Structure and Orientation of Ligands Bound to Membrane Proteins Are Reflected by Residual Dipolar Couplings in Solution NMR Measurements

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1. Introduction

Numerous biological processes involve interactions of proteins with other molecules termed "ligands", which include peptides, nucleic acids, carbohydrates, steroids, vitamins, or even other proteins. Specific interactions between pairs of molecules are the basis for molecular recognition, which is crucial in signal transduction, gene transcription, immune response, or enzymatic regulation. Precise structural data on the bound complex are required to explore the conformation – activity relationship and are of critical importance for the field of drug design.

Tightly bound complexes can be studied with diffraction techniques, provided high quality crystals are available. Multidimensional high-resolution NMR spectroscopy does not require crystals but is restricted to complexes that show sufficiently fast rotational diffusion. The current size limit for analysis of tightly bound complexes with standard solution NMR spectroscopy is on the order of 30 – 50 kDa,[1] although recent developments in methods may push this limit.[2, 3] This weight limit does not apply to solid-state NMR spectroscopy. A rapidly developing suite of solid-state techniques can be successfully used to address focused questions on membrane proteins and tightly bound complexes[4–6].

Weakly bound complexes of large proteins with small ligands that are in rapid exchange between the free and bound state allow the structure of the bound ligand to be studied by standard high-resolution NMR spectroscopy on the free ligand. The traditional transferred NOE (TrNOE) experiment relies on the much higher efficiency of cross relaxation in the slowly tumbling complex compared to the fast-tumbling free ligand and provides proton – proton distance restraints for the bound ligand.[7–10] Transferred cross-correlated relaxation (TrCCR) has recently been measured in the free form of small ligands in weakly bound complexes.[11, 12] Such TrCCR data allow specification of projection angles in the bound ligand.[13] Residual dipolar couplings in fast-exchanging systems, referred to as transferred dipolar couplings (TrDCs), report on the time-averaged orientation of bond vectors in the bound ligand if the complex has a suitable degree of net alignment. Such couplings have been measured for small carbohydrates that weakly bind to toxins or maltose-binding protein, with the water-soluble complex partially aligned in an anisotropic bicelle medium.[14–16] In addition to characterization of the bound structure of the ligand, dipolar couplings provide information on the relative orientation of the interacting molecules. Order matrix analysis or rigid-body minimization might provide the relative orientation if dipolar couplings can be measured for both partners.[17, 18] Use of either method requires prior knowledge of the high-resolution structures of the interacting molecules or molecular fragments and these structures must be at least partially preserved upon complexation. Rigid-body minimization is applicable if the two interacting entities are rigidly oriented with respect to each other, while order matrix analysis permits consideration of dynamically independent units.[19] Favorable symmetry properties may allow determination of the mutual orientation of the protein and ligand from dipolar couplings of the ligand alone.[20]

TrDCs also allow characterization of the bound structure of molecules that rapidly exchange on and off a membrane-anchored protein.[21] Such complexes are of particular interest in cellular signaling. Examples include the interaction of G-protein-coupled receptors with G proteins, hormones, or neurotransmitters. Recently, the rhodopsin-bound structure and orientation of a peptide that represents a binding site from the C terminus of the α subunit of the G protein transducin (G_t) was determined from residual dipolar couplings and TrNOE data and used to propose a model for the mutual orientation of rhodopsin and transducin in their photoactivated complex.[22]

2. Detection of Transferred Dipolar Couplings

Binding of ligands to membrane proteins is likely to induce structural changes in the membrane protein and/or the ligand, at least locally. In particular, small flexible peptides usually lack a

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preferred conformation in solution, but adopt a distinct structure upon binding. The objective of the TrDC experiment is to provide precise information both on the bound structure of transiently binding ligands, and on the orientation of the ligand with respect to the receptor.

In the TrDC experiment the purpose of transient binding of the ligand to a macroscopically aligned membrane protein is twofold. First, it induces the biologically relevant bound structure of the complex, and second, it imparts the small degree of net alignment to the ligand that is required for observation of residual dipolar couplings (Figure 1). Small

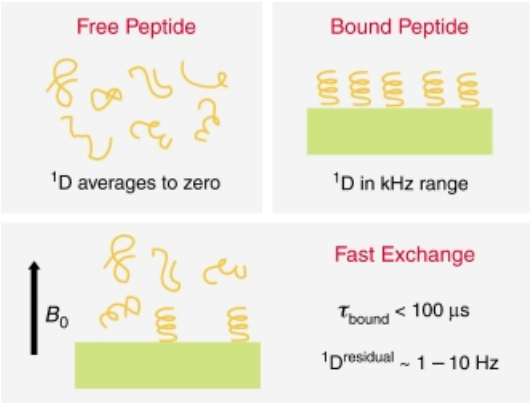

Figure 1. *Schematic representation of the TrDC experiment for a transiently binding peptide. No dipolar couplings are observed for isotropically tumbling, flexible peptides (upper left). Specific binding may induce a distinct peptide structure. The static limit of one-bond $^1H-^{13}C$ and $^1H-^{15}N$ dipolar couplings is in the kHz range; the effective coupling depends on length, orientation, and motional properties of the bond vector (upper right). Residual dipolar couplings are observable with solution NMR spectroscopy in the case of transient binding of the peptide to a macroscopically aligned partner with bound times $< 100\ \mu s$. The observed TrDCs are a time-weighted average of the couplings in the free and bound states, respectively, and carry precise information on structure and orientation of the bound ligand.*

molecules in the free state are subject to fast isotropic tumbling with the effect that all dipolar couplings average to zero. Binding of a small ligand to a macroscopically aligned surface or large particle will pass this alignment on to the bound molecule. A unique set of dipolar couplings is expected in the case of a single binding mode, that is, when the bound ligand has a unique conformation and binds to the aligned target molecule at a single binding site. However, permanent binding of the ligand to a practically immobile large particle would introduce dipolar couplings of up to several kHz and extensive line broadening in the NMR spectrum, an obstacle normally encountered in solid-state NMR spectroscopy but not a suitable situation for rapid detection with standard solution NMR techniques. This unfavorable situation can be avoided if the dipolar couplings are scaled down by a factor of about 10^3 as a result of fast exchange of the ligand between the free and bound forms. Fast exchange within the lifetime of nonequilibrium transverse magnetization results in residual dipolar couplings that represent a time-weighted average of the couplings in the two states. Most importantly, residual dipolar couplings that characterize the bound structure can be detected in the free form of the fast-exchanging ligand by using standard solution-state NMR spectroscopy. Small one-bond residual dipolar couplings are easily detected as variations of the corresponding scalar couplings, ^1J, relative to the spectrum of the unbound ligand without unduly increasing either the complexity or the line width of the high-resolution NMR spectra.[23] Protein-containing membrane fragments or vesicles are very large. To avoid complete dephasing of transverse magnetization of the ligand in the bound state, the duration of a single binding event must not exceed the inverse of the static $^1H-^1H$ dipolar interactions, which is on the order of 100 μs.

TrDC experiments are performed with the membrane protein in its natural environment, a lipid bilayer. There are different ways to achieve the required alignment of the membrane protein. In favorable cases the membrane protein can be prepared from natural sources as part of intact protein-rich membrane fragments that align spontaneously in the strong B_0 field of the NMR spectrometer. A high concentration of transmembrane α helices may cause alignment with the membrane normal, parallel to the B_0 field direction, based on the anisotropic magnetic susceptibility of helices.[24] Indeed, this kind of field-induced alignment has been observed for rhodopsin-rich intact disk membranes[21] and bacteriorhodopsin-rich purple membrane fragments.[25, 26] In other cases it may be necessary to reconstitute the membrane protein into artificial membranes like bicelles that align with their normal perpendicular to B_0 in a cooperative manner and form a nematic liquid crystalline phase.[27]

TrDC studies are particularly suited to molecules that bind weakly to membrane proteins and thus fulfill the critical off-rate (that is, dissociation rate) requirement. Other complexes may be studied if the affinity can be lowered sufficiently by an appropriate choice of experimental conditions like temperature, pH, or ionic strength.[25, 28] Alternatively, minor chemical modifications of the ligand, for example a conservative amino acid replacement in a peptide,[21] might increase the off-rate without compromising structure. In any event, the functional integrity of the complex should be tested to ensure biological relevance of the TrDC-derived bound structure.

A complication arises if the free ligand is structured and unspecific interactions with the aligning medium contribute to the observed couplings. Separation of residual dipolar couplings attributable to the bound conformation of the ligand may be achieved based on the dissociation constant of the complex and the concentration dependence of the observed couplings.[15, 16]

3. Translation of TrDCs into Structure

The alignment of the bound ligand is mathematically described by the molecular alignment tensor.[23] If there is only a single mode for ligand binding to the membrane protein and if the protein undergoes rotational diffusion around the membrane normal that is faster than the inverse of the static limit of any of the observed dipolar interactions (≥ 100 μs), then the alignment tensor will be axially symmetric with the unique axis that points along the local bilayer normal. Indeed, rotational correlation

times of many membrane proteins embedded in a liquid crystalline membrane are in the microsecond time scale and are often shorter than the limiting 100 μs.[29]

The residual dipolar coupling between nuclei P and Q in the bound conformation in the case of axial symmetry is given by Equation (1 a), where $\boldsymbol{S}$ is the generalized order parameter for internal motion of vector PQ[30] and D_a^{PQ} is the magnitude of the residual dipolar coupling tensor, given by Equation (1 b). Here, h is Planck's constant, γ_X the gyromagnetic ratio of nucleus X, μ_0 the magnetic permeability of a vacuum, r_{PQ} the PQ internuclear distance, θ the angle between the orientation of PQ and the unique axis of the molecular alignment tensor $\boldsymbol{A}$, and A_a the axial component of this tensor.

$$D_{PQ}(\theta) = \boldsymbol{S}D_a^{PQ}(3\cos^2\theta - 1) \quad (1\,a)$$

$$D_a^{PQ} = -(\mu_0/4\pi)\gamma_P\gamma_Q h A_a/4\pi^2 r_{PQ}^3 \quad (1\,b)$$

The orientation of the bound ligand relative to the membrane normal (with twofold degeneracy due to the $\cos^2\theta$ dependence) can be obtained from the fit of the set of observed residual dipolar couplings with the couplings predicted for the bound structure of the ligand, that is, by optimization of the orientation of the alignment tensor relative to the coordinate frame of the molecule. The value of A_a is not known a priori but is treated as an adjustable parameter. The fit is often based primarily on a subset of bond vectors for which a similar level of internal mobility is expected. This approach allows use of a uniform generalized order parameter and adsorption of $\boldsymbol{S}$ into D_a^{PQ} in Equation (1a). For example, the assumption of identical $\boldsymbol{S}$ values is justified for backbone bond vectors in structured proteins,[23] and uniform D_a^{NH} and $D_a^{C\alpha H\alpha}$ values can be used for all backbone N–H and C^α–H^α vectors in a bound peptide or protein with an experimentally determined $D_a^{NH}:D_a^{C\alpha H\alpha}$ ratio of -0.48.[31]

The three-dimensional structure of the bound ligand can be refined based on the information on bond vector orientation present in residual dipolar couplings. If a sufficiently large set of qualitatively different residual dipolar couplings is available, it might even be possible to use them as the sole source of experimental data for backbone structure determination as recently demonstrated for a soluble protein.[32] However, up to now TrDCs have been used in concert with NOE-derived distance restraints and dihedral angles, both obtained from TrNOE experiments.[22] Simultaneous structure refinement against restraints from the fundamentally different TrDC and TrNOE experiments minimizes the risk that the derived structure is biased by potential artifacts of TrNOE-derived distances, which can result from intermolecular cross-relaxation or spin diffusion,[33, 34] or errors in predicted TrDCs caused by differential mobility of bond vectors.

Structural resolution of membrane-protein-bound small ligands based solely on TrNOE data is limited. In a small molecule, a large fraction of the protons is exposed to the surface, which reduces the number of observable structurally relevant intramolecular NOEs relative to those seen in a densely packed hydrophobic cluster. The problem is compounded by the complete absence of intermolecular NOESY cross peaks in the complex since the spins of the lipid-bilayer-anchored membrane protein are not observed by the NOESY pulse sequence. Resolution is particularly poor for extended conformations that lack any long-range intramolecular NOEs. NOE-derived distances can be supplemented with residual dipolar couplings to improve the resolution of the local geometry of protein structures.[35] Dipolar couplings restrain bond vector orientation relative to a common reference frame and are therefore global constraints,[23, 36] a feature that is particularly useful in the case of extended structures and for definition of the relative alignment of individual units in large multidomain proteins and macromolecular complexes.[19, 37, 38]

In practice, molecular-dynamics-based simulated annealing combined with a one-dimensional grid search can be used for simultaneous determination of structure and orientation of membrane-protein-bound ligands from TrDC data.[22]

4. How Does a G Protein Bind to Its Receptor? TrDCs Can Tell the Story!

TrDCs were used to determine the high-resolution structure and orientation of the C-terminal binding region of the α subunit of the heterotrimeric G protein transducin (G_t) in the rhodopsin-bound state.[22] A functionally active undecapeptide analogue (referred to as S2 peptide) of the C-terminal $G_t\alpha(340-350)$ fragment was studied. Calculated peptide structures and experimental restraint tables have been deposited with the PDB (accession code 1LVZ).

Binding of G_t to the photoactivated meta II state of rhodopsin catalyzes replacement of $G_t\alpha$-bound guanosine diphosphate (GDP) by guanosine triphosphate (GTP), which causes dissociation of the $G_t\alpha\cdot$GTP and $G_t\beta\gamma$ subunits. These subunits in turn relay the visual signal to downstream effectors.[39] Peptide analogues of the C terminus of $G_t\alpha$, among them the S2 peptide, compete with G_t for binding to meta II and mimic the ability of G_t to stabilize meta II.[40, 41] The exponential decay time of the meta II to meta III transition increases in the presence of the S2 peptide.[21] These observations indicate that the S2 peptide and G_t share a common mode of binding to meta II, that is, the bound S2 structure is of functional relevance for the interaction of G_t with meta II.

Intact individual rhodopsin-rich disk membranes prepared from rod outer segments of bovine retina have the shape of oblate spheroids and spontaneously align with their unique short axis parallel to the direction of the B_0 field of a 14.1 T NMR spectrometer magnet. Transient binding to meta II invokes the small degree of peptide alignment required for detection of TrDCs. Specific binding of S2 to photoactivated rhodopsin is reflected by a sudden decrease in transverse relaxation time, strong relaxation interference (Figure 2), and distinct changes in the observed one-bond J couplings of the peptide. S2 peptide uniformly labeled with ^{13}C and ^{15}N was produced by biosynthesis to enable detection of a large set of TrDCs. A total of nine backbone N–H, nine $C\alpha$–$H\alpha$, and 20 side-chain C–H dipolar couplings were extracted from two-dimensional HSQC experiments (Figure 2) recorded prior to and after photoactivation.

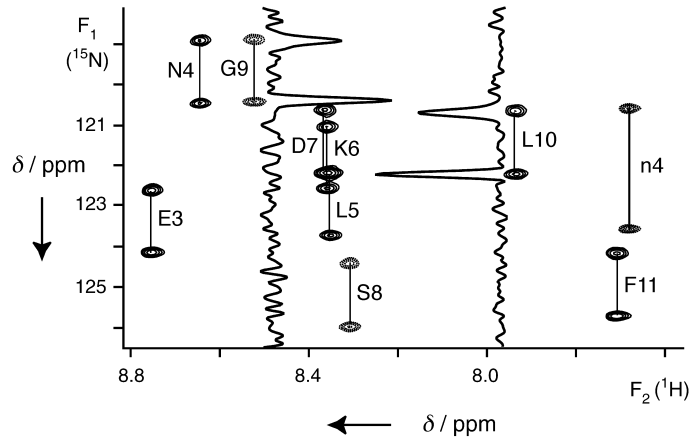

Figure 2. *Section of a 600-MHz $^1H-^{15}N$ HSQC spectrum recorded at 20 °C without ^1H decoupling for the ^{15}N-labeled S2 peptide (2.6 mM) immediately after photoactivation of rhodopsin (63 μM). The inserted F_1 cross sections through the Gly9 and Leu10 resonances show a strong doublet asymmetry caused by relaxation interference between the ^{15}N chemical shift anisotropy and the one-bond $^{15}N-^1H$ dipolar interaction,[52] which proves the transient binding of the small S2 peptide to a very slowly tumbling object, that is, the rhodopsin-bearing disk membrane. Correlations for Gly9, Ser8, and the Asn4 side-chain amide protons (labeled n4) are folded as a result of the small spectral width in F_1. Acquisition times of 120 ms (F_1) and 114 ms (F_2) were used. Data were apodized with 72°-shifted sine-bell (F_1) and squared sine-bell (F_2) windows prior to zero filling (256 × 2048 points) and Fourier transformation.*

The dipolar contributions to the *J* couplings observed after photoactivation show an exponential time dependence with time constants of 15 and 54 min at 20 and 10 °C, respectively, which reflects the decay of the peptide-binding meta II state.[22] This interpretation is in agreement with spectrophotometry measurements under comparable conditions (20 °C; pH 6.7), which gave exponential decay times of 14 ± 2 min (with S2 peptide) and 11 ± 2 min (without peptide) for the meta II to meta III transition.[21]

TrDCs were supplemented with 121 TrNOE-derived distances for structure calculation. The meta-II-bound peptide conformation is characterized by an N-terminal α helix terminated by an α_L-type C cap, with Gly9 in the C′ position.[42] The structure of the three C-terminal residues is relatively open and provides ample opportunity for specific interactions with meta II; for example, the backbone carbonyl groups of Lys6 – Phe11 and the backbone amide of Phe11 are accessible to form intermolecular hydrogen bonds and the hydrophobic side chain of Leu10 points outwards, most likely the result of hydrophobic interaction with meta II. The side chains of Asn4 and Asp7 that are located on one face of the N-terminal helix, might also be in direct contact with meta II as indicated by analysis of intermolecular magnetization transfer upon binding. The meta-II-bound structures of undecapeptides that correspond to the C-terminal binding region of $G_t\alpha$ have been studied previously based on TrNOE data alone.[42, 43] One of these two structures shares the salient features of the bound S2 peptide.[42] However, the Ramachandran score is significantly better for the S2 structure and the conformations differ in a number of details that are certainly relevant for binding meta II, for example, the length of the N-terminal helix, side-chain packing and orientation. The use of both TrDCs and TrNOE-derived distances for structure refinement results in a more detailed conformation that is less susceptible to experimental artifacts. Equally important, the orientation of the bound peptide relative to the membrane normal was extracted from the set of TrDCs, crucial information not available from TrNOE data.

Interpretation of TrNOE and TrDC data is often based on the assumption of a two-state exchange between the free form and a unique bound conformation of the ligand. Transient binding of the S2 peptide to rhodopsin fulfills this condition: the peptide specifically binds to and stabilizes the metarhodopsin II photointermediate[41] and all peptide models generated in molecular dynamics calculations converge to a single and physically reasonable conformation that is in agreement with each of two fundamentally different sets of restraints derived from TrDC and TrNOE data, respectively.[22]

5. Aligning the Pieces of the Puzzle

Although high-resolution x-ray structures are available for GDP-bound transducin ($G_t\alpha\beta\gamma \cdot GDP$) crystallized in the absence of rhodopsin[44] and for dark-adapted rhodopsin,[45] a crystal structure of the active complex has remained out of reach. Several binding sites on the surface of the G protein and on the receptor are known.[40, 46–48] Point-to-point contact sites have been identified by mutagenesis studies[49, 50] and by mass spectrometric identification of chemically cross-linked interaction sites.[51] In particular, close proximity between residues 342 – 345 of the C terminus of $G_t\alpha$ and residue 240 of rhodopsin, located on the third cytoplasmic loop, was observed for the active complex.[51] In this situation TrDCs can provide crucial information on the detailed structure of contact sites and the mutual alignment of the molecular players required to assemble a model of the active complex.

The crystal structure of $G_t\alpha\beta\gamma \cdot GDP$ indicates that helix α5 ends with residue 342 of the $G_t\alpha$ subunit and no electron density is observed for the C-terminal residues 343 – 350. This result contrasts strikingly the well-defined structure of the meta-II-bound S2 peptide. Apparently, intermolecular interactions are required for induction and stabilization of the meta-II-bound conformation of the C terminus of $G_t\alpha$. Most likely, helix α5 gets elongated upon binding to meta II. The short stretch of helical residues common to the x-ray structure of $G_t\alpha\beta\gamma \cdot GDP$ and the NMR structure of S2 allows the two structures to be docked together.[22, 42] A tilt angle of $(40 \pm 4)°$ between the long axis of the helix and the membrane normal was determined from the TrDC data. If it is assumed that the elongated helix α5 is not bent and that binding of $G_t\alpha\beta\gamma \cdot GDP$ to meta II does not cause major rearrangements in the structure of the G protein apart from localized conformational changes in the contact region, the known tilt allows alignment of the G protein relative to the membrane normal. The vectorial orientation of the peptide relative to the membrane is masked by the twofold degeneracy of the axially symmetric alignment tensor. However, only one orientation of the G_t-docked S2 peptide allows interaction with rhodopsin[42] and this orientation is consistent with the inter-

molecular contact sites on S2 predicted by qualitative analysis of the NOESY cross-relaxation pattern.[22]

The experimentally derived orientation of meta-II-bound $G_t\alpha\beta\gamma \cdot$GDP relative to a rhodopsin-containing membrane is shown in Figure 3. In terms of the relative orientation of $G_t\alpha\beta\gamma \cdot$GDP and rhodopsin in the photoinduced complex there remains

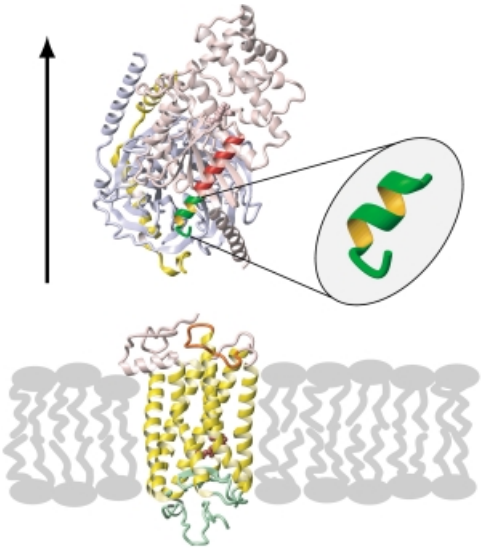

Figure 3. *Orientation of transducin during interaction with rhodopsin. The figure is based on the refined crystal structures of $G_t\alpha\beta\gamma \cdot GDP$[44] and dark-adapted rhodopsin.[45] The insert shows an enlarged ribbon representation of the bound S2 peptide conformation. The orientation of the peptide relative to the membrane normal (black arrow) is precisely defined by the measured TrDCs. The C terminus of $G_t\alpha$, which is not structured in the crystal, was replaced by the meta-II-bound NMR spectroscopy structure of the S2 peptide (green/gold) by using a docking procedure. Binding to meta II is likely to induce elongation of helix α5 (red) in $G_t\alpha$, with the axis of the helix tilted by about 40° relative to the membrane normal. The C terminus of $G_t\alpha$ binds to a site on the third cytoplasmic loop of light-activated rhodopsin (orange) that is close to transmenbrane helix 6 in the sequence.[49, 51] The program MOLMOL was used to prepare this figure.[53]*

only one degree of freedom—a rotation of transducin around the membrane normal. Once a crystal structure of light-activated meta II is available, docking software could be used to predict a model of the activated complex. Precise information on peptides that mimic several independent binding sites may confirm whether the crystal structure of free $G_t\alpha\beta\gamma \cdot$GDP is a reasonable approximation of meta-II-bound transducin. Therefore, TrDC data on the structure and orientation of other contact regions of transducin in the meta-II-bound state are desirable.

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