

Theory and Applications of the Transferred Nuclear Overhauser Effect to the Study of the Conformations of Small Ligands Bound to Proteins

G. M. CLORE AND A. M. GRONENBORN

*Division of Molecular Pharmacology, National Institute for Medical Research,
Mill Hill, London NW7 1AA, United Kingdom*

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The principles, theory, and applications of the transferred proton-proton nuclear Overhauser effect (TRNOE) to the study of the conformations of small molecules to proteins are presented and discussed. The basis of the TRNOE involves the transfer of information concerning cross relaxation between two bound ligand nuclei from the bound to the free state by chemical exchange so that negative NOEs on the easily detectable free or observed ligand resonances may be seen following irradiation of other ligand resonances (free, bound, or observed), thus conveying information on the proximity in space of bound ligand nuclei. In the presence of protein, a negative TRNOE on either the free or observed resonance of nucleus i will be observed following irradiation of either the free, bound, or observed resonance of nucleus j , providing several conditions are met. Methods for obtaining quantitative conformational information from TRNOE measurements are discussed. The TRNOE method is applicable even when no individual proton resonances of either the protein or the bound ligand can be resolved, and is not limited by the molecular weight of the protein, extending the molecular weight range over which ^1H NMR can provide useful conformational information to the very largest systems. This is illustrated by the determination of the glycosidic bond torsion angle of adenosine 5'-monophosphate bound to horse liver alcohol dehydrogenase, yeast alcohol dehydrogenase, and bovine liver glutamate dehydrogenase.

INTRODUCTION

Of the many physical techniques used to study protein-ligand interactions in solution, it is probably true to say that the only method which is potentially capable of determining the conformation of the bound ligand is NMR. The use of paramagnetic relaxation effects is well known but its application is fraught with difficulties as many assumptions have to be made (1, 2). Moreover, in systems without an intrinsic paramagnetic center, extrinsic paramagnetic probes must be used which may severely distort the system that one is studying. The use of three-bond spin-spin coupling constants is familiar in the study of small molecules (3), but in the case of ^1H NMR can only be applied to systems involving small proteins (MW $\leq 20,000$); as for larger proteins the coupling constants are no longer resolvable, the linewidths of the resonances exceeding the values of the coupling constants. Potentially the most direct method of conformational analysis is the use of the proton-proton nuclear Overhauser enhancement (NOE), which can be used to

demonstrate the proximity in space of two nuclei and to determine their separation (4). The power of the NOE in the study of the conformations of small molecules and more recently of small proteins (MW < 10,000), particularly with the advent of the two-dimensional NOE technique, has been amply demonstrated (5-8). However, the extension of such approaches to large molecular weight proteins (MW > 20,000) is severely limited as only a very small number of individual proton resonances are resolvable, and in most cases the signals of bound ligand nuclei cannot be observed. These problems, however, can be overcome by the use of the transferred NOE (TRNOE), the theory and applications of which will be described in the present paper.

In the extreme narrowing limit ($\omega\tau_c \ll 1$), proton-proton NOEs are positive with a maximum value of +0.5; this is the case for small ligand molecules which are characterized by very short correlation times ($\tau_c < 10^{-10}$ sec). In the spin-diffusion limit ($\omega\tau_c \gg 1$), NOEs are negative with a maximum value of -1.0; this is the case for large proteins which are characterized by long correlation times ($\tau_c > 10^{-8}$ sec). The basis of the TRNOE involves making use of chemical exchange between the free and bound ligand to transfer information concerning cross relaxation between two bound nuclei from the bound to the free state. Thus the aim of the TRNOE is to measure negative NOEs on the easily detectable free or observed ligand resonances following irradiation of other ligand resonances (free, bound, or observed) in order to obtain conformational information on the bound ligand.

The TRNOE was initially observed between protein and ligand resonances, and used to demonstrate the proximity of a bound ligand to a particular residue(s) of the protein (9-11). The TRNOE involving bound ligand resonances was first observed by Albrand *et al.* (12) in a complex of NADP⁺ and methotrexate with the enzyme dihydrofolate reductase (MW 18,300). In this particular case, exchange between the free and bound ligand is slow on the NMR time-scale, and the experiments involved observing the change in intensity of the free ligand resonances following irradiation of a bound ligand resonance whose position had been ascertained by transfer of saturation. Irradiation of the bound H1' sugar resonance of the nicotinamide end of NADP⁺ resulted in a decrease of about 20% in the intensity of the free H2 resonance of the nicotinamide ring of NADP⁺, in addition to a substantial decrease in the intensity of the free H1' resonance due to transfer of saturation, from which it was deduced that the conformation about the nicotinamide glycosidic bond must be *anti*. More recently, we have observed TRNOEs in complexes of 3',5'-cyclic AMP (cAMP) with the cAMP receptor protein which has a molecular weight of 45,000 (13). In this case the free and bound cAMP are in fast exchange so that there is only a single set of observed ligand resonances. The experiments were conducted with a 10-fold excess of ligand over protein at very low protein concentrations (~0.1 mM, which corresponds to a concentration of 0.2 mM in cyclic nucleotide binding sites). Irradiation of selected ligand resonances resulted in a specific decrease in the intensity of other ligand resonances. Thus, for example, irradiation of the H1' resonance of the sugar ring of cAMP resulted in a specific decrease of about 20% in the intensity of the H8 resonance of the adenine ring, leaving the intensity of the H2 resonance unchanged. In contrast, irradiation of the H5' sugar resonance resulted in a 20% decrease in the intensity of the H2

resonance but had no effect on the intensity of the H8 resonance. On the basis of these TRNOEs it was deduced that the conformation about the glycosidic bond of cAMP bound to CRP must be *syn*. In the case of both dihydrofolate reductase and the cAMP receptor protein, the effects were extremely specific so that a non-specific spin-diffusion mechanism for the TRNOE can be ruled out.

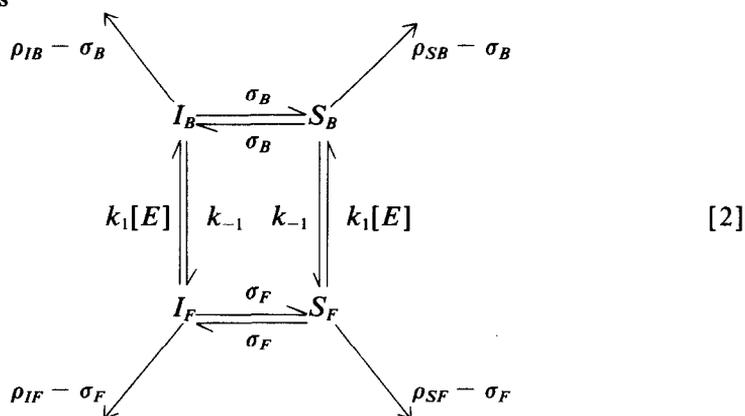
In order to determine the applicability and limitations of the TRNOE method to the determination of the conformations of small ligands bound to proteins, we have carried out a theoretical study to examine how the observed TRNOEs are dependent on the rate constants for exchange between the free and bound states of the ligand, the expected NOEs in the free and bound states of the ligand in the absence of chemical exchange, the relative concentrations of free ligand to protein, the total spin-lattice relaxation rates of the free and bound ligand resonances, and the molecular weight of the protein. This is illustrated by the analysis of the conformation of 5'-AMP when bound to horse liver alcohol dehydrogenase (MW 80,000), yeast alcohol dehydrogenase (MW 150,000), and bovine liver glutamate dehydrogenase (apparent MW $\sim 10^6$).

THEORY

The simple reaction system we will deal with is



where E is free protein, L free ligand, and EL the protein-ligand complex. Consider two nuclei I and S of the ligand related by cross relaxation, such that scheme [1] can be expanded as



where the two nuclei of the ligand in the free state are I_F and S_F , and in the bound state I_B and S_B ; σ_F and σ_B are the cross-relaxation rates relating the magnetization of I and S in the free and bound states, respectively, and ρ_{IF} , ρ_{IB} , ρ_{SF} , and ρ_{SB} are the total spin-lattice relaxation rates of nuclei I_F , I_B , S_F , and S_B , respectively.

The total spin-lattice relaxation rate ρ_i (assuming that the only contributions to ρ_i are from the dipolar local and cross-relaxation rates, and that tumbling is isotropic) is given by

$$\rho_i = \sum_j \frac{\gamma^4 \hbar^2}{r_{ij}^6} \left[\frac{3}{10} \left(\frac{\tau_c}{1 + \omega^2 \tau_c^2} + \frac{4\tau_c}{1 + 4\omega^2 \tau_c^2} \right) + \frac{1}{10} \left(\tau_c - \frac{6\tau_c}{1 + 4\omega^2 \tau_c^2} \right) \right] \quad [3]$$

and the cross-relaxation rate σ_{ij} between the i th and j th nuclei by

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

where r_{ij} is the distance between the i th and j th nuclei, ω the Larmor frequency, τ_c the correlation time, and γ and \hbar have their usual meanings (4, 14, 15). In the extreme narrowing limit ($\omega\tau_c \ll 1$), σ_{ij} is negative and $\sum_j \sigma_{ij}/\rho_i = -0.5$. This will be the case for a small ligand. For $\omega\tau_c = 1.118$, σ_{ij} is zero. In the spin-diffusion limit ($\omega\tau_c \gg 1$), σ_{ij} is positive and $\sum_j \sigma_{ij}/\rho_i = +1$, which will be the case for the ligand bound to a large protein.

The complete set of coupled differential equations describing the evolution of the z component of the magnetization for the nuclei I and S in the free and bound states for scheme [2], based on McConnell's (16) and Solomon's (15) modifications of the Bloch equations for chemical exchange and cross relaxation, are given by

$$\frac{dM_{IF}}{dt} = -\rho_{IF}(M_{IF} - M_{F0}) + \sigma_F(M_{SF} - M_{F0}) + k_{-1}M_{IB} - k'_1M_{IF}, \quad [5]$$

$$\frac{dM_{IB}}{dt} = -\rho_{IB}(M_{IB} - M_{B0}) + \sigma_B(M_{SB} - M_{B0}) - k_{-1}M_{IB} + k'_1M_{IF}, \quad [6]$$

$$\frac{dM_{SF}}{dt} = -\rho_{SF}(M_{SF} - M_{F0}) + \sigma_F(M_{IF} - M_{F0}) + k_{-1}M_{SB} - k'_1M_{SF}, \quad [7]$$

$$\frac{dM_{SB}}{dt} = -\rho_{SB}(M_{SB} - M_{B0}) + \sigma_B(M_{IB} - M_{B0}) - k_{-1}M_{SB} + k'_1M_{SF}, \quad [8]$$

where M_{jF} and M_{jB} are the magnetizations of the j th nucleus in the free and bound states respectively at time t , M_{F0} and M_{B0} are the equilibrium magnetizations of the nuclei in the free and bound states, respectively, prior to perturbation of the system by the application of a radiofrequency field at the position of a particular resonance (note we have set $M_{IF,0} = M_{SF,0}$ and $M_{IB,0} = M_{SB,0}$), and k'_1 is a pseudo first-order rate constant given by $k_1[E]$. We use the normalization $M_{F0} + M_{B0} = 1$, and since in addition $M_{B0}/M_{F0} = [EL]/[L]$,

$$M_{F0} = [L]/L_T, \quad [9]$$

$$M_{B0} = [EL]/L_T. \quad [10]$$

In the case of protein-ligand interactions, the proton resonances of the bound ligand are often difficult to observe directly. Thus, if we are to make use of NOE measurements to obtain structural information on the bound ligand we are limited to observing TRNOEs on either free ligand resonances such as I_F or observed ligand resonances such as I_{obs} (when the free and bound states of nucleus I are in fast exchange on the chemical shift scale), following irradiation of a specific ligand resonance such as S . In such experiments one can irradiate either the free ligand

resonance S_F or the bound ligand resonance S_B (previously located by transfer of saturation experiments) if the resonances S_F and S_B are in slow exchange on the chemical shift scale. Alternatively, if the resonances S_F and S_B are in fast exchange on the chemical shift scale, a single resonance S_{obs} will be observed, and the free and bound states of the ligand nucleus S will be irradiated simultaneously. The steady-state solutions for these experimental cases, derived from Eqs. [5] to [10], are presented below.

Case 1. Free and Bound Resonances of the Irradiated Nucleus S in Fast Exchange on the Chemical Shift Scale

A strong radiofrequency field is applied at $t = 0$. In this situation the initial conditions are $M_{SF} = M_{SB} = 0$, $dM_{SF}/dt = dM_{SB}/dt = 0$. In the steady state (i.e., following irradiation for $t \rightarrow \infty$ when $dM_{SF}/dt = dM_{SB}/dt = 0$), the magnetizations of the nuclei I_F and I_B are given by

$$M_{IF,\infty} = \frac{k_{-1}M_{B0}(\rho_{IB} - \sigma_B) + M_{F0}(\rho_{IF} - \sigma_F)(\rho_{IB} + k_{-1})}{(\rho_{IB} + k_{-1})(\rho_{IF} + k'_1) - k'_1k_{-1}}, \quad [11]$$

$$M_{IB,\infty} = \frac{k'_1M_{F0}(\rho_{IF} - \sigma_F) + M_{B0}(\rho_{IB} - \sigma_B)(\rho_{IF} + k'_1)}{(\rho_{IF} + k'_1)(\rho_{IB} + k_{-1}) - k'_1k_{-1}}. \quad [12]$$

Case 2. Free and Bound Resonances of the Irradiated Nucleus S in Slow Exchange on the Chemical Shift Scale

(1) *Irradiation of the bound ligand nucleus S_B .* The initial conditions are $M_{SB} = 0$, $dM_{SB}/dt = 0$, and in the steady state the magnetizations of the nuclei I_F and I_B are given by

$$M_{IF,\infty} = \frac{M_{F0}(\rho_{IF} - \sigma_F)(\rho_{IB} + k_{-1})(\rho_{SF} + k'_1) + \sigma_F M_{F0}(\rho_{SF} - \sigma_F) \times (\rho_{IF} + k_{-1}) + k_{-1}M_{B0}(\rho_{IB} - \sigma_B)(\rho_{SF} + k'_1)}{(\rho_{IB} + k_{-1})(\rho_{IF} + k'_1)(\rho_{SF} + k'_1) - \sigma_F^2(\rho_{IB} + k_{-1}) - k'_1k_{-1}(\rho_{SF} + k'_1)}, \quad [13]$$

$$M_{IB,\infty} = \frac{M_{B0}(\rho_{IF} - \sigma_F) + k'_1M_{IF,\infty}}{(\rho_{IB} + k_{-1})}. \quad [14]$$

(2) *Irradiation of the free ligand nucleus S_F .* The initial conditions are $M_{SF} = 0$, $dM_{SF}/dt = 0$, and in the steady state the magnetization of the nuclei I_F and I_B are given by

$$M_{IF,\infty} = \frac{M_{F0}(\rho_{IF} - \sigma_F) + k_{-1}M_{IB,\infty}}{(\rho_{IF} + k'_1)}, \quad [15]$$

$$M_{IB,\infty} = \frac{M_{B0}(\rho_{IB} - \sigma_B)(\rho_{IF} + k'_1)(\rho_{SB} + k_{-1}) + k'_1M_{F0}(\rho_{IF} - \sigma_F) \times (\rho_{SB} + k_{-1}) + \sigma_B M_{B0}(\rho_{SB} - \sigma_B)(\rho_{IF} + k'_1)}{(\rho_{IB} + k_{-1})(\rho_{IF} + k'_1)(\rho_{SB} + k_{-1}) - \sigma_B^2(\rho_{IF} + k'_1) - k'_1k_{-1}(\rho_{SB} + k_{-1})}. \quad [16]$$

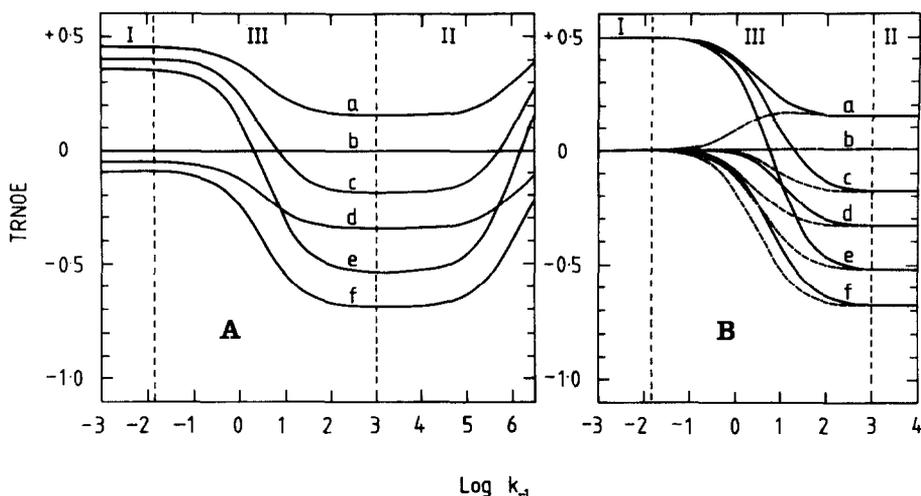


FIG. 1. Dependence of (A) $N_I^{\text{obs}}(S_{\text{obs}})$ (—) and (B) $N_{IF}(S_F)$ (—) and $N_{IF}(S_B)$ (---) on the expected NOEs in the free ($-\sigma_F/\rho_{IF}$) and bound ($-\sigma_B/\rho_{IB}$) states in the absence of chemical exchange, as a function of k_{-1} . $-\sigma_B/\rho_{IB} = 0$ (a, b), -0.5 (c, d), and -1.0 (c, f); $-\sigma_F/\rho_{IF} = 0$ (b, d, f), $+0.5$ (a, c, e). For all curves $L_T = 5 \times 10^{-3} M$ and $\rho_{IB} = \rho_{SB} = 10 \text{ sec}^{-1}$. Values for the other parameters are given in Table 1. The decrease in the TRNOEs observed in Region II for values of $k_{-1} \geq 5 \times 10^4 \text{ sec}^{-1}$, seen both in this figure and in Figs. 2 and 3, is due to the decrease in the concentration of bound ligand and consequent increase in the ratio of free to bound ligand as the value of the association constant falls below $2 \times 10^3 M^{-1}$.

The Observed TRNOE

If the resonances I_F and I_B are in slow exchange on the chemical shift scale, we will observe a steady-state TRNOE on I_F , whose normalized magnitude $N_{IF}(S)$ is given by

$$N_{IF}(S) = (M_{IF,\infty} - M_{F0})/M_{F0} \quad [17]$$

(where S refers to the irradiated nucleus). If, on the other hand, the resonances I_F and I_B are in fast exchange on the chemical shift scale, the normalized magnitude of the observed TRNOE on the resonance I_{obs} , N_I^{obs} , will be given by

$$\begin{aligned} N_I^{\text{obs}}(S) &= [(M_{IF,\infty} + M_{IB,\infty}) - (M_{F0} + M_{B0})]/(M_{F0} + M_{B0}) \\ &= M_{IF,\infty} + M_{IB,\infty} - 1, \end{aligned} \quad [18]$$

From the practical applications point of view the three commonest TRNOEs to be measured are $N_I^{\text{obs}}(S_{\text{obs}})$, where both the resonances of I_F and I_B , and of S_F and S_B are in fast exchange on the chemical shift scale, and $N_{IF}(S_F)$ and $N_{IF}(S_B)$, when both the resonances of I_F and I_B , and of S_F and S_B are in slow exchange on the chemical shift scale. The dependence of $N_I^{\text{obs}}(S_{\text{obs}})$, $N_{IF}(S_F)$, and $N_{IF}(S_B)$ on the NOEs which would be observed in the free ($-\sigma_F/\rho_{IF}$) and bound ($-\sigma_B/\rho_{IB}$) states in the absence of chemical exchange, on the ratio of the total ligand (L_T) to total protein (E_T) concentrations, and on the cross-relaxation rate σ_B between the bound ligand nuclei I_B and S_B are shown in Figs. 1, 2, and 3, respectively, as a function of the dissociation rate constant k_{-1} (at a constant value of the association rate

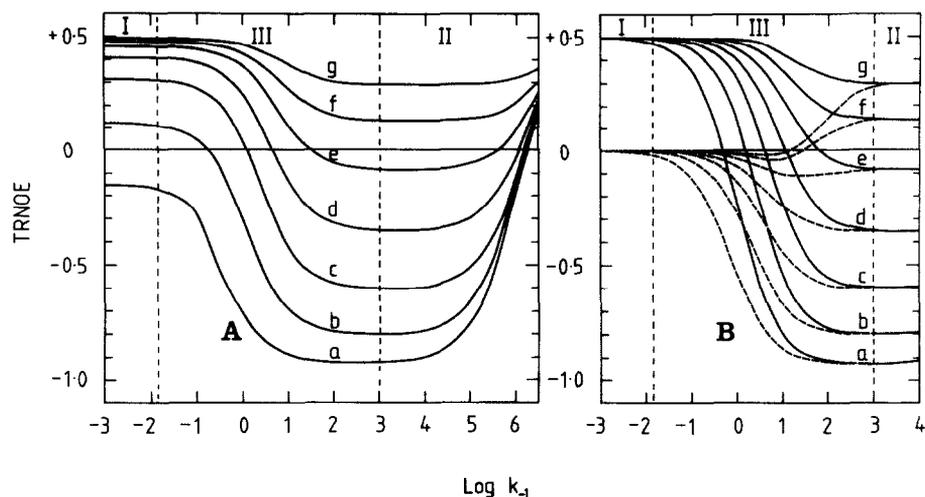


FIG. 2. Dependence of (A) $N_I^{obs}(S_{obs})$ (—) and (B) $N_{IF}(S_F)$ (—) and $N_{IF}(S_B)$ (---) on the ratio of total ligand (L_T) to total protein (E_T) concentration, as a function of k_{-1} . $L_T/E_T =$ (a) 2, (b) 4, (c) 8, (d) 16, (e) 32, (f) 64, and (g) 128. For all curves $-\sigma_F/\rho_{IF} = +0.5$, $-\sigma_B/\rho_{IB} = -1.0$, and $\rho_{IB} = \rho_{SB} = 10 \text{ sec}^{-1}$. Values for the other parameters are given in Table 1.

constant k_1). The parameter values used, chosen to be representative of those in proton NMR experiments on protein-ligand systems, are given in Table 1.

What emerges clearly from Figs. 1 to 3 is that for the three cases considered, two extreme regions, for which considerable simplification in the full expressions

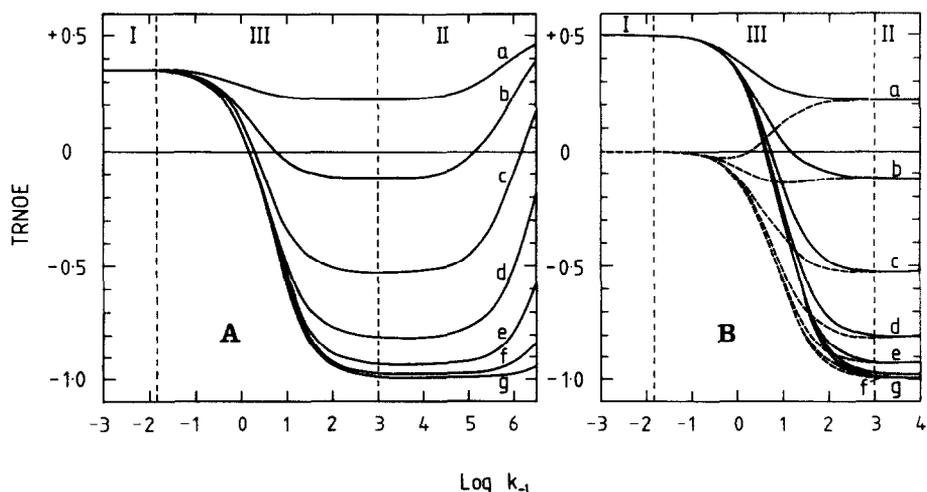


FIG. 3. Dependence of (A) $N_I^{obs}(S_{obs})$ (—) and (B) $N_{IF}(S_F)$ (—) and $N_{IF}(S_B)$ (---) on the cross-relaxation rate σ_B between the bound ligand nuclei I_B and S_B , as a function of k_{-1} . $\sigma_B =$ (a) 1 sec^{-1} , (b) 3.16 sec^{-1} , (c) 10 sec^{-1} , (d) 31.6 sec^{-1} , (e) 100 sec^{-1} , (f) 316 sec^{-1} , and (g) 1000 sec^{-1} . For all curves $-\sigma_F/\rho_{IF} = +0.5$, $-\sigma_B/\rho_{IB} = -1.0$, and $L_T = 5 \times 10^{-3} M$. Values of the other parameters are given in Table 1.

TABLE 1
VALUES OF THE PARAMETERS USED
IN THE CALCULATIONS

Parameter	Value
E_T	$5 \times 10^{-4} M$
L_T	5×10^{-4} to $6.4 \times 10^{-2} M$
k_1	$10^8 M^{-1} \text{sec}^{-1}$
k_{-1}	10^{-3} to 10^6sec^{-1}
$\rho_{IF} = \rho_{SF}$	0.5sec^{-1}
$\rho_{IB} = \rho_{SB}$	1 to 10^3sec^{-1}
$-\sigma_F/\rho_{IF}$	0 to 0.5
$-\sigma_B/\rho_{IB}$	0 to -1.0

[11] to [18] for the normalized magnitudes $N_i(S)$ of the TRNOEs, may be defined in terms of the spin-lattice relaxation rate of the free ligand nucleus I_F :

$$\text{Region I: } k'_1 + k_{-1} \ll \rho_{IF}, \quad [19]$$

$$\text{Region II: } k'_1 + k_{-1} > 10^3 \rho_{IF}. \quad [20]$$

In Region I, the slow exchange limit on the spin-lattice relaxation scale of the free ligand, the following TRNOEs are observed (see Figs. 1 to 3):

$$N_I^{\text{obs}}(S_{\text{obs}}) \sim -[M_{F0} \cdot \sigma_F/\rho_{IF} + M_{B0} \cdot \sigma_B/\rho_{IB}], \quad [21]$$

$$N_{IF}(S_F) \sim -\sigma_F/\rho_{IF}, \quad [22]$$

$$N_{IF}(S_B) \sim 0. \quad [23]$$

(We also note the following TRNOEs and NOEs in Region I: $N_{IF}(S_{\text{obs}}) \sim N_{IF}(S_F)$; $N_{IF}(S_B) \sim 0$; $N_I^{\text{obs}}(S_F) \sim -M_{F0} \cdot \sigma_F/\rho_{IF}$; $N_{IB}(S_{\text{obs}}) \sim N_{IB}(S_B) \sim -\sigma_B/\rho_{IB}$; $N_I^{\text{obs}}(S_B) \sim -M_{B0} \cdot \sigma_B/\rho_{IB}$.) From Eqs. [21] to [23] it is clear that attempts to measure TRNOEs to obtain structural information on the bound ligand will be fruitless in Region I, except in the situation when both the resonances of I_F and I_B , and of S_F and S_B are in fast exchange on the chemical shift scale so that the measured TRNOE is $N_I^{\text{obs}}(S_{\text{obs}})$, and the condition

$$\left| \frac{M_{B0}\sigma_B}{M_{F0}\rho_{IB}} \right| > \left| \frac{\sigma_F}{\rho_{IF}} \right| \quad [24]$$

is fulfilled so that a negative TRNOE is observed. Under most circumstances, however, the only NOE method capable of giving information of the bound ligand in Region I is one where the resonance of a bound ligand nucleus is irradiated and the NOE on the resonance of another bound ligand nucleus is observed. This will only be applicable for very small proteins (MW < 20,000), where the resonances of the bound ligand can potentially be observed and assigned directly.

In Region II, the fast exchange limit on the spin-lattice relaxation scale of the free ligand, only a single TRNOE, $N_i(S)$, will be observed irrespective of the

exchange regions on the chemical shift scale of either I_F and I_B , or S_F and S_B (see Figs. 1 to 3), such that

$$N_I(S) \sim - \frac{M_{F0} \cdot \sigma_F + M_{B0} \cdot \sigma_B}{M_{F0} \cdot \rho_{IF} + M_{B0} \cdot \rho_{IB}} \quad [25]$$

This is in agreement with the result obtained by Schirmer *et al.* (5) for the case of multiple conformations of small molecules in fast exchange on the chemical shift scale in Region II. From Eq. [25] it is clear that the TRNOE can be used to obtain structural information on the bound ligand in Region II providing the relationship

$$|M_{B0} \cdot \sigma_B / M_{F0}| > |\sigma_F| \quad [26]$$

is satisfied so that a negative TRNOE on either the resonance I_F or I_{obs} is observed (see Fig. 1). The enormous potential of this method now becomes immediately obvious. As the molecular weight of the protein increases so the correlation time of the bound ligand becomes longer, and hence from Eq. [4] the value of the cross-relaxation rate σ_B increases. As a result, it is feasible to use a large excess of free ligand over a protein and still obtain substantial negative TRNOEs. Thus, the absolute magnitude of the negative TRNOE may be increased by raising the free ligand concentration, resulting in considerable gains in sensitivity despite the fact that this necessarily results in a reduction in the percentage negative TRNOE observed (see Fig. 2). Further, unlike any other measurement in ^1H NMR, the situation becomes increasingly more favorable as the molecular weight of the protein increases (see Fig. 3). As most proteins of interest have in general molecular weights of 50,000 or more, this is clearly very useful.

Between the two extreme regions I and II lies a third region (Region III) in which exchange is intermediate on the spin-lattice relaxation scale of the free ligand. In Region III the normalized magnitudes $N_I(S)$ of the TRNOEs are intermediate between those in Regions I and II at the same ratio of free to bound ligand, and the full expressions [11] to [18] must be used to calculate their values. However, if, in addition to satisfying relationship [26], the condition

$$k'_1 + k_{-1} \geq 10\rho_{IF} \quad [27]$$

is fulfilled, a negative TRNOE will be observed on the resonances I_F or I_{obs} on irradiation of the resonances S_{obs} , S_F , or S_B in Region III, and thus structural information on the conformation of the bound ligand may be obtained. In addition, for the two cases where the free and bound resonances of both the monitored and irradiated ligand nuclei are in slow exchange on the chemical shift scale, $N_{IF}(S_B)$ is always more negative than $N_{IF}(S_F)$ in Region III. Under conditions where $N_{IF}(S_B)$ is negative, one will usually be able to observe a decrease in the intensity of the free ligand resonance S_F following irradiation of the bound ligand resonance S_B due to the process of transfer of saturation (17), thus providing a means of identifying with certainty the position of the resonance S_B . This is because, in the context of Scheme [2], the only condition required to detect transfer of saturation from the bound ligand resonance S_B to the free ligand resonance S_F is $k'_1 \geq \rho_{SF}$.

The structural information obtained from the observation of a single negative

TRNOE will be of a qualitative nature, simply allowing one to ascertain that two ligand nuclei in the bound state are reasonably close together. To obtain quantitative information one can proceed in one of two ways: (i) the determination of the ratio of distances from two nuclei to a third nucleus; (ii) the determination of the distance between two nuclei. It should be noted, however, that most ligands contain more than two nuclei so that, when the ligand is bound to the protein and rotates very slowly, indirect cross-relaxation between several nuclei may be very effective, and a multinuclear system rather than a two-spin system may have to be considered. Such will be the case when observed steady-state TRNOEs are not highly selective. As in nonexchanging systems (14) this problem may be circumvented by making use of one of the techniques available to observe the initial build-up rates of the TRNOEs.

Determination of the Ratio of the Distance from Two Nuclei to a Third Nucleus

In the absence of chemical exchange, the NOEs that would be measured on the bound ligand nucleus I_B on irradiating the bound ligand nuclei S_B and T_B would simply be $-\sigma_{IS}/\rho_{IB}$ and $-\sigma_{IT}/\rho_{IB}$, respectively (where σ_{IS} and σ_{IT} refer to the cross-relaxation rates between nuclei I_B and S_B , and nuclei I_B and T_B). From Eq. [4] the ratio of the distances from nuclei S_B and T_B to I_B , r_{IS}/r_{IT} , is then given by

$$r_{IS}/r_{IT} = (\sigma_{IT}/\sigma_{IS})^{1/6} = [N_{IB}(T_B)/N_{IB}(S_B)]^{1/6} \quad [28]$$

assuming a single correlation time for the $I_B - S_B$ and $I_B - T_B$ vectors. In the presence of chemical exchange, the TRNOEs obtained will only be equal to those in the bound state in the absence of chemical exchange if, in addition to satisfying condition [20] defining the fast exchange region on the spin-lattice relaxation scale of the free ligand, Region II, the two conditions

$$|M_{B0}\sigma_B/M_{F0}| \gg |\sigma_F| \quad [29]$$

and

$$M_{B0}\rho_{IB}/M_{F0} \gg \rho_{IF} \quad [30]$$

are both fulfilled. Although it is fairly easy to satisfy one of these two conditions, satisfying both of them can prove restrictive in terms of manipulating experimental conditions satisfactorily. However, for the purpose of determining distance ratios such stringent conditions are not required, and, in Regions II and III only conditions [27] and [29] need to be satisfied, as the ratio of the TRNOEs will still be given by

$$N_I(S)/N_I(T) \sim \sigma_{IS}/\sigma_{IT} \quad [31]$$

so that Eq. [28] can be used to calculate distance ratios. (This is easily verified for Region II by looking at Eq. [25].) This is illustrated below by the determination of the glycosidic bond torsion angle of 5'-adenosine monophosphate bound to several large proteins.

If conditions [26], [27], and [29] are not fulfilled, a more complicated approach is required which involves the determination of the NOE in the bound state in the

absence of chemical exchange by measuring $N_I(S)$ at the appropriate number of ligand concentrations and extrapolating back to obtain the value of $N_I(S)$ at zero free ligand concentration which will be equal to $-\sigma_B/\rho_{IB}$. This approach is generally applicable to Regions II and III, and can also be applied to Region I in the situation when both free and bound resonances of both the irradiated and observed nuclei are in fast exchange on the chemical shift scale so that the measured TRNOE, $N_I^{\text{obs}}(S_{\text{obs}})$, is given by Eq. [21].

Determination of the Distance between Two Nuclei

The determination of the distance between two nuclei is a much more complicated procedure than the determination of distance ratios, and is fraught with difficulties. Considering Scheme [2], not only does one have to determine the magnitude of the NOE, $-\sigma_B/\rho_{IB}$, on the bound ligand nucleus I_B following irradiation of the bound ligand nucleus S_B in the absence of chemical exchange by the methods described above, one must also determine the total spin-lattice relaxation rate ρ_{IB} . The determination of the total spin-lattice relaxation rate of a nucleus requires carrying out a selective inversion-recovery experiment (18, 19) in which only the signal of that nucleus is inverted. As the bound ligand resonance cannot usually be observed, it is clear that such a procedure is impractical. As a result, ρ_{IB} can only be determined in Region II by an indirect means involving measuring the selective spin-lattice relaxation rate ρ_{obs} of the observed ligand resonance, which will be given by $\rho_{\text{obs}} = a\rho_{IF} + (1 - a)\rho_{IB}$, where a is the mole fraction of the free ligand. In Regions I and III, however, ρ_{IB} cannot be determined with ease.

Once $-\sigma_B/\rho_{IB}$ and ρ_{IB} have been determined the distance r_{IS} between the two bound ligand nuclei I_B and S_B can be determined using Eqs. [3] and [4], providing a reasonable estimate of the correlation time of nucleus I_B can be made on the basis of the molecular weight of the protein-ligand complex.

EXPERIMENTAL

Horse liver alcohol dehydrogenase, yeast alcohol dehydrogenase, and bovine liver glutamate dehydrogenase were purchased from Sigma Chemicals Comp. Ltd. After extensive dialysis against 20 mM 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. 5'-Adenosine monophosphate (5'-AMP) was also obtained from Sigma Chemicals Comp. Ltd. and used without further purification. All chemicals used were of the highest purity commercially available. Samples for 1H NMR contained 3.33 mM 5'-AMP and between 12 to 16 mg/ml of protein. All experiments were carried out at 20°C.

1H NMR measurements were carried out at 270 MHz using a Bruker WH-270 spectrometer operating in Fourier transform mode. Typically 500 transients were averaged 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 leading to a line broadening of 2 Hz. The pulse sequence used in the TRNOE experiments was $(t_1-t_2-\pi/2-AT)_n$, where the selective irradiation at a chosen frequency was applied during the time interval t_1 (0.5 sec), t_2 is a short delay (2 msec)

to allow for electronic recovery after removal of the selective irradiation, and AT is the acquisition time (0.487 sec). Chemical shifts are given with respect to internal (1 mM) dioxane (3.71 ppm downfield from 2,2-dimethylsilapentane-5-sulphonate).

RESULTS AND DISCUSSION

To illustrate the application of the TRNOE to the study of conformations of ligands bound to proteins, we have used the TRNOE to determine the glycosidic bound torsion angle of the small ligand 5'-AMP when bound to three large proteins, horse liver alcohol dehydrogenase (horse liver ADH), yeast alcohol dehydrogenase (yeast ADH), and bovine liver glutamate dehydrogenase (GDH). Their respective molecular weights are 80,000, 150,000, and 316,000 (20, 21). The effective molecular weight of GDH, however, is much greater than 316,000 owing to the phenomenon of self-association, and at the concentration used in our experiments (~ 12 mg/ml) is approximately 10^6 (21). For all three proteins no individual proton resonances could be resolved at 270 MHz, so that any attempt to obtain structural

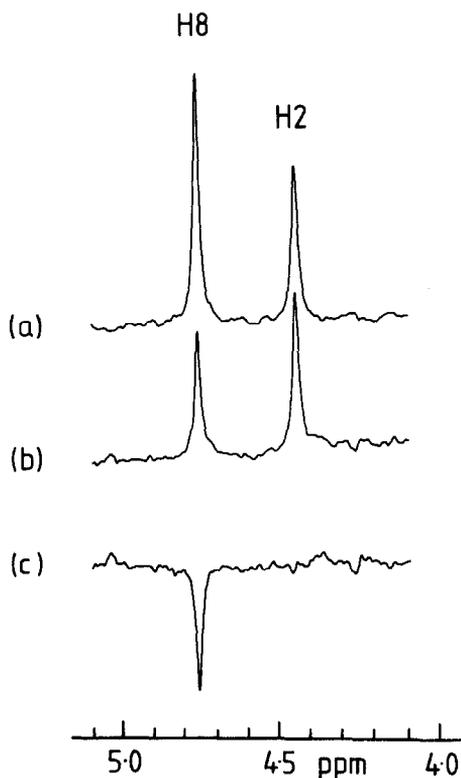


FIG. 4. The aromatic region of the 270-MHz ^1H -NMR spectrum of 3.33 mM 5'-AMP in the presence of 0.1 mM yeast ADH (corresponding to 0.4 mM in 5'-AMP binding sites): (a) control irradiation at -0.46 ppm; (b) irradiation of the observed H2' sugar resonances at 1.06 ppm; (c) spectrum (b) minus spectrum (a). Sample temperature: 20°C . (Under these conditions the positions of the observed resonances of 5'-AMP are at the positions of the corresponding resonances of free 5'-AMP). Chemical shifts are expressed relative to dioxane.

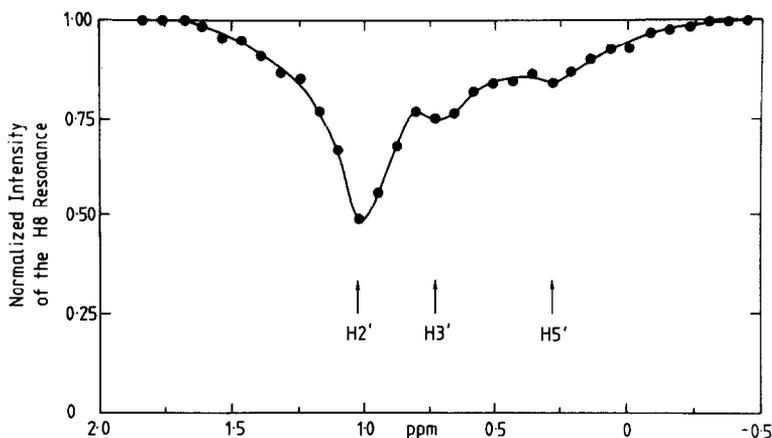


FIG. 5. Normalized intensity of the observed H8 resonance of the adenine ring of 5'-AMP (3.33 mM in the presence of 0.1 mM yeast ADH) as a function of irradiation frequency. Sample temperature: 20°C. The positions of the observed H2', H3', and H5' resonances are at 1.06, 0.76, and 0.25 ppm. (Under these conditions the positions of the observed resonances of 5'-AMP are at the positions of the corresponding resonances of free 5'-AMP). Chemical shifts are expressed relative to dioxane.

information on bound 5'-AMP by conventional NOE measurements would be completely futile.

5'-AMP binds weakly to all three proteins with an equilibrium constant $K_a < 10^5 M^{-1}$ and values for the dissociation rate constant $k_{-1} > 10^3 \text{ sec}^{-1}$ (20, 22) so that exchange between free and bound 5'-AMP is fast on both the spin-lattice relaxation scale (Region II) and the chemical shift scale. Thus, providing relationship [27] is satisfied for any two nonequivalent protons, a negative TRNOE will be seen on the observed resonance of one of the protons following selective irradiation of the observed resonance of the other proton. This is shown in Fig. 4 for the 5'-AMP-yeast ADH system. At an approximately sevenfold molar excess of free over bound 5'-AMP a large negative TRNOE of about 50% is seen on the observed H8 resonance of the adenine ring following irradiation of the observed H2' resonance of the sugar ring of 5'-AMP. (Under these conditions the positions of the observed resonances of 5'-AMP are at approximately the positions of the corresponding resonances of free 5'-AMP.) In addition, smaller TRNOEs on the H8 resonance are observed on irradiating the H3' and H5'/H5'' resonances as shown in Fig. 5, where the normalized intensity of the H8 resonance is plotted as a function of irradiation frequency. However, no changes in intensity of the H8 resonance on irradiation of the H1' resonance, and of the H2 resonance on irradiation of the H1', H2', H3', and H5'/H5'' resonances are seen, demonstrating the specificity of the TRNOE. From these data one can immediately deduce that the conformation of the adenosine glycosidic bond is entirely *anti* when bound to yeast ADH, which is in complete agreement with the X-ray crystallographic data on complexes of 5'-AMP and adenosine diphosphoribose with lactate dehydrogenase and horse liver ADH (24, 25). Similar results were obtained for horse liver ADH and GDH, and these are summarized in Table 2. Looking at Table 2 it can be seen that the sum

TABLE 2

NORMALIZED MAGNITUDES, $N_{\text{H8}}^{\text{obs}}(j_{\text{obs}})$ OF THE TRNOES MEASURED ON THE OBSERVED RESONANCE OF THE H8 PROTON OF 5'-AMP IN THE PRESENCE OF HORSE LIVER ADH, YEAST ADH, AND GDH, TOGETHER WITH THE DISTANCE RATIOS, VALUES OF THE GLYCOSIDIC BOND TORSION ANGLE, AND CONFORMATION OF THE RIBOSE FOR BOUND 5'-AMP^a

	Horse liver ADH (MW 80,000)	Yeast ADH (MW 150,000)	GDH (apparent MW 10 ⁶) ^b
$N_{\text{H8}}^{\text{obs}}(\text{H1}'_{\text{obs}})$	0	0	0
$N_{\text{H8}}^{\text{obs}}(\text{H2}'_{\text{obs}})$	-0.27	-0.48	-0.52
$N_{\text{H8}}^{\text{obs}}(\text{H3}'_{\text{obs}})$	-0.12	-0.21	-0.27
$N_{\text{H8}}^{\text{obs}}(\text{H5}'/\text{H5}''_{\text{obs}})^{\text{c}}$	-0.12	-0.13	-0.21
$\Sigma N_{\text{H8}}^{\text{obs}}(j_{\text{obs}})$	-0.51	-0.82	-1.00
Conformation about the glycosidic bond	<i>anti</i>	<i>anti</i>	<i>anti</i>
$r_{\text{H8-H2}'}/r_{\text{H8-H3}'}$	0.87	0.87	0.90 ^e
$r_{\text{H8-H2}'}/r_{\text{H8-H5}'/\text{H5}''}$	0.87	0.80	0.86 ^e
$\chi(\text{O4}'-\text{C1}'-\text{N9}-\text{C4})^{\text{d}}$	240°	250°	245° ^e
Conformation of ribose	<i>3'-endo</i>	<i>3'-endo</i>	<i>3'-endo</i>

Note. The ratio of the concentration of free to bound 5'-AMP was approximately 7 and the total concentration of 5'-AMP employed was 3.33 mM.

^a The free and bound states of 5'-AMP are in fast exchange on the chemical shift scale for the three systems so that there is only a single set of observed ligand resonances. No TRNOEs could be detected on the observed H2 resonance of 5'-AMP following irradiation of the observed sugar proton resonances in the presence of the three proteins. No NOEs could be detected on either the H8 or H2 resonance of 5'-AMP following irradiation of the sugar proton resonances in the absence of protein.

^b The molecular weight of the bovine liver GDH oligomer composed of six identical subunits is 316,000; however, at high protein concentrations (>1% w/v), the GDH oligomer undergoes self-association to form complexes with a molecular weight of about 10⁶ (21).

^c The resonances of the H5' and H5'' sugar protons are superimposed so that we cannot distinguish whether the TRNOE to the H8 proton arises from the H5' or H5'' proton.

^d The convention used for defining χ is the standard one adopted by Davies (26). The values of χ are estimated by model building on the basis of the values of the distance ratios. These ratios are only consistent with a *3'-endo* conformation of the *N* type for the ribose which is in agreement with the X-ray crystallographic data on coenzyme fragments bound to horse liver ADH (25) and lactate dehydrogenase (24). Further, the distance ratios involving the H5'/H5'' protons are only consistent with either a *gauche-trans* or *trans-gauche* conformation about the C4'-C5' bond (we cannot distinguish between these two possibilities as the resonances of the H5' and H5'' protons are superimposed). From the X-ray crystallographic data, the conformation about the C4'-C5' bond is *gauche-trans* for adenosine diphosphoribose bound to horse liver ADH (25) but *gauche-gauche* for 5'-AMP bound to lactate dehydrogenase (24).

^e It should be noted that each subunit of GDH possesses two binding sites for 5'-AMP which are possibly not identical (21, 22). Thus the values of the distance ratios and χ represent an average of the conformations of 5'-AMP in the two sites. However, from the measured TRNOEs it is obvious that 5'-AMP is bound in the *anti* conformation in both sites.

of the negative TRNOEs from the H2', H3', and H5' resonances to the H8 resonance increases as the molecular weight of the protein increases from -0.5 for horse liver ADH to its maximum attainable value of -1.0 for GDH.

To obtain a quantitative estimate of the glycosidic bond torsion angle χ (O4'-

C1'-N9-C4) for 5'-AMP bound to the three proteins, the distance ratios $r_{\text{H8-H2'}}$ / $r_{\text{H8-H3'}}$ and $r_{\text{H8-H2'}}$ / $r_{\text{H8-H5'/H5''}}$ for bound 5'-AMP must be determined. The value of χ can then be used by simple model building. The distance ratios can be determined directly from the measured TRNOEs on the observed H8 resonance using Eq. [28], providing conditions [27] and [29] are fulfilled. In most cases this is easily verified by making reasonable estimates for the total spin-lattice relaxation rates of the free and bound ligand proton(s) of interest, and for the dissociation rate constant of the ligand-protein complex, and by measuring the relevant NOE in the absence of protein. In the three cases considered here, condition [27] is easily satisfied as the value of ρ_{H8} for free 5'-AMP is 0.6 sec^{-1} (23), and the lower limit of k_{-1} is about 10^3 sec^{-1} (20, 22). Similarly, condition [29] is satisfied since under the experimental conditions used (no EDTA and undegassed samples) we could not detect positive NOEs to the H8 resonance from the H2', H3', or H5'/H5'' resonances for 5'-AMP in the absence of protein. (It should be noted that positive NOEs from the sugar proton resonances to the H8 resonances have been reported for 5'-AMP (23) using degassed samples containing EDTA to remove any dissolved oxygen and trace paramagnetic impurities which may contribute to the spin-lattice relaxation rate of the H8 proton. This is important in the measurement of NOEs in small molecules ($\omega\tau_c \ll 1$), where the cross-relaxation rates are slow, but is of no significance in the measurement of NOEs in large molecules ($\omega\tau_c \gg 1$), where the cross relaxation rates are fast; indeed, addition of EDTA in these measurements makes no difference to the magnitudes of the negative TRNOEs). The values for the distance ratios and χ are given in Table 2. In all three cases the distance ratios are only consistent with a 3'-endo conformation of the N-type for the ribose, in agreement with the X-ray crystallographic data (24, 25), and the values of χ lie in the range 240 to 250°.

CONCLUDING REMARKS

In this paper we presented the theory of the TRNOE and discussed its applications to the analysis of conformations of small ligands bound to proteins. The sensitivity of the TRNOE technique is high. As the TRNOE is observed on free or observed ligand resonances, and as large excesses of ligand over protein can be employed so that only the ligand resonances are resolved, the effects are easy to detect and can be measured with ease. Perhaps the most striking feature of the TRNOE is that its normalized magnitude increases as the molecular weight of the protein increases, reaching its maximum value when the three conditions [20], [29], and [30] are fulfilled. Thus, not only does the TRNOE provide a powerful and unique tool for probing the conformations of small ligands bound to proteins, it also extends the molecular weight range of ligand-protein systems that can be usefully studied by ^1H NMR to the very largest systems.

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