitting caused by J coupling to its geminal ^{19}F, and two reasonably intense components of the H4/H2' doublet cross peak can be seen at each side of the diagonal in the TRNOE spectrum (Fig. 1). In contrast, in the transferred ROESY spectrum (Fig. 2B) these cross peaks are of nearly vanishing intensity. At the contour level shown, only the H4/H2' cross peak below the diagonal is visible, and most importantly, its sign is the same as that for the diagonal resonances and opposite relative to that of direct ROE cross peaks. This indicates that this cross peak is dominated by an indirect interaction. Its intensity is 0.14% relative to the H4 diagonal doublet. As discussed above, the indirect NOE interaction between H4 and H2' cannot be relayed by any of the disaccharide protons and therefore must be due to a Fab' proton which is proximate to both H4 and H2'. Considering the relatively high intensity of the H4/H2' cross peak in the TRNOE spectrum, the protein proton that serves as their relay partner must have only little leakage to the rest of the protein; i.e., it must be relatively remote from other protein protons.

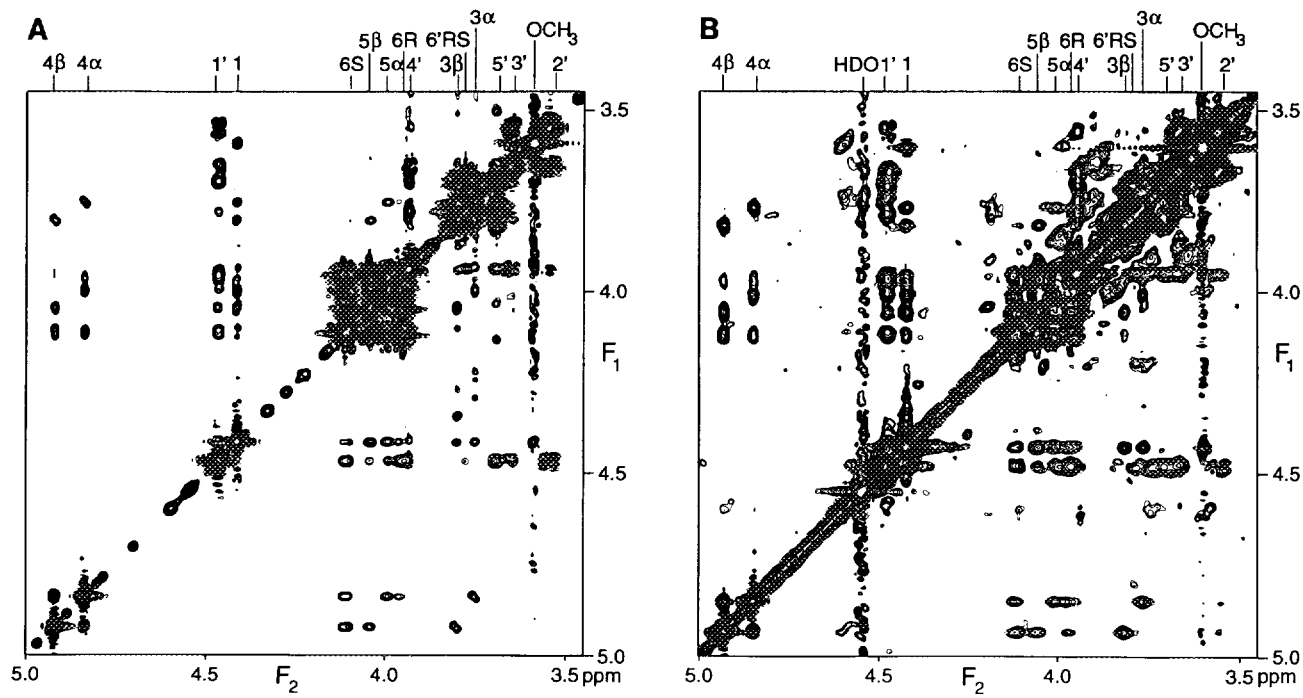

FIG. 2. ROESY spectra (600 MHz, 150 ms mixing time) of the disaccharide (A) in the absence and (B) in the presence of 0.129 mM Fab'. Except for the number of transients per t_1 increment, the spectra have been recorded identically. Spectra were processed nearly identically, using $\sin^{1.5}[\phi + (179° - \phi)t_1/AT_1]$ and $\sin^2[\phi + (177° - \phi)t_2/AT_2]$ digital filtering in the t_1 and t_2 dimensions, respectively, with $\phi = 85°$ for spectrum (A) and $\phi = 76°$ for (B), where AT_1 and AT_2 are the acquisition times in the t_1 and t_2 dimensions. Both spectra are zero filled to a digital resolution of 4.8 Hz (F_2) and 3.3 Hz (F_1). The carrier was positioned at 6 ppm and the strength of the ROESY spin-lock field was 4 kHz. Thick and thin contour lines correspond to negative and positive intensity, respectively. The weak cross peak between 4α/β and H2' (right-hand bottom of (B)) has the same sign as the diagonal resonances, indicating it is caused by an indirect ROE effect. Total measuring times were 11.5 h (A) and 36 h (B).

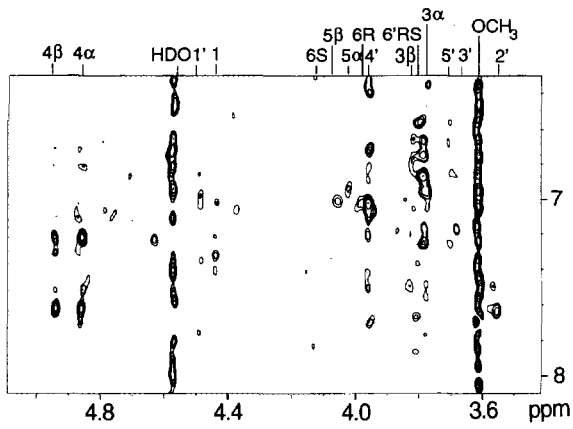

FIG. 3. Small region of the 600 MHz NOESY spectrum (30 ms mixing time) of the disaccharide (5 m*M*) in the presence of 0.129 m*M* Fab', optimized for detection of intermolecular NOEs. The pulse sequence included a 20 ms spin-echo filter immediately prior to data acquisition for suppressing Fab' signals in the F_2 dimension of the 2D spectrum. Total measuring time, 16 h.

To identify which type of proton serves as the relay agent, a NOESY spectrum was recorded which had been optimized to observe intermolecular NOEs, using a short acquisition time in the t_1 dimension, a short (30 ms) NOE mixing time, and a 20 ms spin-echo filter immediately prior to acquisition for attenuating Fab' resonances in the detected dimension. A small region of this spectrum is shown in Fig. 3, which indicates that both H4 and H2' show an NOE interaction to a proton at 7.63 ppm, and a weaker interaction to a proton at 7.51 ppm. H4 also shows a strong NOE interaction with a third protein proton at 7.23 ppm, which does not exhibit an NOE to H2'. As the sample is dissolved in D_2O, the observed protein protons all must be nonexchangeable and their chemical shifts point to aromatic ring protons. Indeed, the Fab' binding pocket contains numerous aromatic rings (*21*) and it is therefore not surprising to find an aromatic protein proton that is proximate to both H4 and H2'.

The present work confirms recent reports which indicate that spin diffusion via the protein can play a significant role in transferred NOE studies. In cases where it is unclear whether an observed TRNOE effect is direct or not, transferred ROESY can answer this question. Disadvantages of transferred ROESY over the TRNOE experiment are its somewhat lower sensitivity and the fact that cross relaxation in the free ligand cannot be neglected and must be measured in a separate experiment before transferred ROESY data can be addressed in a quantitative manner.

ACKNOWLEDGMENTS

We thank Dennis Torchia and Laura Lerner for stimulating discussions and Andy C. Wang for useful suggestions during the preparation of this manuscript. This work was supported by the AIDS Targeted Anti-Viral Program of the Office of the Director of the National Institutes of Health.

REFERENCES

1. J. P. Albrand, B. Birdsall, J. Feeney, G. C. K. Roberts, and A. S. V. Burgen, *Int. J. Biol. Macromol.* **1,** 37 (1979).
2. G. M. Clore and A. M. Gronenborn, *J. Magn. Reson.* **48,** 402 (1982).
3. G. M. Clore and A. M. Gronenborn, *J. Magn. Reson.* **53,** 423 (1983).
4. N. H. Andersen, H. L. Eaton, and K. T. Nguyen, *Magn. Reson. Chem.* **25,** 1025 (1987).
5. S. B. Landy and B. D. N. Rao, *J. Magn. Reson.* **81,** 371 (1989).
6. A. P. Campbell and B. D. Sykes, *J. Magn. Reson.* **93,** 77 (1991).
7. F. Ni, *J. Magn. Reson.* **96,** 651 (1992).
8. R. E. London, M. E. Perlman, and D. G. Davis, *J. Magn. Reson.* **97,** 79 (1992).
9. J. Zheng and C. B. Post, *J. Magn. Reson. B* **101,** 262 (1993).
10. A. A. Bothner-By, R. L. Stephens, J. Lee, C. D. Warren, and R. W. Jeanloz, *J. Am. Chem. Soc.* **106,** 811 (1984).
11. C. P. J. Glaudemans, L. E. Lerner, D. G. Daves, Jr., P. Kovac, R. Venable, and A. Bax, *Biochemistry* **29,** 10,906 (1990).
12. A. Bax, V. Sklenar, and M. F. Summers, *J. Magn. Reson.* **70,** 327 (1986).
13. A. Bax, V. Sklenar, A. M. Gronenborn, and G. M. Clore, *J. Am. Chem. Soc.* **109,** 6511 (1987).
14. C. J. Bauer, T. A. Frenkiel, and A. N. Lane, *J. Magn. Reson.* **87,** 144 (1990).
15. G. M. Clore, A. Bax, and A. M. Gronenborn, *J. Biomol. NMR* **1,** 13 (1991).
16. A. Bax and D. G. Davis, *J. Magn. Reson.* **63,** 207 (1985).
17. T. Scherf and J. Anglister, *Biophys. J.* **64,** 754 (1993).
18. G. D. Daves, Jr., P. Kovac, and C. P. J. Glaudemans, *Carbohydr. Chem.* **9,** 101 (1990).
19. C. P. J. Glaudemans, *Chem. Rev.* **91,** 25 (1991).
20. M. Rance, G. Bodenhausen, G. Wagner, K. Wüthrich, and R. R. Ernst, *J. Magn. Reson.* **62,** 497 (1985).
21. S. W. Suh, T. N. Bhat, A. M. Navia, G. H. Cohen, D. N. Rao, S. Rudikoff, and D. R. Davies, *Proteins Struct. Funct. Genet.* **1,** 74 (1986).