Exchange-transferred NOE spectroscopy and bound ligand structure determination

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The exchange-transferred nuclear Overhauser effect of NMR spectroscopy provides information on small-molecule ligands in association with high-molecular-weight proteins or nucleic acids, or with biomolecular assemblies such as membranes. The method has proved particularly useful for the structural analysis of proton-rich, flexible ligands and for screening mixtures of ligands for binding activity. Recent analysis has established the accuracy of bound peptide structures determined from transferred nuclear Overhauser effect data and that intermolecular spin diffusion effects do not diminish the reliability of the structural result. New applications of the method involve systems of greater complexity, such as membrane-bound receptors and ribosomes. In addition, new experiments have been developed that exploit the transfer of other types of NMR signal (saturation, cross-correlation, dipolar coupling) to obtain structural information.

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Abbreviations

et-NOESY	exchange-transferred NOESY
NOE	nuclear Overhauser effect
NOESY	NOE spectroscopy
STD	saturation transfer difference

Introduction

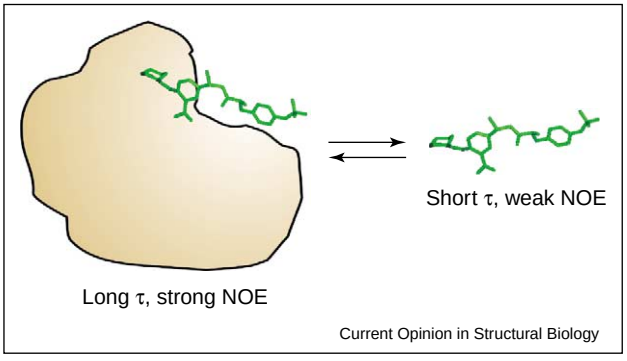
Exchange-transferred nuclear Overhauser effect spectroscopy (et-NOESY) is used to probe the conformation of a ligand while bound to its macromolecular receptor. (Exchange-transferred NOE is abbreviated to et-NOE rather than tr-NOE, as the latter is also used to refer to the transient NOE experiment.) It is primarily applied to systems for which exchange is fast on the chemical shift timescale, so that ligand protons show a single resonance peak averaged over the free and bound states (Figure 1). Observation of the transferred NOE signal requires fast exchange between the bound and free states of the ligand, and has the particular advantage of allowing detailed structural analysis of high-molecular-weight complexes that are not amenable to direct study by NMR due to large line widths or other intractable properties, such as low solubility. Fast exchange generally corresponds to $K_D > 10^{-6}$ M. The et-NOE method uses excess ligand (typical ligand:receptor site ratios range from 10 to 50), so that the resonance line shapes resemble those of the unbound ligand in solution, but the cross-relaxation measured from the NOE spectrum is predominantly determined by internuclear distances within the ligand in the bound state. A recent application of et-NOE is screening mixtures of compounds for binding activity (for a recent review, see [1]), which has clear advantages in drug design efforts (for recent reviews, see [2–4]).

Following a brief description of the experimental requirements and the theoretical treatment of et-NOESY, this review examines the application of the et-NOESY method to conformational analyses of flexible ligands. Three-dimensional structure determination is considered in terms of a bound peptide, but the discussion applies equally well to other proton-rich, flexible ligands, such as oligosaccharides. The most recent applications of et-NOESY to biological complexes in solution are listed in Table 1 and some of these are discussed in the review. Finally, other transfer-based NMR experiments are briefly outlined. Saturation transfer NMR, another method that exploits fast ligand exchange behavior to investigate ligand–macromolecule complexes through dipolar interactions, was recently reviewed [5]. The saturation transfer difference (STD) spectrum provides information on ligand protons in contact with the receptor and thus complements et-NOESY by elucidating the binding surface.

Experimental requirements

The initial observation of the et-NOE phenomenon [6] and definition of the fast exchange limits [7] provide the groundwork for application of the et-NOESY experiment. To observe the transfer of NMR information reflecting the bound state to resonances of the free ligand state, the rate of exchange must be faster than the magnetization life-time in the free state: $k_{off} > T_1^{-1}$. A second requirement sets the fast exchange condition when quantitative information on interproton distances is sought; the initial build-up of cross-peak intensity is proportional to the cross-relaxation rate, σ_{ij}, providing the condition $k_{off} >> \sigma_{ij}$ is met. The value of σ_{ij} is greater than that of T_1^{-1}, so that the latter condition is a more limiting one. Thus, reliable distance estimates require exchange to be significantly faster than the cross-relaxation rate, which becomes a more difficult requirement as the molecular weight of the

Figure 1



et-NOESY. A small molecular ligand, present in molar excess of a macromolecular receptor, binds with $K_D > 10^{-7}$ M. A strong NOE developed in the complex is transferred to the free ligand state and measured from free ligand resonances.

macromolecular receptor increases. The inaccuracies associated with intermediate exchange on the cross-relaxation scale are likely to be reduced when distance estimates are obtained from a ratio of et-NOESY intensities, as opposed to an absolute estimate of σ_{ij}. A rate matrix analysis was conducted to identify errors in distances caused by intermediate exchange [8]. An exchange system with $k_{off} > 300\ s^{-1}$ can be considered a fast exchange system in most circumstances. Values of k_{off} may be estimated from the equilibrium dissociation constant, K_D, when association is assumed to be diffusion limited: $k_{off} \sim 10^8 \times K_D$. To obtain an estimate for σ_{ij}, employ the expression for cross-relaxation rate in the large molecule limit, which is $\sigma_{ij} \approx -57\ \tau_c(ns)/r_{ij}^6(Å)$. In the case of a globular protein, a reasonable estimate for the value of the correlation time, τ_c, is 1 ns per 2.4 kDa. Thus, for a 150 kDa complex, $\tau_c \approx 60$ ns, and the magnitude of σ_{ij} is estimated to be $<50\ s^{-1}$ for r_{ij} distances longer than 2 Å.

The size of the ligand is set by the condition that the dipolar interaction is insignificant for the free ligand, in order that no NOESY cross-peak intensity is observed for this ligand state: $f_b\sigma_b > f_f\sigma_f$. In practice, the ligand rotational correlation time is near the value for the NOE null condition (i.e. $\omega\tau_c = 1.12$) or shorter, which corresponds to a molecular weight of ≤5 kDa. The absence of an NOE signal for the unbound state is easily confirmed by measuring the NOESY spectrum of the free ligand under conditions identical to those used to measure the

Table 1

Recent applications of the et-NOE method to illustrate the diversity of systems that can be investigated.

Ligand	Receptor	Affinity	MW receptor	Description	Refs
Furylacroyloyl amino acid derivatives	Angiotensin-converting enzyme	NR	130 kDa	Screening for specific binding from a mixture	[42]
Decapeptide antibiotic	Lipopolysaccharide	NR	NR	rSA for structure. Conformational averaging not considered	[43]
Bicyclic sulfonium ion	Glucoamylase	$K_I = 1$mM	72 kDa	Restrained MD using eight distances. Docked complex	[44]
Trisaccharide and hexasaccharide from cell-wall polysaccharide of Streptococcus	Monoclonal antibody	$IC_{50} = 135\ \mu$M trisaccharide, 27 μM hexasaccharide	80 kDa	et-NOE restrained modeling for trisaccharide. STD binding surface for trisaccharide and hexasaccharide	[36]
Macrocyclic inhibitor	Farnesyltransferase	$IC_{50} = 0.5\ \mu$M	90 kDa	Restrained energy minimization. Nonspecific binding excluded	[29]
Mannose-based inhibitors	E- and P-selectins	$IC_{50} = 0.2$–$40\ \mu$M	NR	Confirmation of binding	[30]
Pentapeptide	*S. aureus* ribosome	NR	NR	rSA. Nonspecific binding excluded	[45]
^{15}N-labeled cyclic hexapeptide	$\alpha_5\beta_1$ integrin receptor	$IC_{50} = 1.2\ \mu$M	NR	Restrained MD	[32•]
Nonapeptides and their analogs with retro-inverso peptide bonds	Monoclonal antibody	NR	80 kDa	rSA using distance intervals	[23]
Tetrapeptides derived from acetylcholine receptor	Monoclonal antibody	NR	80 kDa	rSA using distance intervals	[24]
Dodecapeptide derived from CD79a co-receptor	Lyn tyrosine kinase– GST fusion	$K_M = 13\ \mu$M	110 kDa	rSA using distance intervals. Nonspecific binding excluded	[21]
Acetylcholine analogs	Acetylcholine receptor	$K_I = 20$–$400\ \mu$M	300 kDa	Restrained modeling. Nonspecific binding excluded	[46]
Nonapeptide derived from cannabinoid receptor CB1	G(a1) protein	NR	NR	rSA using distance intervals	[47]
Antimicrobial 23-residue peptide magainin 2	Phospholipid bilayer vesicles	NR	NR	rSA using distance intervals	[31•]

MD, molecular dynamics; MW, molecular weight; NR, not reported; rSA, restrained simulated annealing.

et-NOESY spectrum of the complex. The macromolecule should be high molecular weight, approximately $\geq$35 kDa, and with no upper limit. The production of isotopically labeled ligand to exploit the numerous multiresonance NMR experiments is advantageous and recent labeling strategies for peptides have appeared [9,10]. Signal-to-noise is often enhanced in et-NOESY spectra from the use of a relaxation filter element in the pulse sequence to suppress unwanted signals from the protein.

Theoretical treatment

Indirect effects are accounted for using the Solomon equations for the time-dependent magnetization including the additional terms for exchange. Simultaneous pairwise dipolar interactions are treated by matrix analysis using both NMR relaxation rates and exchange rates. All indirect effects, intramolecular and intermolecular, are part of the solution. Cross-relaxation from pairwise dipolar relaxation for direct, exchange-transferred, saturation difference and intermolecular magnetization exchange NOE all depend on the same rate matrix analysis and vary only in the initial conditions. Simulation of intensities for these different experiments is therefore possible with the same computational framework. Although the early literature on theoretical analysis of magnetization exchange will not be cited here, we note that a fundamental study related to the simulation of exchange of dipolar relaxation in macromolecular systems describes the symmetrization of the rate matrix containing relaxation and exchange terms, which provided an analytical solution for the time-dependent matrix equation [11]. The first simulations to illustrate the behavior of the et-NOE intensity for parameters such as fraction of bound ligand and molecular size [12], and to probe intermolecular relaxation effects [13] utilized this symmetrization. A recent and thorough simulation study of STD has appeared [14•].

Transferred NOE structure determination

The conformation of the bound ligand state has been analyzed for several complexes using a combination of et-NOESY distance restraints and molecular dynamics following procedures similar to protein structure determination. Interproton distances, r_{ij}, are interpreted from the et-NOESY cross-peak intensities and used in restrained simulated annealing protocols of the molecular dynamics programs XPLOR, CNS, CHARMM and DYANA. Cross-peak intensities are calibrated and restraints defined following the same procedures used for direct NOESY data. Most often, distance restraints are categorized as strong, medium and weak, corresponding to distance intervals for which typical upper limits are 2.8, 3.8 and 5.0 Å. A more quantitative analysis of et-NOESY intensities to achieve higher precision restraints must establish that the system is in the limit of fast exchange on the cross-relaxation timescale, as well as recognize potential effects from spin diffusion and conformational averaging. The effects that influence direct NOE intensities are also present in et-NOESY. (A linear build-up curve does not rule out contributions from spin diffusion for either direct or transferred NOE intensity, in spite of the repeated statements to this effect that appear in the literature.) The fast exchange limit is discussed above. Here, we discuss two factors specific to exchange systems that should be considered in the determination of a bound ligand structure: nonspecific binding and intermolecular spin diffusion.

Nonspecific binding must be ruled out

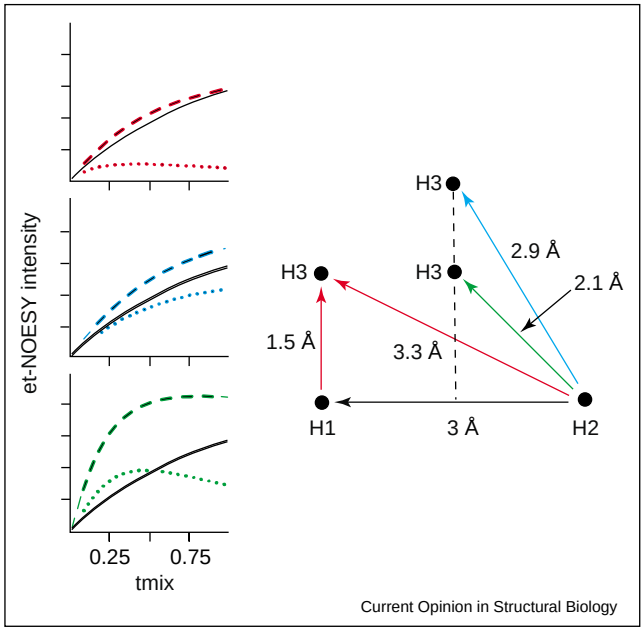
Because the et-NOESY experiment is conducted with high molar excess of ligand over the macromolecule and because the NMR experiment requires near millimolar ligand concentration, there is a strong possibility of nonspecific binding. Any association of the small-molecule ligand with the macromolecule to increase the rotational correlation time of the ligand will lead to NOE cross-peaks that could be incorrectly interpreted in terms of a specific conformer. Several options exist to show specific binding under the conditions of the et-NOESY experiment, perhaps best described by Rao and co-workers [15]. Nonspecific binding is straightforwardly established by demonstrating competitive binding by a second ligand.

Intermolecular spin diffusion

A second factor is the influence of indirect pathways of dipolar relaxation, known as spin diffusion. These pathways may be intramolecular or intermolecular, and are very efficient given the long rotational correlation time of the high-molecular-weight complexes examined by et-NOE. Several studies from 1993 to 1997 examined spin diffusion in exchange systems and noted the potential for efficient indirect relaxation via the protein protons; intermolecular spin diffusion continues to be a concern [3,16,17]. However, it has recently been shown that intermolecular spin diffusion is not a general problem for bound peptide structure determination; this result is briefly reviewed here.

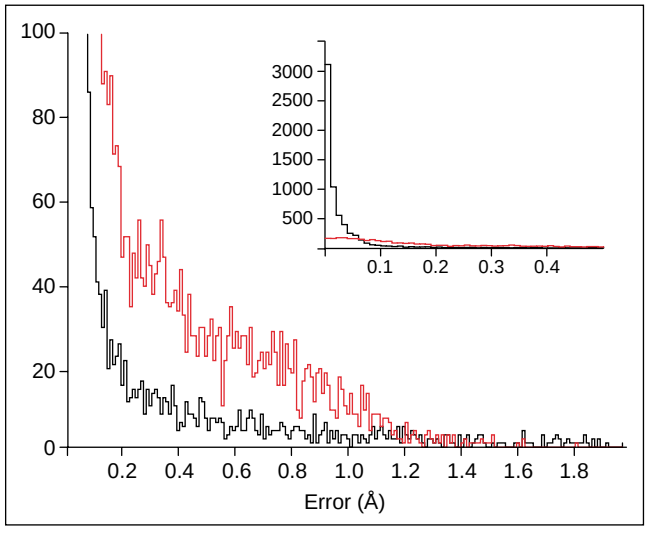
Figure 2 shows the time-dependent et-NOESY intensity simulated with the CORONA program for a three-spin system using a rate matrix including magnetic relaxation and exchange rates. The spatial arrangement of the spins is indicated in Figure 2; the direct NOE interaction is between H1 and H2, while H3 provides an indirect relaxation source as a proton from either the protein or the ligand. Other details of the simulation are described in the original work on intermolecular spin diffusion [13]. An intermolecular spin diffusion effect is shown by the difference between the dashed and solid curves, while that from an intramolecular effect is the difference between the dotted and solid curves. The majority of spatial arrangements involving a third spin give the results shown by the middle plot (cyan) in Figure 2. Thus, most often, the inaccuracies in distance estimates

Figure 2



Time-dependent et-NOESY cross-peak intensity for a three-spin system simulated by a rate matrix approach. The dotted curve is the H1–H2 intensity in the presence of H3 belonging to the ligand (intramolecular spin diffusion), the dashed curve is that for H3 belonging to the protein (intermolecular spin diffusion) and the solid curve is the isolated two-spin intensity shown for reference. Three spatial arrangements are illustrated in green (bottom), cyan (middle), and red (top). Adapted from [13].

Figure 3



Distribution of errors in 19 peptide–protein complexes. Errors due to intermolecular (black line) and intramolecular (red line) spin diffusion are shown. The full-scale inset shows that the vast majority of intermolecular effects are <0.5 Å error. Reproduced with permission from [18••].

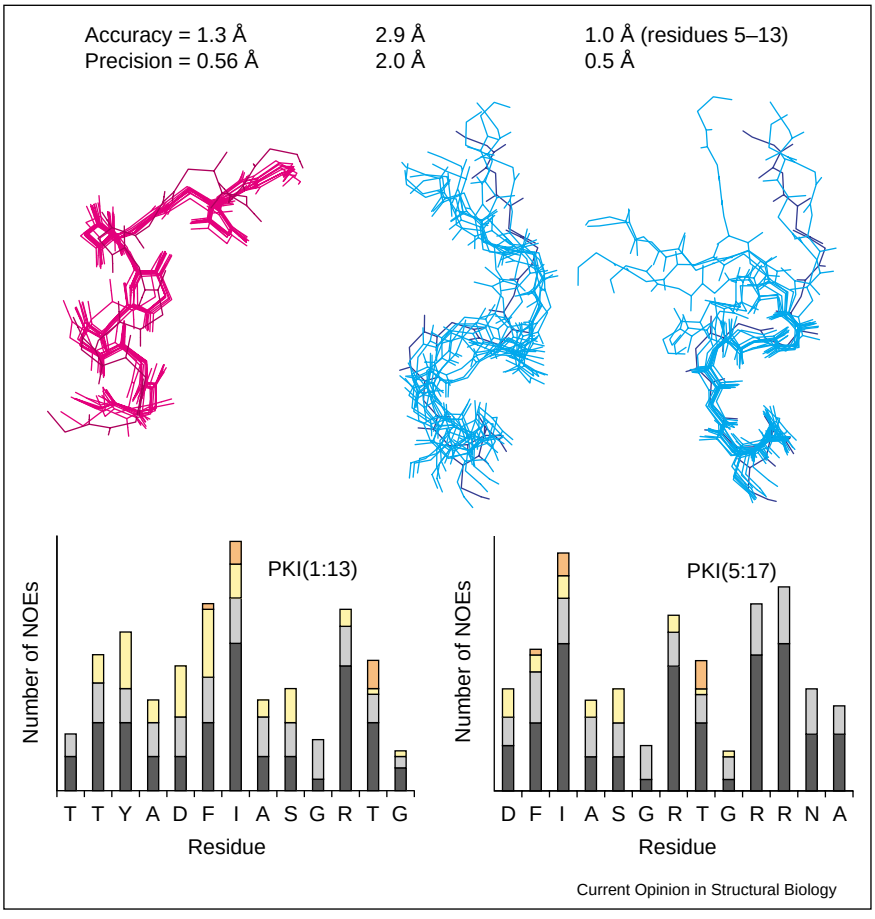
due to spin diffusion between the protein and ligand are similar, or smaller, in magnitude to those that arise from intramolecular effects. The contribution from intermolecular spin diffusion is greater than that from intramolecular spin diffusion only when the indirect third spin is equidistant to the two direct spins and at a shorter distance than that between the two direct spins. These distance errors can be readily accounted for using the procedures established for protein structure determination. In particular, if the lower bounds for the NOE restraint energy term in simulated annealing are set to the van der Waals distance, then errors associated with decreased et-NOESY intensities (e.g. loss of magnetization through spin diffusion, intermediate exchange conditions) will not impose an erroneous penalty function. Only an increase in the observed cross-peak intensity, which defines an upper bound that is too short, is detrimental to structure calculations.

Whereas conformational analysis of carbohydrates and cofactors based on a small number of et-NOE distances demands that the distances be highly accurate, structure determination of peptide ligands involves a relatively larger number of distance restraints and less accuracy is needed. In the case of peptides, and probably any surface-binding ligand, indirect relaxation from the protein has little effect on et-NOESY intensities and does not preclude the reliable determination of bound peptide structures [18••]. The error in calculated intraligand proton distances due to indirect relaxation via protons from either the protein or ligand was evaluated in a simulation study. The error is measured as the difference between the calculated distance, estimated from the ratio of the et-NOE intensity and a reference intensity, and the actual distance. The distribution of the error is plotted in Figure 3 for intermolecular (black) and intramolecular (red) effects. Larger errors ($\geq$0.2 Å) arise from intermolecular relaxation pathways, whereas most errors due to intramolecular pathways are insignificant. The small number of errors with relatively large values (i.e. >1.5 Å) are associated primarily with averaged distances of methyl groups and nonstereospecific assignments.

Bound peptides: the structure of proton-rich, large, flexible ligands

Conformational analyses using et-NOESY have been carried out on numerous peptide–protein complexes. Peptides range in length from pentapeptides up to 20 residues and the protein receptors are generally 50 kDa or larger. Often the protein receptor structure is unknown and the structure calculation is carried out on an 'isolated' bound peptide. Given that the three-dimensional structure of a peptide ligand has relatively few intramolecular tertiary packing interactions and that only ligand–ligand et-NOE cross-peaks are usually observed, an et-NOESY analysis generally provides fewer distance restraints per residue than is obtained for proteins. In addition, the structure calculation for a peptide ligand lacks the

Figure 4



Mainchain superposition of the ten best structures of two peptides bound to cyclic AMP-dependent protein kinase from simulated et-NOESY. The actual structure is shown in bold. Peptide PKI(5–17) is shown in magenta, and peptide PKI(9–21) is shown in cyan using a least-squares superposition of residues 9–21 (left) or residues 9–17 (right). The distribution of NOE interactions is plotted per residue for proton pairs that are intraresidue (black), sequential (dark gray), separated by 2–5 residues in sequence (yellow) and separated by more than 5 residues in sequence (gold). Adapted from [19].

advantage close packing confers by limiting the number of energetically favorable conformations. As such, the reliability of using et-NOESY restraints to determine the structure of ~13-residue peptides bound to proteins was tested using the program CORONA to simulate et-NOE data from a rate matrix, taking into account all dipolar relaxation and exchange rates [19]. Reasonably accurate peptide structures can be defined. Importantly, an indicator of accuracy is suggested from the pattern of NOE interactions; when et-NOE restraints between non-neighboring residues occur over the length of the peptide, the structure is accurate to approximately 1.3 Å (Figure 4, PKI[5–17]). Structures for PKI(9–21) were calculated from only intraresidue and sequential et-NOE interactions for the C-terminal four residues. The accuracy in this case is 2.9 Å overall, although the N-terminal nine residues, for which there are medium-range NOE interactions, are well defined (Figure 4, right set of cyan structures).

Docking flexible ligands using et-NOE restraints

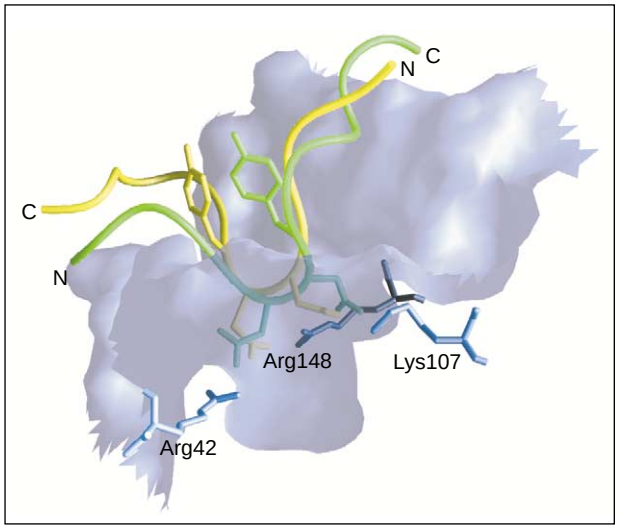
For cases in which the structure of the protein is known from crystallography, additional insight is gained from docking the ligand onto the protein using computational methods that include the distance restraints from et-NOE interactions. The conformational restriction imposed by et-NOE distances greatly facilitates the problem of docking flexible ligands such as saccharides [20] and peptides [21,22]. The docking procedure must include adequate sampling of initial ligand orientations and, in some cases, it may not be possible to distinguish alternative binding modes from the intraligand et-NOE interactions alone, as in the case of docking a 15-residue peptide in a groove-like binding site, shown in Figure 5.

Applications

Peptide–protein complexes

Peptide structures from several peptide–protein complexes have been determined from et-NOESY data. Most

Figure 5



Peptide docked by et-NOE restrained molecular dynamics. Two orientations that differ by an ~180° rotation are favorable (yellow and green). Reproduced with permission from [22].

of the structure calculations were conducted on *in vacuo* peptides. Recent studies include complexes associated with signaling [21], antibodies used to investigate antibody recognition [23] and a peptide–antibody complex intended to mimic structural features of the physiological ion channel [24]. Transferred NOESY experiments were also reported to elucidate the mechanism of antibiotic resistance of peptides bound to the ribosome [25•]. This study illustrates the potential of et-NOE for solution studies of large macromolecular complexes.

Ternary complexes

Examination of both ligands in a ternary complex by transfer experiments is considerably more challenging than binary complexes because the exchange rates of both ligands must be fast on the T_1^{-1} and cross-relaxation timescales. On the other hand, one of the two ligands can be examined providing that exchange of this ligand is fast and the order of binding is either random or the ligand of interest binds second. A theoretical analysis was developed for the evaluation of interligand Overhauser effects in a ternary complex [26] and applied to a ternary complex comprising $NADP^+$ and a folate analog that has a larger number of protons than folate [27].

Inhibitor and drug design

Transferred NOE has been used in drug screening techniques. Preferential binding to a drug-target receptor by one compound from a mixture of compounds can be readily detected. The use of et-NOE to detect low (millimolar to micromolar) affinity binding has been combined with a computational method for lead generation (SHAPES) [28] in drug discovery. The strategy of using et-NOESY with SHAPES has several advantages compared to NMR-based methods that detect the protein target. Most importantly, the size of the protein is not limiting because et-NOESY experiments can be conducted on very high molecular weight receptors. In addition, considerably smaller amounts of protein are needed and the protein is not isotopically labeled. The SHAPES strategy is excellent for identifying leads on potential inhibitors that can be followed up with rigorous binding and structural studies. One example for which information from et-NOE structures aided the design of improved ligands is the efforts to inhibit farnesyltransferase [29]. The et-NOE structure of a conformationally flexible compound aided the development of a macrocyclic analog that exhibited approximately three orders of magnitude enhancement in inhibition.

The transferred NOE experiment also provides a valuable tool for confirming receptor targets implicated in a biological assay for activity. Many biological assays are indirect and do not demonstrate binding to a specific target protein; et-NOESY may be used to confirm actual binding. This type of application of et-NOESY was reported [30] for various mannose-based compounds, which had been shown to have inhibitory activity in an ELISA-based assay. These compounds were found by et-NOESY to indeed bind to the suspected targets, the E- and P-selectins from leukocyte surfaces.

Membrane proteins

The transferred NOE experiment has been applied to the study of membrane-bound peptides and transmembrane receptors, systems not generally amenable to study by NMR. Distances from et-NOESY were used to define the bound structures of megainin, an antimicrobial peptide, bound to lipid vesicles [31•], and a cyclic pentapeptide antagonist associated with the integrin receptor $\alpha_5\beta_1$, which is believed to be involved in tumor metastasis [32•].

Saturation transfer difference spectroscopy

The phenomenon of magnetization transfer or saturation transfer is well known. STD is a recent development of this phenomenon that exploits the same exchange behavior as et-NOESY of a small-molecule ligand present in excess of a high-molecular-weight receptor. A difference spectrum is obtained from spectra collected with and without selective irradiation of a resonance region specific to the macromolecule. Protons near in space to those selectively irradiated will have altered intensity, such that a bound ligand can be identified from the resonances appearing in the difference spectrum. As well as the binding of small-molecule ligands to receptors even as large as a virus [33], the contact surfaces of protein–protein [34] and protein–nucleic acid [35] complexes have been identified by intermolecular transfer of dipolar relaxation. If the system is amenable to both et-NOESY

and STD, the combination of experiments is particularly advantageous for defining both the ligand conformation and the contact surface. Studies of oligosaccharide–protein complexes have exploited this combination [17,36].

Other transfer-based experiments

NMR magnetization may be transferred between the bound ligand state and the easily measured free ligand resonances via mechanisms other than cross-relaxation and saturation. The transfer of cross-correlated relaxation to define dihedral angles was first shown using a partially ^{13}C- and ^{15}N-labeled phosphotyrosine peptide derived from interleukin-4 receptor and ligated to STAT-6 [37]. The approach was subsequently applied to the study of nucleotide cofactors ligated to human recombinant deoxycytidine kinase [38] and epothilone A bound to tubulin [39,40]. Transferred residual dipolar couplings from the transient binding of a selectively labeled transducin undecapeptide to rhodopsin-containing membrane disks defined the angle of N–H vectors with the disk normal. This study demonstrates the potential for determining the conformation and orientation of flexible ligands when bound to integral membrane receptors [41].

Conclusions

The application of et-NOESY has expanded greatly in the past few years and includes investigations of more complex receptor systems, such as membrane bilayers and ribosomes. Notable advances include its use in the high-resolution structure determination of large flexible ligands and in screening mixtures of compounds for specific binding activity. An important consideration in all applications of et-NOESY is the demonstration of specific binding and the exclusion of nonspecific binding. In the case of nonspecific binding, et-NOESY signals can be misinterpreted to define a single rigid conformation when multiple conformations from low-affinity association actually are the source of the signal. It is also now evident that high-resolution structures are reliable and that intermolecular spin diffusion is not generally a significant factor for peptides and other proton-rich, surface-bound ligands.

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