

Supporting Information

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69451 Weinheim, Germany

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

*Youngbok Lee, Haifeng Zeng, Adam Mazur, Melanie Wegstroth, Teresa Carlomagno,
Marcel Reese, Donghan Lee, Stefan Becker, Christian Griesinger, and Christian Hilty**

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Model for Magnetization Transfer

To obtain the expression for the HYPER-BIPO-NOE buildup, we use a three-spin system to derive the analytical solution for signal intensity evolution in a competitive binding experiment.

We consider a competitive binding experiment of two ligands to a protein,



To estimate the signal intensities observed in a NMR experiment, we use one spin for ligand 1, ligand 2 and protein, respectively. Further, we assume that exchange is fast compared to chemical shift and compared to spin relaxation, and that consequently, the system is kinetically at equilibrium at all times.

The vector of signal intensities is

$$\vec{I} = \begin{pmatrix} I_{L1} \\ I_{L2} \\ I_{PL1} \\ I_{L1P} \\ I_{PL2} \\ I_{L2P} \\ I_P \end{pmatrix} \quad (S.3)$$

Here, L1 is unbound ligand 1, L2 is unbound ligand 2, PL1 is the protein when bound to ligand 1, L1P is ligand 1 when bound to protein, PL2 is protein when bound to ligand 2, L2P is ligand 2 when bound to protein, and P is unbound protein.

The relaxation matrix is given by

$$R = \begin{pmatrix} \rho_{L1} & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & \rho_{L2} & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & \rho_{PL1} & \sigma_1 & 0 & 0 & 0 \\ 0 & 0 & \sigma_1 & \rho_{L1P} & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & \rho_{PL2} & \sigma_2 & 0 \\ 0 & 0 & 0 & 0 & \sigma_2 & \rho_{L2P} & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & \rho_P \end{pmatrix} \quad (\text{S.4})$$

The terms ρ are auto-relaxation rate constants for the respective species. σ_1 is the cross-relaxation rate constant between ligand 1 and protein, and σ_2 the cross-relaxation rate constant between ligand 2 and protein.

The kinetic matrix is

$$K = \begin{pmatrix} kon_1[P] & 0 & 0 & -koff_1 & 0 & 0 & 0 \\ 0 & kon_2[P] & 0 & 0 & 0 & -koff_2 & 0 \\ 0 & 0 & koff_1 & 0 & 0 & 0 & -kon_1[L1] \\ -kon_1[P] & 0 & 0 & koff_1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & koff_2 & 0 & -kon_2[L2] \\ 0 & -kon_2[P] & 0 & 0 & 0 & koff_2 & 0 \\ 0 & 0 & -koff_1 & 0 & -koff_2 & 0 & (kon_1[L1] + kon_2[L2]) \end{pmatrix} \quad (\text{S.5})$$

Quantities in square brackets are concentrations, which are constant, since the system is kinetically in equilibrium state. Using the above definitions, the time evolution of signal intensity is given by

$$\bar{I} = e^{-(K+R)t} \bar{I}_0 \quad (\text{S.6})$$

Loosely following,^[1] since exchange is on a faster time scale than spin relaxation, Equation S.6 can be simplified by finding a matrix V that diagonalizes K . An explicit solution is

$$V = \begin{pmatrix} 0 & 0 & \frac{koff_1}{[P]kon_1} & -1 & 0 & 0 & 0 \\ 0 & \frac{koff_2}{[P]kon_2} & 0 & 0 & -1 & 0 & 0 \\ \frac{[L1]kon_1}{koff_1} & 0 & 0 & 0 & 0 & \frac{-a_1+b}{a_2-b} & \frac{-a_1-b}{a_2+b} \\ 0 & 0 & 1 & 1 & 0 & 0 & 0 \\ \frac{[L2]kon_2}{koff_2} & 0 & 0 & 0 & 0 & \frac{-2[L2]kon_2}{a_2-b} & \frac{-2[L2]kon_2}{a_2+b} \\ 0 & 1 & 0 & 0 & 1 & 0 & 0 \\ 1 & 0 & 0 & 0 & 0 & 1 & 1 \end{pmatrix} \quad (S.7)$$

with

$$a_1 = koff_1 - koff_2 + [L1]kon_1 - [L2]kon_2 \quad (S.8)$$

$$a_2 = koff_1 - koff_2 + [L1]kon_1 + [L2]kon_2 \quad (S.9)$$

$$b = \sqrt{(koff_1 + koff_2 + [L1]kon_1 + [L2]kon_2)^2 - 4([L1]koff_2kon_1 + koff_1(koff_2 + [L2]kon_2))} \quad (S.10)$$

Using Equation S.7, it is found that K has three eigenvalues that are equal to zero, such that

$$K^\dagger = V^{-1}KV = \begin{pmatrix} 0 & & & & & & \\ & 0 & & & & & \\ & & 0 & & & & \\ & & & \lambda_1 & & & \\ & & & & \lambda_2 & & \\ & 0 & & & & \lambda_3 & \\ & & & & & & \lambda_4 \end{pmatrix} = \begin{pmatrix} 0 & 0 \\ 0 & \Lambda \end{pmatrix} \quad (S.11)$$

By the same transformation, R is not diagonalized:

$$R^\dagger = V^{-1}RV = \begin{pmatrix} R_A & R_B \\ R_C & R_D \end{pmatrix} \quad (S.12)$$

in which R_A is a 3×3 block, R_B a 3×4 block, R_C a 4×3 block, and R_D a 4×4 block.

Equation S.6 becomes

$$\vec{I} = e^{-(K+R)t} \cdot \vec{I}_0 = V \cdot e^{-V^{-1}(K+R)Vt} \cdot V^{-1} \cdot \vec{I}_0 = V \cdot e^{-(K^\dagger+R)^\dagger t} \cdot V^{-1} \cdot \vec{I}_0 \quad (\text{S.13})$$

The matrix exponential in Equation S.13 would be simplified by a second transformation that block diagonalizes $K^\dagger + R^\dagger$ into a 3×3 and a 4×4 block. Analogous to the treatment in,^[2] but with higher dimensionality, we consider R^\dagger as a small perturbation on K^\dagger . As in perturbation theory,^[2] we write

$$\begin{aligned} (K^\dagger + \zeta R^\dagger)(E + \zeta W + \zeta^2 W' + \zeta^3 W'' + \dots) \\ = (E + \zeta W + \zeta^2 W' + \zeta^3 W'' + \dots)(K^\dagger + \zeta D + \zeta D' + \zeta D'' + \dots) \end{aligned} \quad (\text{S.14})$$

ζ is a parameter to keep track of the order, and the matrices W are the corrections to the eigenvectors of K^\dagger . By definition, the matrices D are block diagonal, and the diagonal block elements of W are chosen to be 0. The first order correction is found by comparing the terms in ζ on both sides of Equation S.14.

$$\zeta(R^\dagger + K^\dagger W) = \zeta(WK^\dagger + D) \quad (\text{S.15})$$

Writing every matrix in block form

$$\begin{pmatrix} R_A & R_B \\ R_C & R_D \end{pmatrix} + \begin{pmatrix} 0 & 0 \\ 0 & \Lambda \end{pmatrix} \begin{pmatrix} 0 & W_A \\ W_B & 0 \end{pmatrix} = \begin{pmatrix} 0 & W_A \\ W_B & 0 \end{pmatrix} \begin{pmatrix} 0 & 0 \\ 0 & \Lambda \end{pmatrix} + \begin{pmatrix} D_A & 0 \\ 0 & D_B \end{pmatrix} \quad (\text{S.16})$$

The result shows that

$$D_A = R_A, D_B = R_D \quad (\text{S.17})$$

and

$$W_{A_i,j} = R_{B_i,j} / \Lambda_{j,j}, W_{B_i,j} = R_{C_i,j} / \Lambda_{i,i} \quad (\text{S.18})(\text{S.19})$$

If the elements in Λ are much larger than the elements of R , the first order correction W to the eigenvectors is negligible. Therefore, to first order approximation of eigenvalues and zero order correction of eigenvectors,

$$\exp(-(K^\dagger + R^\dagger)t) \approx \exp\left(-\left(\begin{array}{c|c} R_A & 0 \\ \hline 0 & \Lambda + R_D \end{array}\right)t\right) \approx \exp\left(-\left(\begin{array}{c|c} R_A & 0 \\ \hline 0 & \Lambda \end{array}\right)t\right) \quad (\text{S.20})$$

with

$$R_A = (V^{-1}RV)_{1..3,1..3} \quad (\text{S.21})$$

the 3x3 matrix taken from the top left corner of $V^{-1}RV$, and

Then, Equation S.13 becomes

$$\vec{I} \approx V \cdot \left(\begin{array}{c|c} e^{-R_A t} & 0 \\ \hline 0 & e^{-\Lambda t} \end{array}\right) \cdot V^{-1} \cdot \vec{I}_0 \quad (\text{S.22})$$

To calculate the NMR signal under fast exchange, it is necessary to sum the signals for the bound and free form of each species, since those appear at the same chemical shift. The summation can be accomplished by a transformation

$$\vec{J} = C \cdot \vec{I} \quad (\text{S.23})$$

with

$$C = \begin{pmatrix} 1 & 0 & 0 & 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 1 & 0 & 1 & 0 & 1 \\ 0 & 0 & 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 1 & 0 & 0 & 0 & 0 \end{pmatrix} \quad (\text{S.24})$$

and

$$\vec{J} = \begin{pmatrix} S_{L1} \\ S_{L2} \\ S_P \\ I_{L1P} \\ I_{PL2} \\ I_{L2P} \\ I_{PL1} \end{pmatrix} \quad (\text{S.25})$$

In J , only the first three elements are of interest, designating the NMR signal from ligand 1, ligand 2, and protein, respectively. The last four elements simply remain to complete the basis. Using Equation S.22,

$$\vec{J} \approx C \cdot V \cdot \left(\begin{array}{c|c} e^{-R_A t} & 0 \\ \hline 0 & e^{-\Lambda t} \end{array} \right) \cdot V^{-1} \cdot C^{-1} \cdot \vec{J}_0 \quad (\text{S.26})$$

To calculate the matrix product in Equation S.26, it is convenient to write the constituents in block form:

$$C \cdot V = \left(\begin{array}{c|c} (CV)_{1..3,1..3} & 0 \\ \hline (CV)_{4..7,1..3} & (CV)_{4..7,4..7} \end{array} \right) \quad (\text{S.27})$$

and

$$V^{-1} \cdot C^{-1} = \left(\begin{array}{c|c} (V^{-1}C^{-1})_{1..3,1..3} & 0 \\ \hline (V^{-1}C^{-1})_{4..7,1..3} & (V^{-1}C^{-1})_{4..7,4..7} \end{array} \right) \quad (\text{S.28})$$

The property that the upper right block of 3x4 elements in Equations S.27 and S.28 is equal to zero appears non-trivial, but can be verified explicitly from Equations S.7 and S.24. Then,

$$\vec{J} \approx \left(\begin{array}{c|c} (CV)_{1..3,1..3} \cdot e^{-R_A t} \cdot (V^{-1}C^{-1})_{1..3,1..3} & 0 \\ \hline \dots & \dots \end{array} \right) \cdot \vec{J}_0 \quad (\text{S.29})$$

For obtaining the first three elements of J , it is only necessary to consider the upper row of blocks in Equation S.29:

$$\vec{S} \approx (CV)_{1..3,1..3} \cdot e^{-R_A t} \cdot (V^{-1}C^{-1})_{1..3,1..3} \cdot \vec{S}_0 \quad (\text{S.30})$$

with

$$\vec{S} = \begin{pmatrix} s_{L1} \\ s_{L2} \\ s_P \end{pmatrix} \quad (\text{S.31})$$

From Equations S.7 and S.24, it can further be found that in the basis that was used, fortuitously $((CV)_{1..3,1..3})^{-1} = (V^{-1}C^{-1})_{1..3,1..3}$. In this case, Equation S.30 can be re-written as

$$\vec{S} \approx e^{-Mt} \cdot \vec{S}_0 \quad (\text{S.32})$$

with

$$M = (CV)_{1..3,1..3} \cdot R_A \cdot (V^{-1}C^{-1})_{1..3,1..3} \quad (\text{S.33})$$

The explicit form of M obtained using Equations S.7 and S.24 are

$$M = \begin{pmatrix} \frac{koff_1 \rho_{L1} + [P]kon_1 \rho_{L1P}}{koff_1 + [P]kon_1} & 0 & \frac{[L1]kon_1 koff_2 \sigma_1}{[L1]kon_1 koff_2 + koff_1 koff_2 + [L2]kon_2 koff_1} \\ 0 & \frac{koff_2 \rho_{L2} + [P]kon_2 \rho_{L2P}}{koff_2 + [P]kon_2} & \frac{[L2]koff_1 kon_2 \sigma_2}{[L1]kon_1 koff_2 + koff_1 koff_2 + [L2]kon_2 koff_1} \\ \frac{[P]kon_1 \sigma_1}{koff_1 + [P]kon_1} & \frac{[P]kon_2 \sigma_2}{koff_2 + [P]kon_2} & \frac{[L1]kon_1 koff_2 \rho_{PL1} + koff_1 koff_2 \rho_P + [L2]kon_2 koff_1 \rho_{PL2}}{[L1]kon_1 koff_2 + koff_1 koff_2 + [L2]kon_2 koff_1} \end{pmatrix} \quad (\text{S.34})$$

Since the system is kinetically in the steady state,

$$koff_1[PL1] = kon_1[P][L1] \quad (\text{S.35})$$

and

$$koff_2[PL2] = kon_2[P][L2] \quad (\text{S.36})$$

Consequently, M can be written in terms of concentrations

$$M = \begin{pmatrix} \frac{[L1]\rho_{L1} + [PL1]\rho_{L1P}}{[L1] + [PL1]} & 0 & \frac{[PL1]\sigma_1}{[P] + [PL1] + [PL2]} \\ 0 & \frac{[L2]\rho_{L2} + [PL2]\rho_{L2P}}{[L2] + [PL2]} & \frac{[PL2]\sigma_2}{[P] + [PL1] + [PL2]} \\ \frac{[PL1]\sigma_1}{[L1] + [PL1]} & \frac{[PL2]\sigma_2}{[L2] + [PL2]} & \frac{[P]\rho_P + [PL1]\rho_{PL1} + [PL2]\rho_{PL2}}{[P] + [PL1] + [PL2]} \end{pmatrix} \quad (S.37)$$

It may further be useful to define the parameters that describe the concentration fraction of species that are bound or unbound:

$$\text{Fraction of ligand 1 bound to protein} \quad pb_1 = \frac{[PL1]}{[PL1] + [L1]} \quad (S.38)$$

$$\text{Fraction of ligand 1 not bound to protein} \quad pf_1 = \frac{[L1]}{[PL1] + [L1]} = 1 - pb_1 \quad (S.39)$$

$$\text{Fraction of ligand 2 bound to protein} \quad pb_2 = \frac{[PL2]}{[PL2] + [L2]} \quad (S.40)$$

$$\text{Fraction of ligand 2 not bound to protein} \quad pf_2 = \frac{[L2]}{[PL2] + [L2]} = 1 - pb_2 \quad (S.41)$$

$$\text{Fraction of protein bound to ligand 1} \quad p_1 = \frac{[PL1]}{[P] + [PL1] + [PL2]} \quad (S.42)$$

$$\text{Fraction of protein bound to ligand 2} \quad p_2 = \frac{[PL2]}{[P] + [PL1] + [PL2]} \quad (S.43)$$

$$\text{Fraction of protein not bound to any ligand} \quad p = \frac{[P]}{[P] + [PL1] + [PL2]} = 1 - p_1 - p_2 \quad (S.44)$$

Then, Equation S.37 simplifies to

$$M = \begin{pmatrix} pf_1\rho_{L1} + pb_1\rho_{L1P} & 0 & p_1\sigma_1 \\ 0 & pf_2\rho_{L2} + pb_2\rho_{L2P} & p_2\sigma_2 \\ pb_1\sigma_1 & pb_2\sigma_2 & p\rho_P + p_1\rho_{PL1} + p_2\rho_{PL2} \end{pmatrix} \quad (S.45)$$

From Equation S.45, it can be seen most clearly that the evolution of NMR signal in Equation S.32 is governed by rate constants, which consist of the auto- and cross-relaxation rates of the individual species weighted by the corresponding concentration fractions.

Solution of simplified equations

Since magnetization of the hyperpolarized ligand L1 is much larger than the magnetization transferred to L2, back-transfer of magnetization can be ignored. Then, M becomes

$$M = \begin{pmatrix} r_1 & 0 & 0 \\ 0 & r_2 & \sigma_2^{**} \\ \sigma_1^* & 0 & r_p \end{pmatrix} \quad (\text{S.46})$$

The elements of M are,

$$r_1 = pf_1\rho_{L1} + pb_1\rho_{L1P} \quad (\text{S.47})$$

$$r_2 = pf_2\rho_{L2} + pb_2\rho_{L2P} \quad (\text{S.48})$$

$$r_p = p\rho_p + p_1\rho_{PL1} + p_2\rho_{PL2} \quad (\text{S.49})$$

$$\sigma_1^* = pb_1\sigma_1 \quad (\text{S.50})$$

$$\sigma_1^{**} = p_1\sigma_1 \quad (\text{S.51})$$

$$\sigma_2^* = pb_2\sigma_2 \quad (\text{S.52})$$

$$\sigma_2^{**} = p_2\sigma_2 \quad (\text{S.53})$$

In this form, the analytical solutions to Equation 1 are considerably simplified, such that

$$I_{L1}(t) = I_{L1,H} \cdot \exp(-r_1 \cdot t) \quad (\text{S.54})$$

$$I_{L2}(t) = -\frac{(r_1 - r_2) \cdot \exp(-r_p \cdot t) + (r_2 - r_p) \cdot \exp(-r_1 \cdot t) + (r_p - r_1) \cdot \exp(-r_2 \cdot t)}{(r_1 - r_2) \cdot (r_2 - r_p) \cdot (r_p - r_1)} \sigma_1^* \cdot \sigma_2^{**} \cdot I_{L1,H} \quad (\text{S.55})$$

Equations S.54 and S.55 are valid in the absence of radio-frequency pulses. For experimental measurement of I_{L1} and I_{L2} , however, a RF pulse with flip angle α converts a fraction $\sin(\alpha)$ of the total longitudinal magnetization into a coherence for detection. A fraction $\beta = \cos(\alpha)$ of the longitudinal magnetization is preserved for following scans. Additionally, depending on the conditions for sample injection prior to the NMR experiment, mixing between the sample inside and outside the active coil region can result in a factor β' that is larger than β .^[8a] The experimental signal intensities of the k^{th} scan then become:

$$s_{L1}^k = I_{L1,H} \cdot \exp(-r_1 \cdot t) \cdot \prod_i^{k-1} \beta_i' \cdot \sin\alpha \quad (\text{S.56})$$

$$s_{L2}^k = - \frac{(r_1 - r_2) \exp(-r_p t) + (r_2 - r_p) \exp(-r_1 t) + (r_p - r_1) \exp(-r_2 t)}{(r_1 - r_2) \cdot (r_2 - r_p) \cdot (r_p - r_1)} \sigma_1^* \sigma_2^{**} I_{L1,H} \prod_i^{k-1} \beta_i' \sin\alpha \quad (\text{S.57})$$

where β_i' is the fraction of polarization preserved in scan i . The additional terms containing α and β' , which are introduced due to the experiment, cancel if considering the relative HYPER-BIPO-NOE signal intensity

$$s_{L2,\text{rel}} = s_{L2} / s_{L1} \quad (\text{S.58})$$

If Equation S.58 is expanded for small times t , a quadratic dependence

$$s_{L2,\text{rel}} = \frac{1}{2} \sigma_1^* \cdot \sigma_2^{**} \cdot t^2 = \frac{1}{2} p b_1 \cdot p_2 \cdot \sigma_1 \cdot \sigma_2 \cdot t^2 \quad (\text{S.59})$$

is found. Further, in the limit that $r_p \gg r_1, r_2$ and $r_1 \approx r_2$, Equation S.59 can be simplified to

$$s_{L2,\text{rel}} = \frac{\sigma_1^* \cdot \sigma_2^{**} \cdot t}{r_p} = \frac{p b_1 \cdot p_2 \cdot \sigma_1 \cdot \sigma_2 \cdot t}{p \rho_p + p_1 \rho_{PL1} + p_2 \rho_{PL2}} \quad (\text{S.60})$$

predicting a linear dependence of the signal on time, which also holds for longer times.

Calculation of Transferred Magnetization

To validate the model, numerical calculations were carried out using Equations S.3–S.6 (“ 7×7 matrix”), as well as using Equations 1–3 from the text (“ 3×3 matrix”). These calculations

were compared with results by Equations S.54–S.58 (“Formula”) and Equation 4 (“Linear”), and results are shown in Figures S1-S2.

Primary parameters used for approximation

Total concentrations

$$c_{L1} = 448 \mu\text{M}, c_{L2} = 329 \mu\text{M}, c_P = 19 \mu\text{M}$$

Kinetic parameters for the binding of ligand 1 or 2 to protein

$$kon_1 = kon_2 = 1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} = 100 \mu\text{M}^{-1} \text{ s}^{-1}, koff_1 = 300 \text{ s}^{-1}, koff_2 = 400 \text{ s}^{-1}$$

Auto relaxation rates of protein and protein bound ligands

$$\rho_P = \rho_{PL1} = \rho_{PL2} = \rho_{L1P} = \rho_{L2P} = 8 \text{ s}^{-1}$$

Auto relaxation rates of free ligands

$$\rho_1 = \rho_2 = 0.25 \text{ s}^{-1}$$

Cross relaxation rates between ligand 1 or 2 and protein

$$\sigma_1 = \sigma_2 = -1.4 \text{ s}^{-1}$$

Derived parameters

Equilibrium concentrations

$$[L_1] = 436 \mu\text{M}, [L_2] = 322 \mu\text{M}, [PL_1] = 12.2 \mu\text{M}, [PL_2] = 6.75 \mu\text{M}, [P] = 0.0838 \mu\text{M}$$

Dissociation constants for binding of ligand 1 and 2 to protein

$$K_{D1} = 3 \mu\text{M}, K_{D2} = 4 \mu\text{M}$$

Fractions of free and bound ligand

$$pb_1 = 0.027, pb_2 = 0.021, pf_1 = 0.97, pf_2 = 0.98$$

Fractions of free and bound protein

$$p = 0.0044, p_1 = 0.64, p_2 = 0.36$$

Apparent auto relaxation rates for ligand and protein

$$r_1 = 0.46 \text{ s}^{-1}, r_2 = 0.41 \text{ s}^{-1}, r_P = 8 \text{ s}^{-1}$$

Apparent cross relaxation rates between ligand 1 or 2 and protein

$$\sigma_1^* = -0.038 \text{ s}^{-1}, \sigma_1^{**} = -0.90 \text{ s}^{-1}, \sigma_2^* = -0.029 \text{ s}^{-1}, \sigma_2^{**} = -0.50 \text{ s}^{-1}$$

Supplementary Figures

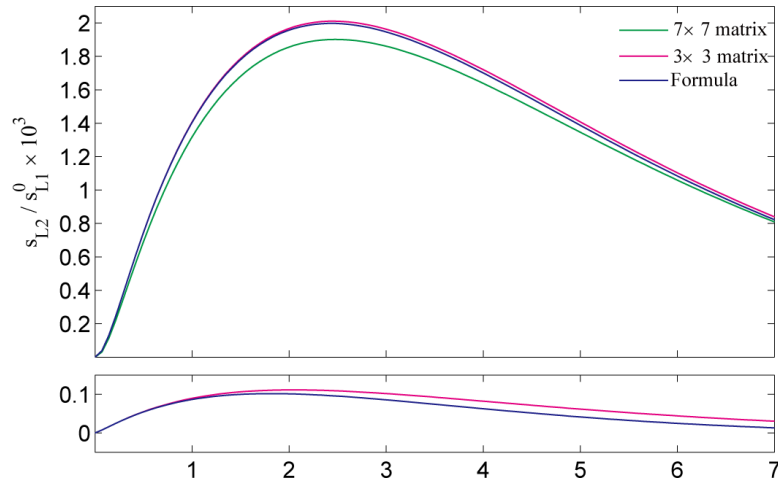


Figure S1: Simulated signal intensities of L_2 with the 7×7 evolution matrix (green), 3×3 evolution matrix (violet) and Equations S.54–S.58 (blue). In the 7×7 evolution matrix method, $s_{L1} = I_{L1} + I_{L1P}$, $s_{L2} = I_{L2} + I_{L2P}$. The lower panel shows the difference from the 7×7 evolution matrix method.

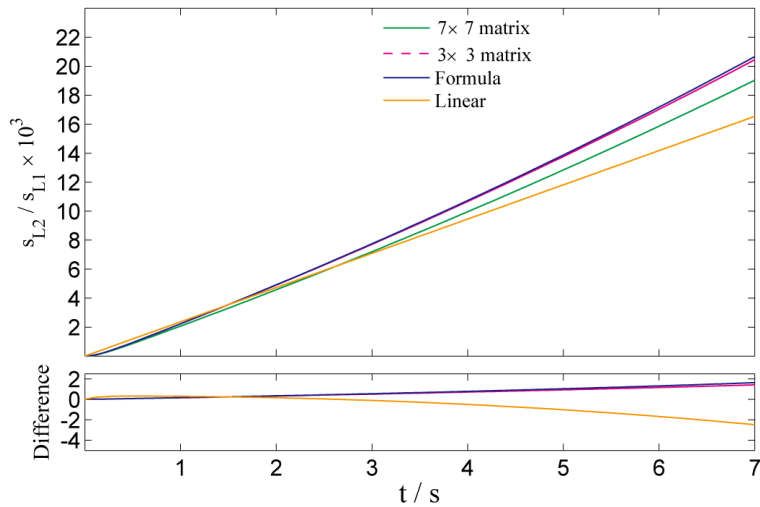


Figure S2: Simulated signal intensities of L_2 relative to the signal intensity of L_1 with the 7×7 evolution matrix (green), 3×3 evolution matrix (violet), Equations S.54–S.58 (blue) and Equation (4) (yellow). The lower panel shows the difference from the calculation using the 7×7 evolution matrix.

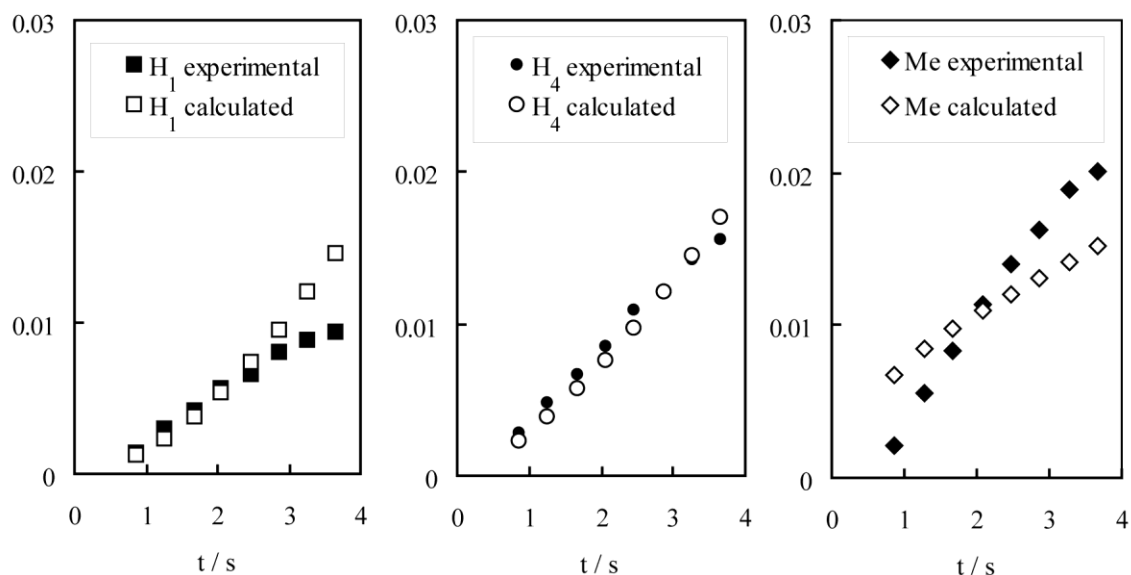


Figure S3: Buildup of the calculated (unfilled symbols) and experimental (filled symbols) signal integrals for the best pair which is described in the main text. The calculated peak integrals were calculated with our home written program. The integrals of peaks of ligand 2 were normalized as described in the caption of Figure 3d).

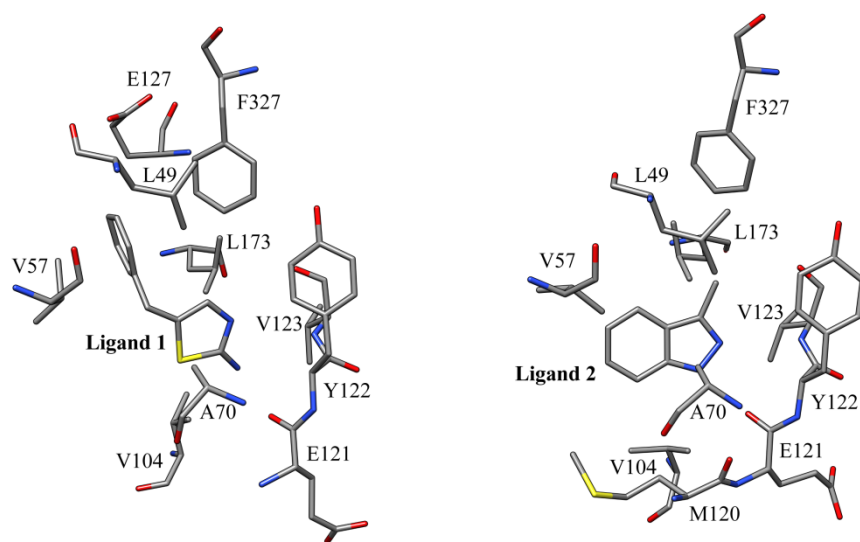


Figure S4: Structures of PKA complexes with ligand 1 (left) and ligand 2 (right). The protein residues within 3 Å distance from the ligands are shown.

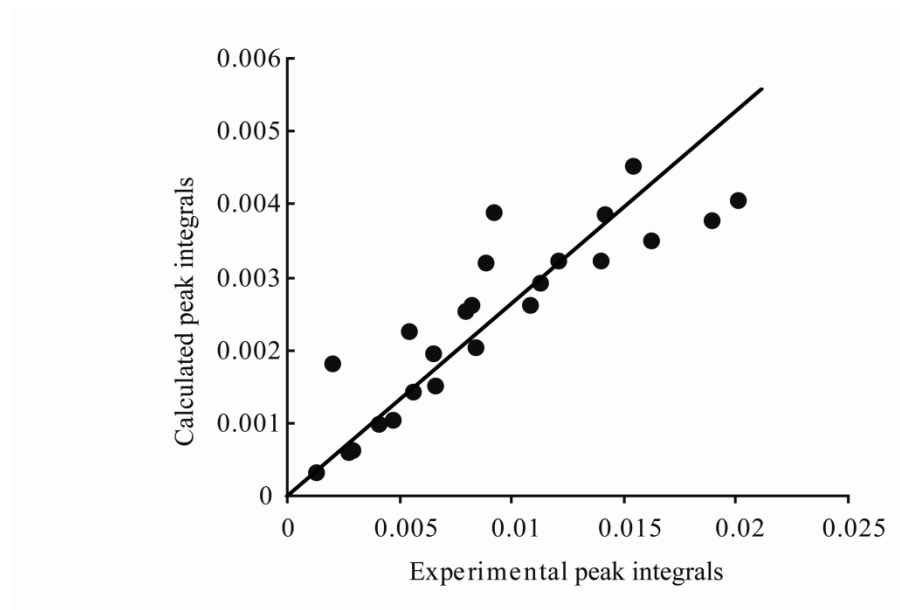


Figure S5: Correlation plot of experimental and calculated normalized peak integrals.

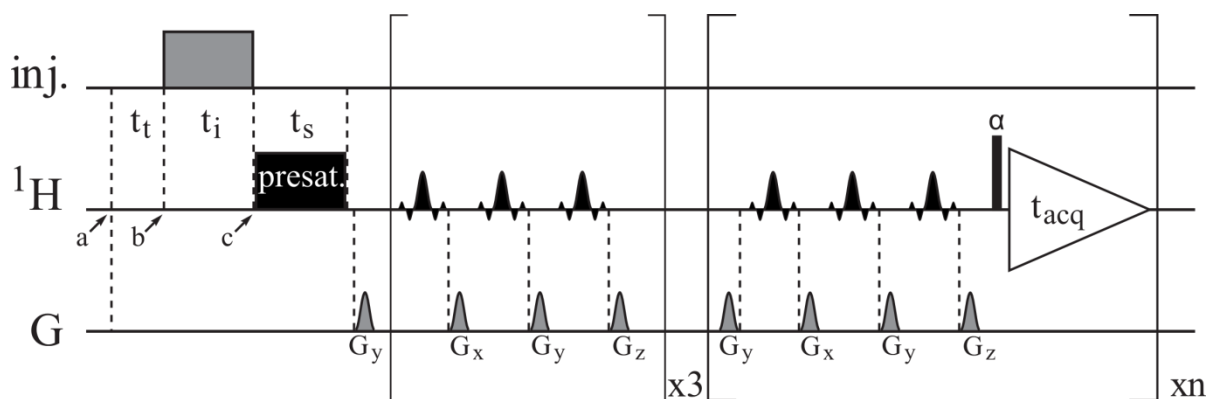


Figure S6: The INPHARMA buildup curve was measured by small flip angle pulses. The polarized sample was transferred from the polarizer to the home-built sample injector for a transfer time (t_t). The sample was injected from the injection loop to a 5 mm NMR tube, which was preinstalled in a 400 MHz NMR spectrometer. NMR experiment was triggered after an injection time (t_i) of 445 ms. After injection and sample mixing, a waiting time of 2 s in single scan experiments (Figure 2), and 400 ms in small flip angle excitation experiments (Figure 3) allowed for sample stabilization (t_s) and NOE transfer. During the stabilization times, presaturation was applied for 400 ms at the resonance frequency of DMSO at 2.68 ppm. The carrier frequency was set to the resonance frequency of water. The water resonance was selectively excited by EBURP2 shaped $\pi/2$ pulses of 20 ms duration, and dephased by

randomized pulsed field gradients G_x , G_y or G_z (25..35 G/cm, 1 ms). This solvent suppression scheme was sufficiently selective, since the resonance frequency difference between water and the nearest protons of interest was 250 Hz. After a hard $\pi/2$ pulse or 20.7° (α_x) pulse, 4096 data points were collected over 320 ms. Time interval between acquisitions was 0.4 s. The NMR data was processed using the TOPSPIN 3.0 program (Bruker Biospin).

		RMSD values [\AA] for all structures		RMSD values [\AA] for the best pair	
	Superimposed atoms	Ligand 1	Ligand 2	Ligand 1	Ligand 2
all residues	bb ^a + ligand	1.96 \pm 0.21	2.10 \pm 0.20	2.15	1.62
	heavy ^b + ligand	2.67 \pm 0.22	2.81 \pm 0.27	2.88	2.14
binding site residues	bb + ligand	1.41 \pm 0.28	1.09 \pm 0.14	1.33	0.68
	heavy + ligand	1.68 \pm 0.22	1.51 \pm 0.18	1.71	1.04

Table S1: RMSD values for the PKA-ligand complex structures.

^a backbone atoms

^b all atoms except hydrogens

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