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Theory of the Time Dependent Transferred Nuclear Overhauser Effect: Applications to Structural Analysis of Ligand–Protein Complexes in Solution

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The theory of the time dependent transferred nuclear Overhauser effect (TRNOE) for the generalized case of an exchanging system containing multiple spins as applied to a ligand–protein complex is presented and discussed. It is shown that cross-relaxation rates between pairs of bound ligand protons and between a bound ligand proton and a proton of the protein in the ligand–protein complex can be determined directly and with ease from the initial slopes of the time dependent TRNOEs, thus enabling distance ratios between any two such pairs of protons or, if one of the interproton distances is known, interproton distances to be calculated (on the assumption of a single correlation time for all the corresponding interproton distance vectors). By this means the conformation of a ligand bound to a protein can be determined with great precision. Because the cross-relaxation rates in the ligand–protein complex are directly proportional to the correlation time τ_c of the ligand–protein complex, this technique is particularly suitable to large proteins because its sensitivity increases as the molecular weight of the protein increases, enabling large ratios of free over bound ligand protons to be employed. This is illustrated by the determination of the conformation of NAD^+ bound to yeast alcohol dehydrogenase.

INTRODUCTION

The proton–proton transferred nuclear Overhauser effect (TRNOE) (*1*) involves the extension of nuclear Overhauser enhancement (NOE) measurements on biological macromolecules (*2–12*) to exchanging systems such as ligand–protein complexes, and has proved extremely powerful in the elucidation of the conformations of small molecules bound to proteins, particularly large ones of MW $\gtrsim$ 40,000 (*1, 13–23*). The basis of the TRNOE involves the transfer of magnetic information concerning cross-relaxation between two bound ligand protons from the bound state to the free state by chemical exchange so that negative NOEs on the easily detectable free or averaged ligand resonances may be observed following irradiation of other ligand resonances (free, bound, or averaged), thus providing information on the proximity in space of two bound ligand protons (*1*). In the presence of a protein for which $\omega\tau_c \gg 1$ (the spin diffusion limit), a negative TRNOE, $N_i(j)$, on either the free or averaged resonance of the ligand proton i will be observed following irradiation of either the free, bound, or averaged resonance of the ligand proton j providing the two conditions

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$$k > 10\rho_{iF} \quad [1]$$

and

$$|(1 - a)\sigma_{ij}^{BB}| > |a\sigma_{ij}^{FF}| \quad [2]$$

are met, where k is the chemical exchange rate between the free and bound states of the ligand, ρ_{iF} the spin–lattice relaxation rate of proton i in the free state, a the mole fraction of free ligand, and σ_{ij}^{FF} and σ_{ij}^{BB} the cross-relaxation rates between protons i and j in the free bound states, respectively (*1*).

The theory of the steady state TRNOE has been developed for a system with two spins in the bound state and two corresponding spins in the free state (*1*), and it was shown that in the steady state

$$N_i(j) \propto \sigma_{ij}^{BB} \quad [3]$$

providing the additional conditions

$$k \gtrsim 100\rho_{iF} \quad [4]$$

$$|(1 - a)\sigma_{ij}^{BB}| \gg |a\sigma_{ij}^{FF}| \quad [5]$$

are satisfied. It should be noted, however, that the constant of proportionality relating $N_i(j)$ to σ_{ij}^{BB} is *not* the same as that relating $N_k(j)$ to σ_{kj}^{BB} except in the fortuitous case where the total spin–lattice relaxation rates of protons i and k are equal both in the free and bound states. Thus if Eq. [3] applies for any two pairs of ligand protons i and j, and i and k, the ratio of the distances from the two protons j and k to the third proton i in the bound state, r_{ij}^{BB}/r_{ik}^{BB}, will simply be given by

$$r_{ij}^{BB}/r_{ik}^{BB} = (\sigma_{ik}^{BB}/\sigma_{ij}^{BB})^{1/6} = [N_i(k)/N_i(j)]^{1/6} \quad [6]$$

(assuming the same correlation time for the two distance vectors).

In practical cases involving large proteins (MW > 20,000), however, the steady state TRNOE is not selective owing to the phenomenon of spin diffusion (*24*) which arises from highly effective indirect cross-relaxation between many protons, so that Eq. [3] and, consequently, Eq. [6] are no longer valid in the steady state, and a multiple spin system has to be considered. The approach we have previously used to circumvent this problem consists of carrying out systematic pre-steady state TRNOE measurements, irradiating at 10–20 Hz intervals throughout a region of interest, using a constant short irradiation time, typically 0.5 sec (*1*, *20–23*). Under such conditions a plot of the intensity of a particular resonance as a function of irradiation frequency yields an "action spectrum" and apparent selectivity is maintained. Thus, for example, in the case of the 5′-AMP-yeast and horse liver alcohol dehydrogenase systems, a plot of the intensity of the averaged H8 resonance of the purine ring of 5′-AMP as a function of irradiation frequency (systematic irradiation being carried out throughout the sugar proton region of the spectrum) shows specific decreases in intensity at the positions of the averaged H2′, H3′, and H5′/H5″ sugar resonances but no decrease in intensity at the position of the averaged H1′ sugar resonance, whereas the equivalent plot for the H2 resonance of the purine ring of 5′-AMP shows no change in intensity at any of the positions of the averaged sugar proton resonances (*1*). From such experiments, it can be immediately deduced from qualitative considerations alone that the conformation about the glycosidic bond of bound 5′-AMP is *anti* and the

ribose conformation *3'-endo* of the N type. To obtain more quantitative conformational information, it was assumed that, providing condition [5] is satisfied, Eq. [3] is approximately correct for short irradiation times (as would be the case for the analogous pre-steady state NOE experiment in a nonexchanging system containing multiple spins (*5, 11*)), so that Eq. [6] could still be used to determine the distance ratio from two protons to a third proton. (Note that condition [5] is easily verified by measuring the pre-steady state NOEs on the free ligand in the absence of protein using the same irradiation time as that used for the TRNOE measurements in the presence of protein: if no corresponding positive NOE is seen for the free ligand (for which $\omega\tau_c < 1$), then condition [5] is easily shown to hold.) Such an approach, however, is severely limited since (a) one can only determine the distance ratio from two protons to a third proton because the cross-relaxation rates between pairs of bound ligand protons cannot be obtained from such measurements at a single irradiation time; and (b) if condition [5] is not satisfied (which is quite frequently the case), the dependence of the TRNOEs on the ratio of the concentrations of free to bound ligand must be determined in order to obtain by extrapolation the values of the TRNOEs at zero free ligand concentration (i.e., when all the ligand present is in the bound state) which are proportional to the relevant cross-relaxation rates in the bound state.

In the present paper, we develop the theory of the time dependent TRNOE for the generalized case of an exchanging system containing multiple spins as applied to ligand–protein interactions, and show that the cross-relaxation rates between pairs of bound ligand protons and between a bound ligand proton and a proton of the protein in the ligand–protein complex can easily be determined from the initial buildup rates of the time dependent TRNOEs, thus circumventing the above limitations and enabling distance ratios between any two such pairs of protons to be determined (assuming the same correlation time for the two distance vectors). This is illustrated by the use of the time dependent TRNOE to determine the conformation of NAD^+ bound to yeast alcohol dehydrogenase (MW 150,000).

THEORY

We will consider the simple reaction scheme

$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL \qquad [7]$$

where E is the free protein, L the free ligand and EL the ligand–protein complex. For a multiple spin system, there will be four groups of protons in scheme [7]: i_B, the ith bound ligand proton and i_F the corresponding free ligand proton; l_X, the lth proton of the protein in the protein–ligand complex, and l_Y, the corresponding proton in the free protein. For each group of protons there will be a corresponding set of total spin–lattice relaxation rates, ρ_{i_B}, ρ_{i_F}, ρ_{l_X}, and ρ_{l_Y}, respectively. The magnetization of these protons will be connected to each other by the following cross-relaxation rates: σ_{ij}^{BB}, the cross relaxation rate between bound ligand protons i and j, and σ_{ij}^{FF}, the cross-relaxation rate between the corresponding pair of free ligand protons; σ_{lk}^{XX}, the cross-relaxation rate between protons l and k of the protein in the ligand–

protein complex, and σ_{lk}^{YY}, the cross-relaxation rate between the corresponding protons in the free protein; and σ_{il}^{BX}, the cross-relaxation rate between the bound ligand proton i and proton l of the protein in the ligand–protein complex.

The complete set of coupled ordinary differential equations describing the evolution of the z component of the magnetization for the protons i_B, i_F, l_X, and l_Y for the generalized case of an exchanging system [7] containing multiple spins, based on McConnell's (*25*) and Solomon's (*26*) modifications of the Bloch equations for chemical exchange and cross-relaxation, and neglecting cross-correlation (see Ref. (*27*) for the justification of this simplifying assumption), are given by

$$\frac{dM_{i_B}}{dt} = -\rho_{i_B}(M_{i_B} - M_{B0}) + \sum_{j,j\neq i}^{n} \sigma_{ij}^{BB}(M_{j_B} - M_{B0}) + \sum_{l}^{m} \sigma_{il}^{BX}(M_{l_X} - M_{X0}) - k_{-1}M_{i_B} + k_1[E]M_{i_F} \quad [8]$$

$$\frac{dM_{i_F}}{dt} = -\rho_{i_F}(M_{i_F} - M_{F0}) + \sum_{j,j\neq i}^{n} \sigma_{ij}^{FF}(M_{j_F} - M_{F0}) + k_{-1}M_{i_B} - k_1[E]M_{i_F} \quad [9]$$

$$\frac{dM_{l_X}}{dt} = -\rho_{l_X}(M_{l_X} - M_{X0}) + \sum_{k,k\neq l}^{m} \sigma_{lk}^{XX}(M_{k_X} - M_{X0}) + \sum_{i}^{n} \sigma_{il}^{BX}(M_{i_B} - M_{B0}) - k_{-1}M_{l_X} + k_1[L]M_{l_Y} \quad [10]$$

$$\frac{dM_{l_Y}}{dt} = -\rho_{l_Y}(M_{l_Y} - M_{Y0}) + \sum_{k,k\neq l}^{m} \sigma_{lk}^{YY}(M_{k_Y} - M_{Y0}) + k_{-1}M_{l_X} - k_1[L]M_{l_Y} \quad [11]$$

where M_{i_B}, M_{i_F}, M_{l_X}, and M_{l_Y} are the magnetizations of the z component of protons i_B, i_F, l_X, and l_Y, respectively, at time t, and M_{B0}, M_{F0}, M_{X0}, and M_{Y0} are the corresponding equilibrium magnetisations prior to perturbation of the system by the application of a radiofrequency field at the position of a particular resonance, defined by

$$M_{B0} = M_{X0} = [EL]/L_T \quad [12]$$

$$M_{F0} = [L]/L_T \quad [13]$$

$$M_{B0} + M_{F0} = 1 \quad [14]$$

$$M_{Y0} = M_{X0}[E]/[EL] \quad [15]$$

where L_T is the total ligand concentration. (Note that (a) we have set the value of all $M_{i_B,0}$ to M_{B0}, and similarly for the other three sets of protons, and (b) the sign convention of Kalk and Berendsen (*24*) is used for the cross-relaxation rates such that σ_{ij} is negative for $\omega\tau_c < 1.118$, zero for $\omega\tau_c = 1.118$, and positive for $\omega\tau_c > 1.118$.)

The Initial Buildup Rates of the Time Dependent TRNOEs

For practical purposes there are only two types of proton resonances whose intensity may be monitored in a TRNOE experiment: (a) an averaged ligand proton resonance I_{av} when the free (I_F) and bound (I_B) ligand proton resonances are in fast exchange on the chemical shift scale, and (b) the free ligand proton resonance I_F when I_F and I_B are in slow exchange on the chemical shift scale. Similarly, there are four groups of proton resonances which may be irradiated: (i) an averaged ligand proton resonance S_{av} when the free (S_F) and bound (S_B) ligand proton resonances are in fast exchange on the chemical shift scale; (ii) the free ligand proton resonance S_F or (iii) the bound ligand proton resonance S_B when S_F and S_B are in slow exchange on the chemical shift scale; and (iv) an averaged (T_{av}) or bound (T_X) proton resonance of the protein. Expressions for the initial rate of change of the intensity of I_{av} or I_F (i.e., for the initial buildup rates of the time dependent TRNOEs) for these four cases are given below and are derived directly by modifying Eqs. [8]–[11] appropriately for the corresponding initial conditions.

In all the cases considered we have assumed that saturation of the irradiated resonance is instantaneous so that at $t = 0$, the magnetization of the z component of the saturated proton resonance is zero. In practice of course, saturation is not instantaneous, and, in the high power limit, the decay of the magnetization of the z component of the saturated proton resonance, M_i, is given by

$$M_i = M_0 \exp[-Rt] \cos(\gamma B_2 t) \qquad [16]$$

where the decay rate constant R is a function of the spin–spin relaxation rate, the spin–lattice relaxation rate, and the main field (B_0) inhomogeneities, and $\cos(\gamma B_2 t)$ is the Torrey oscillation term which causes the saturated resonance to undergo a sinusoidal oscillation with a frequency proportional to the applied radiofrequency field strength B_2 (*28, 29*). Fortunately, the effect of the Torrey oscillation term at high values of B_2 is such as to make the time development of an NOE in the high power limit virtually indistinguishable from that calculated using the simplifying instantaneous saturation assumption (*11*).

The initial rate of change in the intensity of the averaged proton ligand resonance I_{av} following irradiation of another proton resonance of any one of the four groups considered above, is given by

$$\left.\frac{dM_{I_{av}}}{dt}\right|_{t=0} = \left.\frac{dM_{I_F}}{dt}\right|_{t=0} + \left.\frac{dM_{I_B}}{dt}\right|_{t=0}. \qquad [17]$$

When chemical exchange between the free and bound states of the ligand is fast on the cross-relaxation scale of the relevant pairs of protons, that is to say when

$$k_{-1} + k_1[E] \gg |\sigma_{IS}^{BB}| + |\sigma_{IS}^{FF}| \qquad [18]$$

in the case where either the free (S_F), bound (S_B), or averaged (S_{av}) ligand resonance of proton S is irradiated, or when

$$k_{-1} + k_1[E] \gg |\sigma_{IT}^{BX}| \qquad [19]$$

in the case where the averaged (T_{av}) or bound (T_X) proton resonance of the protein

is irradiated, the initial rate of change in the intensity of the free proton ligand resonance I_F is given by

$$\left.\frac{dM_{I_F}}{dt}\right|_{t=0} = \left.\frac{d(M_{I_F} + M_{I_B})}{dt}\right|_{t=0} = \left.\frac{dM_{I_{av}}}{dt}\right|_{t=0}. \quad [20]$$

Case I. Irradiation of the averaged ligand proton resonance S_{av}. The radiofrequency field B_2 is applied at $t = 0$, so that the initial conditions are $M_{S_B} = M_{S_F} = 0$, and all other magnetizations are equal to their equilibrium magnetizations prior to the perturbation by the radiofrequency field.

The initial rate of change in the intensity of the averaged ligand proton resonance I_{av}, and, in the case of fast exchange on the cross-relaxation scale (cf. Eq. [18]), of the free ligand proton resonance I_F, is given by

$$\left.\frac{dM_{I_{av}}}{dt}\right|_{t=0} = -(M_{B0}\sigma_{IS}^{BB} + M_{F0}\sigma_{IS}^{FF}) = -[(1 - a)\sigma_{IS}^{BB} + a\sigma_{IS}^{FF}] \quad [21]$$

(where a is the mole fraction of free ligand), providing that, in the case of the bound ligand proton I_B, either

$$|\sigma_{IS}^{BB}| \geq |\sigma_{Ij}^{BB}|, |\sigma_{Ik}^{BX}| \quad [22]$$

or

$$|\sigma_{IS}^{BB}| \geq |\sigma_{Sj}^{BB}|, |\sigma_{Sk}^{BX}| \quad [23]$$

and, in the case of the free ligand proton I_F, either

$$|\sigma_{IS}^{FF}| \geq |\sigma_{Ij}^{FF}| \quad [24]$$

or

$$|\sigma_{IS}^{FF}| \geq |\sigma_{Sj}^{FF}|. \quad [25]$$

It is clear from Eq. [21] that under these conditions the cross-relaxation rate σ_{IS}^{BB} between the two bound ligand protons I_B and S_B is easily obtained providing the cross-relaxation rate σ_{IS}^{FF} between the corresponding free ligand protons I_F and I_S has been previously determined (e.g., from the initial buildup rate of the NOE between protons I_F and I_S of the free ligand in the absence of protein). Alternatively, σ_{ij}^{BB} and σ_{ij}^{FF} can be determined simultaneously by measuring the initial buildup rate of the TRNOE at two different ratios of free to bound ligand.

If, on the other hand,

$$k_{-1} + k_1[E] < |\sigma_{IS}^{BB}| + |\sigma_{IS}^{FF}| \quad [26]$$

so that chemical exchange is slow on the cross-relaxation scale, the initial rate of change in the intensity of the free ligand resonance I_F is given by

$$\left.\frac{dM_{I_F}}{dt}\right|_{t=0} = (1 - a)k_{-1} - a(\sigma_{IS}^{FF} + k_1[E]) = -a\sigma_{IS}^{FF} \quad [27]$$

and no information on the cross-relaxation rate σ_{IS}^{BB} between the bound ligand protons I_B and S_B can be obtained.

Case 2. Irradiation of the bound ligand proton resonance S_B. In this case the initial conditions are $M_{S_B} = 0$ and all other magnetizations are equal to their equilibrium magnetizations.

The initial rate of change in the intensity of the averaged ligand proton resonance I_{av}, and, in the case of fast exchange on the cross-relaxation scale (cf. Eq. [18]), of the free ligand proton resonance I_F, is given by

$$\left.\frac{dM_{I_{av}}}{dt}\right|_{t=0} = -a\sigma_{IS}^{BB} \qquad [28]$$

(providing either Eq. [22] or Eq. [23] holds), so that the cross-relaxation rate σ_{IS}^{BB} between the two bound ligand protons I_B and S_B can be obtained directly from the initial slope.

If exchange is slow on the cross-relaxation scale (cf. Eq. [26]), the initial rate of change in intensity of the free ligand resonance I_F is zero:

$$\left.\frac{dM_{I_F}}{dt}\right|_{t=0} = (1 - a)k_{-1} - ak_1[E] = 0. \qquad [29]$$

In addition to monitoring the intensity of I_{av} or I_F, the intensity of the free ligand proton resonance S_F can be monitored. This constitutes the transfer of saturation experiment (*30*), and allows one to obtain information on the chemical exchange rate as

$$\left.\frac{dM_{S_F}}{dt}\right|_{t=0} = -ak_1[E] = -(1 - a)k_{-1}. \qquad [30]$$

Case 3. Irradiation of the free ligand proton resonance. In this case the initial conditions are $M_{S_F} = 0$ and all other magnetizations are equal to their equilibrium magnetizations.

Initial rate measurements for this particular TRNOE experiment yield no information on cross-relaxation between the bound ligand protons I_B and S_B when exchange is slow on the cross-relaxation scale (cf. Eq. [26]) as

$$\left.\frac{dM_{I_{av}}}{dt}\right|_{t=0} = \left.\frac{dM_{I_F}}{dt}\right|_{t=0} = -a\sigma_{IS}^{FF}. \qquad [31]$$

If, on the other hand, exchange is fast on the cross-relaxation scale is fast (cf. Eq. [18]) then the initial slope will be given by Eq. [21]).

Case 4. Irradiation of the averaged (T_{av}) *or bound* (T_X) *proton resonance of the protein.* In this case the initial conditions are $M_{T_X} = 0$ and $M_{T_Y} = 0$ if the averaged proton resonance T_{av} is irradiated, and $M_{T_X} = 0$ if the bound proton resonance T_X is irradiated; all other magnetizations are equal to their equilibrium magnetizations.

Providing either Eq. [22] or Eq. [23] holds, the initial rate of change in the intensity of the averaged ligand proton resonance I_{av}, and, in the case of fast exchange on the cross-relaxation scale (cf. Eq. [19]), of the free ligand resonance I_F, is given by

$$\left.\frac{dM_{I_{av}}}{dt}\right|_{t=0} = -(1 - a)\sigma_{IT}^{BX} \qquad [32]$$

so that the cross-relaxation rate σ_{IT}^{BX} between the bound ligand proton I_B and the proton T_X of the protein in the ligand–protein complex can be obtained directly from the initial slope.

CALCULATIONS

To illustrate the effect of spin diffusion on the time dependence of the TRNOE we have carried out a series of simulations for a simple scheme consisting of three ligand protons which exist in the bound (I_B, J_B, and S_B) and free (I_F, J_F, and S_F) states (see Fig. 1). For the sake of simplicity we have only considered the case where chemical exchange between the free and bound states is fast on the chemical shift scale for all three ligand protons, and the values of the cross-relaxation rates between the free ligand protons, I_F, J_F, and S_F, are zero. In all the simulations the intensity of the averaged ligand proton resonance I_{av} is calculated as a function of the time t of irradiation of the averaged ligand proton resonance J_{av} or S_{av}. For each time dependent TRNOE simultation, we have carried out a corresponding calculation for the time dependence of the NOE which would be observed on the bound ligand proton I_B following irradiation of the bound ligand proton J_B or S_B in the absence of chemical exchange. The parameter values used, chosen to be representative of those in ^{1}H NMR experiments on ligand–protein systems, are given in Table 1. The simulations were carried out by numerical integration of the coupled simultaneous ordinary differential equations describing the scheme in Fig. 1 using Curtis' modification (*31*) of Gear's backward difference method (*32*).

The effect of (a) direct cross-relaxation between the bound ligand protons I_B and S_B in the absence of indirect cross-relaxation (i.e., $\sigma_{IJ}^{BB} = \sigma_{SJ}^{BB} = 0$), (b) indirect cross-relaxation in the presence of direct cross-relaxation between the bound ligand protons I_B and S_B, and I_B and J_B (i.e., σ_{IS}^{BB} and σ_{IJ}^{BB} are nonzero), and (c) indirect cross-

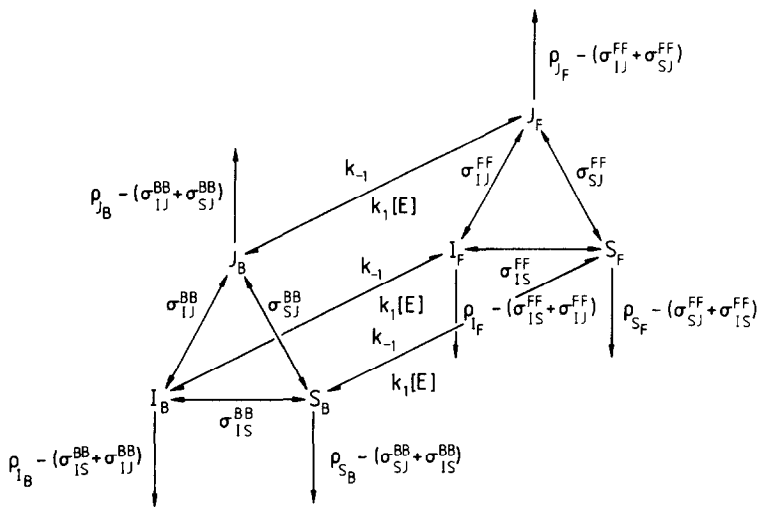

FIG. 1. Scheme used for the simulations to illustrate the effect of spin diffusion on the time dependence of the TRNOE.

TABLE 1

VALUES OF PARAMETERS USED IN THE CALCULATIONS

Parameter	Value
E_T[a]	5×10^{-4} M
L_T[a]	5.5×10^{-3} M
k_1[a]	10^8 M^{-1} sec^{-1}
k_{-1}[a]	100 sec^{-1}
$\sigma_{IS}^{FF} = \sigma_{IJ}^{FF} = \sigma_{SJ}^{FF}$	0 sec^{-1}
σ_{IS}^{BB}, σ_{IJ}^{BB}, σ_{SJ}^{BB}	0–20 sec^{-1}
$\rho_{I_F} = \rho_{J_F} = \rho_{S_F}$	0.5 sec^{-1}
$\rho_{I_B} - (\sigma_{IS}^{BB} + \sigma_{IJ}^{BB})$	0.5 sec^{-1}
$\rho_{J_B} - (\sigma_{IJ}^{BB} + \sigma_{SJ}^{BB})$	0.5 sec^{-1}
$\rho_{S_B} - (\sigma_{IS}^{BB} + \sigma_{SJ}^{BB})$	0.5 sec^{-1}

[a] For these values of E_T, L_T, k_1, and k_{-1}, the ratio of free to bound ligand, $[L_F]/[L_B]$, is 10, and $k_1[E]$ is 10 sec^{-1}.

relaxation in the absence of direct cross-relaxation between the bound ligand protons I_B and S_B (i.e., $\sigma_{IS}^{BB} = 0$), on the time dependence of the TRNOE on the averaged ligand proton resonance I_{av} is shown in Figs. 2A, 3A, and 4A, respectively, and on

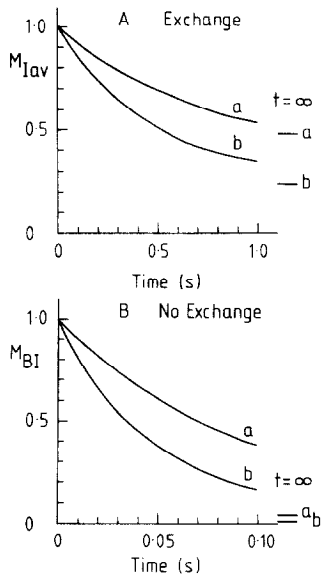

FIG. 2. Effect of direct cross-relaxation in the absence of indirect cross-relaxation on the time dependence of (A) the TRNOE on the averaged ligand proton resonance I_{av} following irradiation of the averaged ligand resonance S_{av}, and of (B) the corresponding NOE on the bound ligand proton resonances I_B following irradiation of the bound ligand proton resonance S_B in the absence of chemical exchange (i.e., $k_{-1} = 0$). σ_{IS}^{BB} = (a) 10 sec^{-1} and (b) 20 sec^{-1}; $\sigma_{IJ}^{BB} = \sigma_{SJ}^{BB} = 0$ sec^{-1}. Values of the other parameters are given in Table 1.

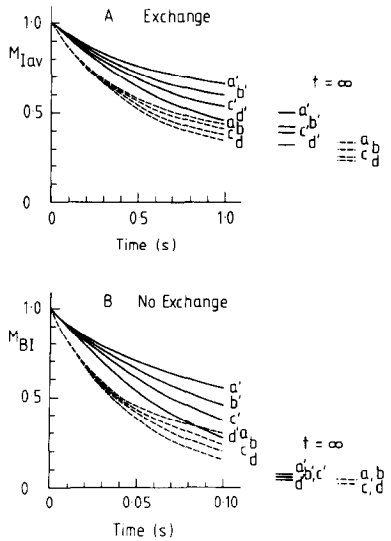

FIG. 3. Effect of indirect cross-relaxation in the presence of direct cross-relaxation on the time dependence of (A) the TRNOE on the averaged ligand proton resonance I_{av} following irradiation of the averaged ligand proton resonance S_{av} (——) and J_{av} (– – –), and of (B) the corresponding NOE on the bound ligand proton resonance I_B following irradiation of the bound ligand proton resonances S_B (——) and J_B (– – –) in the absence of chemical exchange (i.e., $k_{-1} = 0$). σ_{SJ}^{BB} = (a, a') 0 sec^{-1}, (b, b') 5 sec^{-1}, (c, c') 10 sec^{-1}, and (d, d') 20 sec^{-1}; $\sigma_{IS}^{BB} = 20$ sec^{-1}; $\sigma_{JJ}^{BB} = 10$ sec^{-1}. Values of the other parameters are given in Table 1.

the time dependence of the corresponding NOE which would be observed on the bound ligand proton resonance I_B in the absence of chemical exchange in Figs. 2B, 3B, and 4B, respectively. A comparison of Figs. 2A, 3A, and 4A with Figs. 2B, 3B, and 4B shows that:

(i) The initial slope of both the time dependent TRNOE and NOE provides a good measure of direct cross-relaxation (see Figs. 2 and 3) even in the presence of significant indirect cross-relaxation (see Fig. 3). Thus, in the examples given in Figs. 2 and 3 where chemical exchange is fast on the chemical shift scale, the initial slope of the TRNOE is given by the weighted average of the direct cross-relaxation rates in the free and bound states (cf. Eq. [21]), whereas, for the corresponding NOE in the absence of chemical exchange, it is given by the direct cross-relaxation rate in the bound state. A natural consequence of this is that the time development of the TRNOE is slower than that of the corresponding NOE (in the calculations presented here by an order of magnitude). This has the advantage that it allows one to use longer irradiation times in time dependent TRNOE measurements than in the corresponding time dependent NOE measurements, and, therefore, to preserve greater selectivity of the applied radiofrequency field (as the bandwidth of the latter is given by $\sim 1/t$ where t is the time for which it is applied). It should be noted from the experimental point of view that in time dependent TRNOE measurements the molar ratio of free to bound ligand can *always* be chosen such that the initial slope can be measured from irradiation times of ≥ 50 msec (cf. Eqs. [21], [28], and [33]) such that conditions for both selectivity and high power of the saturating pulse can be achieved.

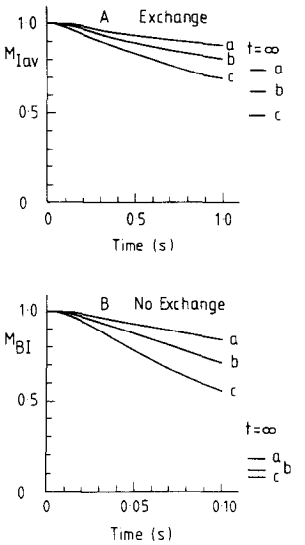

FIG. 4. Effect of indirect cross-relaxation in the absence of direct cross-relaxation on the time dependence of (A) the TRNOE on the averaged ligand proton resonance I_{av} following irradiation of the averaged ligand proton resonance S_{av}, and of (B) the corresponding NOE on the bound ligand proton resonance I_B following irradiation of the bound ligand proton resonance S_B in the absence of chemical exchange (i.e., $k_{-1} = 0$). σ_{SJ}^{BB} = (a) 5 sec^{-1}, (b) 10 sec^{-1}, and (c) 20 sec^{-1}; $\sigma_{IJ}^{BB} = 20$ sec^{-1}; $\sigma_{IS}^{BB} = 0$ sec^{-1}. Values of the other parameters are given in Table 1.

Given that the interproton distances for which the TRNOEs are going to be observed lie in the range of 2 to 4 Å, the choice for the molar ratio of free to bound ligand can be estimated on the basis of an approximate value for the rotational correlation time of the protein calculated using the Stokes–Einstein equation.

(ii) Indirect cross-relaxation in the absence of direct cross-relaxation is easily detected by the presence of a lag phase in the time development of both the TRNOE and NOE.

(iii) Providing either Eq. [5] or the condition

$$(1 - a)\rho_{i_B} \gg a\rho_{i_F} \qquad [33]$$

is not fulfilled, the value of the steady state TRNOE (i.e., at $t \rightarrow \infty$) is sensitive to the value of the direct cross-relaxation rate σ_{ij}^{BB} in the bound state (see Figs. 2A and 3A, and Ref. (*1*)). This has the major advantage that the sixth root of the ratio of two TRNOEs from two protons to a third proton can still give an approximate estimate of the corresponding distance ratio at relatively long irradiation times, providing direct cross-relaxation between the two pairs of protons predominates over indirect cross-relaxation (see Figs. 2A and 3A) or the contribution from indirect cross-relaxation can be eliminated (see Results section and Figs. 5C and 6). When both conditions [33] and [34] are fulfilled, however, the value of the steady state TRNOE will be equal to that of the corresponding steady state NOE which would be observed in the absence of chemical exchange, namely ~ -1 when $\omega\tau_c \gg 1$ (see Figs. 2B and 3B).

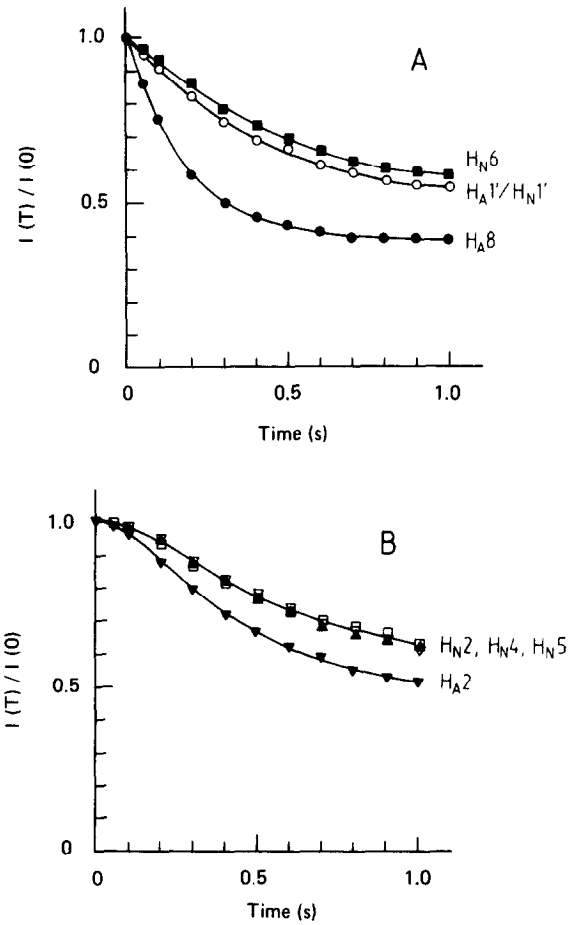

FIG. 5. Time dependence of the TRNOEs observed on the averaged ligand resonances of NAD^+ following irradiation of the averaged H_A2'/H_N2' resonance of NAD^+ at 1.00 ppm in the presence of yeast ADH at a ratio of free to bound NAD^+ of 10. In A and B, $I(t)/I(0)$, and in C and D $[I(t) - I_c(t)]/I_c(t)$ are plotted as a function of time where $I(t)$ and $I(0)$ are the intensities of the averaged ligand resonance at time t and 0, respectively, and $I_c(t)$ is the intensity of the same averaged ligand resonance following irradiation for a time t at a control frequency of −0.53 ppm which is placed within the protein envelope approximately 0.9 ppm upfield from the highest field sugar proton resonance of NAD^+. ●, H_A8; ○, H_A1'/H_N1'; ■, H_N6; ▼, H_A2; □, H_N2; ▲, H_N4; ▽, H_N5. The experimental conditions are given in the Experimental section. (Chemical shifts are expressed relative to dioxane which is 3.71 ppm downfield from 2,2 dimethylsilapentane-5-sulphonate).

EXPERIMENTAL

Yeast alcohol dehydrogenase (ADH) was purchased from Sigma Chemical Company Ltd. After dialysis against 20 m*M* potassium phosphate pH* 7.0 (meter reading uncorrected for the isotope effect on the glass electrode) in D_2O, the solutions were clarified by centrifugation and used without further purification. NAD^+ was also obtained from Sigma Chemical Company Ltd., lyophilized from D_2O, and used

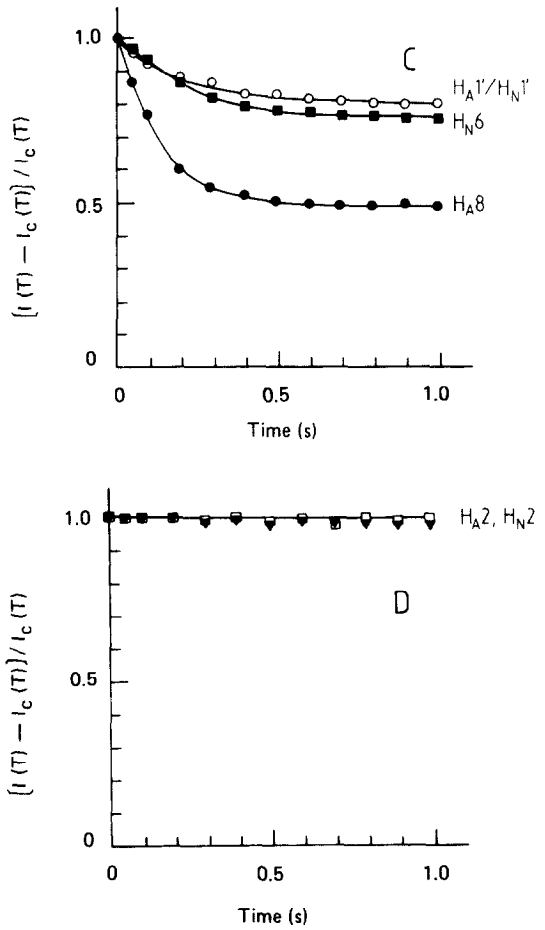

FIG. 5—*Continued.*

without further purification. All chemicals used were of the highest purity commercially available. Samples for ^{1}H NMR contained 8.8 m*M* NAD^+ and 0.2 m*M* yeast ADH (corresponding to 0.8 m*M* in NAD^+ binding sites) so that the ratio of free to bound NAD^+ was 10, 20 m*M* potassium phosphate pH* 7.0, 3.3 μM EDTA and 1 m*M* dioxane (as an internal standard). All experiments were carried out at 20°C.

^{1}H NMR measurements were carried out at 270 MHz using a Bruker WH-270 spectrometer operating in Fourier transform mode. Five hundred transients were averaged for each spectrum using 4096 data points for a 4.2 kHz spectral width, and, prior to Fourier transformation, the free induction decay was multiplied by an exponential function equivalent to a line broadening of 2 Hz. The pulse sequence used in the time dependent TRNOE experiments was $(t_1\text{–}t_2\text{–}\pi/2\text{—AT–}t_3)_n$ where the selective irradiation at a chosen frequency was applied during the time interval t_1 (0.002–1 sec), t_2 is a short delay (2 msec) to allow for electronic recovery after removal of the selective irradiation, AT is the acquisition time (0.487 sec), and t_3 is a delay (4 sec) to allow for complete recovery of the magnetization of all protons to their

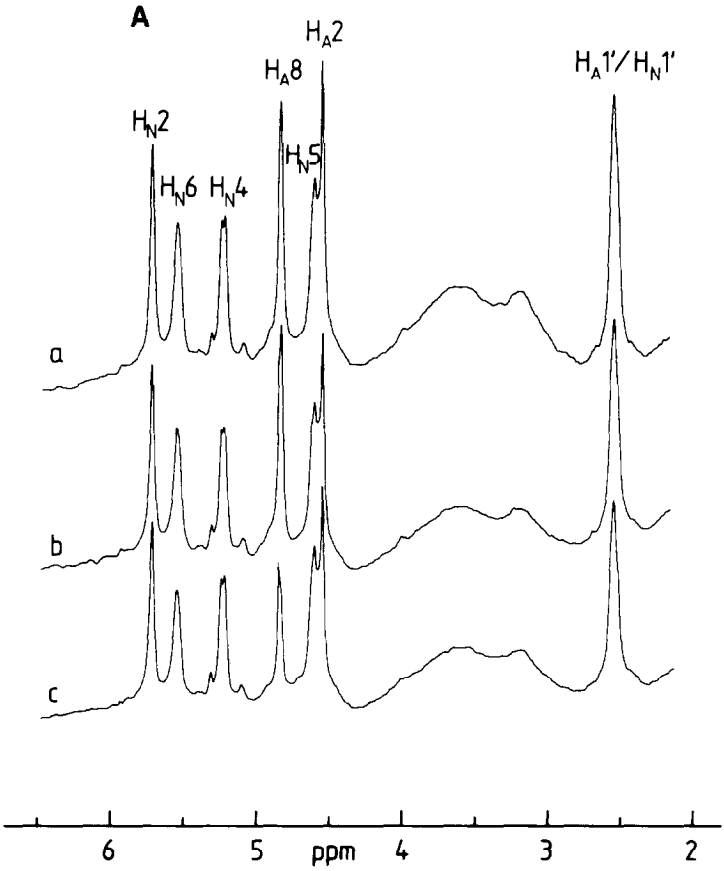

FIG. 6. The low field region of the 270 MHz ^{1}H-NMR spectrum of 8.8 mM NAD^+ in the presence of 0.2 mM yeast ADH (corresponding to 0.8 mM in NAD^+ binding sites). (A) Spectrum with (a) no irradiation, (b) irradiation for 0.4 s at a control frequency (−0.53 ppm) placed within the protein envelope, and (c) irradiation of the averaged H_A2'/H_N2' resonance at 1.00 ppm for 0.4 s. (B) Difference spectra of (b) minus (a), (c) minus (a) and (c) minus (b). The experimental conditions are given in the Experimental section. (Chemical shifts are expressed relative to dioxane).

equilibrium values prior to perturbation by the selective radiofrequency field. Chemical shifts are expressed relative to internal (1 m*M*) dioxane (3.71 ppm downfield from 2,2-dimethylsilapentane-5-sulphonate).

RESULTS AND DISCUSSION

To illustrate the applications of time dependent TRNOE measurements to the conformational analysis of ligands bound to proteins we have used the NAD^+–yeast ADH system as an example. Yeast ADH is a large protein of molecular weight 150,000 composed of four identical subunits, each of which possesses a NAD^+ binding site (*33*). Because NAD^+ binds weakly to yeast ADH with an equilibrium association constant of $1.4 \times 10^3\ M^{-1}$ (*34*) and exchange between the free and bound states of NAD^+ is fast on the chemical shift scale (*21*), the time dependent TRNOE experiment

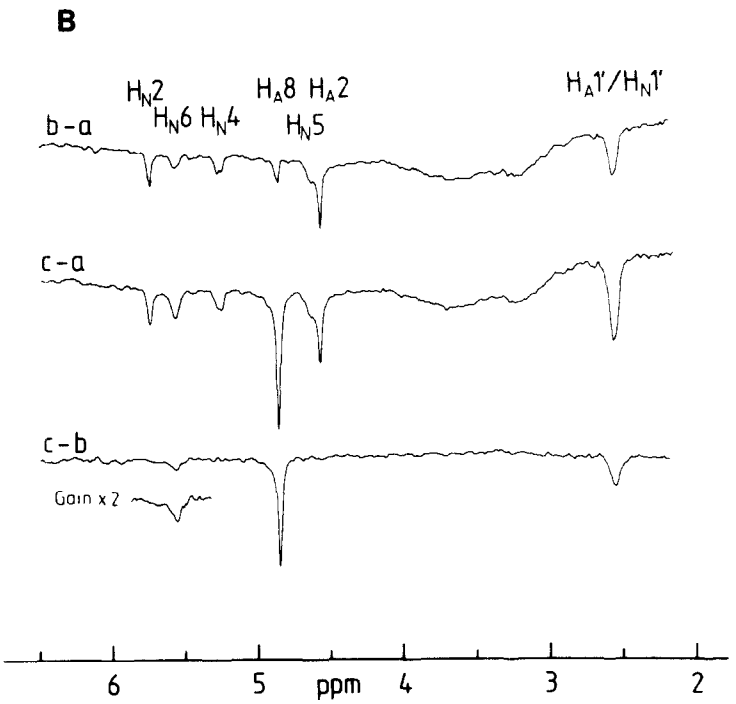

FIG. 6—*Continued.*

consists of irradiating a particular averaged ligand resonance and monitoring the intensity of the other averaged ligand resonances as a function of the time t for which the radiofrequency field is applied. All measurements were carried out with a ratio of free to bound NAD^+ of 10.

Under these conditions the contribution to the initial buildup rate of the TRNOE from cross-relaxation between free ligand protons can be neglected (i.e., Eq. [5] is satisfied) since (a) no NOEs between any pair of protons could be observed for free NAD^+ or NAD^+ in the presence of denatured yeast ADH in 3M guanidinium chloride for irradiation times as long as 1 sec, and (b) it can be calculated using the equation (*24, 26*)

$$\sigma_{ij} = \frac{1}{10}\frac{\gamma^4 h^2}{r_{ij}^6}\left(\tau_c - \frac{6\tau_c}{1 + 4\omega^2\tau_c^2}\right) \qquad [34]$$

that for any given distance r_{ij}, the absolute value of the cross-relaxation rate σ_{ij} for free NAD^+ will be between 100 and 170 times smaller than that for bound NAD^+ at a spectrometer frequency of 270 MHz using $\tau_c = 2\text{–}3 \times 10^{-10}$ sec for free NAD^+ (*35*) and $\tau_c = 6\text{–}10 \times 10^{-8}$ sec for the NAD^+–yeast ADH complex (calculated using the Stokes–Einstein equation). Consequently, from Eq. [21], the initial buildup rates of the TRNOEs in the presence of direct cross-relaxation between the relevant pairs of protons will simply be given by $-(1 - a)\sigma_{ij}^{BB}$.

The time dependence of the TRNOEs observed on the averaged H_A8, H_A2, H_A1'/H_N1', H_N6, H_N5, H_N4, and H_N2 resonances of NAD^+ following irradiation of the

averaged H_A2'/H_N2' resonance of NAD^+ is shown in Fig. 5. (Note that the subscripts A and N used in the numbering of the NAD^+ protons refer to the adenine and nicotinamide moieties, respectively, and that the positions of the sugar proton resonances of the nicotinamide ribose closely overlap the corresponding sugar proton resonances of the adenine ribose, and therefore cannot be distinguished.) It can be clearly seen from the absence of a lag phase for the $I(t)/I(0)$ versus t plots for the averaged H_A8, H_N6, and H_A1'/H_N1' ligand resonances (where $I(t)$ and $I(0)$ are the intensities of the averaged ligand resonances at time t and 0, respectively), that significant direct cross-relaxation occurs between the H_A2' proton and the H_A8 and H_A1' protons, and between the H_N2' proton and the H_N6 and H_N1' protons (see Fig. 5A). (Any contribution from direct cross-relaxation between protons of the nicotinamide moiety and protons of the adenine moiety is insignificant and can be neglected as NAD^+ is known to be bound to alcohol dehydrogenase in the extended conformation with the nicotinamide and adenine rings separated by 10–12 Å (*36–38*).) Conversely, the presence of a lag phase in the case of the $I(t)/I(0)$ versus t plots for the averaged H_N2, H_N4, H_N5, and H_A2 ligand resonances indicates that direct cross-relaxation to the H_N2' or H_A2' proton is insignificant and that indirect cross-relaxation predominates (see Fig. 5B). However, no distinction could be made between the above two groups of ligand protons on the basis of a difference spectrum obtained by subtracting a spectrum without irradiation (Fig. 6, spectrum a) from one with irradiation of the averaged H_A2'/H_N2' ligand resonance for, say, $t = 0.4$ sec (Fig. 6, spectrum c) as a decrease in the intensity of all the averaged ligand resonances is observed (see Fig. 6, difference spectrum c minus a). The predominant source of indirect cross-relaxation arises via the protons of the protein (i.e., generalized spin diffusion) and can be virtually eliminated by plotting $[I(t) - I_c(t)]/I_c(t)$ as a function of t, where $I_c(t)$ is the intensity of the averaged ligand resonance following irradiation for a time t at a control frequency placed within the protein envelope. This is illustrated in Figs. 5C and D with the control irradiation frequency at −0.53 ppm which is within the β-CH_2 region of the protein spectrum approximately 0.9 ppm upfield from the highest field sugar proton resonance of NAD^+. The initial slopes of the $[I(t) - I_c(t)]/I_c(t)$ versus t plots for the averaged H_A8, H_N6, and H_A1'/H_N1' resonances remain unchanged from those of the corresponding $I(t)/I(0)$ versus t plot; at $t \rightarrow \infty$, however, the values of $[I(t) - I_c(t)]/I_c(t)$ differ from the corresponding values of $I(t)/I(0)$ owing to the elimination of the contribution from indirect cross-relaxation at long values of t (see Fig. 5C). In contrast, no change in the value of $[I(t) - I_c(t)]/I_c(t)$ as a function of t is observed for the averaged H_A2, H_N2, H_N4 and H_N5 ligand resonances; this is illustrated in Fig. 5D for the averaged H_A2 and H_N2 resonances. Consequently, a difference spectrum taken at, say, $t = 0.4$ sec of a spectrum with the irradiation frequency at the position of the averaged H_A2'/H_N2' resonance (Fig. 6, spectrum c) minus a control spectrum using the same irradiation frequency (−0.53 ppm) as that used in Figs. 5C and D (Fig. 6, spectrum b) only shows a specific decrease in the intensity of the averaged resonances of those ligand protons where significant direct cross-relaxation to the H_A2' or H_N2' ligand protons occurs in the bound state, namely the H_A8, H_N6, and H_A1'/H_N1' protons (see Fig. 6, difference spectrum c minus b). Thus, a useful initial procedure to determine between which pairs of protons significant direct cross-relaxation occurs is to obtain a set of "action" spectra by plotting the intensities of the averaged ligand resonances as a function of irradiation frequency

(*1, 19–23*). In this manner selectivity of the observed TRNOEs is achieved since, in general, the extent of spin diffusion arising from indirect cross-relaxation via the protons of the protein is approximately independent of the irradiation frequency providing this is placed within the protein envelope (see, for example, Fig. 5 in Ref. (*1*) and Fig. 1 in Ref. (*22*)). Nevertheless, small effects seen in such "action" spectra should be treated with caution as they may still arise from indirect cross-relaxation, and should therefore be checked by measuring the time dependence of the TRNOE.

The values of all the direct cross-relaxation rates which could be measured between pairs of bound ligand protons in the NAD^+–yeast ADH system are given in Table 2. From the cross-relaxation rates, the distance ratios, r_{ij}/r_{kl}, between pairs of bound ligand protons can be determined as, from Eq. [34], $\sigma_{ij} \propto (r_{ij})^{-6}$ (see Table 2). In the case of the NAD^+–yeast ADH system interproton distances between bound ligand protons can also be calculated (see Table 2) as the distances r_{H_N5}–r_{H_N6} and r_{H_N5}–r_{H_N4} are fixed and have a values of 2.48 Å. Given both the value of the distance r_{H_N5}–r_{H_N6} and of the cross-relaxation rate $\sigma^{BB}_{H_N5,H_N6}$, the value of τ_c computed for the NAD^+–yeast ADH complex using Eq. [34] is $\sim 8 \times 10^{-8}$ sec which is within the range ($6–10 \times 10^{-8}$ sec) predicted for the rotational diffusion time of a protein of MW 150,000 on the basis of the Stokes–Einstein equation.

It should be noted that the values of 0.89 and 0.77 for the two distance ratios $r_{H_A8-H_A2'}/r_{H_A8-H_A3'}$ and $r_{H_A8-H_A2'}/r_{H_A8-H_A5'/H_A5''}$ respectively, in NAD^+ bound to yeast ADH, obtained by Gronenborn and Clore (*21*) using the ratios of the corresponding TRNOEs observed in an "action" spectrum with an irradiation time of 0.5 sec, are approximately the same as those obtained from the cross-relaxation rates given in Table 2, namely 0.87 and 0.81, respectively. Thus, providing one is sure that the TRNOEs observed from two bound ligand protons to a third bound ligand proton in an "action" spectrum arise from direct cross-relaxation, a reasonable estimate of the corresponding distance ratio may be obtained (providing an appropriately short irradiation time is employed).

Based on the large number of interproton distance ratios and distances in bound NAD^+ derived from the time dependent TRNOE measurements (see Table 2), the conformation of NAD^+ bound to yeast ADH can be determined with much greater precision than was possible on the basis of the TRNOEs measured by Gronenborn and Clore (*21*) from an "action" spectrum obtained using a single irradiation time (see Fig. 7). The distance ratios and interproton distances given in Table 2 are only consistent with an *anti* conformation about both the adenosine and nicotinamide glycosidic bonds with torsion angles of χ_A (04′–C1′–N9–C4) $\sim 270°$ and χ_N (04′–C1′–N1–C2) $\sim 240°$, a *3′ endo* conformation of the N type for both the adenine and nicotinamide ribose, and either a *gauche–trans* or *trans–gauche* conformation about the C4′–C5′ bond of the adenine ribose (we cannot distinguish between these two possibilities as the averaged ligand resonances of the H_A5' and H_A5'' protons are superimposed). Moreover, given the distances between the H_N6 proton and the H_N2' and H_N3' protons (see Table 2), the absence of significant direct cross-relaxation from either the H_N5' or H_N5'' proton to the H_N6 proton, as evidenced by a distinct lag phase in the time dependent TRNOE, indicates that the corresponding distances are greater than 4 Å and, therefore, that the conformation about the C4′–C5′ bond of the nicotinamide ribose is likely to be *gauche–gauche*. These findings are entirely consistent with the available crystallographic data on coenzymes and coenzyme frag-

TABLE 2

CROSS-RELAXATION RATES BETWEEN PAIRS OF BOUND LIGAND PROTONS IN THE NAD$^+$–YEAST ADH COMPLEX DETERMINED FROM TIME DEPENDENT TRNOE MEASUREMENTS TOGETHER WITH THE INTERPROTON DISTANCE RATIOS AND DISTANCES DERIVED FROM THEM

Irradiated resonance[a]	Observed resonance	σ_{ij}^{BB} (sec^{-1})[b]	Distance ratios[c]	r_{ij}^{BB} (Å)[d]
A. Adenine moiety			$r_{H_A8-H_A2}^{BB}/r_{ij}^{BB}$	
H_A1'	H_A8	3.8	0.72	3.27
H_A2'	H_A1'[e]	9.5	0.84	2.81
H_A2'	H_A8	27.0	1.00	2.36
H_A3'	H_A8	11.8	0.87	2.71
H_A5'/H_A5''[f]	H_A8	7.8	0.81[f]	2.90[f]
B. Nicotinamide moiety			$r_{H_N1'-H_N2}^{BB}/r_{ij}^{BB}$	
H_N1'	H_N2	20.0	1.00	2.48
H_N1'	H_N6	4.8	0.79	3.15
H_N2'	H_N1'[e]	9.5	0.88	2.81
H_N2'	H_N6	7.9	0.86	2.90
H_N3'	H_N6	22.7	1.02	2.43
H_N5	H_N6	20.0	1.00	2.48
H_N5	H_N4	20.0	1.00	2.48

[a] Although the positions of the sugar proton resonances of the adenine ribose closely overlap the corresponding sugar proton resonances of the nicotinamide ribose so that they cannot be distinguished, any contribution from direct cross-relaxation between protons of the adenine ribose and protons of the nicotinamide ribose, protons of the adenine ribose and protons of the nicotinamide ring, and protons of the nicotinamide ribose and protons of the adenine ring is insignificant and can be neglected since NAD$^+$ is known to be bound to alcohol dehydrogenases in the extended conformation with the adenine and nicotinamide rings 10–12 Å apart (*36–38*).

[b] The relative errors, $\Delta\sigma_{ij}^{BB}/\sigma_{ij}^{BB}$, in the values of the cross-relaxation rates are $\leqslant \pm 0.10$.

[c] The interproton distance ratios are calculated using Eq. [34] on the assumption of a single correlation time for all the interproton distance vectors of the adenine moiety and a single correlation time for all the interproton distance vectors of the nicotinamide moiety. The errors in the values of the distance ratios are $\leqslant \pm 0.03$.

[d] The interproton distances are calculated relative to the two distances $r_{H_N5-H_N6}$ and $r_{H_N5-H_N4}$ which have a value of 2.48 Å (calculated on the basis of standard bond lengths and angles for the nicotinamide ring), using Eq. [34] on the assumption of a single correlation time for all the interproton distance vectors of the adenine and nicotinamide moieties. Assuming an error of ± 0.05 Å in the estimated value of $r_{H_N-H_N6}$ and $r_{H_N-H_N4}$, the errors in the values of the other interproton distances are $\leqslant \pm 0.15$ Å.

[e] As the H_N1' and H_A1' resonances are superimposed, individual effects on the H_N1' and H_A1' protons cannot be distinguished. It was therefore assumed that the contributions to the initial slope of the TRNOE from cross-relaxation between the H_N1' and H_N2' protons and between the H_A1' and H_A2' protons were equal.

[f] The H_A5' and H_A5'' resonances are superimposed so that one cannot distinguish whether the TRNOE observed on the averaged H_A8 resonance arises from the H_A5' or H_A5'' proton. In calculating the distance ratio $r_{H_A8-H_A2}^{BB}/r_{H_A8-H_A5'/H_A5''}^{BB}$ and the distance $r_{H_A8-H_A5'/H_A5''}^{BB}$ we have assumed that the conformation about the C4′–C5′ bond of bound NAD$^+$ exists in only one form so that the TRNOE arises from only one of these two protons.

ments bound to a number of dehydrogenases including horse liver ADH (*36–44*). It should be noted, however, that there are no crystallographic data at present on the NAD$^+$–yeast ADH complex.

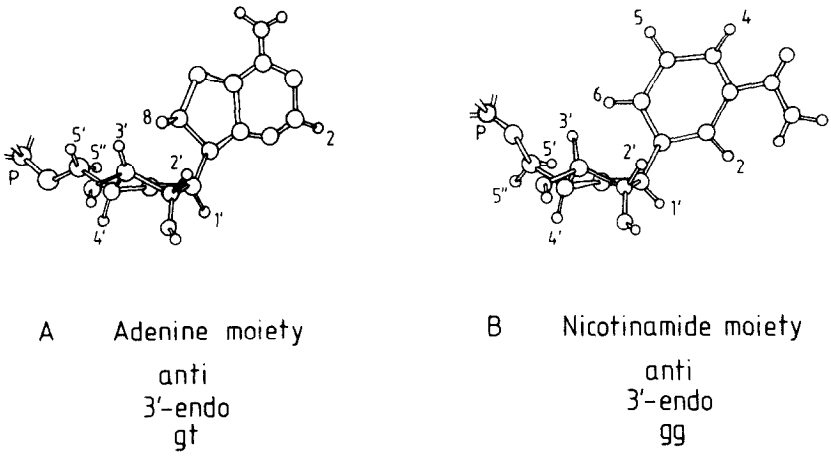

FIG. 7. Conformation of the adenine (A) and nicotinamide (B) moieties of NAD^+ bound to yeast ADH deduced from the distances between pairs of bound ligand protons given in Table 2 derived from time dependent TRNOE measurements (see text for further details). (Note that the conformation about the C4'–C5' bond of the adenine ribose is drawn in the *gauche–trans* conformation; we cannot, however, distinguish between this conformation and the *trans–gauche* conformation on the basis of our TRNOE measurements.) Abbreviations used: gg, *gauche–gauche;* gt, *gauche–trans.*

CONCLUDING REMARKS

In this paper we have discussed the theory and applications of time dependent TRNOE measurements to the study of the conformations of ligands bound to proteins. We have shown that cross-relaxation rates between pairs of bound ligand protons and between a bound ligand proton and a proton of the protein in the ligand–protein complex can be determined with ease from the initial buildup rates of the TRNOEs, thus enabling either distance ratios between any two such pairs of protons or, if a particular interproton distance is known, interproton distances to be calculated directly. The large number of interproton distance ratios or distances obtained in this manner enable one to define with precision the conformation of the bound ligand and potentially its location with respect to neighboring amino acid residues of the protein (providing the resonance positions of the latter's protons are known).

The measurement of time dependent TRNOEs is a technique of wide applicability since only conditions [1] and [2] need be fulfilled for a negative TRNOE to be observed. Moreover, the time dependent TRNOE is particularly suitable to the study of ligands bound to large proteins because (a) only free or averaged ligand resonances need be observed, and (b) the sensitivity of the technique increases as the molecular weight of the protein increases owing to the fact that the cross-relaxation rates in the ligand–protein complex are directly proportional to the correlation time τ_c of the ligand–protein complex (since $\omega\tau_c \gg 1$), thus enabling large excesses of free over bound ligand to be employed.

ACKNOWLEDGMENTS

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