NMR Spectroscopy in Solution

DOI: 10.1002/anie.201201003

# Hyperpolarized Binding Pocket Nuclear Overhauser Effect for Determination of Competitive Ligand Binding**

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Nuclear magnetic resonance (NMR) spectroscopy has long been used to identify the binding of ligands to biological macromolecules for drug discovery or other applications.[1] Apart from NMR parameters such as chemical shift or spin relaxation,[2] transfer of magnetization between a protein and ligand can be used to determine binding sites and binding modes. In the saturation transfer difference (STD) experiment, radio-frequency irradiation is applied to a resonance on the protein, spreads by spin diffusion, and transfers to a ligand.[3] In the case of competitive binding, more specific information on the binding pocket can be obtained by observing protein-mediated magnetization transfer between the two ligands. This experiment, termed "interligand NOEs for pharmacophore mapping" (INPHARMA) has recently been introduced by several of the authors of this work (Figure 1a).[4] It relies on a nuclear Overhauser effect spectroscopy (NOESY) experiment, where non-Boltzmann polarization of the spins of a ligand can be traced back to its origin on the other ligand.[5] Given that direct NOEs rarely exceed 10% of the diagonal peak intensity, the two-step transfer yields low sensitivity.[4c]

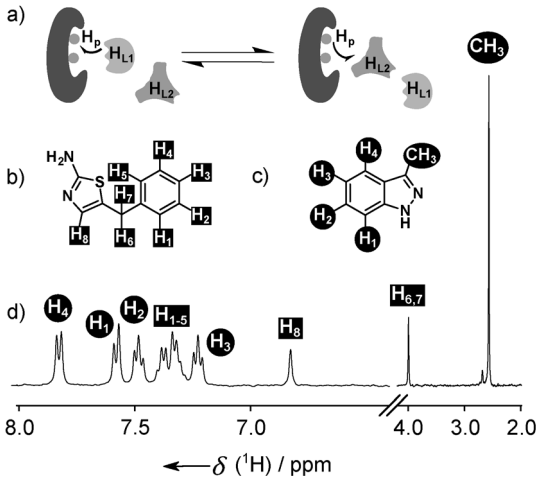


***Figure 1.*** a) Transfer of magnetization from protons of ligand 1 ($H_{L1}$) to ligand 2 ($H_{L2}$) through a protein ($H_P$) by means of competitive binding. b) Structure of ligand 1. c) Structure of ligand 2. d) $^1$H NMR spectrum (not hyperpolarized) of a mixture of ligands 1 and 2. Chemical shift assignments are indicated.

Hyperpolarization enables the generation of non-Boltzmann polarization that is enhanced by several orders of magnitude compared to the simple population inversion achievable in a conventional NOESY experiment.[6] Here, we use dynamic nuclear polarization (DNP)[7] to polarize the $^1$H spins[8] of one of the two ligands. The transfer of a fraction of this polarization through the binding pocket to the second ligand is termed here "hyperpolarized binding pocket NOE" (HYPER-BIPO-NOE). It is related to the spin polarization-induced NOE[9] for hyperpolarized xenon, however, is based on more selective ligand binding.[10]

The HYPER-BIPO-NOE effect is observed for the ligands 1 (5-benzyl-1,3-thiazol-2-amine) and 2 (3-methyl-1*H*-indazole), which bind competitively to protein kinase A (PKA) (Figure 1). An aliquot of ligand 1 was hyperpolarized by DNP in the solid state, dissolved, and rapidly mixed with ligand 2 and protein in the NMR spectrometer. Due to an enhancement greater than 620, when compared to thermal polarization in the NMR magnet, the resulting spectra predominantly show the signals from ligand 1 (Figure 2a). The transfer of signal to the second ligand can be observed in the top trace of Figure 2b, where the peaks for $H_4$, $H_1$, and $CH_3$ groups of ligand 2 have become enhanced. The transferred polarization is sufficiently large to observe the inter-

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[**] C.H. thanks the Camille and Henry Dreyfus Foundation for a New Faculty Award. Support from the National Science Foundation (grant number CHE-0846402), and from the Texas A&M University startup funds is gratefully acknowledged. C.G. thanks the Max Planck Society for support. This project was also performed under the auspices of the BMBF project ("Neue Verfahren der Bio-NMR zur Optimierung und Beschleunigung strukturbasierter Wirkstoffentwicklung") and the EU funded FP7-Bio-NMR project (project number: 261863).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201201003.

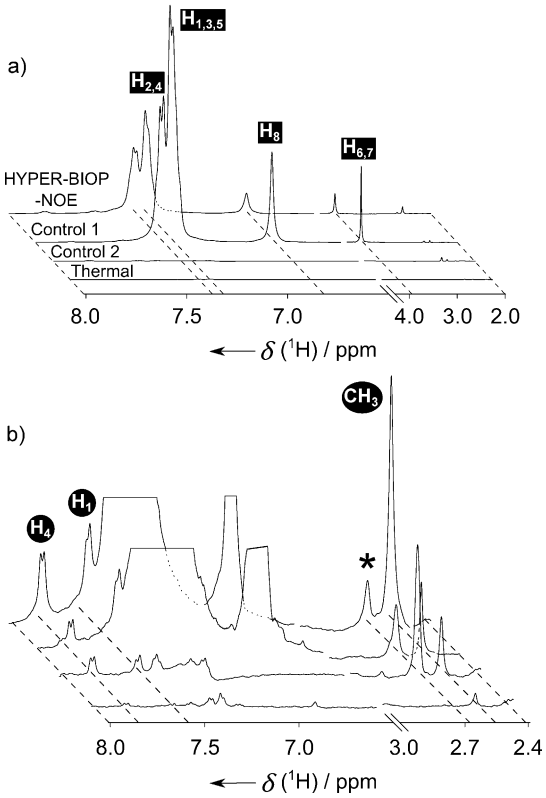


***Figure 2.*** 1D HYPER-BIPO-NOE spectra, a) in full scale and b) expanded to show transferred signals. Stacked spectra are, from top to bottom: hyperpolarized ligand 1 with ligand 2 and protein (HYPER-BIPO-NOE); hyperpolarized ligand 1 with ligand 2, but without protein (Control 1); hyperpolarized [$D_6$]DMSO/$D_2O$, with only ligand 2 and protein (Control 2); Thermal spectrum of the HYPER-BIPO-NOE sample (Thermal). The resonance from DMSO, which was suppressed using presaturation, is designated by *.

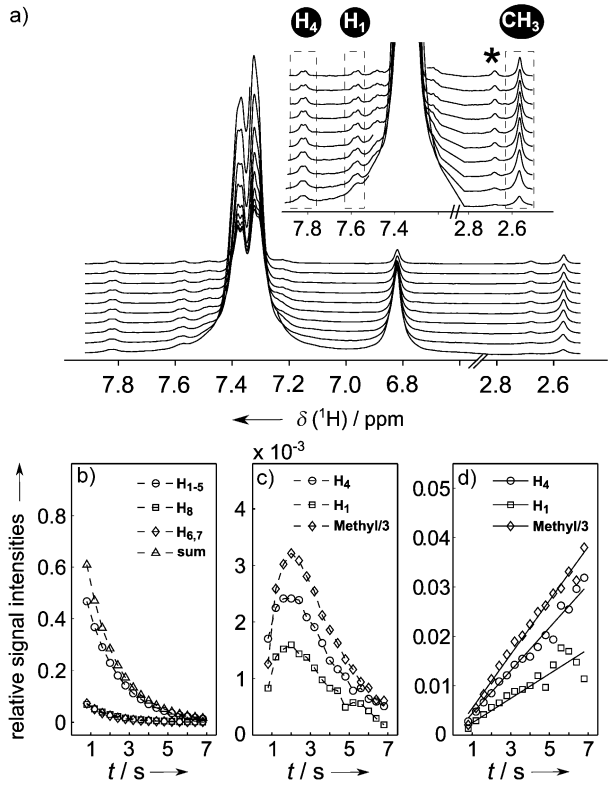


***Figure 3.*** Build-up of the HYPER-BIPO-NOE signal as a function of time. a) Series of spectra recorded from a single sample of hyperpolarized ligand 1 mixed with ligand 2 and protein. Spectra were recorded with a 20.7° flip angle at intervals of 0.4 s. b) Integrals of ligand 1 and c) integrals of ligand 2 signals taken from spectra in (a), normalized per proton. d) Integrals of ligand 2 signals divided by the sum of the integrals of ligand 1 signals and fitted with a linear function. The solvent resonance that was suppressed using presaturation is given by *.

ligand NOE in a single scan. Additionally, a non-specific enhancement was observed in hyperpolarized control experiments in the absence of protein or ligand 1 (second and third traces in Figure 2b). From these spectra, it can be estimated that the specific enhancement of the HYPER-BIPO-NOE over nonspecific enhancement is a factor of 4.7, 3.4, and 5.7 for the $H_4$, $H_1$, and $CH_3$ groups, respectively. The kinetics of the HYPER-BIPO-NOE transfer and potential information about the binding modes of the two ligands can be obtained from measuring the build-up of signal on ligand 2 as a function of time. This measurement is accomplished from a single hyperpolarized sample by a series of small flip angle excitations (Figure 3a). Integrated signal intensities of the resolvable protons of the hyperpolarized ligand 1 and of the nonpolarized ligand 2 are shown in Figure 3b and c, respectively. Neglecting nonspecific enhancement, the course of signal intensity in function of time $t$ can be modeled by relaxation networks as described previously [Eq. (1)],[4b]

$$\overrightarrow{(I_z(t)-I_B)} = e^{-(R+K)t}\overrightarrow{(I_z(t)-I_B)} \quad (1)$$

where $\overrightarrow{(I_z(t)-I_B)}$ is the deviation of $z$ magnetization from the Boltzmann equilibrium and $R$ and $K$ describe the relaxation and kinetics of the system, respectively. In the 1D HYPER-BIPO-NOE experiment, we observe a bulk transfer of magnetization according to Equation (1) with $\vec{I}_z(0) = \vec{I}_z^{\,\text{hyperpolarized}}$ where $\vec{I}_z^{\,\text{hyperpolarized}}$ reflects the $z$ magnetizations on each of the spins of ligand 1. These polarizations can then be put into programs calculating INPHARMA transfer.[4b]

Also qualitatively, we can derive some important properties concerning the HYPER-BIPO-NOE effect. We assume only three spins, namely one for each ligand ($L_1$ and $L_2$) and one for the protein (P). Since each of these species can be present both in free and bound form, a $7\times7$ matrix is required for the $R+K$ term to describe the relaxation and kinetics of the system. Under the assumption of fast kinetics, the evolution matrix $M = R + K$ can be approximated with a $3\times3$ matrix [Eq. (2)] using a procedure similar to Ref. [11] (see the Supporting Information).

$$M = \begin{pmatrix} r_1 & 0 & \sigma_1^{**} \\ 0 & r_2 & \sigma_2^{**} \\ \sigma_1^{*} & \sigma_2^{*} & r_p \end{pmatrix} \quad (2)$$

  

In Equation (2), the three dimensions correspond to the signal intensities observed from ligand 1, protein, and ligand 2, respectively. The diagonal elements of $M$, $r_1 = pf_1{\cdot}\rho_{L1} + pb_1{\cdot}\rho_{L1P}$, $r_2 = pf_2{\cdot}\rho_{L2} + pb_2{\cdot}\rho_{L2P}$, $r_P = p{\cdot}\rho_P + p_1{\cdot}\rho_{PL1} + p_2{\cdot}\rho_{PL2}$, and off-diagonal elements $\sigma_1^* = pb_1 \cdot \sigma_1$, $\sigma_1^{**} = p_1 \cdot \sigma_1$, $\sigma_2^* = pb_2 \cdot \sigma_2$, and $\sigma_2^{**} = p_2 \cdot \sigma_2$ are averages of auto- and cross-relaxation rate constants weighted with corresponding concentration fractions. The fractions of the free and bound form of ligands 1 and 2 are $pf_{1,2}$ and $pb_{1,2}$. The fractions of the protein in the free form is $p$, and $p_1$, $p_2$ in bound form $L_1$ and $L_2$, respectively. The cross-relaxation rates of protons of ligand 1 and ligand 2 with protein protons are $\sigma_1$ and $\sigma_2$, respectively. The parameters $\rho$ are auto-relaxation rates of the species designated by the subscript, where L1P stands for ligand 1 when bound to protein, and PL1 for the protein when bound to ligand 1, and the same notation is used for ligand 2.

In the HYPER-BIPO-NOE experiment, the signal from ligand $L_1$ is hyperpolarized (index H), whereas the polarization of the protein P and ligand $L_2$ are from Boltzmann (index B) magnetization, so that initial conditions are given by Equation (3).

$$\vec{I}(0) = \begin{pmatrix} I_{L1,H} \\ I_{L2,B} \\ I_{P,B} \end{pmatrix} \tag{3}$$

Additionally, comparing Figure 3b and c, it can be seen that the transferred magnetization for the entire build-up time is much smaller than the initial magnetization. In this case, the back-transfer of magnetization can be ignored, and equations become considerably simplified. Under the assumption that $r_P \gg r_1$, $r_2$, and $r_1 \approx r_2$, the solutions are approximated by Equation (4) (see the Supporting Information).

$$s_{L2,rel} = \frac{\sigma_1^* \sigma_2^{**} t}{r_P} = \frac{pb_1 p_2 \sigma_1 \sigma_2 t}{p\rho_P + p_1\rho_{PL1} + p_2\rho_{PL2}} \tag{4}$$

Equation (4) illustrates that the transferred signal is proportional to the product of cross-relaxation rates between both ligands and the protein. Since the cross-relaxation rates depend on the distances of the proton in a ligand to the protons in the protein, a more efficient transfer indicates a closer contact. Since $r_1$ and $r_2$ are averages of bound and free relaxation rates, the condition $r_1 \approx r_2$ is fulfilled if the fraction of bound state is similar for ligand 1 and ligand 2. This is approximately true for the ligands used here. If this is not the case, Equations (S.54–S.58) (see the Supporting Information) should be used instead. Curves calculated for parameters similar to those expected in the present system are shown in Figures S1 and S2 (see the Supporting Information).

In the experimental data (Figure 3d), the integrals of resonances from ligand 2 were divided by the sum of integrals of ligand 1, which per proton showed a normalized enhancement ratio of $H_{1-5}$:$H_8$:$H_{6,7}$ = 1.4:1.0:0.6. This treatment is nevertheless reasonable under the assumption that magnetization transferred from ligand 1 to the protein is equally distributed through spin diffusion. Individual differences in polarization and cross-relaxation rates of protons in ligand 1 are then assumed to be averaged. The relative intensities that are obtained can be compared to Equation (4), and primarily contain information on the mode of binding of ligand 2. The data from Figure 3d indicate that the methyl group of ligand 2 has the most efficient magnetization exchange with ligand 1, followed by protons $H_4$ and then $H_1$. This methyl group should therefore have a close contact to the protein, while proton $H_1$ should be more distant from or less surrounded by protons of the protein. This finding is in agreement with the previously determined structures of protein kinase A with the ligand 3-pyridin-4-yl-1*H*-indazole,[4c] which features a piperidyl instead of the methyl of ligand 2. In the crystal structure and according to INPHARMA data,[4c] this pyperidyl group is buried in the protein comprising an equivalent binding epitope as suggested here for ligand 2. Additionally, full relaxation matrix calculations were performed with $10^6$ pairwise combinations of the structures of the PKA in complex with the ligands (see Figure S3 in the Supporting information) using our home-written program. The structures were generated by performing low-temperature (300 K) molecular dynamics simulations starting from the crystal structures to sample the local conformational space. The backbone root-mean-square deviation (RMSD) values are $(1.41 \pm 0.28)$ and $(1.09 \pm 0.14)$ Å for PKA complexes with ligands 1 and 2, respectively (Table S1 in the Supporting Information), using the residues within the binding sites (< 5 Å from the ligands). The calculated and experimental peak integrals were correlated and Pearson's correlation coefficient ($R$) and $Q$-factor values were used as criteria for discerning between the structure pairs. The best pair of structures (Figure S4 in the Supporting Information) was identified with $R = 0.88$ and $Q = 0.23$ (Figure S5). The RMSD values between this best pair and the crystal structures are 1.33 and 0.68 Å for ligand 1 and 2 complexes, respectively. This result shows that the analytical solution is in an excellent agreement with the presented numerical result.

In summary, using a new experiment termed HYPER-BIPO-NMR, protein-mediated interligand NOEs were observed. Dynamic nuclear polarization induced hyperpolarization of one of the ligands provided a sensitivity contrast sufficiently large to avoid the need of using spin-state-selective or two-dimensional NMR experiments for observing the transferred magnetization. In screening experiments for ligand binding, the observation of the transferred magnetization can be used to rapidly identify that both ligands bind to the same protein. Additionally, the build-up of transferred signal intensity from ligand 1 to ligand 2 as a function of time can be obtained from a single hyperpolarized experiment. The magnitude of the build-up rate is a function of the contact between individual spins on the receiving ligand and the protein, and as such provides limited structural information on the binding epitope. The information obtained from the HYPER-BIPO-NOE experiment may be contrasted to that available from the related STD and INPHARMA experiments. The characterization of the binding mode of ligand 2 in the present experiments is similar to what may be obtained with STD. However, unlike STD, the HYPER-BIPO-NOE based experiment uses ligand 1 for a selective enhancement

  

of the binding pocket. Therefore, we expect this experiment to show a degree of selectivity towards a pair of ligands binding in the same pocket. On the other hand, since the present experiment does not distinguish between different spins on ligand 1, the correlation between spins on the two ligands that are in equivalent positions when binding to the protein, as in INPHARMA, is not observed. Such information could potentially be recovered by applying selective inversion[12] or 2D gradient encoding techniques.[13] Even though the present report focuses on the observation of competitive binding of two ligands to a protein, the HYPER-BIPO-NMR experiment is not limited to this application. Rather, it presents a more general way to enhance the magnetization of the binding pocket in a protein. In other experiments, the enhanced signal could be used to selectively assign and observe those resonances. Especially for larger proteins, where full assignments cannot easily be obtained, the selective enhancement may greatly facilitate the study of an active site.

## Experimental Section

Samples of 342 mM ligand 1 and 15 mM 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl free-radical in 80% [$D_6$]dimethyl sulfoxide (DMSO) and 20% $D_2O$ were polarized in a HyperSense system (Oxford Instruments, Tubney Woods, UK) by irradiating 100 mW microwave power at 94.005 GHz frequency for 30 min, at a temperature of 1.4 K. Polarized samples were dissolved by a stream of heated $D_2O$ and injected into a 400 MHz NMR spectrometer (Bruker Biospin, Billerica, MA) containing a 5 mm NMR tube pre-loaded with 25 μL of 350 μM protein kinase A (PKA[14]), 150 mM NaCl, 2 mM tris(2-carboxyethyl)phosphine, and 2 mM $NaN_3$ in $D_2O$ phosphate buffer (10 mM phosphate, pH 7.0).[15] Estimated final concentrations after dissolution were 448, 329, and 19 μM for ligand 1, ligand 2, and protein, respectively. All of the spectra were measured at a temperature of 25 °C, using a single π/2 excitation pulse or a sequence of small flip angle excitation pulses. Solvent suppression was achieved by presaturation of DMSO and selective excitation of water (Figure S6 in the Supporting Information).

Intensities of interligand peaks were calculated using a complete relaxation matrix approach implemented in an in-house C++ program. Protein protons within 8 Å of bound ligands were included. Parameters were: correlation time of 17 ns for complexes and free PKA, and of 0.1 ns for free ligands; $K_{D(L1)} = 3.0$ μM and $K_{D(L2)} = 4.0$ μM; diffusion limited $k_{on}$ rates of $10^8\,M^{-1}s^{-1}$. The structures of PKA and the ligands were generated by molecular dynamics simulations using the Gromacs program.[16] The AMBER99SB[17] and General Amber Force Field (GAFF)[18] force fields were used for the protein and ligands, respectively. Calculated peak intensities were normalized as in Figure 3d, to remove the effect from depletion of magnetization due to previous scans. They were correlated with experimental values using Pearson's correlation coefficient and $Q$-factor given by Equation (5).

$$Q = \sqrt{\sum \left(I_{exp} - I_{calc}\right)^2 \Big/ \sum I_{exp}^2} \qquad (5)$$

Received: February 7, 2012
Published online: April 12, 2012

**Keywords:** hyperpolarization · ligands · NMR spectroscopy · proteins

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