A $T_{1\rho}$-filtered two-dimensional transferred NOE spectrum for studying antibody interactions with peptide antigens

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ABSTRACT Transferred nuclear Overhauser effect (TRNOE) spectroscopy can be used to study intra- and intermolecular interactions of bound ligands complexed with large proteins. However, the 2D NOE (NOESY) spectra of large proteins are very poorly resolved and it is very difficult to discriminate the TRNOE cross peaks, especially those due to intermolecular interactions, from the numerous cross peaks due to intramolecular interactions in the protein. In previous studies we measured two-dimensional difference spectra that show exclusively TRNOE and exchange cross-peaks (Anglister, J., 1990. *Quart. Rev. Biophys.* 23:175–203). Here we show that a filtering method based on the difference between the $T_{1\rho}$ values of the ligand and the protein protons can be used to directly obtain a two-dimensional transferred NOE spectrum in which the background cross-peaks due to intramolecular interactions in the protein are very effectively removed. The usefulness of this technique to study protein ligand interactions is demonstrated for two different antibodies complexed with a peptide of cholera toxin (CTP3). It is shown that the $T_{1\rho}$-filtering alleviates the problems encountered in our previous measurements of TRNOE by the difference method. These problems were due to imperfections in the subtraction of two spectra measured for two different samples.

INTRODUCTION

One of the most fascinating areas of investigation in structural biology today is the understanding of the specific binding of ligands to their receptor proteins. Especially intriguing is the capability of the immune system to raise highly specific antibodies against virtually any foreign substance. To understand how changes in the primary structure of proteins modulate their specificities we need to know the three-dimensional structure of complexes between proteins and their ligands at atomic resolution. The only techniques that can be used for this purpose are x-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy.

Current NMR techniques enable us to determine the complete three-dimensional structure of relatively small proteins, up to a molecular weight of about 30,000 D (2, 3). However, there are NMR methods that use ^{13}C, ^{15}N or ^{2}H isotope substituted ligands and isotope editing or difference spectra measurements to study the interactions of ligands with larger proteins and intramolecular interactions in bound ligands (4–6). Unfortunately, even these techniques suffer from poor signal-to-noise ratios as the molecular weight of the protein increases. Moreover, as the T_2 relaxation time of tightly bound ligand protons is comparable to that of the protein protons, common sequential assignment techniques that use coherence transfer, are not applicable for ligands complexed with large proteins and the assignment may require expensive specific labeling of the ligand (7).

TRNOE spectroscopy (8–12) is a very effective technique for studying *intramolecular* interactions in bound ligands if their lifetime in the bound state is considerably shorter than the proton T_1 relaxation times in the bound and free ligand, and the mixing period of the NOESY experiment. TRNOE measurements alleviate many of the problems encountered with the isotope edited methods as they detect intramolecular interactions in the bound ligand through cross-peaks between the free ligand protons. Therefore the analysis of these interactions requires only the assignments of the free ligand resonances. The measurements of intramolecular TRNOE use very large excess of the ligand and low protein concentration to discriminate the intra-ligand NOE from cross-peaks due to intramolecular interactions in the protein. The intramolecular NOE interactions in the free form of the ligand usually do not interfere with the TRNOE measurements, as the free ligand which is much smaller than the protein is characterized by a short correlation time ($\omega\tau_c \leq 1$) for which the NOE is positive or approaches zero while the TRNOE is negative like the intramolecular NOE in the protein. The conformation of bound ligands complexed with big proteins such as whole antibodies (13) and acetylcholine receptor (14), has been studied using TRNOE.

Only few studies have employed TRNOE spectroscopy to observe *intermolecular* interactions between proteins and their ligands (15–17) as it was difficult to discriminate between intermolecular TRNOE cross-peaks and the numerous cross-peaks due to intramolecular interactions in the protein. While intramolecular interactions in a bound ligand are manifested by cross-peaks between the narrow resonances of its free form that are usually very well resolved from the numerous broader and much weaker cross-peaks due to intramolecular interactions in the protein, intermolecular interactions are evident by cross-peaks between the broad resonances of the protein protons and the sharp resonances

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of the ligand protons and are usually weaker than the intra-ligand TRNOE cross-peaks. This makes them more comparable in intensity and linewidth to the NOEs of the protein. To extract the intermolecular TRNOE cross-peaks from the background, we previously used two dimensional (2D) difference spectroscopy to study the interactions of Fab fragments of three antibodies with their peptide antigen (18, 19). The difference between the NOESY spectrum of the Fab in the presence of peptide excess and that of the peptide saturated Fab revealed only transferred NOE and exchange cross-peaks. These interactions were assigned to their corresponding protons using specific deuteration of the antibody and a predicted model for its binding site structure (20, 21). Although the difference method was successful in elucidating the intermolecular interactions between the aromatic amino acids of the three antibodies and the peptide antigen, they were quite cumbersome and required good matching of spectra measured for two different samples.

The fact that the T_2 relaxation times of the free peptide protons are much longer than those of the protein protons suggests that filtering techniques could be used to simplify the spectra and discern TRNOE from the numerous intramolecular NOE cross-peaks of the protein. Recently, Glaudemans et al. (13) used a T_2-filtering technique to remove the diagonal and the protein cross-peaks in transferred NOE measurements of intramolecular interactions in a carbohydrate molecule bound to an antibody. No intermolecular TRNOE cross-peaks were observed in this study. Here we present direct measurements of intermolecular transferred NOE interactions in Fab-peptide complexes. The technique is based on $T_{1\rho}$-filtering using a spin-lock pulse before the evolution period in a NOESY experiment, such that the broad resonances of the protein protons decay before the evolution period. In most cases this technique is more powerful than the T_2-filtering technique (13) for both inter- and intramolecular TRNOE measurements.

MATERIALS AND METHODS

Two anti-CTP3 antibodies, TE33 and TE34, were purified from the supernatant of hybridoma cells (17). In all measurements antibody Fab fragments were used. Fab labeling and preparation, the synthesis and purification of the peptides CTP3 (VEVPGSQHIDSQKKA) and CTP3-amide (VEVPGSQHIDSQKKA-NH_2), and the procedure for NOESY measurements and difference spectra calculations were described previously (18, 19). CTP3-amide was used with TE34 to get a faster off-rate (19). Fab concentrations were between 2.5 to 3.0 mM in 0.01 M phosphate-buffered D_2O. Spectra were measured on a Bruker AM500 spectrometer (Bruker, Karlsruhe, Germany) at 315°K and 310°K for TE33 and TE34, respectively.

In order to calculate the 2D TRNOE difference spectrum, NOESY spectra of the TE33/CTP3 complex were measured with a mixing time, τ_m, of 100 ms and at pH = 7.15. One spectrum was measured with equimolar concentrations of Fab and peptide and a second spectrum was measured with a Fab solution containing a 4-fold peptide excess. It is important that the pH and salt concentration are exactly the same for the two samples and that the addition of the peptide causes only a minimal dilution of the Fab solution. For each of the two NOESY spectra 64 scans of 2K data points in t_2 were collected for 256 t_1 increments.

The $T_{1\rho}$ relaxation times of the protons of Fab, free peptide and free peptide in the presence of Fab were measured using the pulse sequence:

$$90^\circ_x - SL_y - Acq_x$$

and following the decrease in resonance intensity as a function of the duration of the spin-lock pulse.

A $T_{1\rho}$-filtered NOESY spectrum was measured using the following pulse sequence:

$$90^\circ_{\phi 1} - SL_{\phi 2} - t_1 - 90^\circ_{\phi 3} - \tau_m - 90^\circ_{\phi 4} - Acq.$$

with phases: $\phi 1 = x, -x$; $\phi 2 = y, -y$; $\phi 3 = 8(x), 8(-x)$; $\phi 4 = 2(x), 2(-x), 2(y), 2(-y)$; Acq $= x, 2(-x), x, y, 2(-y), y, -x, 2(x), -x, -y, 2(y), -y$. The transmitter in a low power mode was used for both the 90° pulses and the spin-lock pulse. The 90° pulse duration was 72–78 μsec. A 10-fold excess of CTP3 and a 15-fold excess of CTP3-amide was used with TE33 and TE34, respectively. This excess is larger than that used for the 2D TRNOE difference spectra measurements in order to get better discrimination between the peptide and protein resonances on the basis of their $T_{1\rho}$ relaxation times. Under these conditions a 20 ms spin-lock pulse was found to be long enough to remove the Fab resonances without considerably affecting the peptide resonances. For TE33 the mixing time was 70 ms, and 96 scans of 2K data points were recorded for each of 140 t_1 increments. The spectra were measured at pH 7.15 and at 315°K.

The $T_{1\rho}$-Filtered NOESY spectra of TE34 in the presence of excess CTP3-amide were measured with a mixing period of 100 ms. For each of 256 t_1 increments, 64 or 80 scans with 2K data points in t_2 were collected. Two differently labeled TE34 Fab were prepared: in one of them all tyrosine and phenylalanine residues were perdeuterated, and in the other, all tryptophan and phenylalanine residues were perdeuterated while tyrosine residues were deuterated at $C_{\delta 1}$ and $C_{\delta 2}$ positions leaving $C_{\epsilon 1}H$ and $C_{\epsilon 2}H$ unlabeled. The spectrum of the first sample was measured at pH 8 while the second one was measured at pH 6.0. These pH values were chosen to prevent an overlap between the resonances of the histidine imidazole protons of the free peptide and the resonances of Fab protons interacting with the peptide. In all measurements continuous selective irradiation during the preparation time was used to presaturate the solvent.

RESULTS AND DISCUSSION

The $T_{1\rho}$ relaxation times of the protons of TE33 Fab, free CTP3, and free CTP3 in the presence of TE33 were measured. Because of a poor resolution in the one-dimensional NMR spectrum of the Fab, its measured $T_{1\rho}$ values represent an averaging of several resonances. The $T_{1\rho}$ relaxation time of the Fab protons varies from 8–12 ms, while the $T_{1\rho}$ value of the free peptide protons varies between 130–500 ms, except His $C_{\delta 1}$ and $C_{\epsilon 1}$ protons with $T_{1\rho}$ of approximately 1 s. The protons of the free peptide in the presence of the Fab can be divided into two groups: *a*) protons that are in the epitope recognized by the antibody and their $T_{1\rho}$ is slightly lower than that of the same protons of the free peptide in the absence of the Fab. For most of them $T_{1\rho}$ is ~100 ms, except for $C_{\delta 1}$ and $C_{\epsilon 2}$ of His 8, which have a $T_{1\rho}$ of approximately 500 ms. *b*) protons that are outside the epitope, retain their mobility after binding and their $T_{1\rho}$ is comparable to that of the peptide protons in the absence of the Fab.

The 10-fold difference in the $T_{1\rho}$ relaxation times between the Fab and the peptide protons enabled us to use a spin-lock pulse after the first 90° pulse to destroy both transverse and longitudinal magnetization of the protein protons (i.e., they become "saturated") without considerably affecting the free peptide resonances. Therefore, no protein magnetization is labeled during the subsequent evolution period, t_1, and it is not effected by the second 90° pulse. During the mixing period the magnetization of the protein protons partially relax to equilibrium due to cross-relaxation with the peptide and other protein protons. The partially recovered longitudinal magnetization, M_z, is rotated to the y axis after the third 90° pulse and it is detected during the acquisition period of the individual FIDs. As this magnetization has not been labeled during t_1 it could potentially appear as axial cross-peaks ($\omega_{F1} = 0$) which are canceled out by the phase cycle of the NOESY experiment (22).

In the absence of exchange, the fate of the bound peptide magnetization is the same as the protein magnetization. However in the presence of exchange the bound peptide magnetization, in the direction of the effective field is partially replenished by that of the free peptide which decays at a much slower rate. Based on McConnell's (23) modifications of the Bloch equations for chemical exchange, bringing into account the presence of the spin-lock RF field and neglecting cross-relaxation terms, the evolution of magnetization of the bound and free peptide and the antibody protons (iB, iF, and jA, respectively) in the direction of the effective field is described by the following coupled equations:

$$dM_{iB}/dt = -M_{iB}/T^0_{1\rho,iB} + k'_1 M_{iF} \cos\phi - k_{-1} M_{iB}; \quad (1a)$$

$$dM_{iF}/dt = -M_{iF}/T^0_{1\rho,iF} + k_{-1} M_{iB} \cos\phi - k'_1 M_{iF}; \quad (1b)$$

$$dM_{jA}/dt = -M_{jA}/T^0_{1\rho,jA}. \quad (1c)$$

Where M_{iB}, M_{iF}, and M_{jA} are the magnetizations of the iB, iF, and jA protons, respectively, in the direction of the effective field experienced by them, $T^0_{1\rho,iB}$, $T^0_{1\rho,iF}$, and $T^0_{1\rho,jA}$ are the intrinsic longitudinal relaxation times of the iB, iF, and jA protons in the rotating-frame in the absence of exchange, and ϕ is the angle between the vectors of the different effective fields experienced by iB and iF as a result of having different chemical shifts offsets, δ_{iB} and δ_{iF}, respectively, relative to the carrier frequency of the spin-lock pulse, k_{-1} and k'_1 are the off-rate and the pseudo on-rate, respectively ($k'_1 = k_1$ [Fab], where k_1 is the reaction on-rate and [Fab] is the equilibrium free Fab concentration). In the presence of a large peptide excess, and when the binding constant $K_b > 10^4\ M^{-1}$ and the Fab concentration is in the mM range, all the Fab is practically bound and the free Fab concentration and its magnetization can be neglected. The fact that the direction of the effective field deviates from the axis of the spin-lock pulse SL_y will cause some minor reduction in the observed transverse magnetization (a factor of $\sin^2\theta$ where $\theta = \text{arctg}(\nu/\delta)$). Under a strong spin-lock field ($\nu \gg \delta$) $\sin^2\theta \approx 1$ and therefore this type of loss in the observed transverse magnetization is negligible. Similarly, under this condition, $\cos\phi$ in Eqs. 1a and 1b is also approximated by 1 and therefore each exchange event will not cause any loss of coherence.

In the limit of a strong spin-lock pulse and a fast exchange, $k_{-1} \gg \delta_{iF} - \delta_{iB}$, when averaging of the bound and free peptide resonances occurs, the contribution of the exchange to the relaxation rate is canceled out and one obtains for the averaged longitudinal relaxation time of peptide proton i in the rotating-frame:

$$1/T_{1\rho,iF} = 1/T_{1\rho,iB} = (N_F/T^0_{1\rho,iF}) + (N_B/T^0_{1\rho,iB}) \quad (2)$$

where N_F and N_B are fractions of the peptide population in the free and bound states, respectively. It can be shown, by solving the coupled differential Eqs. 1a and 1b that the relationship in Eq. 2 holds also for the hypothetical case in which k_{-1}, $k'_1 \gg 1/T^0_{1\rho,iB}$, $1/T^0_{1\rho,iF}$ and the exchange is slow relative to the change of chemical shift of peptide proton i upon binding.

In the limit of slow exchange-rate relative to both chemical shift difference and the reciprocals of the relaxation times, $k_{-1} \ll \delta_{iF} - \delta_{iB}$ and $k_{-1} \ll 1/T^0_{1\rho,iF}$; $1/T^0_{1\rho,iB}$, there is no averaging of the relaxation times and the free and bound peptide are characterized by their intrinsic relaxation times $T^0_{1\rho,iB}$ and $T^0_{1\rho,iF}$.

To compare the $T_{1\rho}$-filter to the T_2-filter, the respective relaxation times have to be compared. In fast exchange when averaging of the bound and free resonances occurs and in the limit when the averaged resonances are narrow:

$$1/T_{2,i} = (N_F/T^0_{2,iF}) + (N_B/T^0_{2,iB}) \quad (3)$$

where $T^0_{2,iF}$ and $T^0_{2,iB}$ are the intrinsic transverse relaxation times of protons iF and iB without exchange. This relationship is analogous to that obtained for $T_{1\rho}$ under the same conditions. For fast exchange rate relative to the reciprocals of the transverse relaxation times, k_{-1}, $k'_1 \gg 1/T^0_{2,iF}$, $1/T^0_{2,iB}$ however slow relative to the change in chemical shift upon binding we get:

$$1/T_{2,iF} = [1/T^0_{2,iF}] + N_B/N_F\tau_B \quad (4)$$

where $\tau_B = 1/k_{-1}$. Comparison of Eqs. 2 and 4 shows that in case $\tau_B \ll T^0_{1\rho,iB}$ and $N_F\tau_B/N_B \ll T^0_{2,iF}$, the transverse relaxation time of the free peptide, $T_{2,iF}$ is much shorter than its longitudinal relaxation time in the rotating frame under the same conditions. The underlying reason for the different behavior of T_2 and $T_{1\rho}$ is that in the absence of a spin-lock field and for exchange-rates slower than those leading to narrowing of the resonance which is the average of the bound and the free form, each exchange event results in a destruction of coherence, while with a strong spin-lock field the magnetization conserves its coherence in each exchange process.

The second 90° pulse of the NOESY experiment, applied after the evolution period, inverts the magnetiza-

tion of the free peptide and the remaining bound peptide magnetization along the z axis. If the peptide exchange-rate is fast relative to the reciprocals of both the T_1-cross-relaxation-rate and the NOESY mixing period, then during the mixing the free peptide molecules exchange with bound molecule. In the bound state the peptide protons transfer magnetization to proximal protein protons and bound peptide protons. The transferred magnetization due to intermolecular interactions is then detected during the acquisition period after a third 90° pulse converts the z-magnetization into an observable transverse magnetization. The observation of TRNOE due to intramolecular interactions in the bound ligand requires a second exchange between bound and free peptide. Detailed treatments of the 1D and 2D TRNOE were given by Clore and Gronenborn (10, 11) and by Campbell and Sykes (12).

The measured $T_{1\rho}$-filtered spectra should contain symmetrical TRNOE cross-peaks due to intramolecular interactions in the bound peptide, and asymmetrical cross-peaks due to TRNOE from free peptide to protein protons. The exchange cross-peaks contributed by peptide molecules that are in the free state during the evolution period and are bound during the acquisition period (t_2) will be stronger than those contributed by molecules that are in the bound state during the evolution period as the magnetization of the later is partly attenuated by the spin-lock pulse. The exact ratio between the intensities of the symmetrical exchange cross peaks will depend on how fast the exchange rate is relative to the longitudinal relaxation rate of the bound peptide protons in the rotating frame. Since the peptide rotational correlation time fulfills the relationship $\omega\tau_c \approx 1$, the intramolecular NOE in the free molecule vanishes under the experimental conditions used. In principle, the filtered spectra could contain also NOE cross-peaks between the bound peptide and the protein resonances, however the bound peptide resonances are considerably attenuated by the filtering and they are much broader than the free peptide resonances, causing these cross-peaks to be of vanishingly small intensity in comparison to the inter- and intramolecular TRNOE cross-peaks.

Fig. 1 shows the $T_{1\rho}$-filtered TRNOE spectrum of specifically deuterated preparations of TE34 Fab in the presence of a 15-fold excess CTP3-amide. Fig. 1 *A* shows a spectrum of TE34 Fab in which all the tyrosine and phenylalanine residues of the Fab were perdeuterated. It therefore shows interactions of Fab tryptophan and histidine residues with the peptide. The spectrum reveals interactions of two tryptophan protons of the Fab with Asp 10, His 8, and Ile 9 protons of CTP3-amide. These assignments are based on our previous studies using difference spectroscopy (19). Interference from t_1 noise at the chemical shift of the His 8 imidazole protons is observed in both Fig. 1, *A* and *B* (6.95 and 7.68 ppm). All cross-peaks due to intramolecular interactions in the Fab have been removed. Among these are several cross-peaks that previously appeared in the 2D TRNOE difference spectrum but did not have chemical shifts of free peptide protons (19). Fig. 1 *B* shows a spectrum of TE34 Fab in which tryptophan and phenylalanine residues of the Fab have been perdeuterated and tyrosine residues have been deuterated at $C_{\delta 1}$ and $C_{\delta 2}$ positions. Only interactions of $C_{\epsilon 1}H$ and $C_{\epsilon 2}H$ of the Fab tyrosine residues, as well as interactions of the imidazole protons of the Fab histidine residues with the peptide antigen, are therefore observed. These spectra are very similar to those previously obtained using the 2D TRNOE difference spectroscopy (19) except two symmetrical cross-peaks labeled Y that are observed in the $T_{1\rho}$-filtered spectrum but not in the 2D TRNOE difference spectrum. As the two chemical shifts of the extra cross-peak differ from the chemical shifts of the free peptide resonances, and as these cross-peaks disappear upon deuteration of the Fab tyrosine as shown in Fig. 1 *A*, they are assigned to inter-residue intramolecular interactions in the Fab involving at least one tyrosine residue. This tyrosine residue has a longer $T_{1\rho}$ relaxation time relative to the others most likely due to its increased mobility.

Fig. 2 shows a comparison between a $T_{1\rho}$-filtered 2D TRNOE spectrum (*A*) and a 2D TRNOE difference spectrum measured for TE33 Fab interacting with the peptide CTP3 (*B*). Ten-fold peptide excess was used for the $T_{1\rho}$-filtered TRNOE measurements, while only 4-fold excess was used for the difference spectrum measurements. The larger excess in the $T_{1\rho}$-filtered spectrum was used to get a better discrimination on the basis of $T_{1\rho}$ between the Fab and peptide protons. The assignments of the cross-peaks to their corresponding protons are based on our previous studies using difference spectroscopy (18, 20). All the interactions that are observed in the difference spectrum appear also in the $T_{1\rho}$-filtered spectrum, except three cross-peaks, designated Y2,h8 and that were assigned to interactions of antibody tyrosine with the peptide His 8 (3.08;6.53, 6.53;7.82, and 6.90;7.82 ppm). Almost no interference from t_1 noise is observed in the $T_{1\rho}$-filtered spectrum (Fig. 2 *A*) while some interference from t_1 noise is observed in the difference spectrum (Fig. 2 *B*). In the section of the $T_{1\rho}$-filtered TRNOE spectrum showing aromatic-aromatic proton cross-peaks we observe an interaction between antibody tryptophan and His 8 of the peptide (W1,h8). In addition, this part of the spectrum contains several cross-peaks that are not observed in the difference spectrum. As the chemical shifts of these additional cross-peaks differ from the chemical shifts of the peptide protons they are assigned to intramolecular interactions in the Fab and are contributed by antibody residues with long $T_{1\rho}$. All these cross-peaks are attributed to residues in the combining site region (Scherf, T., and J. Anglister, unpublished results).

The mixing time used in our experiments is relatively long, considering the molecular weight of the complex, and could in principle represent interference from spin

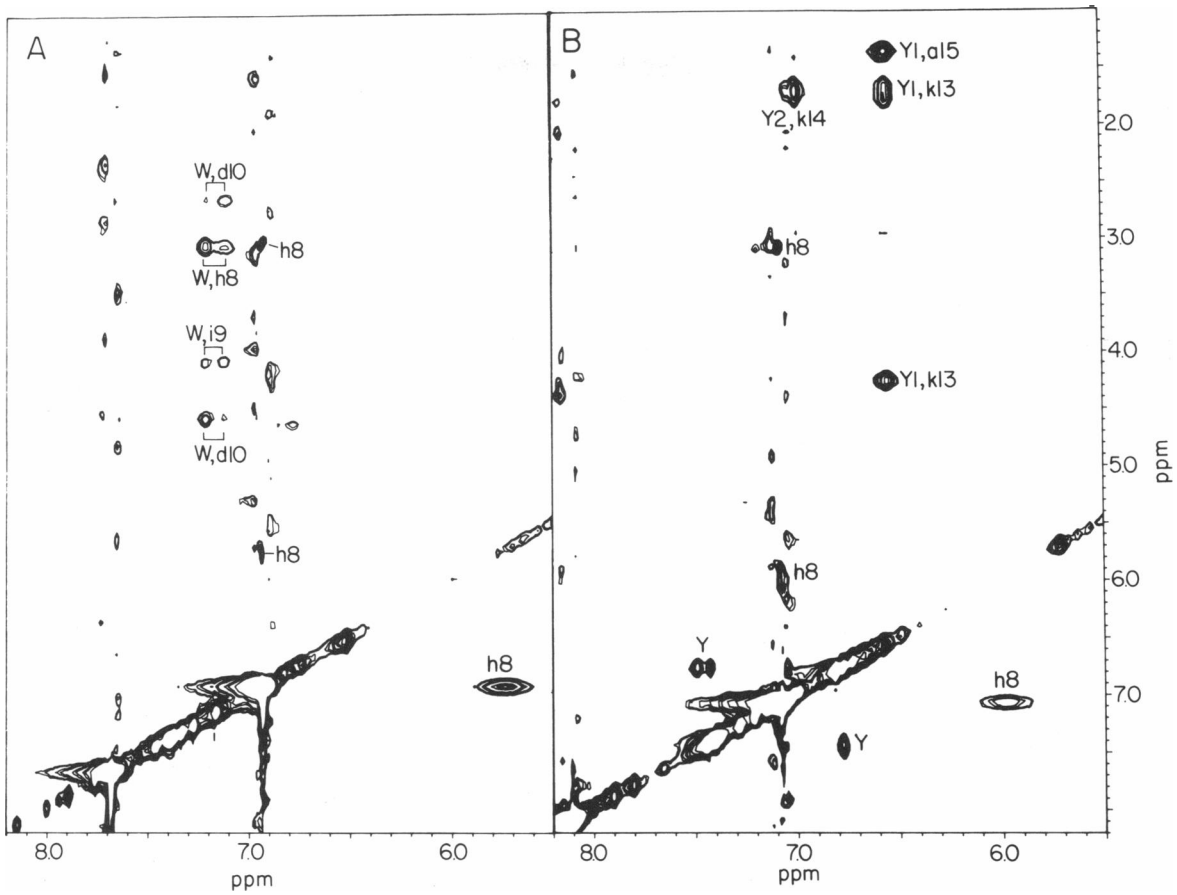

FIGURE 1 $T_{1\rho}$-filtered NOESY spectrum of specifically deuterated TE34 Fab in the presence of 15-fold excess of its peptide antigen, CTP3-amide. Measurements were carried out at 310°K with a mixing time of 100 ms and 20 ms spin lock pulse. (*A*) A spectrum of Fab showing interactions of Fab tryptophan and histidine residues with CTP3-amide measured at pH 8. All antibody phenylalanine and tyrosine residues are perdeuterated. (*B*) A spectrum of Fab showing interactions of Fab tyrosine and histidine residues with the peptide. All antibody phenylalanine and tryptophan residues are perdeuterated, while tyrosine residues are deuterated at the C_δ positions. The spectrum was measured at pH 6. Assigned Fab residues are marked by capital letters and arbitrary numbers, while peptide residues are marked by lower-case letters and numbers referring to their sequential assignment.

diffusion. However the effective mixing time for intra-ligand interactions is the actual time that a bound ligand molecule spends in the complex. For TE34 the bound time is approximately 2 ms, however, since we use only 15-fold excess of the ligand, each molecule is bound about three times during a mixing period of 100 ms and therefore the actual mixing is approximately 6 ms, for which only minimal spin diffusion in the ligand is expected. The effective mixing time experienced by the Fab protons is the actual mixing time used in the experiment and magnetization transferred from bound peptide proton to a protein proton could in principle be transferred by spin diffusion to other Fab protons. The extensive labeling of the aromatic amino acids that we have been using in our TRNOE measurements considerably limits spin diffusion pathways as the binding site of the antibody is highly aromatic. In previous studies (24) we have shown that deuteration of the tryptophan and phenylalanine residues of the Fab as well as at C_δ positions of tyrosine resulted in drastic narrowing of the linewidth of C_ϵ protons of tyrosine residues in the binding site region to values of 4–10 Hz. For TE33, the bound time is approximately 30 ms and with the excess used each molecule is bound only once during the mixing time. In principle, this could lead to some spin diffusion in the bound ligand, however it seems that the effect is only minimal, as we do not see Fab protons interacting with more than one proton of a neighboring peptide residue, except a single tryptophan proton that interacts with two protons of His 8 of the peptide.

Fig. 3 shows a comparison of ω_2-cross-sections of the difference spectrum with the cross-sections of the $T_{1\rho}$-filtered spectrum. This comparison shows that the difference spectrum suffers from imperfections in the subtraction and sometimes a distorted baseline. The $T_{1\rho}$-filtered spectrum gives a flat baseline with less interference from cross-peaks other than TRNOE cross-peaks. Almost all background signals can be eliminated by using long

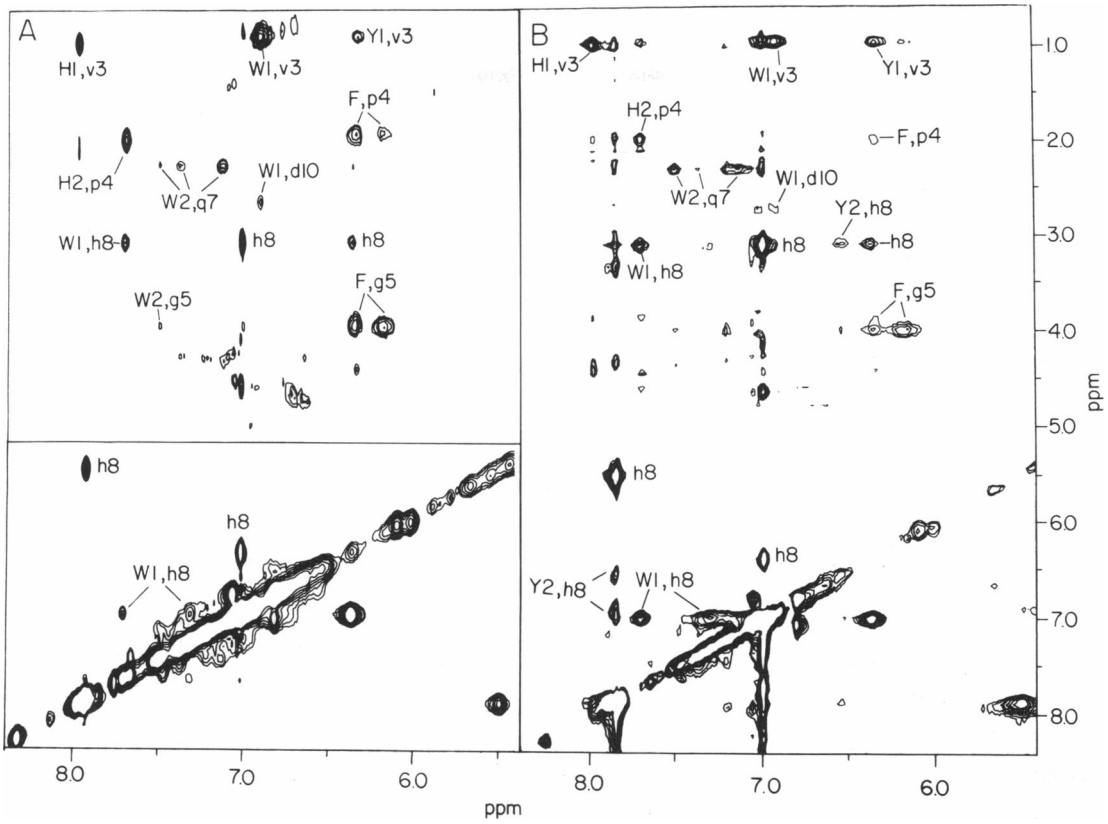

FIGURE 2 TRNOE spectra of TE33 Fab interacting with excess CTP3 showing the interactions of aromatic amino acids of the Fab with CTP3 protons as well as exchange cross-peaks due to the imidazole protons of peptide histidine (designated h8). Measurements were carried out at 315°K and pH = 7.15. (*A*) A $T_{1\rho}$-filtered NOESY spectrum of the TE33 Fab in the presence of 10-fold excess CTP3. A 20 ms spin-lock was applied and the mixing time was 70 ms. (*B*) 2D TRNOE difference spectrum of the complex. A mixing time of 100 ms was used. Assigned antibody residues are marked by capital letters and arbitrary numbers, while peptide residues are marked by lower-case letters and numbers referring to their sequential assignment.

enough spin-lock pulses. However, if one considers only the high frequency noise, then the signal-to-noise ratio in the $T_{1\rho}$-filtered spectrum is slightly worse. This happens because of the partial dephasing of the peptide resonances occurring during the application of the spin-lock pulse, while in the difference spectrum there is no loss of the signal intensity due to filtering. Thus, the efficient elimination of cross-peaks due to intramolecular interactions is achieved at the cost of reduction in the signal-to-noise ratio of the transferred NOE cross-peaks. Improved signal-to-noise can be obtained by increasing the ratio between the free and bound peptide, resulting in longer $T_{1\rho}$ relaxation time. However the increase in peptide concentration results in more severe interference from t_1 noise, which is especially bad in the upfield part of the spectrum. As a rule of thumb, in case of relatively slow exchange-rate as observed for the TE33 Fab (30 s^{-1}), we calculate a concentration for which each ligand molecule is bound once during the mixing period of the NOESY experiment and use a slightly higher concentration to lower the probability that a peptide molecule will be bound twice during the mixing period of the NOESY experiment. In case of TE34 for which the peptide off-rate is 460 s^{-1}, an excess of 15 fold is sufficient to give a good signal-to-noise ratio for the intermolecular transferred NOE cross-peaks although it is lower than the optimal excess.

The slightly lower signal-to-noise ratio in the presented $T_{1\rho}$-filtered spectra relative to the 2D TRNOE difference spectra is probably also due to the use of 3.3 kHz spin-lock field which is too weak for resonances with a large chemical shift offset from the carrier frequency of the RF field and for protons experiencing large changes in chemical shift between the bound and free states. This may explain why in the $T_{1\rho}$-filtered spectrum we do not observe the weak cross-peaks due to the interaction of an antibody tyrosine with His 8 of the peptide. It should be mentioned that the measurement time of the $T_{1\rho}$-filtered spectrum is approximately the same as the measurement time for each of the two spectra recorded for the difference spectra calculation. The slight reduction in the signal-to-noise ratio could be more than offset if the measurement time of the $T_{1\rho}$-filtered spectrum was the same as the total measurement

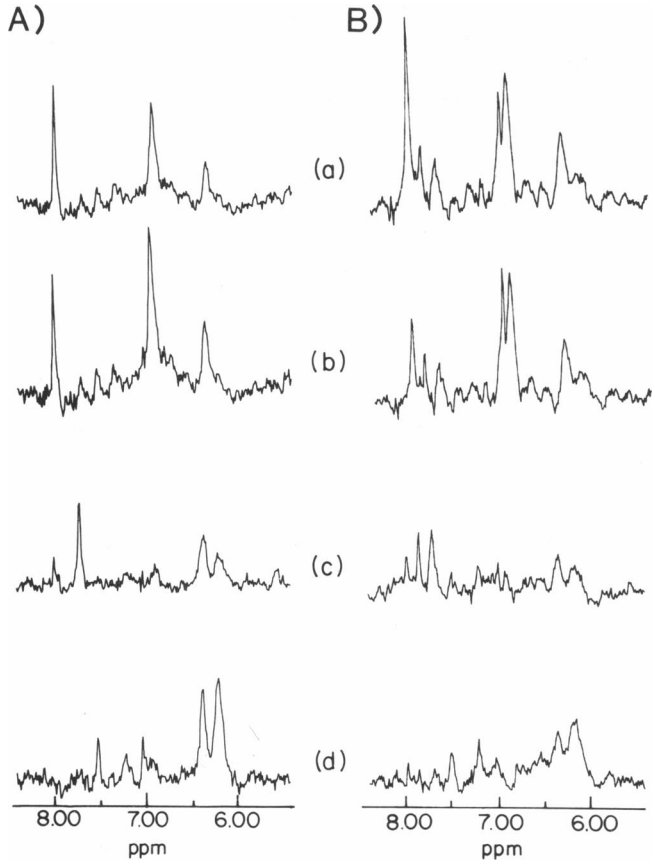

FIGURE 3 Cross sections (ω_2) of the 2D TRNOE spectra showing intermolecular interactions in TE33 Fab complex with CTP3 obtained by two different methods. Fig. 3 *A* shows cross sections of the $T_{1\rho}$-filtered NOESY spectrum (Fig. 2 *A* above), and Fig. 3 *B* shows cross sections of the 2D TRNOE difference spectrum of the same complex (Fig. 2 *B* above). (*a*) An interaction Val 3 methyl groups of the peptide with a histidine proton of the Fab designated H1,v3 in Fig. 2. (*b*) Interactions of the second methyl group of the peptide Val 3 with a tryptophan and a tyrosine proton of the Fab designated W1,v3 and Y1,v3 respectively, in Fig. 2. (*c*) Interactions of the β and γ protons of peptide Pro 4 with two phenylalanine protons of the Fab designated F,p4 and with a histidine proton of the Fab designated H2,p4 in Fig. 2. (*d*) Interactions of an α proton of the peptide Gly 5 with two antibody phenylalanine protons. This interaction is designated F,g5 in Fig. 2.

time used for obtaining the difference spectrum. In optimal conditions the $T_{1\rho}$-filtered TRNOE spectrum should give two times better signal-to-noise ratio in comparison to the 2D TRNOE difference spectrum for the same measurement time.

Nevertheless, the TRNOE difference spectroscopy was found to yield better results in the region displaying nonaromatic proton interactions (results not shown) because of more severe problems of t_1 noise in the $T_{1\rho}$-filtered spectrum caused probably by the larger excess of the peptide and by possible heating due to the spin-lock pulse. In this region we previously observed TRNOE cross-peaks due to intramolecular interactions between nonaromatic protons of the bound peptide (25). The only interaction that can be clearly observed in the present experiment is between the methyl protons of Val 3 and the Asp 10 β protons.

CONCLUSION

We have shown that the $T_{1\rho}$-filtering can be used to record 2D TRNOE spectra of protein ligand complexes that are almost completely free of cross-peaks due to intramolecular interactions in the protein. This method alleviates many of the problems encountered in difference spectra calculations and is much simpler to implement. The $T_{1\rho}$-filtering technique can be applied to study the interactions of large proteins with their ligands as long as the ligand exchange-rate is faster than the reciprocals of the T_1 relaxation time of the ligand protons, the mixing period of the NOESY experiment, and the ^{1}H-^{1}H cross relaxation rates of the bound ligand. The conservation of coherence during the exchange under a spin-lock RF field is the main advantage of our filtering technique over the T_2-filtering approach used by Glaudemans et al. The T_2-filtering is only efficient in cases where the T_2 of the protein is comparable to or shorter than the lifetime of the ligand in the bound state, and is especially inefficient when the T_2 of the protein is considerably larger than the lifetime of the ligand in the bound state, as is the case for the TE34 antibody. To prevent the decay of the free ligand magnetization in case of fast off-rate (k_{-1}) very large excess of the ligand may be required. This may not be feasible in studies of *intermolecular* interaction by TRNOE which require protein concentration of 1–3 mM.

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