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

S. Rao Arepalli, Cornelis P. J. Glaudemans, G. Doyle Daves, Jr., Pavol Kovac, and Ad Bax

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, \( \sigma_b \). 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 (9), 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, \( k_{ab} \), is much larger than the difference, \( \delta \), in \( ^1H \) 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, \( \sigma_b \). 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 \( \sigma_{B(3 \lambda)} \). 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 \( 1/\sigma_{B(3 \lambda)} \). 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 (\( p_b + p_t = 1 \), where \( p_t \) 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 \( \sigma_{B(3 \lambda)} \). 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 attend-
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 \( \omega \) is the angular \(^1\text{H}\) Larmor frequency and \( \tau_c \) is the rotational correlation time. In this \( \tau_c \) 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 \( \sim 30\% \) (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 mM methyl \( O-\beta-D\)-galactopyranosyl-(1,6)-4-deoxy-2-deuterio-4-fluoro-\( \beta-D\)-galactopyranoside (18) and 130 \( \mu \)M Fab', derived from the monoclonal antibody IgA X24, which is a prototype of the anti-(1,6)-\( \beta-D\)-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 mM), 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 mM 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 \( 300^* \times 1024^* \) 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 instabil-

ities 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 (\( \gamma B_1 = 10 \) 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.

![Figure 1](image-url)
some intrar residue 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). Intra- molecular 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 T1p 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 19F, 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 t2 increment, the spectra have been recorded identically. Spectra were processed nearly identically, using sin1/2(φ + (179° - φ)/AT1) and sin1/2(φ + (177° - φ)/AT2) digital filtering in the t1 and t2 dimensions, respectively, with φ = 85° for spectrum (A) and φ = 76° for (B). Where AT1 and AT2 are the acquisition times in the t1 and t2 dimensions. Both spectra are zero filled to a digital resolution of 4.8 Hz (F1) and 3.3 Hz (F2). 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).
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