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Use of the Transferred Nuclear Overhauser Effect To Determine the Conformations of Ligands Bound to Proteins

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Proteins and other biomacromolecules express their unique functions by interacting with other proteins or ligands such as peptides, nucleic acids, and polysaccharides. An understanding of the specificity of biomolecular interactions, and ultimately the design of specific inhibitors, requires a detailed characterization of the dynamic structures of proteins, ligands, and their complexes. Many natural ligands or functional peptides form specific, but rapidly associating and dissociating complexes with large biomolecules such as proteins, enzymes, antibodies, and cell receptors. The equilibrium binding constants and the exchange rates can be determined through studies of the changes in the NMR frequencies and the relaxation times of the ligand or the protein nuclei during complex formation.[1-4] Information about the structures of protein-ligand complexes can be obtained from binding-induced NMR relaxation enhancements including transferred nuclear Overhauser effects (NOEs).[5-7]

Transferred NOE experiments have been used to determine the conformations of ligands in the protein-bound state by focusing on the easily detected NMR signals of the free ligands.[8-11] During these experiments, geometric information about the bound ligands is transferred to the free ligands by the ligand association with, and dissociation from, the binding protein. In combination with two-dimensional NMR spectroscopy, transferred NOE experiments have become particularly useful for deriving ligand conformations bound to protein targets that are too large (>40 kDa) to be studied directly by NMR. Recent applications in this area include proteolytic enzymes and proenzymes such as thrombin or prothrombin (40-75 kDa),[12-15] the Fab fragments of natural antibodies (50 kDa),[16] protein assemblies such as the *Escherichia coli* ribonucleotide reductase (171 kDa),[17] the molecular chaperone GroEL (840 kDa),[18,19] and even the intact photon receptor rhodopsin situated on a membrane surface.[20] Precise structural information about the bound ligands can be obtained from approximate-distances derived from transferred NOEs between ligand protons distant in the sequential chemical structure.[12,13,16,20-26] Knowledge of the structures of protein-bound ligands may aid in the design of small and novel molecules that are of therapeutic value as drugs.

Feng Ni was born in Henan Province, China, in 1958. He received his B.Sc. in physical chemistry at Lanzhou University in 1982 and his Ph.D. in biophysical chemistry (with Prof. Harold A. Scheraga) at Cornell University in 1988. Following a brief postdoctoral period at Cornell University with Prof. Scheraga, Dr. Ni joined the Biotechnology Research Institute, National Research Council of Canada, Montréal, where he is presently a research officer and project leader in NMR spectroscopy. He also holds an adjunct professorship with the Department of Biochemistry, McGill University. His research focuses on the study of protein-ligand interactions by NMR spectroscopy and structure-function relationships of proteins and bioactive peptides.

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Transferred NOE experiments have also been applied to explore the interactions of peptides with membrane vesicles along with the study of protein–ligand interactions, as documented in recent reviews.[8-11,27] The importance of transferred NOEs has prompted a detailed review and analysis of both the theory and the associated experimental implementations.[28] Methods have been developed and are now available for the quantitative analysis of transferred NOEs in order to enhance the information content of this technique. In this Account, we will focus on the applications of these recent developments for the determination of the dynamic structures of peptide ligands in complexes with large protein targets.

Binding-Induced Relaxation Enhancements and Differential Line Broadening Effects

In protein-ligand interactions, we usually deal with three species: the ligand, L, the protein, P, and the reversible molecular complex, LP (Figure 1). The binding equilibrium is characterized by a thermodynamic dissociation constant, $K_d = k_{off}/k_{on}$, where k_{on} and k_{off} are the specific rate constants for the ligands to associate with the free protein and to dissociate from the protein–ligand complex, respectively. The most obvious evidence for protein–ligand interactions is the binding-induced line broadening in the NMR spectra of the free ligands (Figure 2). Upon complex formation, a ligand nucleus often experiences a different magnetic environment depending on the location of this nucleus in relation to other nuclei in the bound ligand and in the bound protein. The environmental differences are reflected as differences in both the NMR transverse relaxation times and the frequency separation for different ligand nuclei, thereby causing the observed differential line broadening

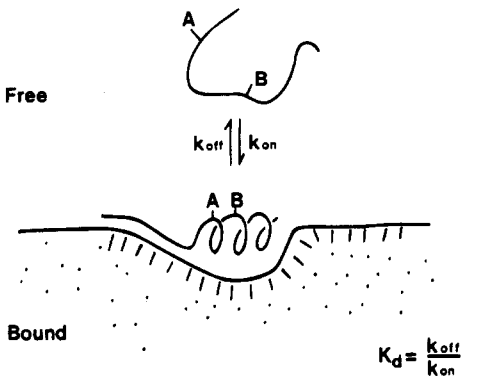

Figure 1. Schematic illustration of a protein–ligand complex (LP) in dynamic exchange with a large excess of the free ligand (L). Protons A and B exchange magnetization in the bound ligand, but measurements are made in the free state with magnetization transfers achieved through combinations of chemical exchange and cross-relaxation mechanisms. The unbound protein molecules (P) are not shown here since the protein is saturated by a large excess of the ligand ($[L]_0/[P]_0 > 10$) with a reasonable binding affinity ($K_d = 10^{-3}$–10^{-6} M) for the protein–ligand complex.[13]

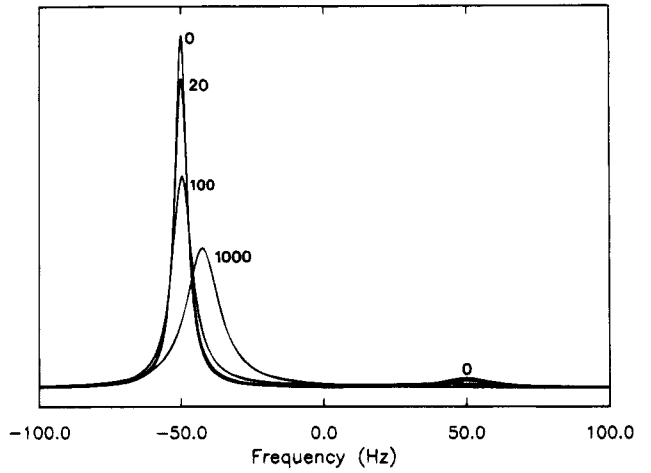

Figure 2. Line-broadening effects on ligand resonances induced by protein binding. The resonance frequencies of the free and the bound ligand nuclei are −50 and 50 Hz with line widths of 5 and 20 Hz, respectively, in the absence of exchange ($k_{off} = 0$). Line shapes were calculated for a singlet resonance with a ligand concentration of 5 mM, a protein concentration of 0.5 mM, and a K_d value of 0.01 mM. Because the ligand is present in large excess, the observed resonances are dominated by the sharper signal from the free ligand. The sharper resonance is progressively broadened with increased values of the k_{off} (s^{-1}) exchange rate (indicated by the numbers) until the exchange rate is much larger than the frequency separation $\delta\omega_b$ (eq 1).

effects on ligand resonances.[5,6] Differential resonance broadening has been used in various interacting systems to identify the contact sites on the ligands that are interacting with the protein.[28] If necessary, contact residues can also be established through a study of the change of the selective longitudinal relaxation times (T_1) of the ligand protons in the absence and presence of the binding protein.[29]

In general, the resonance line shapes can be very complicated depending on the exchange kinetics and the concentrations of the exchanging species.[28] When a large excess of the free ligand is present, or if the fraction of free ligand (α_f) is ~1, the resonance of each ligand nucleus is predominately a single signal (for

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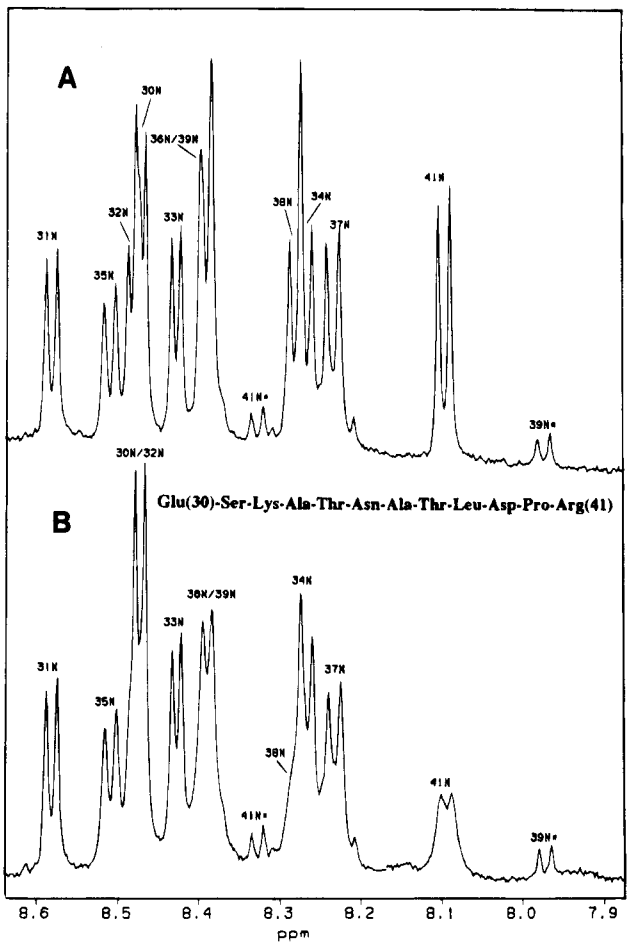

Figure 3. Selective peptide line broadening (relaxation enhancements) induced by protein binding (see Figure 2). Shown here is the amide proton NMR spectrum of a linear peptide derived from a platelet receptor that is activated by thrombin.[14] (A) Spectrum of the free peptide at a concentration of 5 mM. (B) The same spectrum in the presence of 0.4 mM thrombin. The minor resonances (*) are the result of the cis–trans isomerization of Pro(40).

an uncoupled nucleus) with a line width Δ given by[30]

$$\pi\Delta = \frac{1}{T_{2,f}} + \alpha_b k_{off} \frac{\frac{1}{T_{2,b}}\left(\frac{1}{T_{2,b}} + k_{off}\right) + \delta\omega_b{}^2}{\left(\frac{1}{T_{2,b}} + k_{off}\right)^2 + \delta\omega_b{}^2} \quad (1)$$

where $T_{2,f}$ and $T_{2,b}$ are the NMR transverse relaxation times of the nuclei in the free and bound states, respectively, α_b is the fraction of bound ligand, and $\delta\omega_b/2\pi$ is the frequency separation between the resonances of the free and bound nuclei. Figure 2 and eq 1 indicate that exchange line broadening is mediated by the value of k_{off}, by the difference between the frequency shift between the free and bound ligands, and by the increased NMR relaxation (smaller $T_{2,b}$) for the bound ligand in the usually large protein–ligand complex. In particular, eq 1 implies that more pronounced line broadening would be introduced by a larger difference between the resonance frequencies of the free and bound ligands regardless of the exchange kinetics.

Figure 3 shows the NH region of the proton NMR spectrum of a platelet-receptor peptide in the absence (Figure 3A) and in the presence (Figure 3B) of bovine α-thrombin.[14] It is seen that some peaks are more affected by thrombin binding than others. The NH peaks of Leu(38) and Arg(41) are significantly broadened while the broadening of the NH peak of Thr(37) is less severe (Figure 3B). On the other hand, thrombin has minimal effects on the the NH peaks of Glu(30) [and/or Lys(32)], Ser(31), Ala(33), Thr(34), and Asn(35). In correlation with NH line broadening, there is also broadening of the side-chain proton resonances of Leu(38), Asp(39), Pro(40), and Arg(41).[14] Therefore, residues Glu(30) to Thr(37) of the peptide may be more mobile than residues Leu(38) to Arg(41) in the thrombin–peptide complex. The differential mobilities of the residues in the bound peptide were confirmed by following the buildup of the resolved transferred NOEs between adjacent residues.[14] While the magnitudes of the sequential NOEs for residues Glu(30) to Thr(37) continued to increase up to an NOE mixing time of 500 ms, there were already significant decays at 350 ms for the corresponding NOEs between residues Leu(38) and Asp(39) and between Pro(40) and Arg(41). This kind of NOE decay indicates an increased extent of spin diffusion as a result of restricted mobilities of residues Leu-Asp-Pro-Arg upon binding to thrombin.

Similar NMR binding studies were carried out for the interaction of thrombin with peptides derived from the Aα chain of human fibrinogen and from a leech-derived anticoagulant protein, hirudin.[12,31] For human fibrinopeptide A (FpA), in particular, it was found that up to 10 residues (7–16 of the fibrinogen Aα chain) are required for thrombin binding in contrast to only four residues from the platelet-receptor peptide. Within these 10 residues, there is a mutation of Gly(12) to Val(12) associated with a bleeding disorder in human fibrinogen Rouen.[32] We designed analogs of normal FpA and FpA Rouen that included a Pro(15) residue to replace the natural Val(15) at this position to resolve resonance overlaps between Gly(13) and Gly(14) in natural FpA peptides.[32–34] Interestingly, there is essentially no broadening of the proton resonances of P15-FpA compared to significant broadening effects on the resonances of P15-FpA Rouen induced by thrombin binding.[34] Either peptide P15-FpA does not show significant binding to thrombin or the bound peptide does not dissociate from the complex fast enough to effect line broadening of the resonances of the free peptide. However, line-broadening effects start to appear with increasing sample temperatures from 25 to 35 °C (corresponding to increased k_{off}), suggesting that the thrombin–peptide complex exhibits a slow binding behavior on the time scale of the chemical shift separation (Figure 2). The peptide P15-FpA Rouen, on the other hand, must have a larger k_{off} compared to P15-FpA based on the increased line-broadening effect with similar concentrations of thrombin and peptides.[34]

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Transferred NOEs

Transferred NOEs were first reported during early biochemical applications of NMR spectroscopy in systems involving the interaction of peptide hormones with neurophysin II.[5,6] The transferred NOE experiment, in particular intraligand transferred NOEs,[7] has since found wide applications for the determination of substrate and inhibitor conformations bound to proteins.[8-11,27] In some cases, this technique is the only applicable method since the protein–ligand complexes are too large to be studied directly by NMR spectroscopy.[12-15,17-20] If the proton resonances on the ligand are well-resolved, selective irradiation methods can be employed in 1D NMR experiments to observe NOEs between selected proton pairs.[8,35-37] For larger ligands such as peptides, there are often increased degrees of spectral overlap where selective NOE experiments become difficult. In such cases, two-dimensional phase-sensitive NOE (2D NOESY) techniques are the optimal methods so that transferred NOEs between all the protons in the ligands can be obtained in a single experiment.[31-33,38-42]

Transferred NOEs are usually observed between protons on small ligands in the presence of less than stoichiometric amounts of a large binding protein. In the bound state, the range of conformations accessible to the ligand is restricted by the geometry of the binding site on the protein. Information about the spatial proximity between the ligand protons in the bound state is transferred to the free ligands whose proton resonance signals are monitored and studied (Figure 1 and 2). The observable NOEs involving the free ligand include (1) the exchange connectivities between the free (A_f or B_f) and the bound resonances (A_b or B_b), (2) the exchange-relayed NOE (to A_f) between a bound ligand proton A_b and another bound ligand proton B_b or some nearby protein proton(s) (intermolecular transferred NOEs), (3) the exchange-transferred NOE (to A_f and B_f) between the bound ligand protons A_b and B_b (intraligand transferred NOEs), and (4) the exchange-transferred NOE between A_b and B_b relayed by some protein proton(s) (protein spin diffusion). Intermolecular NOEs carry information about the contact sites between the protein and the ligand upon complex formation[5,6] whereas the intraligand transferred NOEs can be used to derive the bound conformations of ligands interacting with the protein.[7] Finally, the exchange cross peaks can be used to determine the exchange kinetics (k_{on} and k_{off})[43] of the protein–ligand complexes.

Intensities $\mathbf{Q}$ for a given mixing time τ_m in a 2D NOE spectrum of protein–ligand complexes are determined by the following equations:

$$\mathbf{Q}(\tau_m) = \exp[-(\mathbf{R}+\mathbf{K})\tau_m]\mathbf{M}_0 \tag{2a}$$

$$\mathbf{R} = \begin{bmatrix} \mathbf{R}_L{}^f & \mathbf{0} & \mathbf{0} & \mathbf{0} \\ \mathbf{0} & \mathbf{R}_L{}^b & \mathbf{R}_{LE}{}^b & \mathbf{0} \\ \mathbf{0} & \mathbf{R}_{EL}{}^b & \mathbf{R}_E{}^b & \mathbf{0} \\ \mathbf{0} & \mathbf{0} & \mathbf{0} & \mathbf{R}_E{}^f \end{bmatrix}$$

$$\mathbf{K} = \begin{bmatrix} q_L k_{off}\mathbf{I} & -k_{off}\mathbf{I} & \mathbf{0} & \mathbf{0} \\ -q_L k_{off}\mathbf{I} & k_{off}\mathbf{I} & \mathbf{0} & \mathbf{0} \\ \mathbf{0} & \mathbf{0} & k_{off}\mathbf{I} & -q_E k_{off}\mathbf{I} \\ \mathbf{0} & \mathbf{0} & -k_{off}\mathbf{I} & q_E k_{off}\mathbf{I} \end{bmatrix} \tag{2b}$$

$$\mathbf{Q} = \begin{bmatrix} \mathbf{Q}_{ff} & \mathbf{Q}_{fb} & \mathbf{Q}_{fE} \\ \mathbf{Q}_{bf} & \mathbf{Q}_{bb} & \mathbf{Q}_{bE} \\ \mathbf{Q}_{Ef} & \mathbf{Q}_{Eb} & \mathbf{Q}_{EE} \end{bmatrix} \tag{2c}$$

where $\mathbf{R}_L{}^f$, $\mathbf{R}_L{}^b$, $\mathbf{R}_E{}^f$, and $\mathbf{R}_E{}^b$ describe the proton–proton relaxation pathways in the ligand and the protein molecules in the free and bound states and $\mathbf{R}_{EL}{}^b$ and $\mathbf{R}_{LE}{}^b$ are the cross-relaxation matrices between the ligand and protein protons in the protein–ligand complex.[44] The exchange matrix $\mathbf{K}$ represents the microscopic kinetics of the two-component, three-species equilibrium system (Figure 1), with q_L being the ratio of the equilibrium concentrations of the ligand in the bound and the free states and q_E that for the protein. The diagonal matrix $\mathbf{M}_0$ is the population of equivalent protons for each component of magnetization. The free protein has been combined with the protein–ligand complex since the protein is saturated by the large excess of ligands used in most practical applications. The submatrix $\mathbf{Q}_{ff}$ in eq 2c contains NOEs (including transferred NOEs) between the resonances of the free ligands while $\mathbf{Q}_{bf}$ ($\mathbf{Q}_{fb}$) and $\mathbf{Q}_{Ef}$ ($\mathbf{Q}_{fE}$) represent exchange-relayed NOEs from the free ligand to the protein–ligand complex. The NOEs $\mathbf{Q}_{EE}$, $\mathbf{Q}_{bE}$ ($\mathbf{Q}_{Eb}$), and $\mathbf{Q}_{bb}$ are usually not resolvable due to the very low concentration and the high complexity of the large protein–ligand complex.

Figure 4 illustrates the relative magnitudes of various types of NOEs in protein–ligand complexes with a large excess of the ligand molecules. The direct NOEs between the bound ligand protons (bb) are effectively 0 throughout the mixing period after an initial buildup of very weak NOE intensities. In contrast, NOEs between the free ligand protons (ff) should be more easily observable because of their large magnitude (Figure 4) and also because of the relatively sharp resonance lines of the free ligands (Figures 2 and 3). Figure 4 also shows that intermolecular transferred NOEs (fE) have magnitudes only slightly smaller than the largest intraligand transferred NOEs (ff). However, experimental observations of these intermolecular transferred NOEs are usually more difficult because of the low concentrations of the protein and the faster decays of the NMR resonances of large protein–ligand complexes. Thus, intermolecular transferred NOEs between the ligand and the binding protein can be observed only with a higher concentration (1–3 mM) of the binding protein and

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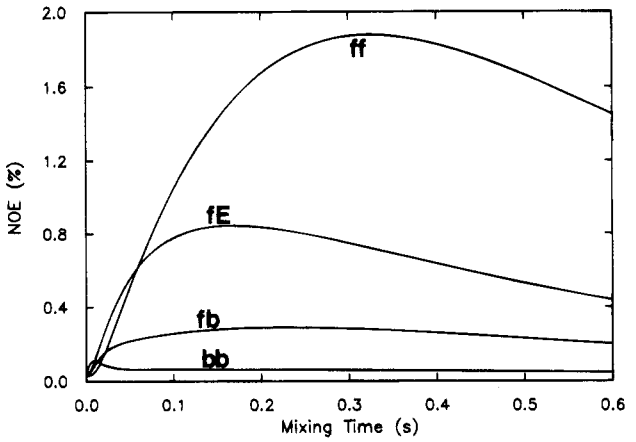

Figure 4. Magnitudes of transferred NOEs in comparison with direct NOEs in the protein–ligand complex for a pair of protons separated by 2.7 Å. NOEs were calculated with a ligand concentration of 5 mM, a protein concentration of 0.5 mM, and a K_d value of 0.01 mM. The protein–ligand complex was assumed to tumble slowly in solution with a correlation time of 20 ns. The tumbling correlation time of the peptide was set to 0.356 ns, which coincides with null NOEs with a spectrometer frequency of 500 MHz. The k_{off} rate of chemical exchange was set to 100 s^{-1}. The NOE calculation was carried out with a relaxation matrix analysis of transferred NOEs incorporating the rates of chemical exchange and the geometry of the ligand binding site in the protein–ligand complex.[44,48] ff: intraligand transferred NOE. fE: intermolecular transferred NOE. fb: exchange-relayed NOE. bb: direct NOE between the bound pair of ligand protons.

with a larger fraction of bound ligands.[9,11,45] Thus, the easily observable intraligand transferred NOEs (ff) have been used widely to study the conformations of ligands bound to proteins, as documented in recent reviews.[4,8,10,27] In favorable cases, intermolecular transferred NOEs have been used to dock bound ligands to combining sites that are constructed by model building based on homologous protein structures.[9,11,16,25] More recently, intermolecular transferred NOEs are being used to study protein hydration and to locate specifically bound water molecules in protein structures.[46,47]

In practice, the ligand molecules must dissociate from the protein–ligand complexes fast enough to effect the efficient transfer of NOE information from the bound to the free state. Consequently, in a 1D transferred NOE experiment employing selective saturation, the ligand k_{on} or k_{off} rates should be larger than the cross-relaxation rates of the ligand protons in both the free and bound states.[35,36] In two-dimensional NOESY experiments, sizable transferred NOEs can be observed as long as the ligand molecules can associate with the binding protein and dissociate from the complex at least a few times during the NOE mixing time.[48,49] When the ligand off rate is small relative to the inverse of the NOE mixing time (5–10 s^{-1} or τ_m = 100–200 ms), it becomes necessary to increase the exchange rates through changes of the pH, salt concentration, or the sample temperature[2,50] at which the transferred NOE experiments are carried out. Minor modifications of the ligand may also decrease its binding affinity and enhance its off rate. For example, conversion of the C-terminal carboxyl group of a peptide ligand into an amide decreased the association constant of the peptide to an antibody by 2 orders of magnitude while increasing its off rate similarly and allowing transferred NOE spectra to be obtained with excellent signal-to-noise ratio.[11,49] Conversely, amino acid substitutions in peptide ligands may also increase the binding affinities of weak-binding ligands, thus allowing the observation of transferred NOEs relevant to specific interactions in protein–ligand complexes.[34] These strategies may be used to extend the applicability of transferred NOEs to the study of protein–ligand complexes involving slow ligand off rates.

The detection of transferred NOEs can be complicated if the free ligands themselves show some kind of NOE cross peaks especially with a large excess of the free ligands.[40,51,52] With the normal NOESY technique, positive NOEs are expected for small ligand molecules while negative NOEs can be seen for larger peptide ligands.[28] Thus, NOE contributions from the free ligands can be minimized through a choice of the appropriate sizes of the ligand molecules so that the corresponding tumbling correlation times approach the NOE null with a particular spectrometer frequency. An increase in the sample temperature can also speed up the tumbling motion of larger ligands, thus decreasing the contribution of free-ligand NOEs to the observed NOE intensities. the analysis of transferred NOEs may therefore be simplified considerably since the cross peaks in NOESY spectra are now dominated by NOEs arising from the bound ligand. Otherwise, control experiments may be necessary to assess the effects of both viscosity changes and nonspecific binding, and to obtain and incorporate the relaxation properties of both the free and bound ligands in the quantitative interpretation of transferred NOEs.[13,21,31,34,40] These experiments may involve NOE and binding measurements with denatured proteins[53] or with proteins modified by a tight- or covalent-binding ligand.[13,25,40] Effects of weak nonspecific binding can be reduced by decreasing the concentrations of both the ligand and the protein while keeping a fixed ratio of the protein and ligand concentrations.[54]

Bound Structures of Ligands by Transferred NOEs

The applications of transferred NOEs for structure determination usually rely on the assumption of the fast exchange conditions.[10] In such cases, the observed intraligand NOEs may be interpreted by the isolated spin-pair/initial-slope approximation to yield distances between bound ligand protons.[35,36,51,55] However, slower exchange and spin-diffusion effects often result in a compression of the apparent distances derived from transferred-NOE experiments (Figure 5). In particu-

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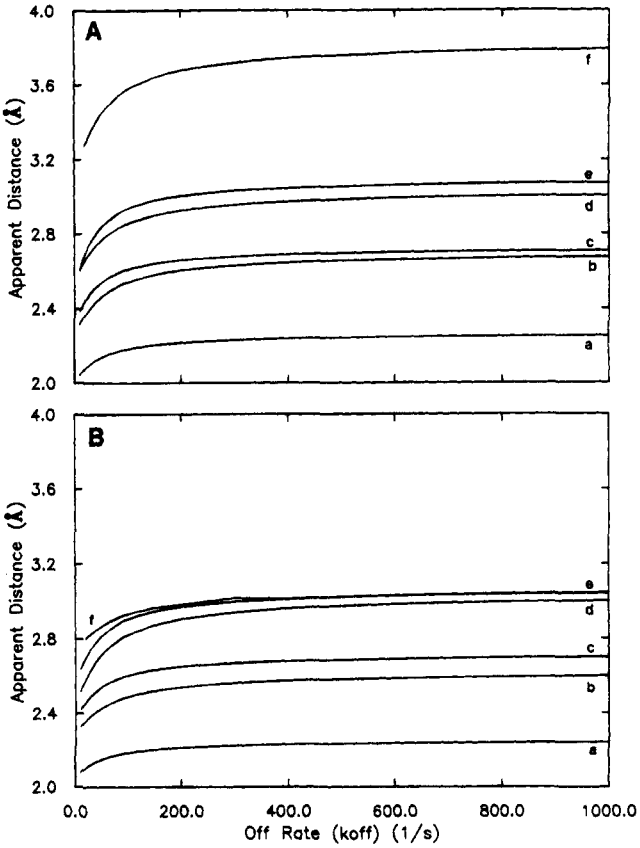

Figure 5. Effects of the exchange off rates on the determination of interproton distances in bound ligands for a complex between thrombin and fibrinopeptide A. The apparent distances were estimated from normalized NOE intensities with a reference distance of 1.79 Å for a geminal proton pair as in Gly. The actual distances were (a) 2.29 Å, (b) 2.73 Å, (c) 2.79 Å, (d) 3.09 Å, (e) 3.19 Å, and (f) 3.87 Å. (A) NOE intensities were computed in the absence of thrombin protons. (B) NOE intensities were computed with the thrombin protons included. An NOE mixing time of 0.2 s was used in all simulations. Small off rates and protein spin diffusion would lead to overly compact ligand structures as a result of shortened apparent distances.[44]

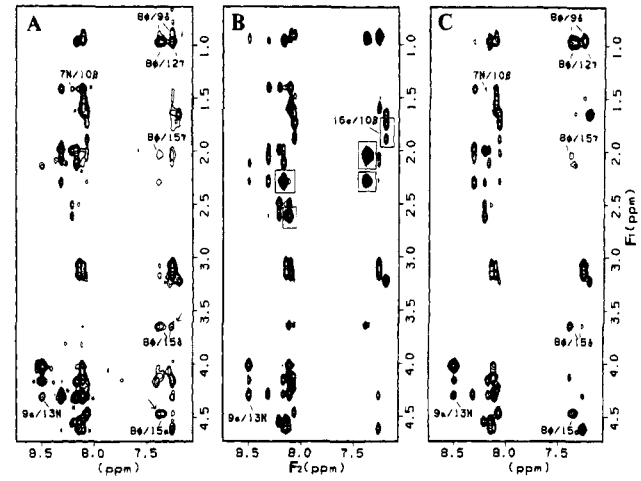

Figure 6. Comparison of the experimental and the predicted transferred NOEs involving the backbone NH and the side-chain protons of the peptide P15-FpA Rouen. (A) Experimental transferred NOEs with an NOE mixing time of 0.2 s. (B) Computed transferred NOEs using a structure model of P15-FpA Rouen constructed from a model of FpA Rouen.[32] No thrombin residues were included in the NOE calculations at this stage since the initial model of the peptide could not be docked into the active site of thrombin without refinement. (C) Computed transferred NOEs based on a refined (Figure 7B) or a docked (not shown) model of P15-FpA Rouen including some thrombin residues. Asterisks (*) in spectrum A label those NOE cross peaks whose intensities come partially from the free peptide. These NOE intensities from the free peptide were subtracted from spectrum A during spectral comparison. The arrows in A indicate the NOEs missing in the computed spectrum B. The boxed peaks in B, on the other hand, represent the NOEs predicted by the starting model, but not observed experimentally A.[34]

lar, relatively distant ligand protons may appear to be close to each other (Figure 5B) because there can be transferred NOEs of sizable intensities if spin diffusion occurs through some unknown protein protons.[44] In these situations, transferred NOEs can be interpreted only conservatively, with, for example, an upper bound of <4 Å assigned for all the ligand protons exhibiting transferred NOEs (FIgure 5), leading to a severe loss of information obtainable from transferred NOE experiments.

Relaxation matrix analysis of transferred NOEs can be used to recover the loss of information by incorporating the k_{on} and k_{off} exchange rates, and the selective longitudinal relaxation times for resolved ligand resonances.[14,34,54,56-59] The goal is to find, iteratively, refined distances and structure models such that the *computed* transferred NOEs match experimental data to within the noise level. The exchange rates and the relaxation times can be determined as part of the fitting process or obtained independently by magnetization transfer or from quantitative measurements of binding-induced relaxation exhancements.[28] The NOE distances are processed by distance geometry and then subjected to energy minimization[13,22] or restrained molecular dynamics[21,38] for structural refinement. Additional NOEs identified on the basis of the resulting model are then included in the iterative procedure until agreement is achieved between the predicted and observed transferred NOEs.[14,34,54,56-59]

The structures of thrombin–FpA complexes in solution have been investigated by use of NMR relaxation enhancements and transferred NOE spectroscopy.[31-33] The identification of a few side-chain to side-chain NOEs was enough to establish the existence of a hydrophobic cluster formed by the nonpolar side chains of the highly conserved residues Phe(8), Leu(9), and Val(15) that interact with thrombin.[13,31] Distance estimates were derived from transferred NOEs using the initial slope or isolated spin pair approximation.[32,33] Conservative distance ranges were chosen to ensure that distance assignments were not affected by differences in local correlation times, by residual NOEs from the free ligand, and/or by protein-mediated indirect effects. Furthermore, the proton resonances of natural FpA analogs were found to be partially overlapped especially for residues Gly(13) and Gly(14), resulting in degeneracies even among the small number of distance constraints.[32,33] Despite this, the available transferred NOE distances allowed the structure of the helical portion of FpA to be determined with an accuracy approaching those in the

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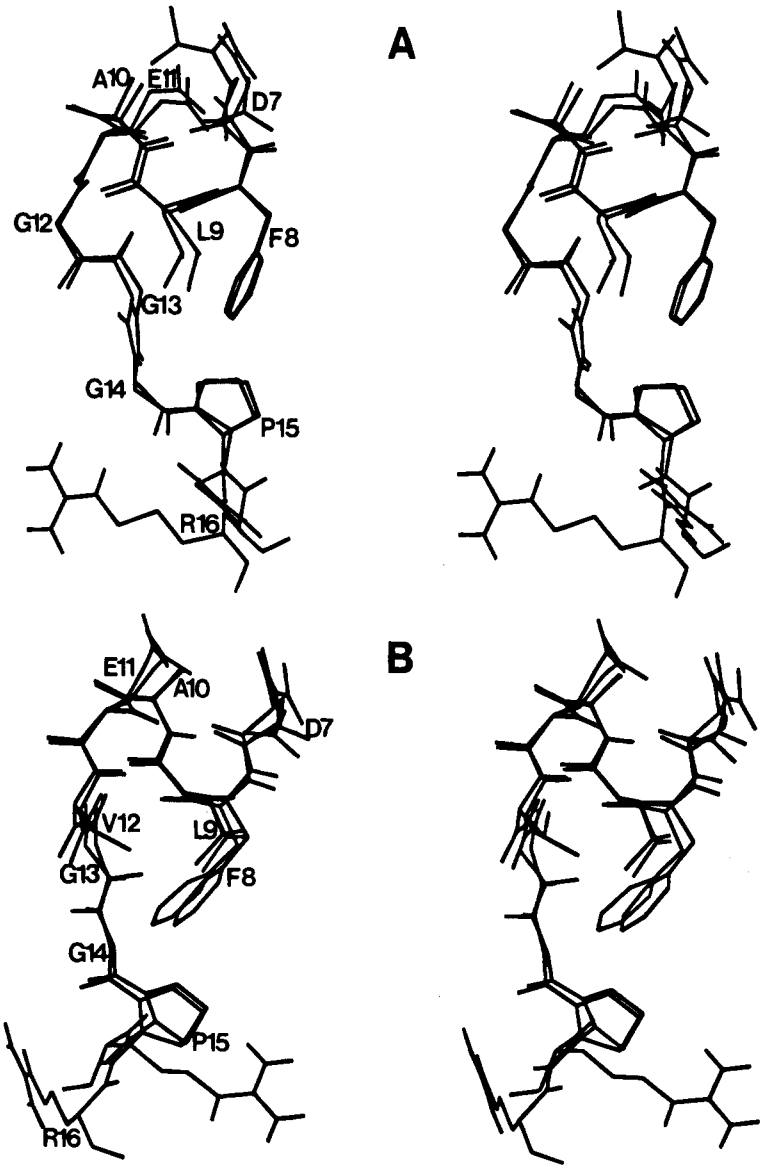

Figure 7. Representative distance geometry structures of the fibrinopeptide analogs P15-FpA (A) and P15-FpA Rouen (B). The superposition between each converged structure was carried out using all the backbone atoms of the peptides. No prochiral assignments were assumed during the distance geometry calculations. All the converged structures predicted the experimentally observed (or absent) transferred NOEs (Figure 6). No thrombin residues were considered at this stage of structure refinement. The orientation of the Arg(16) side chain could be defined further by an explicit consideration of the geometry of the thrombin active site in distance geometry calculations.[14,34]

crystal structures of thrombin–FpA complexes available after NMR studies.[13]

In our recent work, designed FpA analogs with a Pro(15) replacing Val(15) in natural FpAs allowed the identification of more resolved NOEs, resulting in better defined distance constraints involving residues Gly(13), Gly(14), and Pro(15). The bound structures of P15-FpA and P15-FpA Rouen were determined iteratively incorporating transferred NOE information identified with the analog peptides. Initial models of the peptides were constructed by replacing residue Val(15) with Pro(15), starting from the bound structures of FpA analogs proposed previously.[32,33] For example, the initial structure of P15-FpA Rouen showed reasonable agreement with experimental data, but the side chain of Arg(16) had a wrong orientation since there were predicted NOEs between the ϵNH proton and the βCH_2 protons of Arg(16) (Figure 6B) that were not observed in the experimental spectra (Figure 6A). The distance constraints and the initial structures were subsequently refined so that the recomputed transferred NOE spectra were free from the aforementioned artifacts while the valid NOE cross peaks were preserved. After a few refinement cycles, the transferred NOE distances constrained residues Asp(7) to Pro(15) of P15-FpA Rouen to almost unique conformations with root mean square deviations of less than 0.4 Å for the C^{α} atoms among the various converged structures. Residue Arg(16), on the other hand, demonstrated greater flexibility (Figure 7) because of the absence of NOE contacts between its side-chain protons and the rest of the peptide.[32,34] The conformation of Arg(16) may be defined further by incorporation of the available structural information for the active site of thrombin during distance geometry calculations. For example, with some adjustments of the Arg(16) side chain, P15-FpA Rouen docked successfully with the structures of the peptide in the complex reproducing the observed transferred NOEs (Figure 6C).

To define the bound ligand structures, the usual focus is to achieve a good fitting of the observed transferred NOE intensities through relaxation matrix analysis.[54,56-58] However, it is not clear to what extent the fitted structures reproduce the *entire* experimental data, including the absence of transferred NOEs between specific proton pairs. "Absent NOE" distance constraints can limit the conformational freedom of protein or ligand molecules, especially when used in combination with distance constraints derived from NOE intensities.[33,60-62] However, absent NOEs may also be a result of local mobility, bleaching of ligand resonances from solvent saturation transfer, or spectral artifacts such as base-plane distortions. If the NOE spectra are acquired free from experimental artifacts, absent NOEs and associated lower bounds can be identified by a comparison of experimental and simulated transferred NOE intensities (Figure 6). The issue of local mobility can be circumvented by focusing on those protons that participate in many other NOE interactions in a series of experimental NOE spectra with different mixing times. A limited number of conservative distance lower bounds (r_{low} = 3-4 Å) were included in the initial stages of structure calculations for fibrinopeptides to eliminate many false NOEs predicted by the model structures (Figure 6B). The validity of some essential "absent NOE" distance constraints can be confirmed by independent data, e.g., through a comparison of transferred NOEs acquired with both the NOESY and ROESY techniques.[26]

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Concluding Remarks

The successful applications of transferred NOE experiments clearly indicate that this method has become one of the tools for investigating the structure of bound ligands in large protein-ligand complexes. Future research calls for more accurate determinations of the structures of protein-ligand complexes based on distance constraints that can be deduced from transferred NOE studies. For iterative structure refinement, it is important to incorporate the motional properties of the bound ligand either from experimental NMR measurements or from molecular dynamics simulations of the model complex. A fruitful field for application of the transferred NOE technique concerns the studies of peptide hormones or other ligands when complexed with membrane-bound protein receptors.[20] Since, in most current applications, the protein receptors are not available in pure form, it would be worthwhile to attempt to extend the transferred NOE technique to the study of whole cell membranes.

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