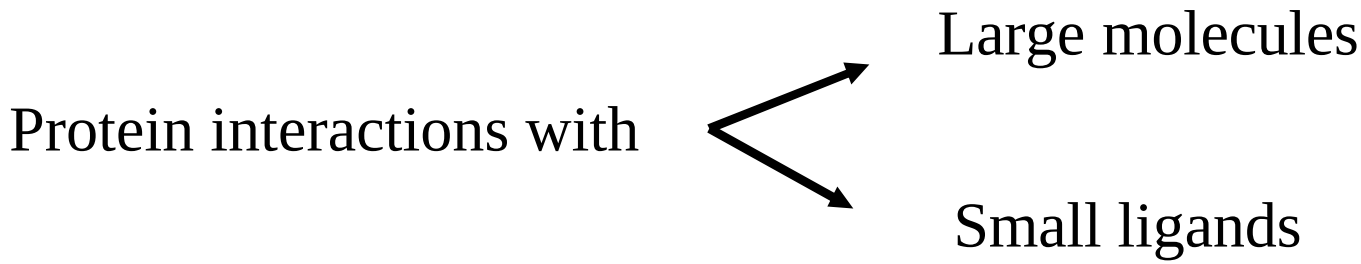
NMR relaxation and cross-relaxation

to study protein interactions

Pavia, September 7th 2007



NMR: more easily applicable to protein-small ligand interactions

Protein-small ligand interactions: relevant for drug discovery

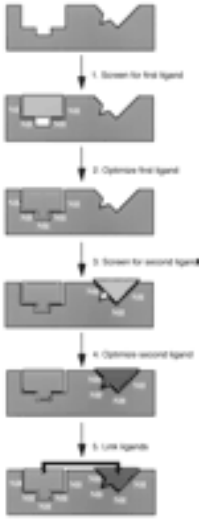
1) Lead generation	Identification of suitable ligands with specific activity and structure activity relationship (SAR)
2) Lead optimization	Improving *in vitro* performance by design-synthesis-assay cycles based on 3D structure
3) Preclinical development	Improving *in vivo* performance and bioavailability - ADMET: absorption, distribuition, metabolic stability, excretion, toxicity

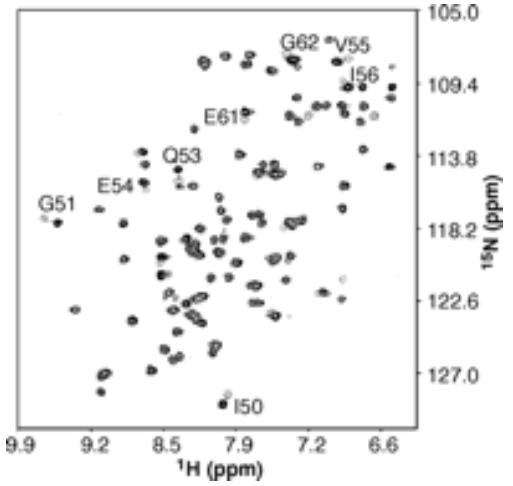
Initially NMR appeared ideally suited for stage 2, i.e. lead optimization, but NMR 3D structure determinations are time-consuming and routinely feasible for molecules below 10-12 kDa, which does not fit with the requirements of pharmaceutical industry research.

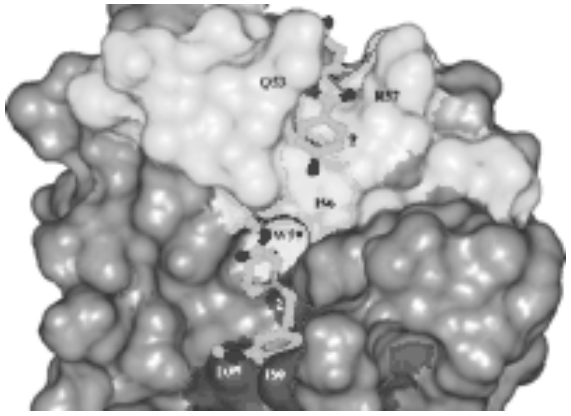
In mid 1990s it was shown that NMR could be profitably used for lead generation with the SAR-by-NMR approach.

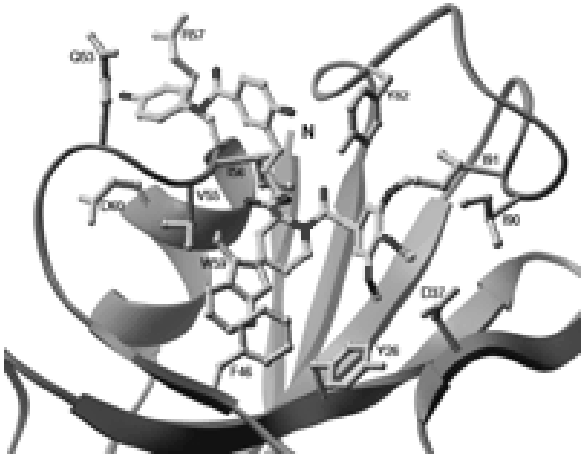
NMR was successfully applied, for specific issues, also to stage 3.

SAR by NMR



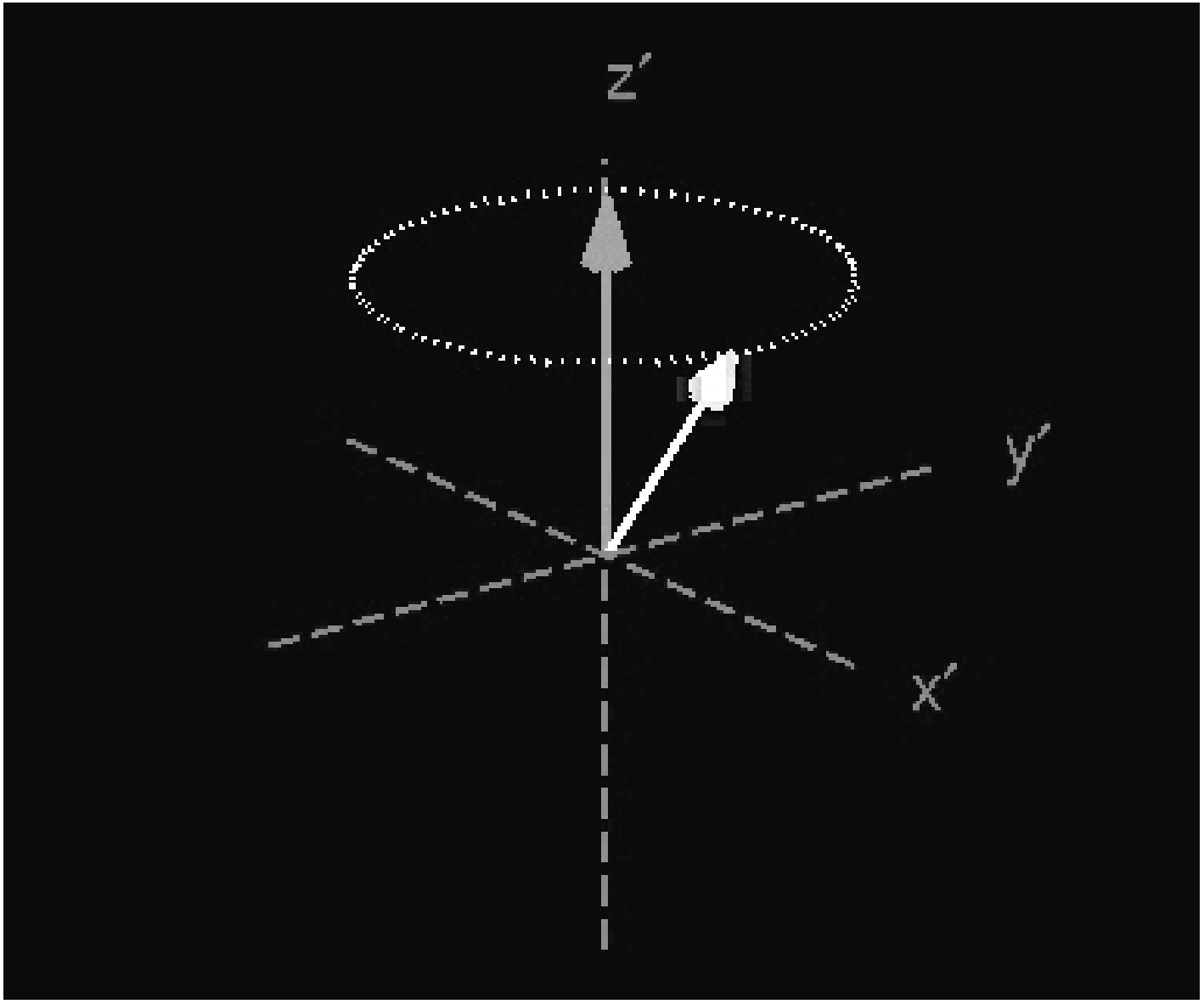






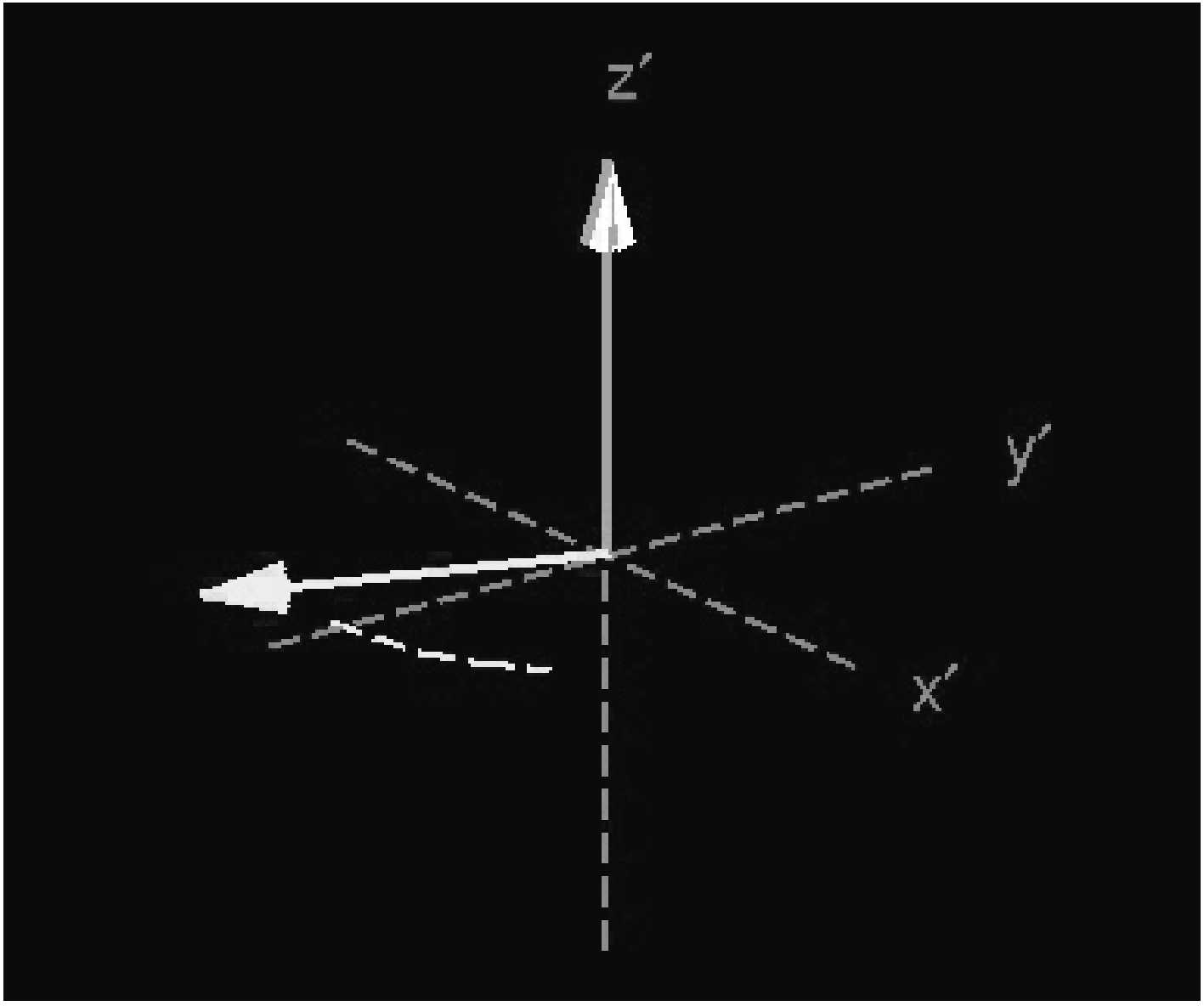
Ligand library screening by NMR.
Additional nearby binding site.
NMR assay of double-binding-site ligands.

Shuker, Hajduk, Meadows & Fesik, *Science*, 1996



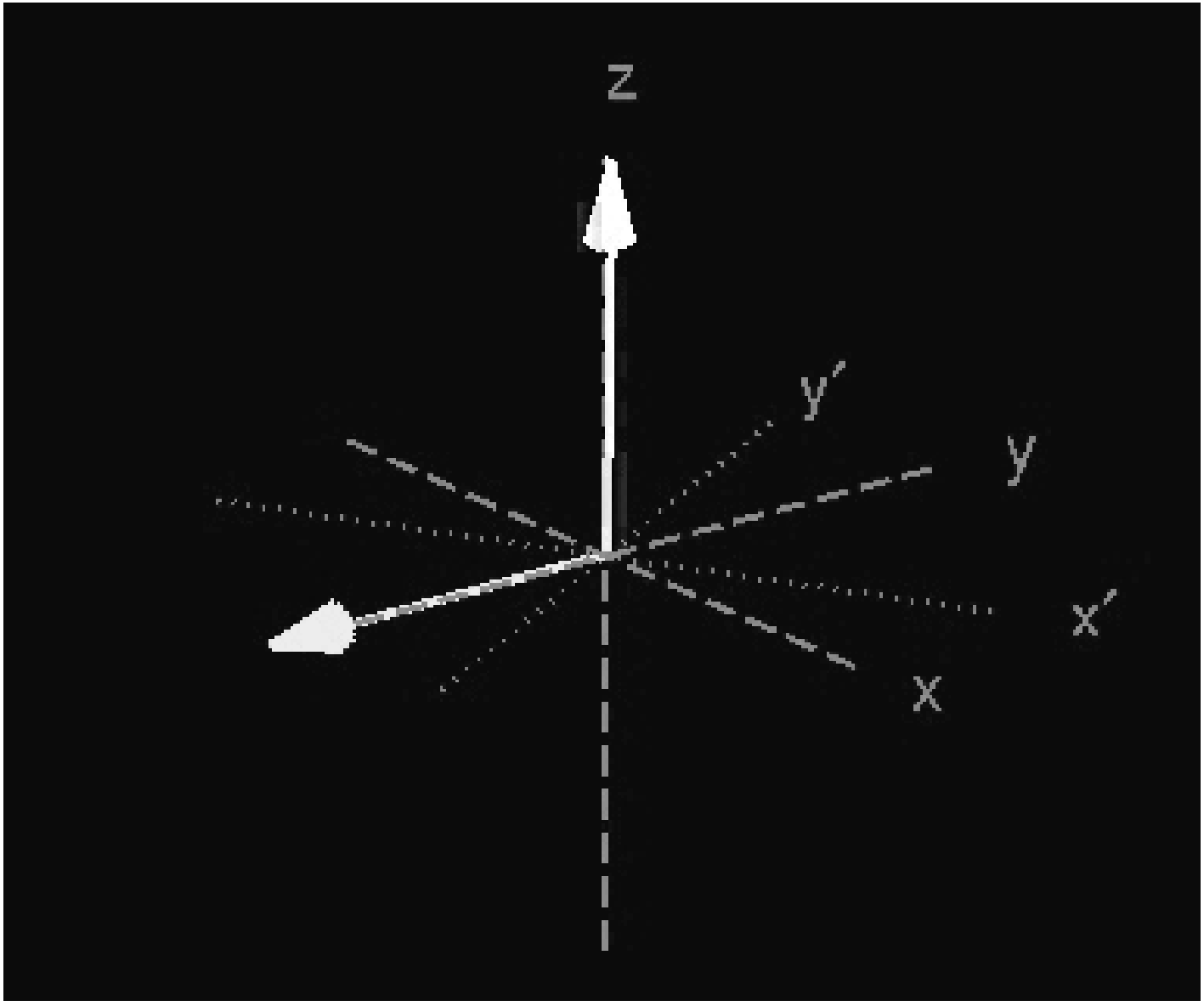
Movie: *Brian Hargreaves*

Equilibrium



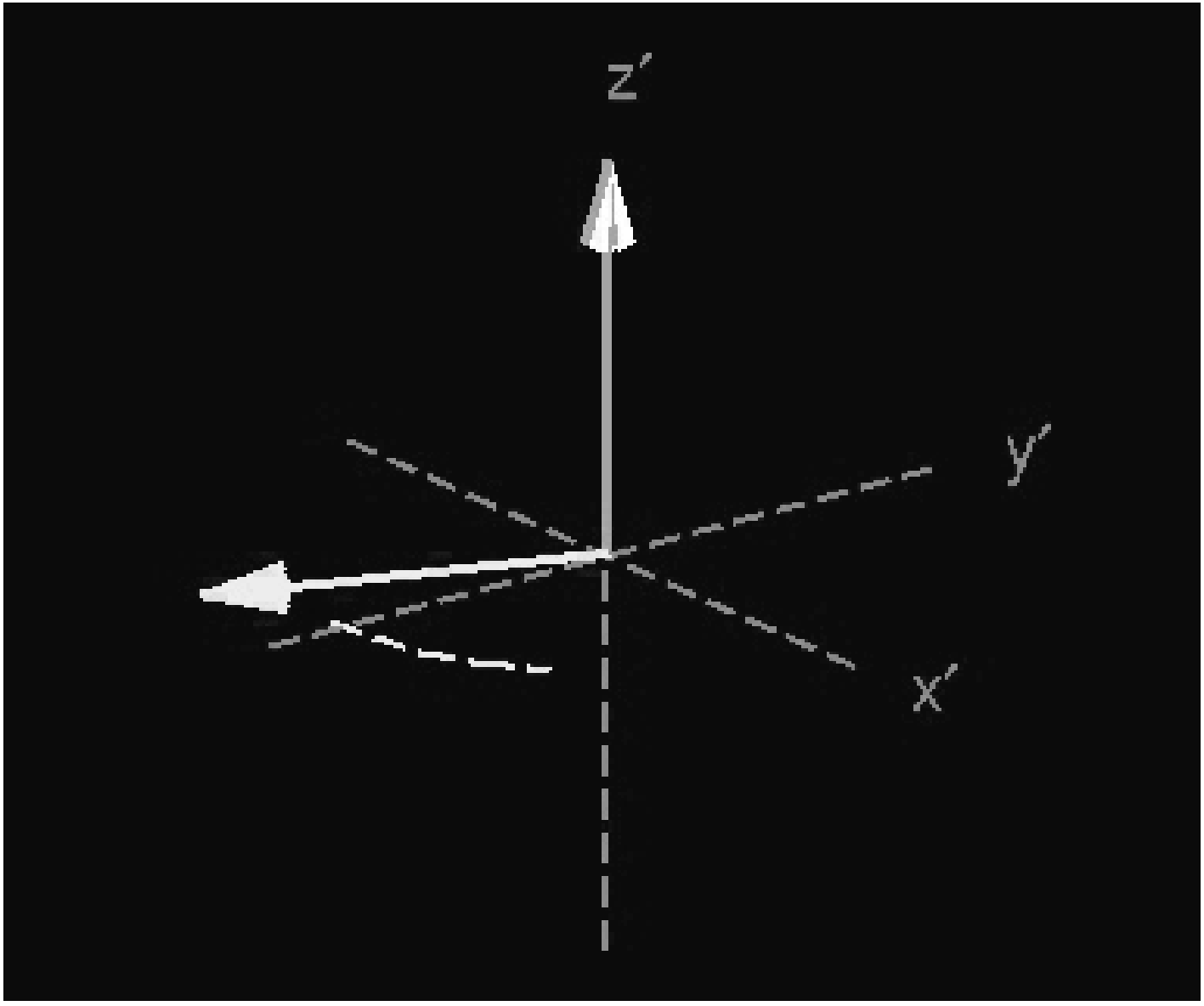
Movie: *Brian Hargreaves*

Excitation: laboratory frame



Movie: *Brian Hargreaves*

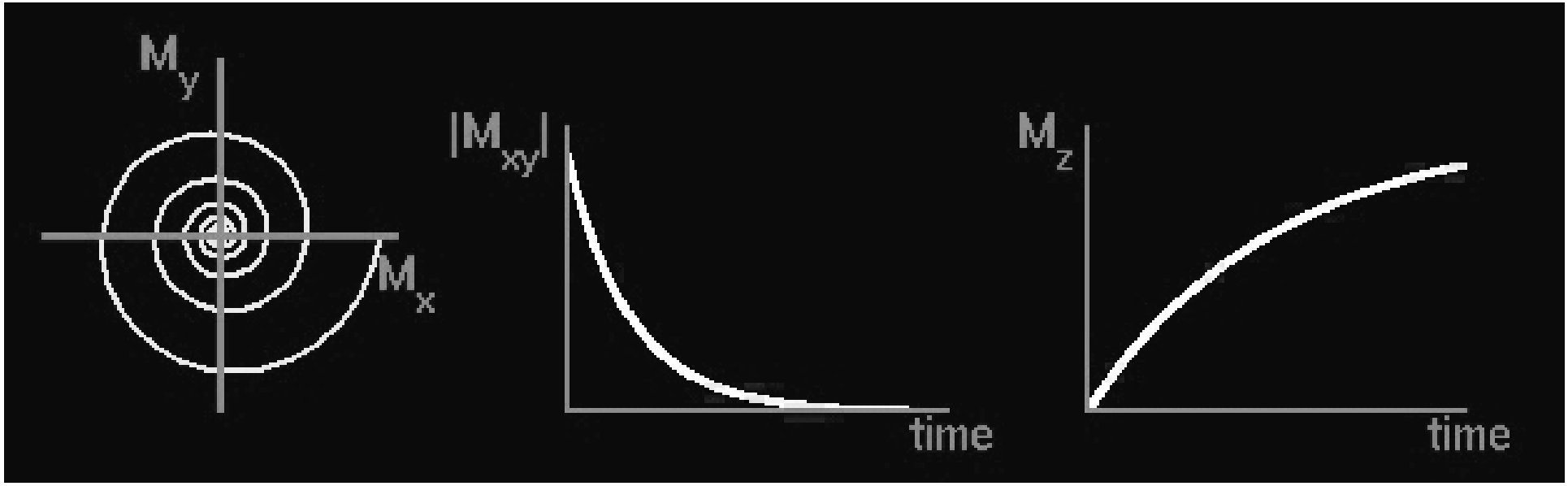
Excitation: rotating frame



Movie: *Brian Hargreaves*

Excitation: laboratory frame

Relaxation = restoring equilibrium



Loss of *x*-*y* coherence = transverse relaxation T_2

Recovery of *z* magnetization = longitudinal ralaxation, T_1

Movie: *Brian Hargreaves*

Relaxation

The event that ultimately determines relaxation is the transition of the nuclear magnetic moment that occurs at a precise frequency value.

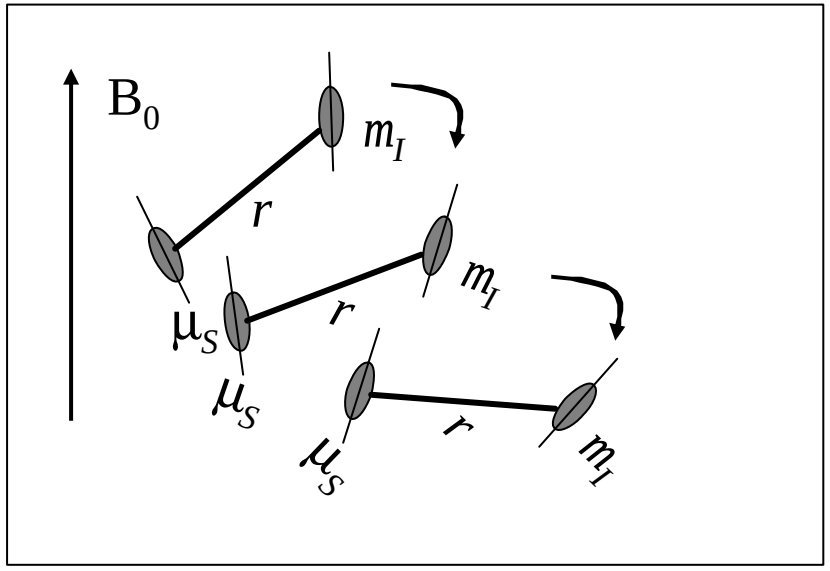
$$\nu = \frac{g}{2p} B$$

This frequency can be reached by fluctuations, due to molecular motions, of the local magnetic field.

One of the most relevant sources of fluctuating local magnetic fields are the dipole-dipole interactions among different spins.

Dipole-Dipole interaction

In isotropic liquids, the longitudinal and trasverse components of B^{dip} with respect to the static field are modulated by the molecular motions and thus generate local fluctuating magnetic fields.

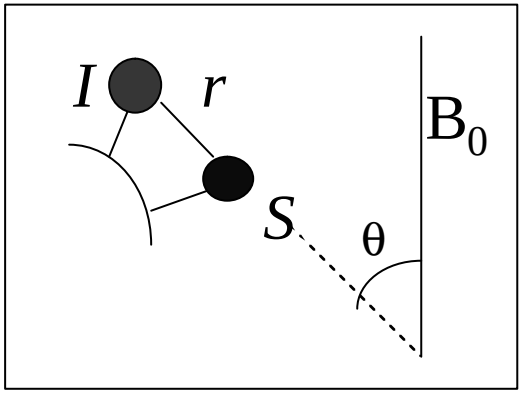


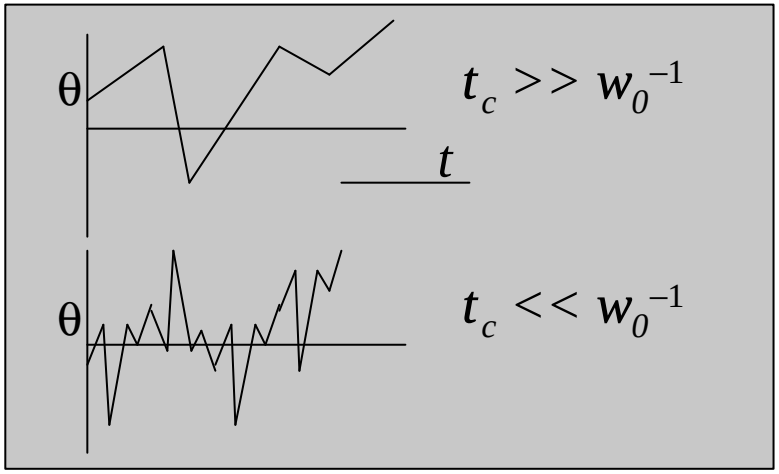
If the molecular motion frequency will be appropriate, the ensuing fluctuations of the local magnetic fields will be able to induce transitions, that is inducing relaxation:

$$n = \frac{g}{2p} B^{dip}$$

D-D interactions, cross-relaxation and nuclear Overhauser effect - NOE

The effects of the dipolar interaction between two nuclei in a magnetic field depend on the internuclear separation and on the reorientation speed of the internuclear vector with respect to the external magnetic field, i.e. the frequency of θ change.





NOE, molecular motion and internuclear distance

$$h = f\left(t_c, r_{IS}^{-6}\right)$$

Relaxation parameters

For homonuclear D-D relaxation (I = 1/2)

$$s_{ij} = \frac{\hbar^2 g^2}{20 r_{ij}^6}\left[6J_{ij}(2w) - J_{ij}(0)\right]$$

$$R_1^{ns} = \frac{\hbar^2 g^2}{20}\sum_{j\neq i}\frac{1}{r_{ij}^6}\left[3J_{ij}(w) + 12J_{ij}(2w)\right]$$

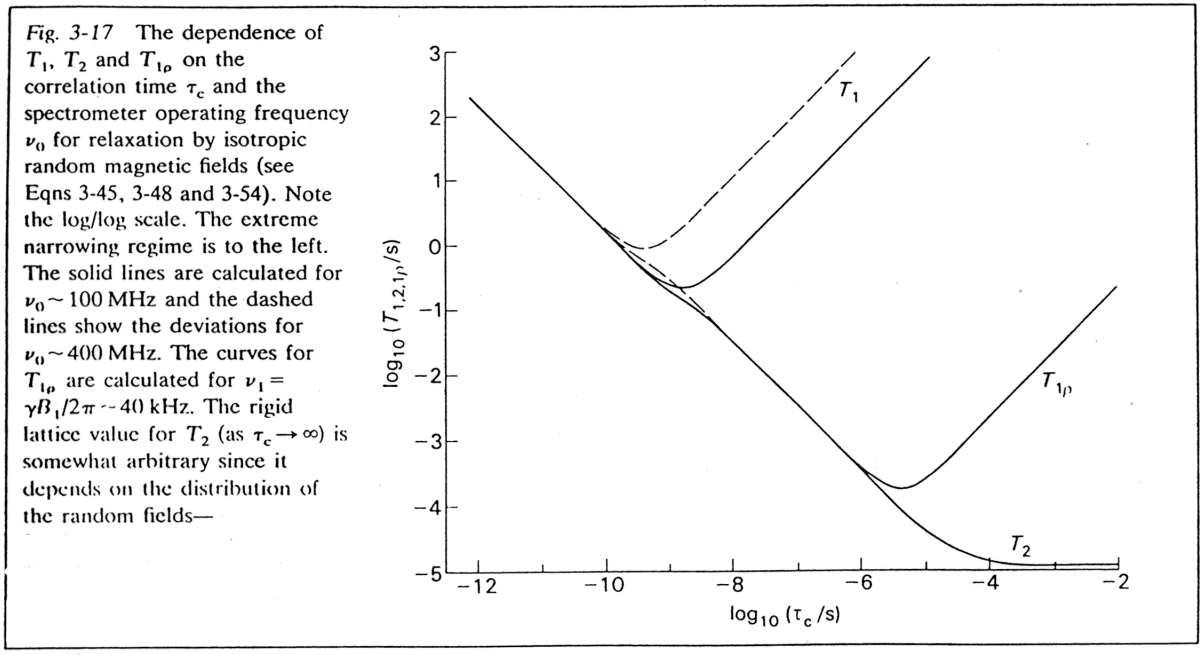
$$R_1^{sel} = \frac{\hbar^2 g^2}{20}\sum_{j\neq i}\frac{1}{r_{ij}^6}\left[J_{ij}(0) + 3J_{ij}(w) + 6J_{ij}(2w)\right]$$

$$R_2 = \frac{\hbar^2 g^2}{40}\sum_{j\neq i}\frac{1}{r_{ij}^6}\left[5J_{ij}(0) + 9J_{ij}(w) + 6J_{ij}(2w)\right]$$

$$R_{1r} = \frac{\hbar^2 g^2}{40}\sum_{j\neq i}\frac{1}{r_{ij}^6}\left[5J_{ij}(2w_1) + 9J_{ij}(w) + 6J_{ij}(2w)\right]$$

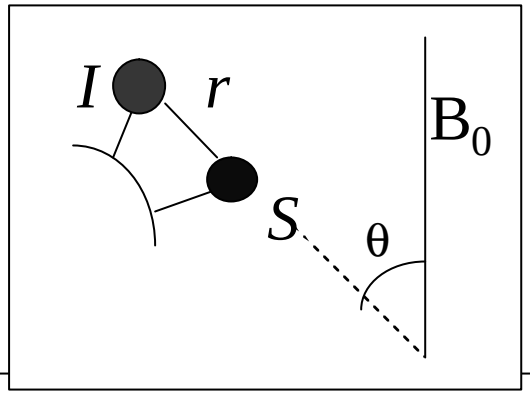
$$J(nw) = \frac{2t_c}{1+(nwt_c)^2}$$

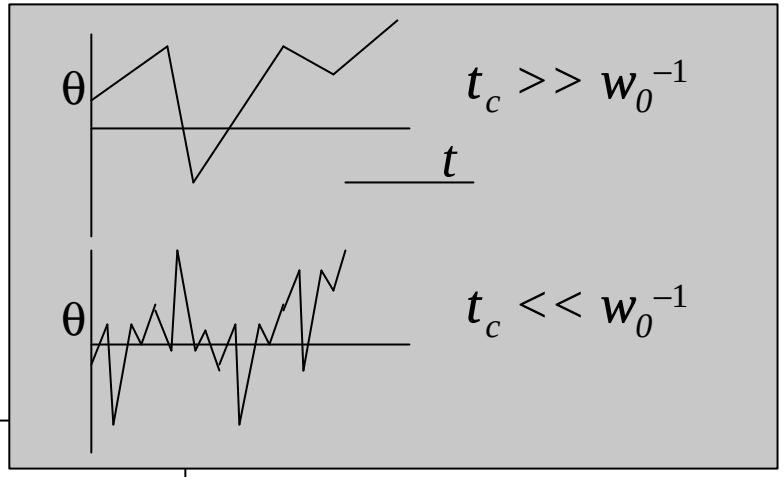
Molecular mobility and relaxation

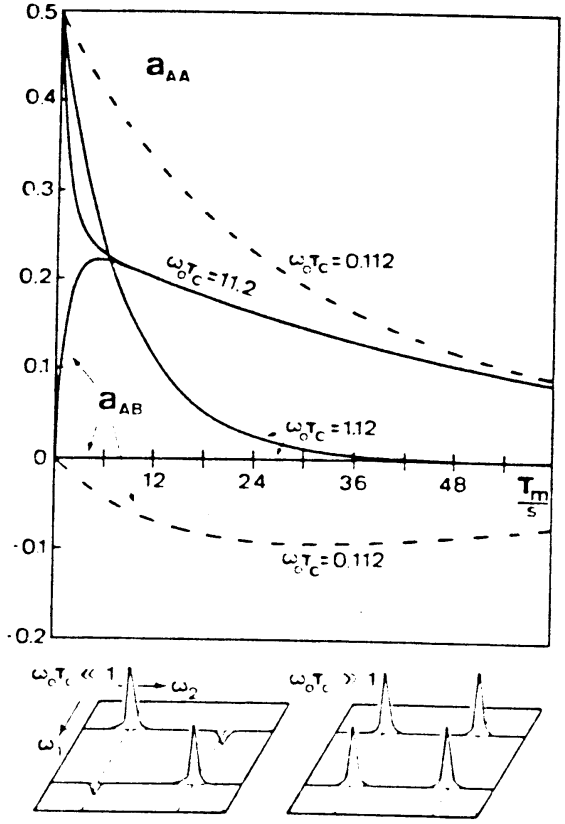


Harris, 1983

The dependence of relaxation on molecular reorientation translates into the measured values of T_1 and T_2, that are equal for small molecules (fast motions) – *extreme narrowing limit* – and divergent (with $T_1 > T_2$) for large molecules (slow motions) – *spin diffusion limit*.



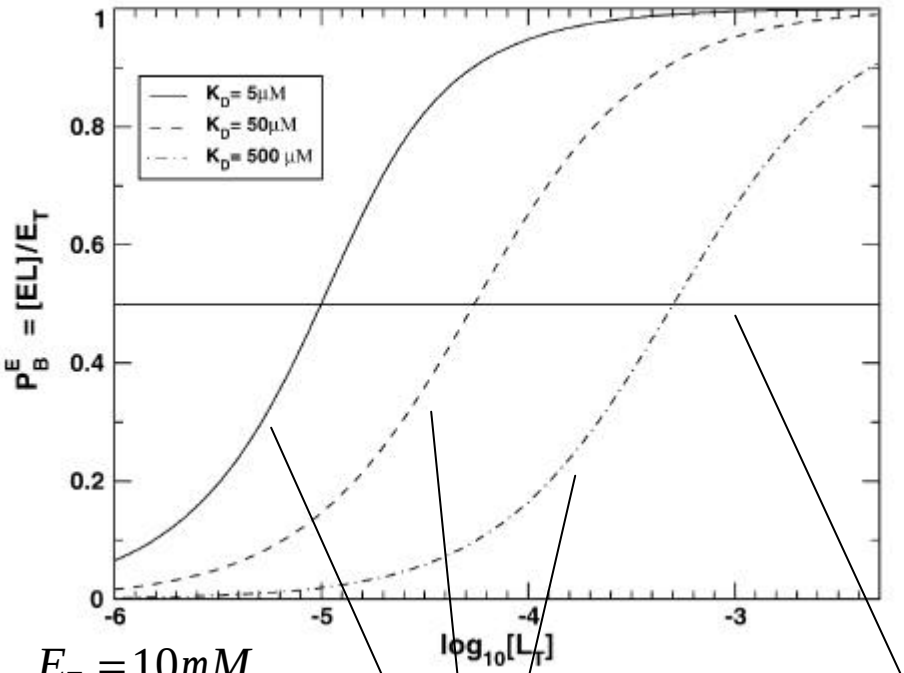




$$s_{ij} = \frac{\hbar^2 g^2}{20 r_{ij}^6}\left[6J_{ij}(2w) - J_{ij}(0)\right]$$

Macura & Ernst, 1980

Receptor-ligand binding equilibria



$$E + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EL \qquad K_D = \frac{[E][L]}{[EL]} = \frac{k_{off}}{k_{on}}$$

$$E_T = [EL] + [E]; L_T = [EL] + [L]; e = \frac{[L_T]}{[E_T]}$$

$$P_B^E = \frac{[EL]}{[E_T]} \qquad \left(P_B^L = \frac{[EL]}{[L_T]} \right)$$

$$P_B^E = \frac{[L]}{[L] + K_D} \qquad \left(P_B^L = \frac{[E]}{[E] + K_D} \right)$$

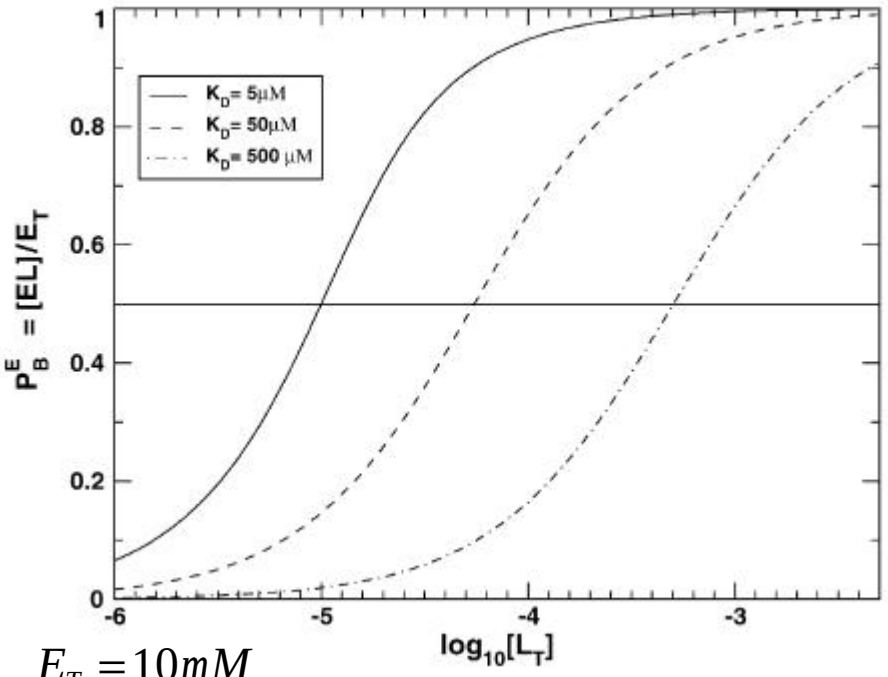
$E_T = 10mM$

$k_{on} = 1 \times 10^8 M^{-1} s^{-1}$ (diffusion limited)

$k_{off} = 5 \times 10^2 - 5 \times 10^4 s^{-1}$

$P_B^E = 0.5 \Rightarrow [L] = K_D$

Receptor-ligand binding equilibria



$E_T = 10mM$

$k_{on} = 1\times10^8 M^{-1}s^{-1}$ (diffusion limited)

$$E + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EL \qquad K_D = \frac{[E][L]}{[EL]} = \frac{k_{off}}{k_{on}}$$

$$P_B^E = \frac{[EL]}{[E_T]} \qquad\qquad P_B^E = \frac{[L]}{[L]+K_D}$$

The fraction of ligand-bound receptor can be calculated in terms of known quantities.

$$P_B^E = \frac{[EL]}{[E_T]} \quad vs \quad L_T$$

$$K_D = \frac{[E][L]}{[EL]} = \frac{[E_T - EL][L_T - EL]}{[EL]}$$

$$[EL] = \frac{1}{2}(L_T + E_T - K_D) - \frac{1}{2}\sqrt{(L_T + E_T - K_D)^2 - 4L_T E_T}$$

The NMR spectrum of a system undergoing binding exchange equilibria exhibits changes of the observable NMR parameters, *Q*, i.e. chemical shifts (*W*), relaxation rates (*R*), cross-relaxation rates (σ) diffusion constants (*D*).

This is accounted for by the modified Bloch equations:

$$\frac{d}{dt}\left(\mathbf{?M_z}\right) = -\left\{\mathbf{R}+\mathbf{K}\right\}\left(\mathbf{?M_z}\right)$$

$$\frac{d}{dt}\left(\mathbf{M_+}\right) = -\left\{\mathbf{R}+\mathbf{K}-i\mathbf{O}\right\}\mathbf{M_+}$$

The solutions of the modified Bloch equations give the time course of free and receptor-bound ligand magnetizations.

$$\frac{d}{dt}\begin{bmatrix} M_{+F} \\ M_{+B} \end{bmatrix} = -\left\{ \begin{bmatrix} R_{2F} & 0 \\ 0 & R_{2B} \end{bmatrix} + \begin{bmatrix} P_B k_{ex} & -P_F k_{ex} \\ -P_B k_{ex} & P_F k_{ex} \end{bmatrix} - i \begin{bmatrix} \Omega_F & 0 \\ 0 & \Omega_B \end{bmatrix} \right\} \begin{bmatrix} M_{+F} \\ M_{+B} \end{bmatrix}$$

with $F, B = \mathit{free}, \mathit{bound}; R_2 = T_2^{-1}; k_{ex} = k_{on}[E] + k_{off}$

$$P_B = \frac{[EL]}{L_T}; P_F = \frac{[L]}{L_T}; k_{ex} P_B = k_{on}[E]; k_{ex} P_F = k_{off}$$

By symmetrization and diagonalization of **R**, W and **K** matrices, one obtains the exchange-modulated relaxation rates and precession frequencies.

Analytical solutions are available (Hahn, Maxwell, McConnel solution or Swift-Connick formula).

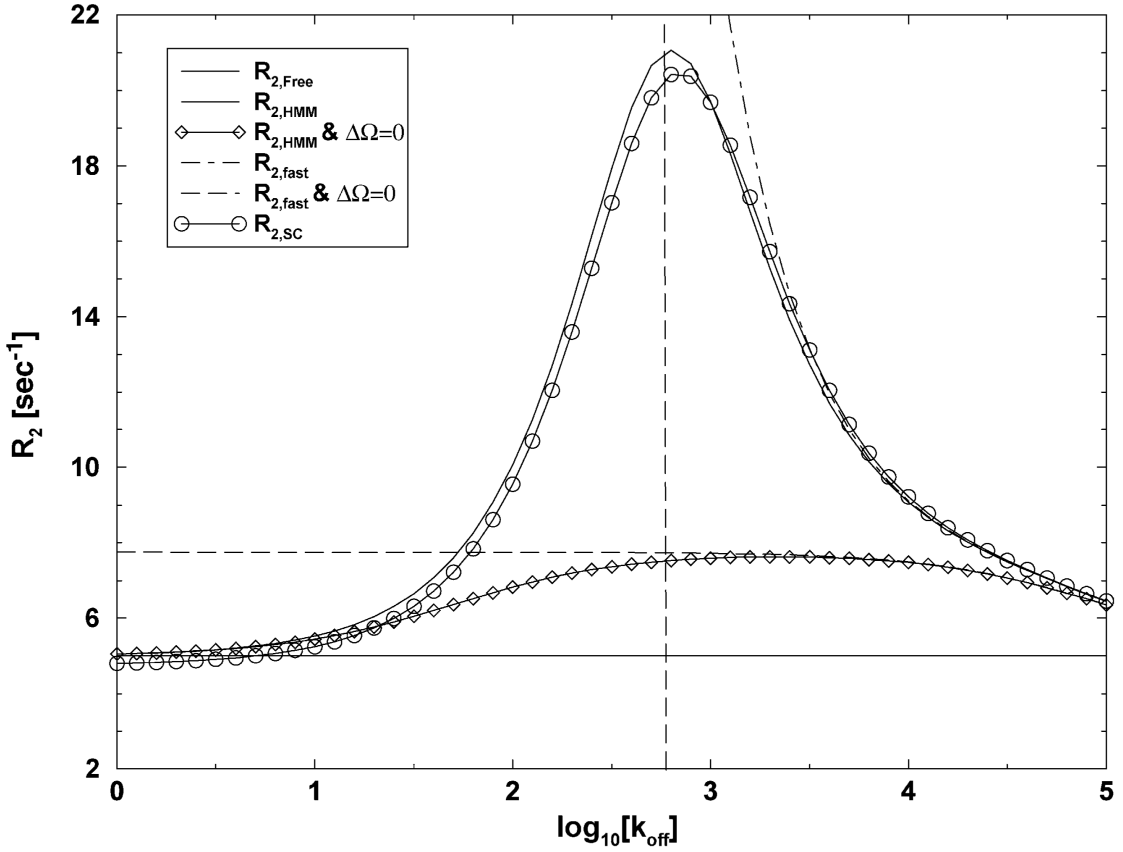


Fig. 3. Simulation of transverse relaxation rate constant R_2 for a single uncoupled ligand spin as a function of the off-rate, k_{off} using the Eqs. (23)–(26) in the main text. The figure assumes $\text{L}_\text{T} = 1$ mM, $\text{E}_\text{T} = 50$ μM, $k_{\text{on}} = 1 \times 10^8$ M^{-1} s^{-1}, $\Delta\Omega/2\pi = 100$ Hz, $\text{R}_{2\text{F}} = 5$ s^{-1}, and $\text{R}_{2\text{B}} = 60$ s^{-1}. Slow and fast exchange limits prevail on the left and right respectively. Curve traces are as follows: solid flat trace $= \text{R}_{2\text{F}}$; solid peaked trace $= R_2^{\text{HMM}}$; sigmoidal diamond trace $= R_2^{\text{HMM}}$ with $\Delta\Omega = 0$; dashed-dotted curve = fast exchange result $R_{2,\text{fast}}$; long dashes = fast exchange $R_{2,\text{fast}}$ with $\Delta\Omega = 0$; open circle trace = Swift–Connick R_2^{SC}.

Peng et al., 2004

Slow-exchange limit

The exchange matrix **K** introduces only a small perturbation to (**R** – *i*W): $\sqrt{P_F P_B}\,k_{ex} = \left(R_{2F} - R_{2B}\right), \left(\Omega_F - \Omega_B\right)$

The free and bound ligand signals retain their precession frequency and have modified transverse relaxation rates:

$$R_{2F,sl} = R_{2F} + P_B k_{ex} \qquad R_{2B,sl} = R_{2B} + P_F k_{ex}$$

Typically R_{2B} ? R_{2F} and $P_B = P_F$

Hence $R_{2F,sl}$; R_{2F} $\Rightarrow$ it may be hard to distinguish slow exchange and lack of binding.

Fast-exchange limit

Fast exchange on the chemical shift and relaxation time scales means that the term (**R** – *i*W) becomes a small perturbation to the exchange matrix **K**.

A single averaged value is observed for chemical shift and transverse relaxation relaxation rate:

$$\Omega_{avg} = P_F \Omega_F + P_B \Omega_B$$

$$R_{2,avg} = P_F R_{2F} + P_B R_{2B} + R_{ex} \quad \text{with} \quad R_{ex} = \left(\Omega_F - \Omega_B\right)^2 \frac{P_F P_B}{k_{ex}}$$

For very fast exchange $R_{ex} = 0$.

The information on the bound state is encoded by the averaged relaxation rate of a single resonance.

Fast-exchange limit

The conditions of fast-exchange limit are quite usual and convenient.

$$E + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EL \qquad K_D = \frac{[E][L]}{[EL]} = \frac{k_{off}}{k_{on}}$$

With a typical value of K_D = 100 μM, if k_{on} is in the range 10^7-10^9 $M^{-1}s^{-1}$, then $10^3 < k_{off} < 10^5$ s^{-1}.

This value of exceeds most differences of transverse relaxation rates, rotating frame relaxation rates, cross-relaxation rates, chemical shifts, diffusion coefficients i.e. ΔQ.

Fast-exchange limit

A single averaged value of a generic NMR parameter may be observed as:

$$Q_{avg} = P_F Q_F + P_B Q_B$$

$$Q_{avg} = P_F Q_F + P_B Q_B + Q_{ex}$$

and compared with the corresponding value in the absence of binding, i.e. Q_F.

Observing Q_{avg} is convenient when $Q_B >> Q_F$ because typically screening conditions imply:

$$e = \frac{L_T}{E_T} \; ? \; 1 \quad \Rightarrow P_B = \; P_F$$

Under these conditions it is convenient to consider $\left(Q_{avg} - Q_F\right)$

Fast-exchange limit

$$E + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EL \qquad K_D = \frac{[E][L]}{[EL]} = \frac{k_{off}}{k_{on}}$$

$$e = \frac{L_T}{E_T} \qquad P_B = \frac{P_B^E}{e}$$

$$P_B^E = \frac{[EL]}{[E_T]}; \quad P_F^E = \frac{[E]}{[E_T]}; \quad P_B^E + P_F^E = 1$$

$$P_B = \frac{[EL]}{[L_T]}; \quad P_F = \frac{[L]}{[L_T]}; \quad P_B + P_F = 1$$

$$Q_{avg} = P_B Q_B + P_F Q_F = P_B Q_B + (1 - P_B) Q_F$$

$$Q_{avg} - Q_F = P_B (Q_B - Q_F) = \frac{P_B^E}{e} (Q_B - Q_F)$$

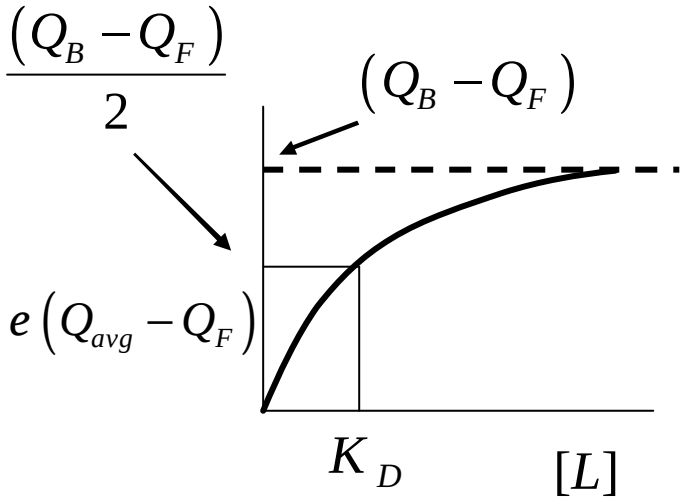
$$e (Q_{avg} - Q_F) = P_B^E (Q_B - Q_F) = \frac{[EL]}{[E] + [EL]} (Q_B - Q_F)$$

$$\boxed{e (Q_{avg} - Q_F) = \frac{[L]}{[L] + K_D} (Q_B - Q_F)}$$

This is a dose-response hyperbolic curve.

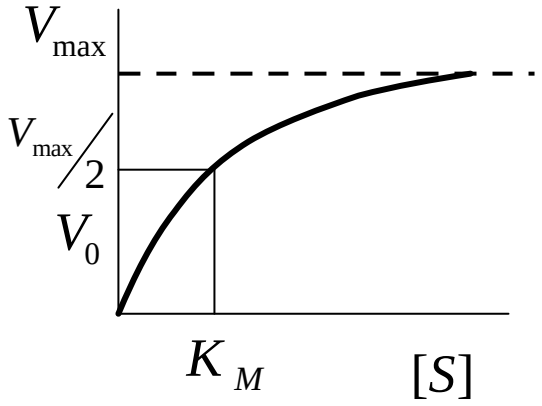
Fast-exchange limit

$$e\left(Q_{avg}-Q_F\right)=\frac{[L]}{[L]+K_D}\left(Q_B-Q_F\right)$$



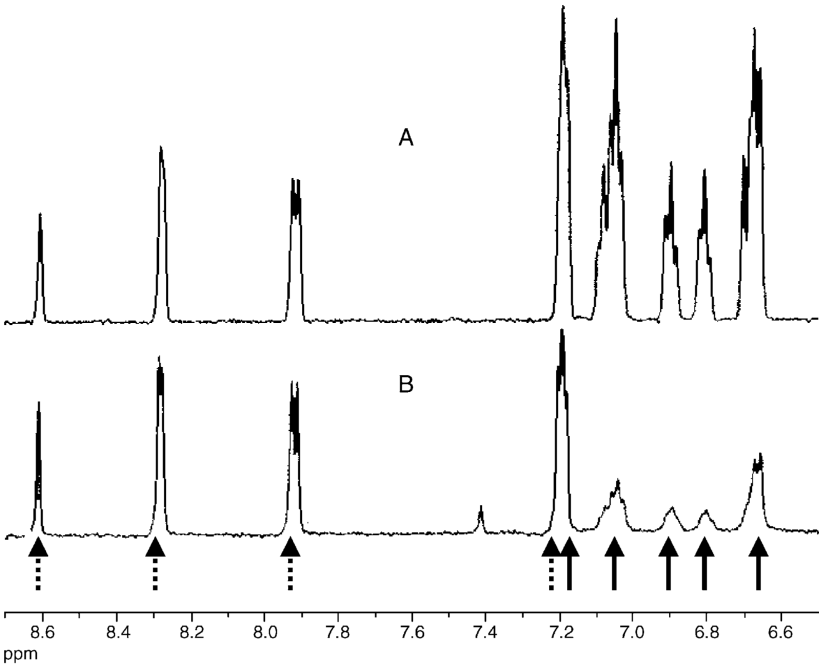
Analogy with Michaelis-Menten steady state kinetics equation:

$$V_0=\frac{[S]}{[S]+K_M}\cdot V_{\max}$$



By measuring Q_{avg} and correcting for Q_F, if $\varepsilon >> 1$, $[L] \sim [L_T]$ and the curve yields estimates for K_D and the plateau value.

Binding and line broadening



Peng et al., 2004

Broadening, i.e. increased R_2, can reveal binding. Mixture of small ligands (A) in the absence and (B) in the presence of p38 MAP kinase receptor. Solid arrows highlight broadened signals.

Relaxation parameters

For homonuclear D-D relaxation (I = 1/2)

$$J(nw) = \frac{2t_c}{1+(nwt_c)^2}$$

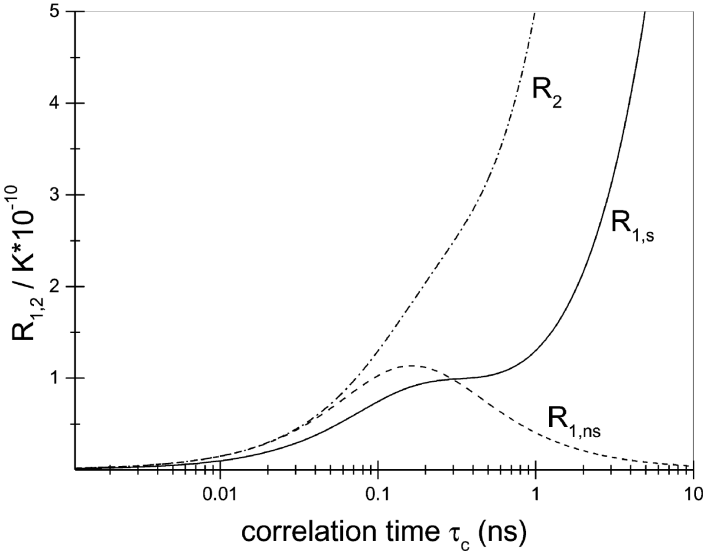
$$s_{ij} = \frac{h^2 g^2}{20 r_{ij}^6}\left[6J_{ij}(2w) - J_{ij}(0)\right]$$

$$R_1^{ns} = \frac{h^2 g^2}{20}\sum_{j\neq i}\frac{1}{r_{ij}^6}\left[3J_{ij}(w) + 12J_{ij}(2w)\right]$$

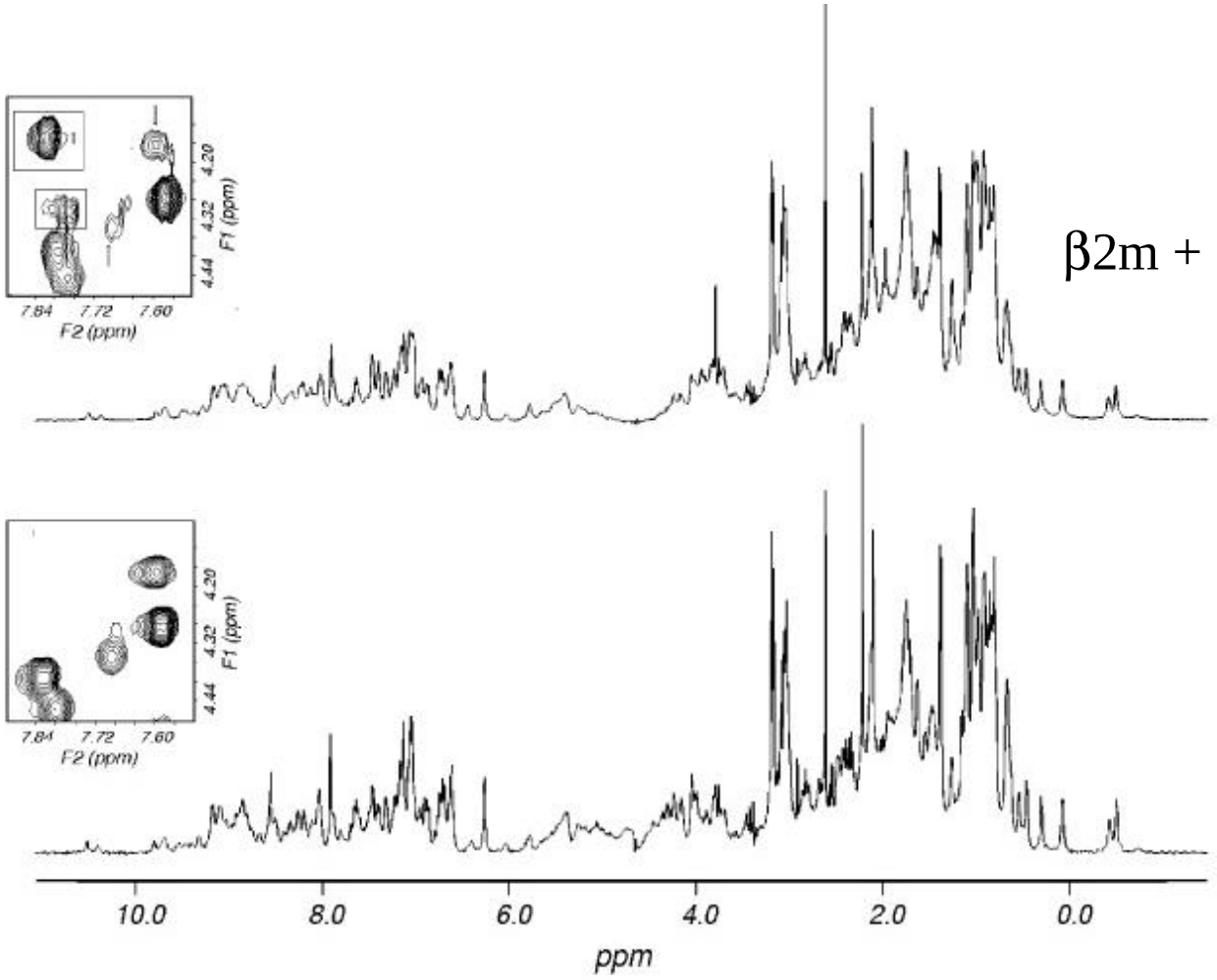
$$R_1^{sel} = \frac{h^2 g^2}{20}\sum_{j\neq i}\frac{1}{r_{ij}^6}\left[J_{ij}(0) + 3J_{ij}(w) + 6J_{ij}(2w)\right]$$

$$R_2 = \frac{h^2 g^2}{40}\sum_{j\neq i}\frac{1}{r_{ij}^6}\left[5J_{ij}(0) + 9J_{ij}(w) + 6J_{ij}(2w)\right]$$

$$R_{1r} = \frac{h^2 g^2}{40}\sum_{j\neq i}\frac{1}{r_{ij}^6}\left[5J_{ij}(2w_1) + 9J_{ij}(w) + 6J_{ij}(2w)\right]$$



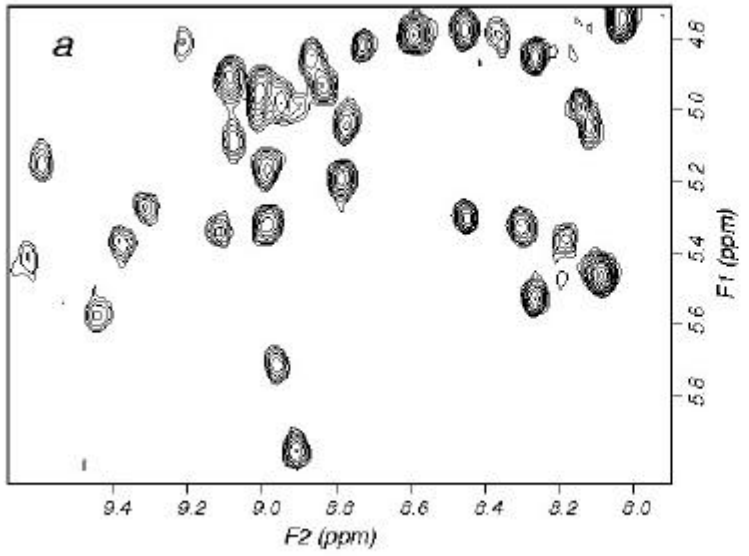
Binding and line broadening



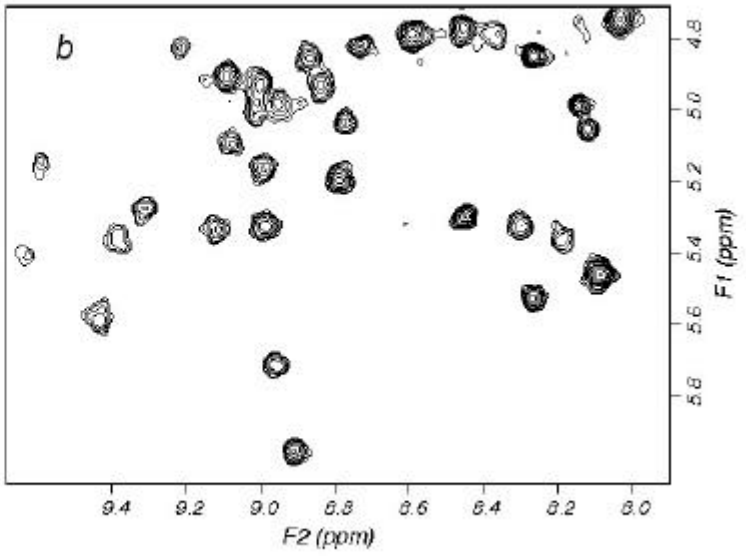
β2m + αBcrystallin

β2m

Binding and line broadening

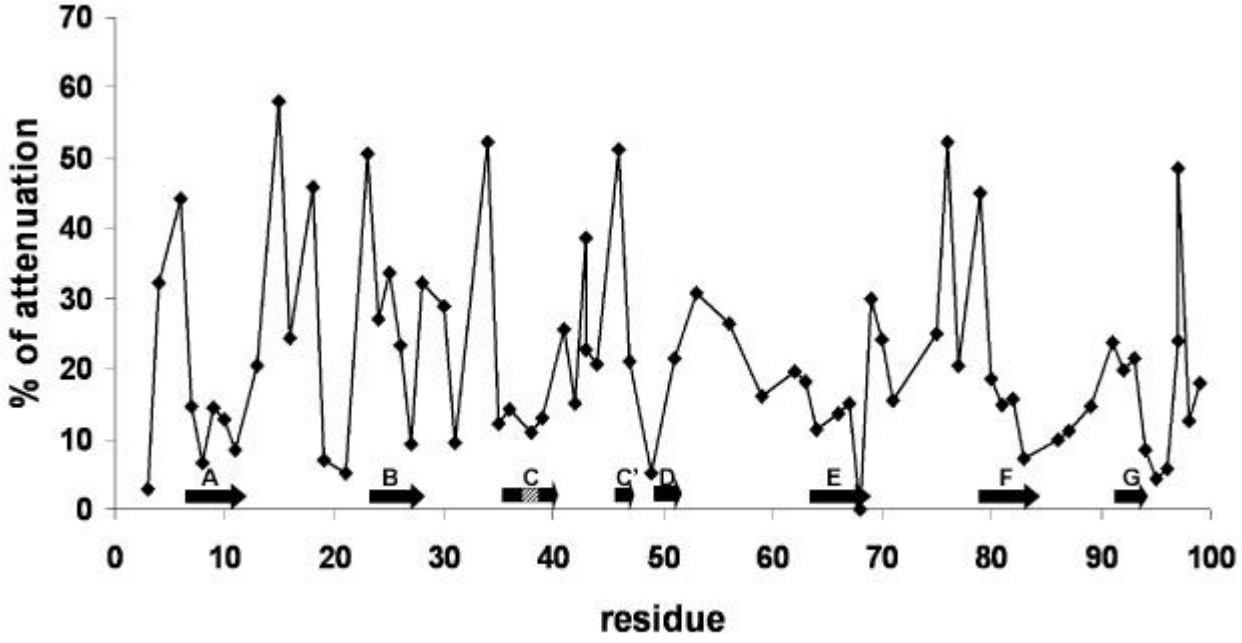


β2m

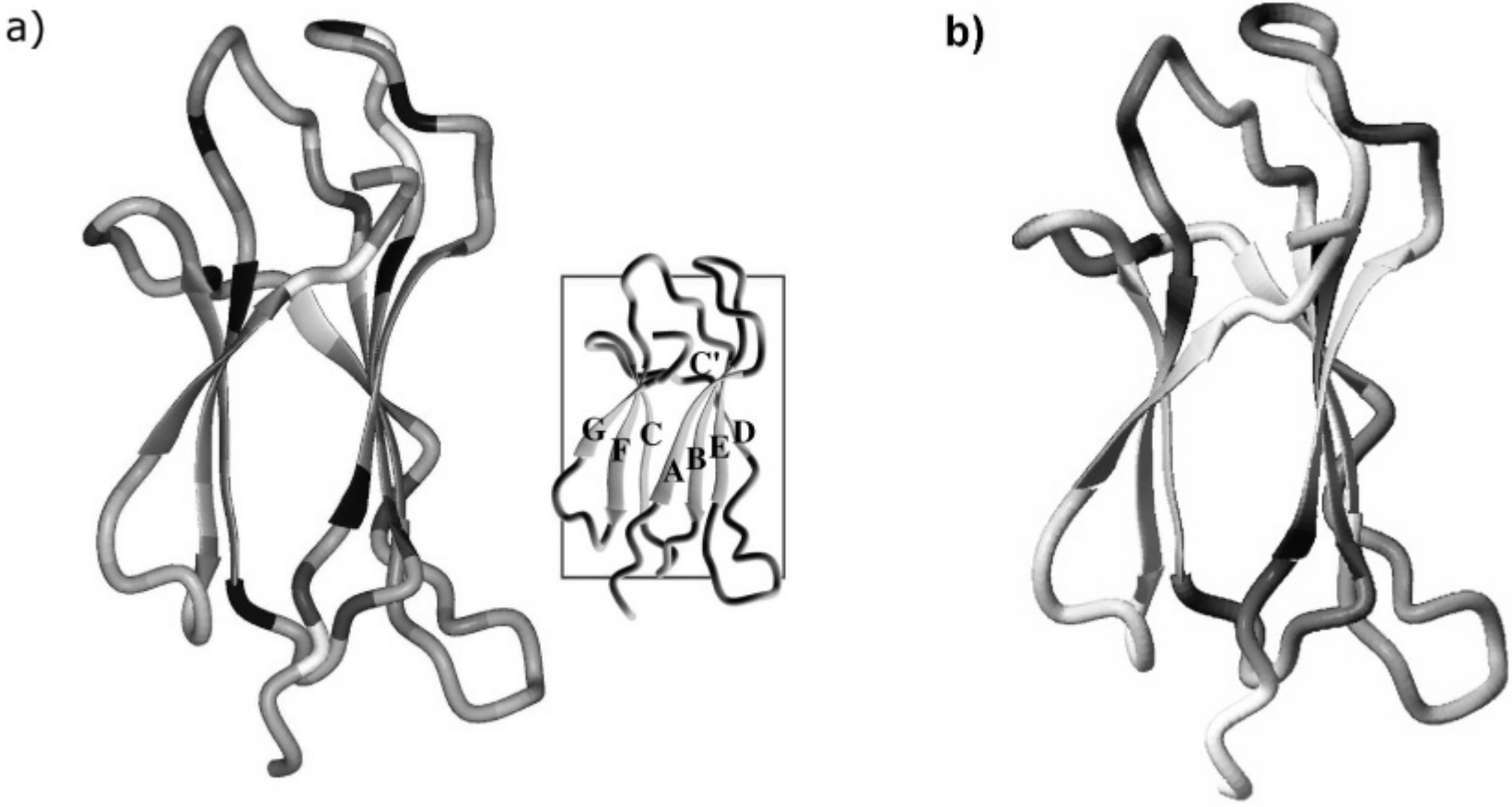


β2m + αBcrystallin

Binding and line broadening

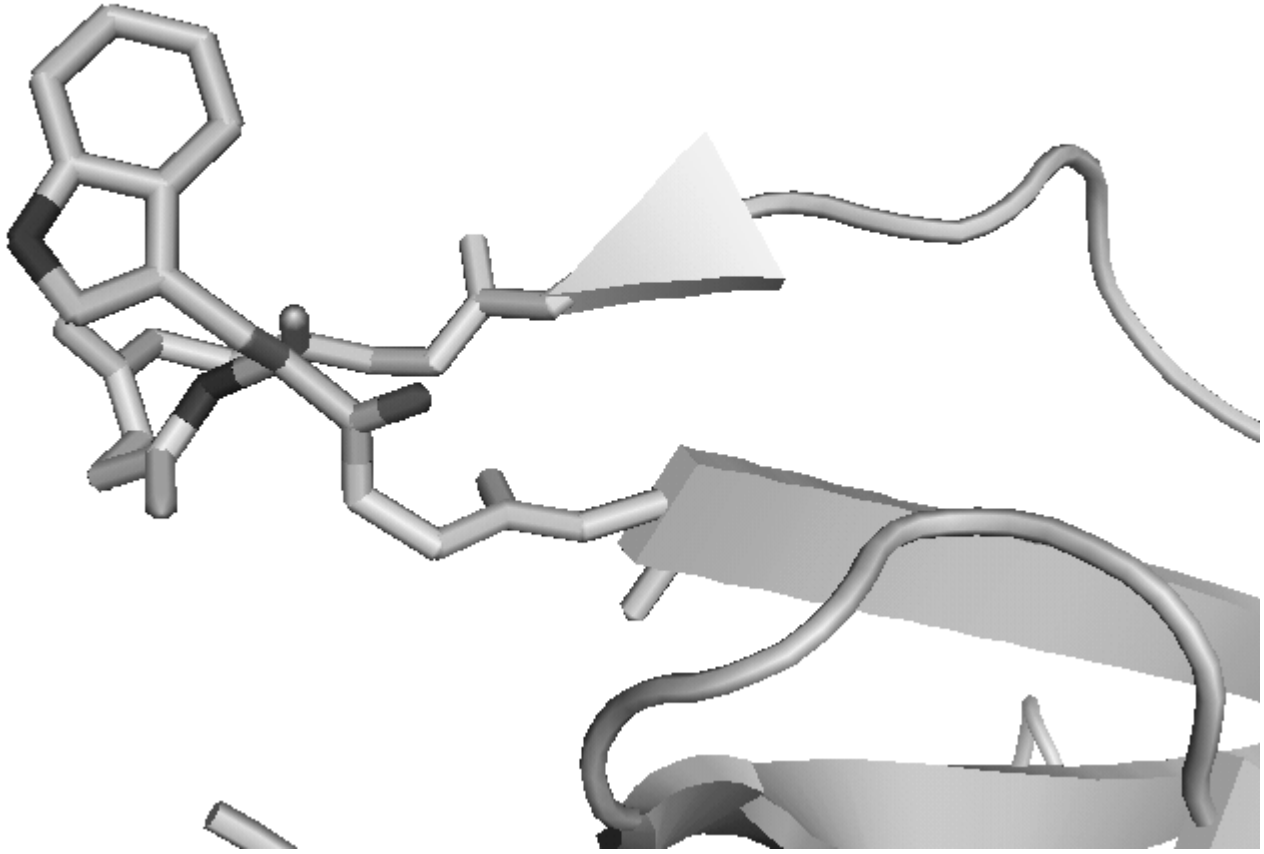


Binding and line broadening



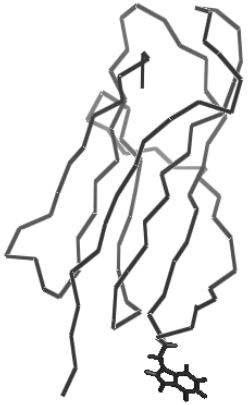
Transverse relaxation and aggregation

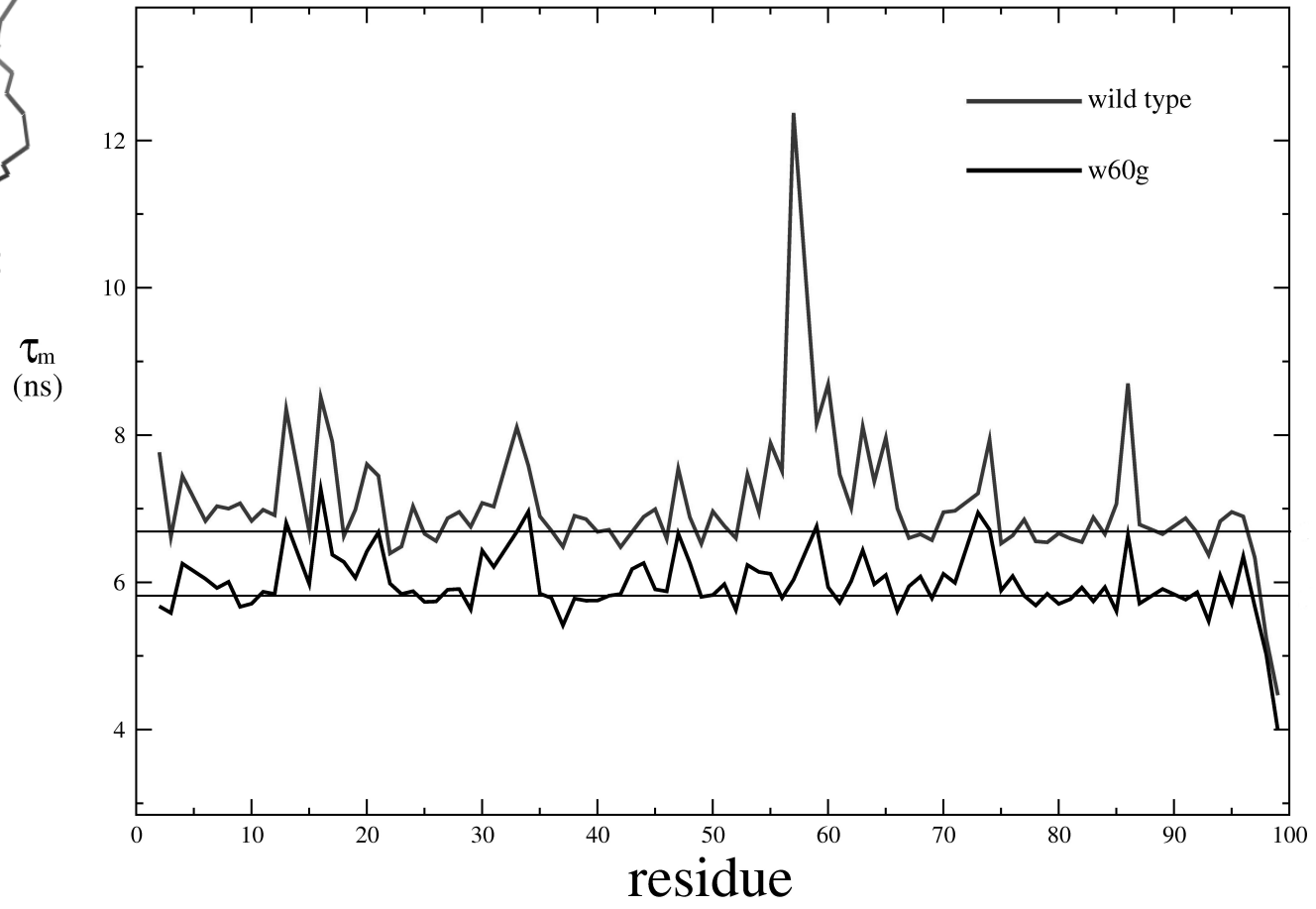
Trp60 occurs in α_L backbone conformation in β2m



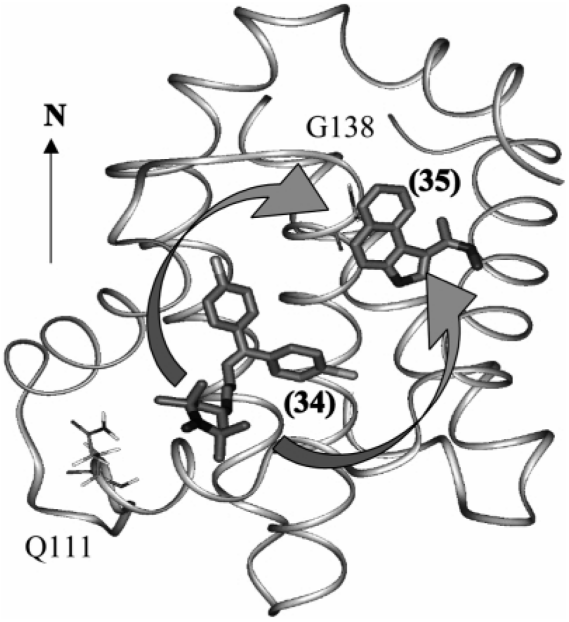
Transverse relaxation and aggregation

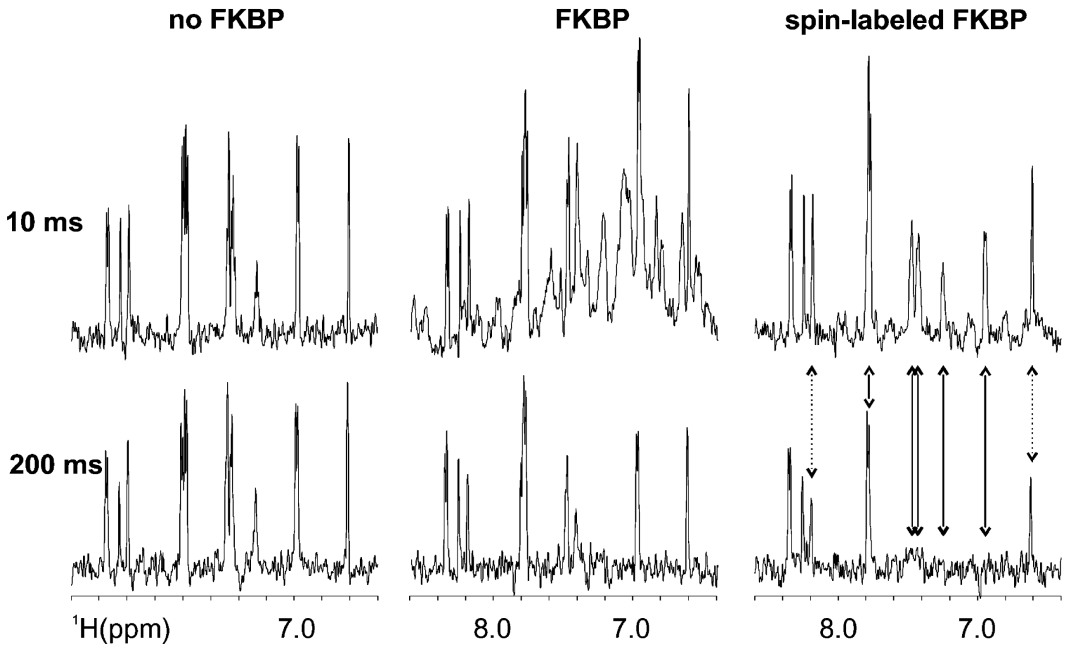
NMR: ^{15}N relaxation measurements





Transverse relaxation editing by paramagnetic labeling



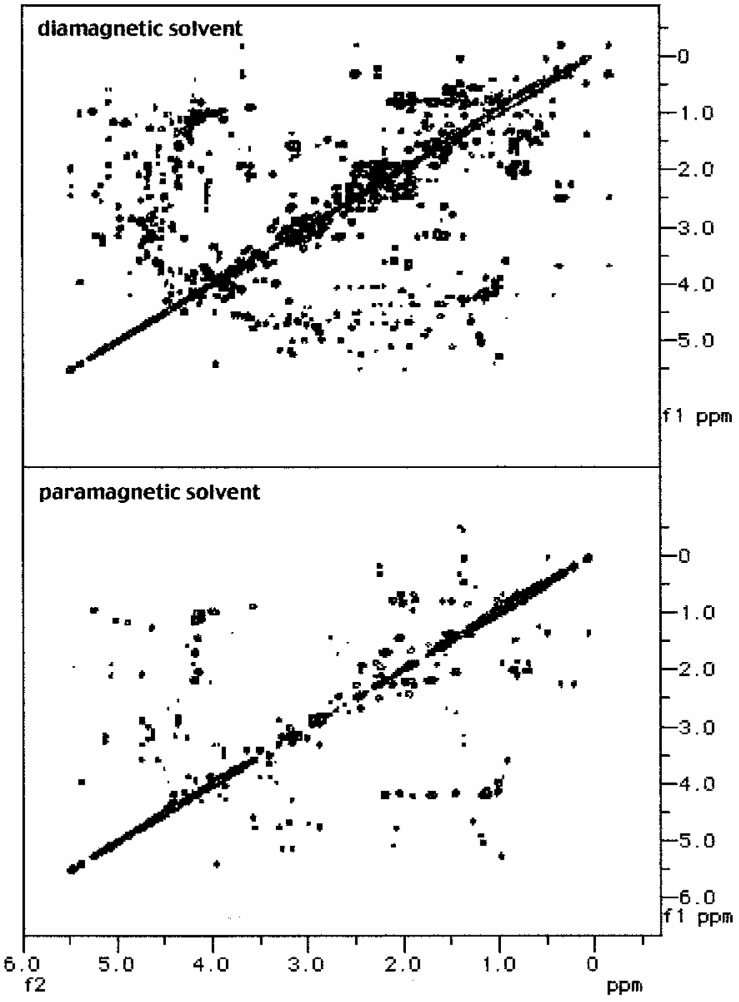


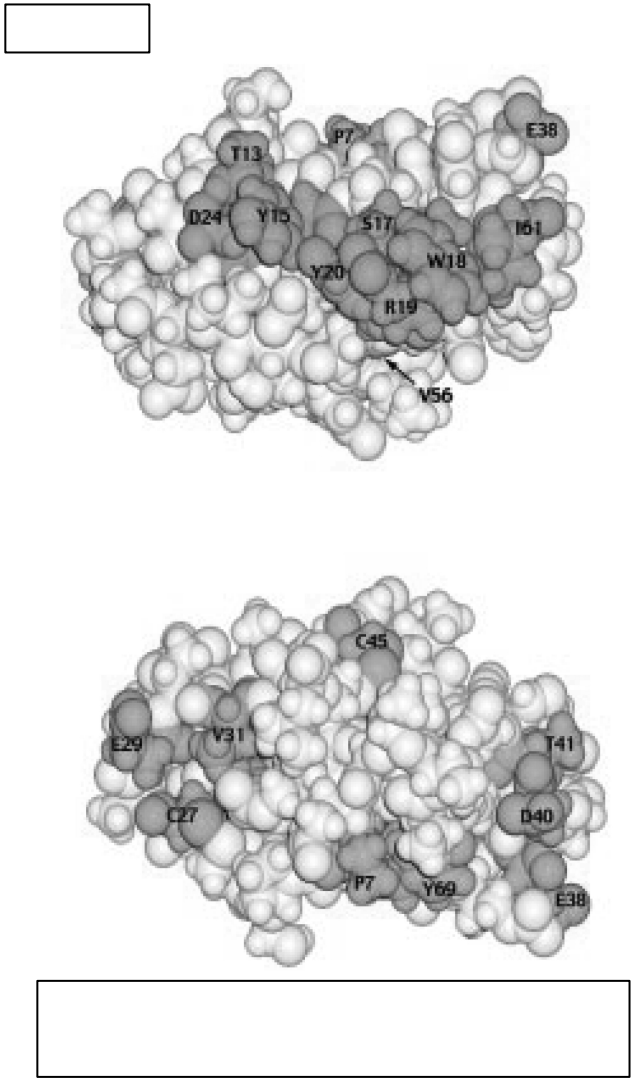
A paramagnetic labelled ligand (34) magnifies the tranverse relaxation enhancement of a adjacent ligand (35).

T_2-edited spectra of a ligand mixture without and with FKBP protein and with spin-labeled FKBP.

Stockman & Dalvit, 2002

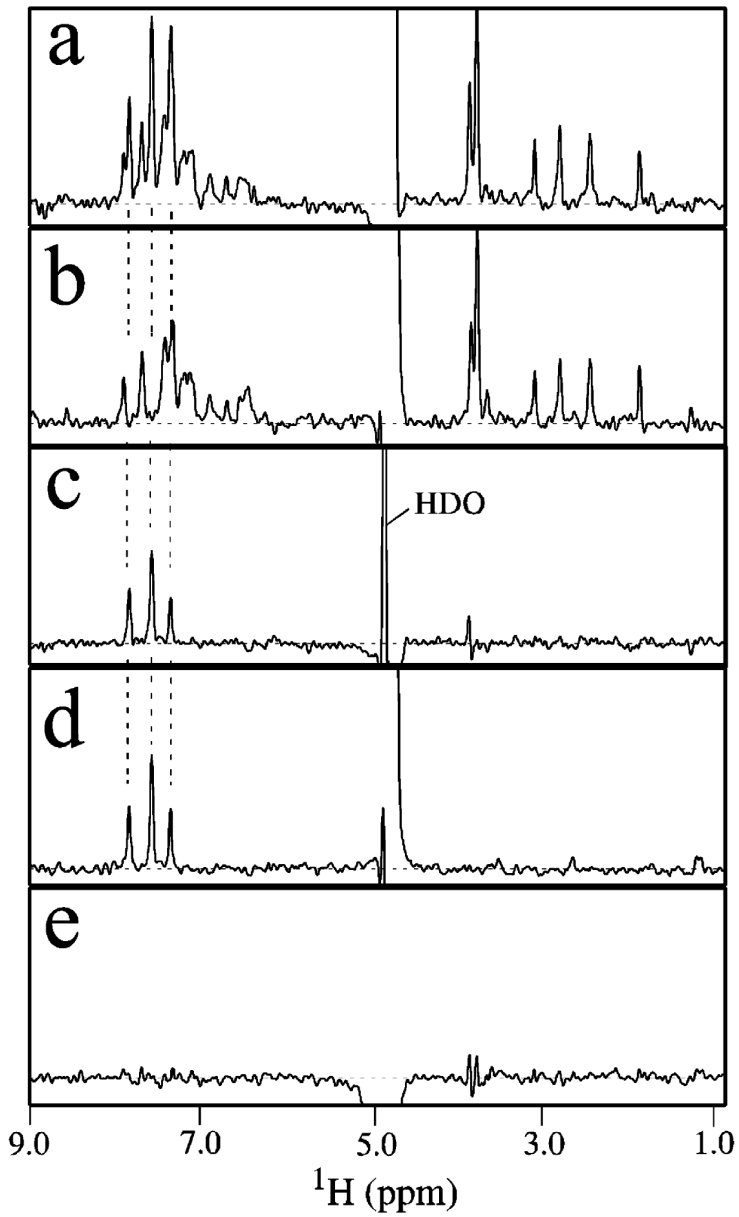
Transverse relaxation editing by paramagnetic labeling





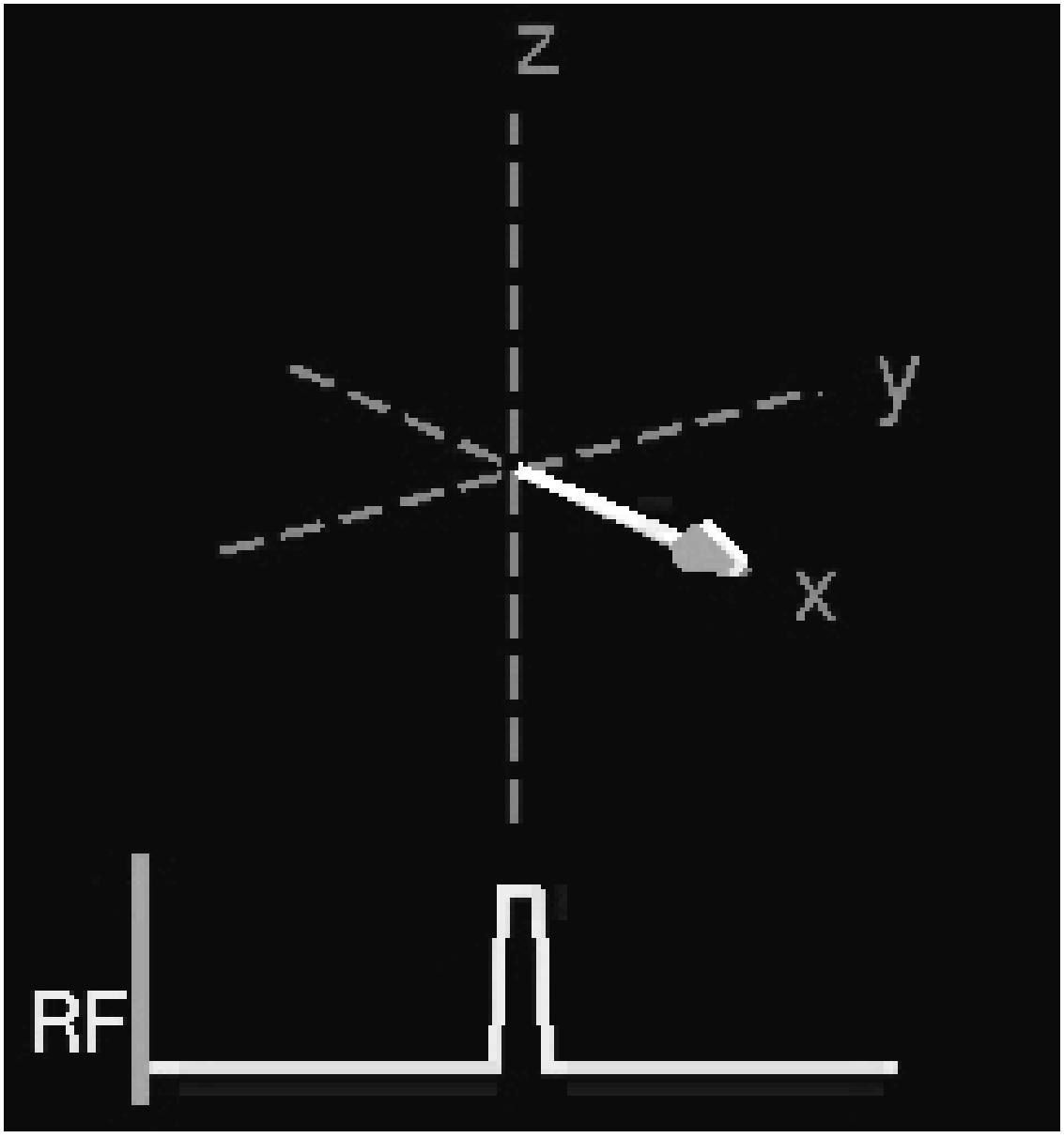
Niccolai et al., 2001

Transverse relaxation filtering



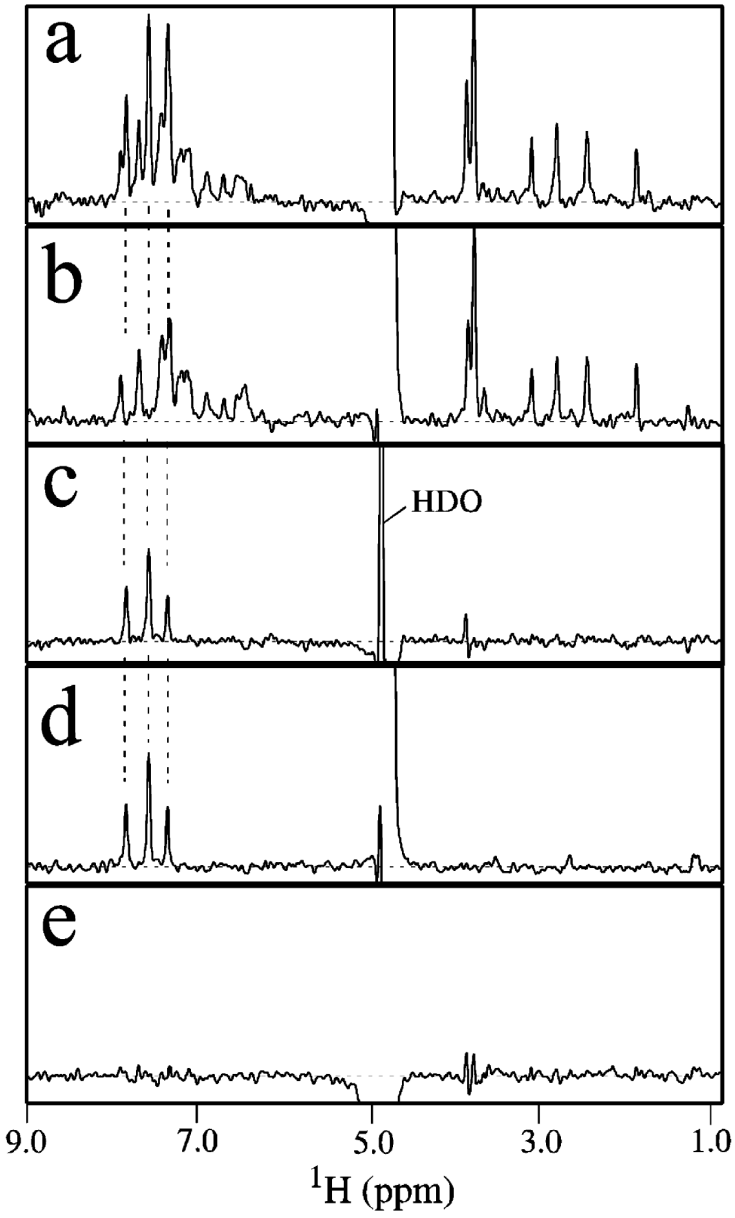
Stockman & Dalvit, 2002

A single FKBP binding ligand is identified in a mixture of 9 compounds by transverse relaxation editing.



Movie: *Brian Hargreaves*

Transverse relaxation filtering



Stockman & Dalvit, 2002

a) transverse-relaxation-edited spectrum of 9 compounds without FKBP.

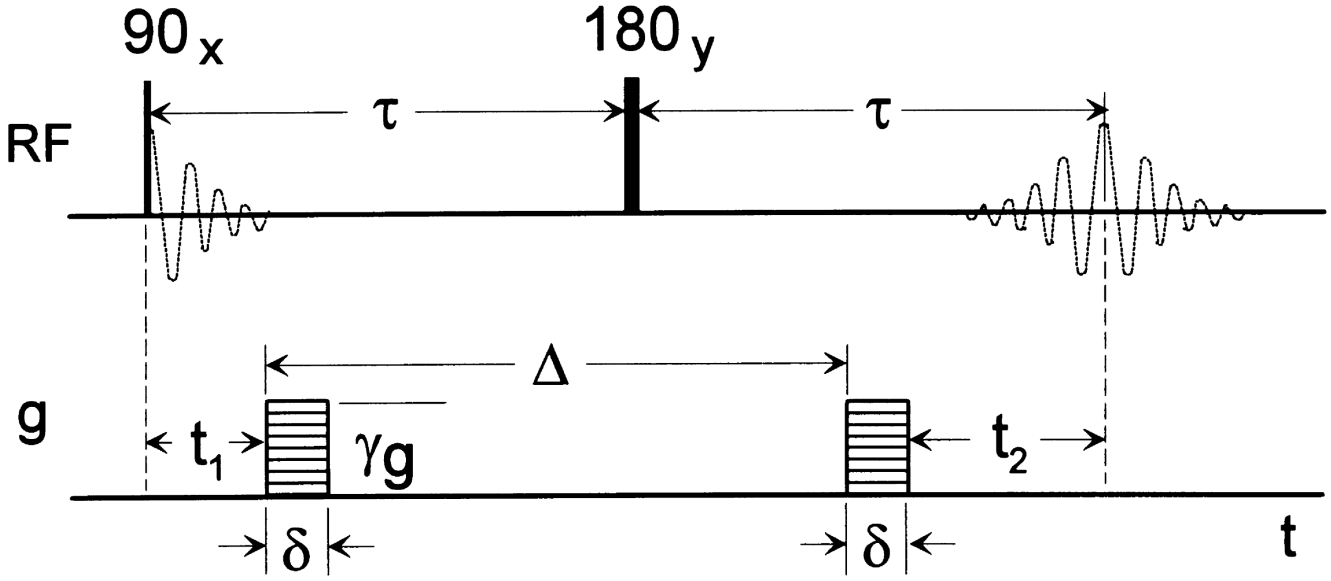
b) transverse-relaxation-edited spectrum of 9 compounds with FKBP minus the same editing for FKBP alone.

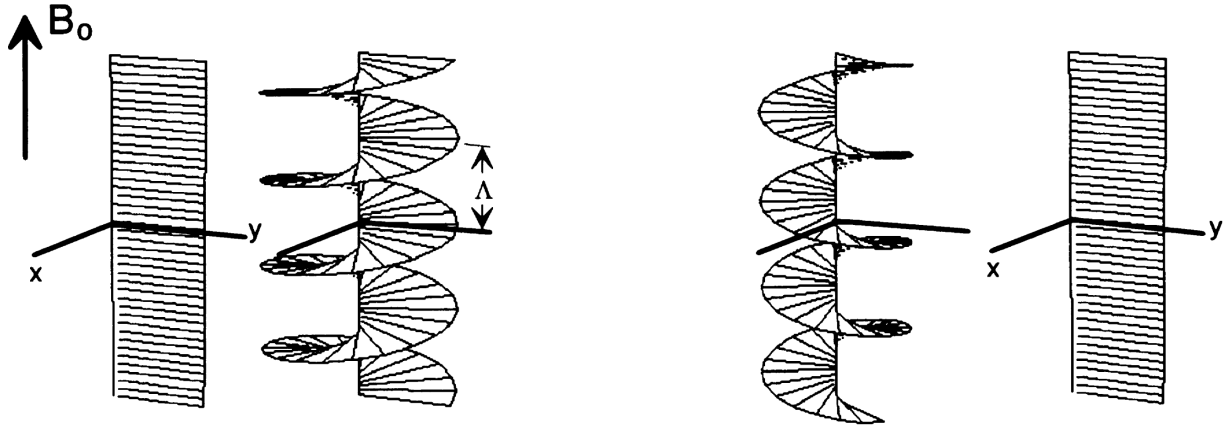
c) difference spectrum from a) minus b).

d) reference spectrum of the ligand.

e) same difference spectrum as in c) obtained with a mixture of the 8 non-binding ligands

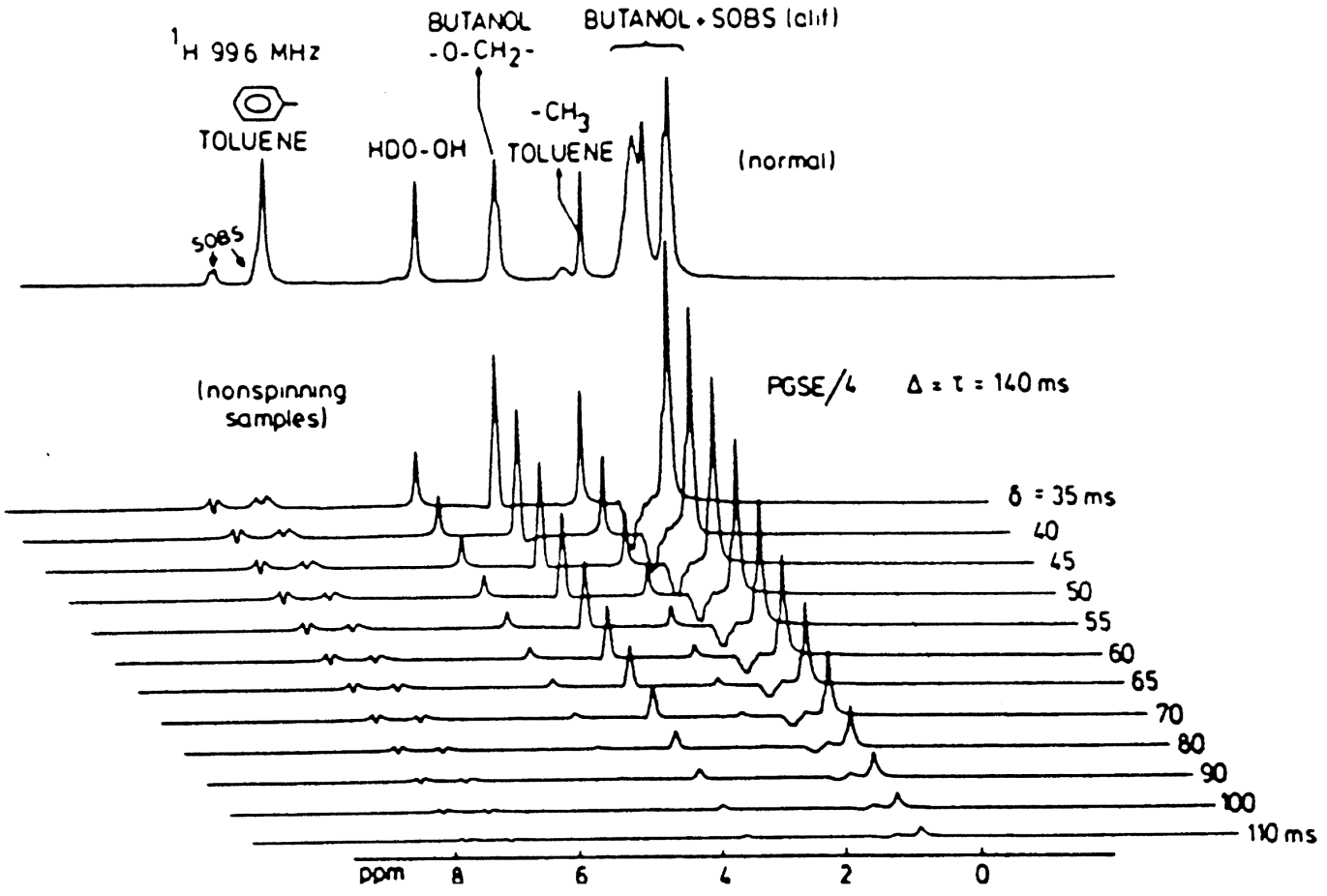
Diffusion filtering





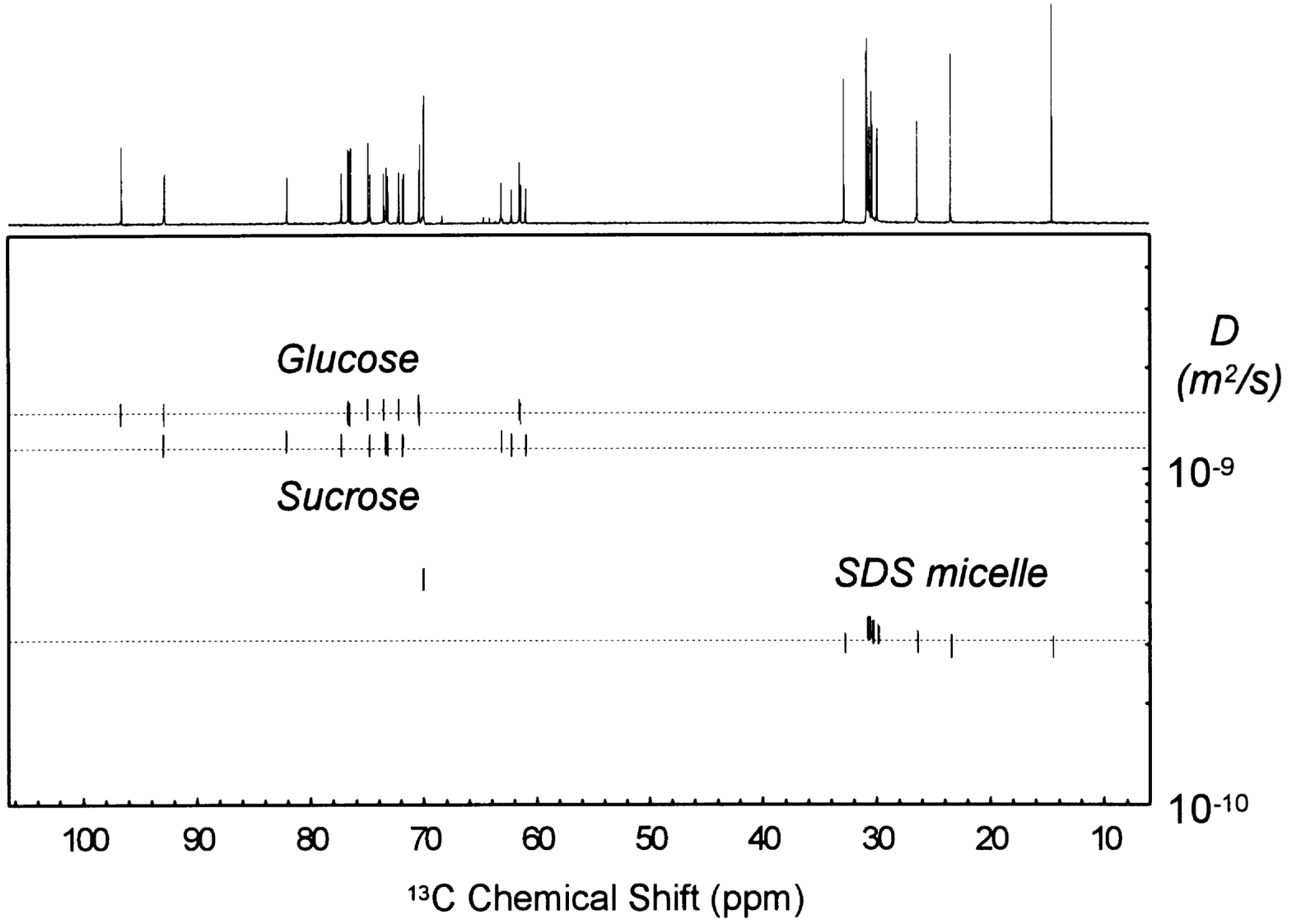
Johnson, 1999

Diffusion filtering



Johnson, 1999

Diffusion filtering - DOSY



Johnson, 1999

Saturation transfer difference

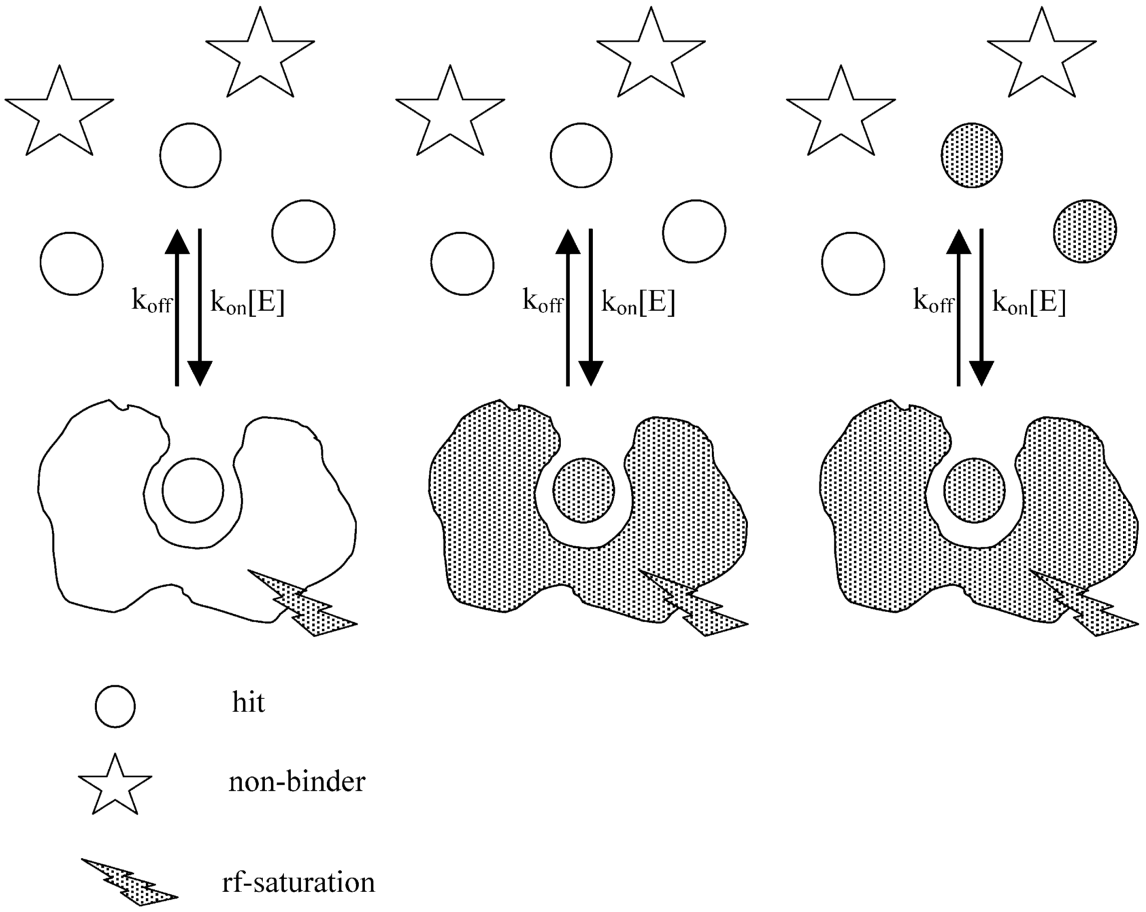
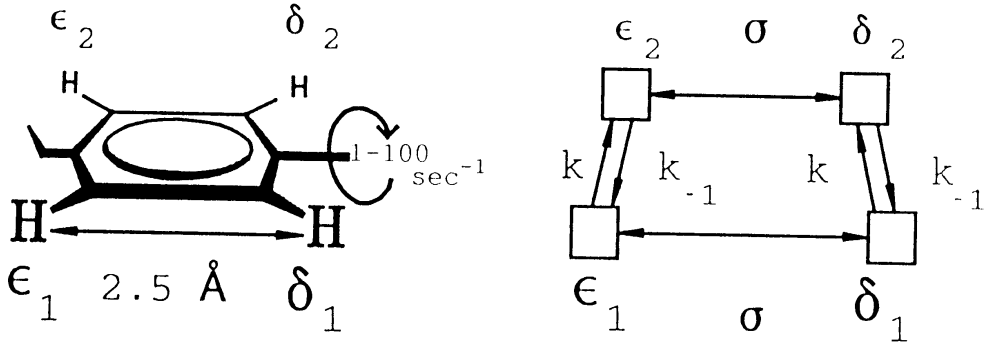


Fig. 5. Detection of binders using the Saturation Transfer Difference (STD) experiment [55]. Frequency selective irradiation (lightning bolt) cause selective ^{1}H saturation (shading) of the target receptor (e.g. protein, nucleic acid). The irradiation is applied for a sustained interval during which saturation spreads throughout the entire receptor via $^{1}H-^{1}H$ cross-relaxation (spin-diffusion). Saturation is transferred to binding compounds (circles) during their residence in the receptor binding site. The number of ligands having experienced saturation transfer increases as more ligand exchanges on and off the receptor during the sustained saturation period. Non-binding compounds are unaffected (stars).

Peng et al., 2004

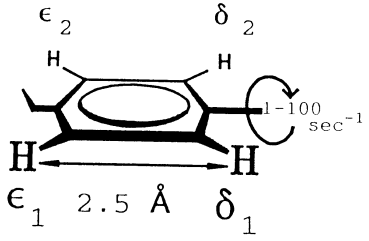
Exchange driven spin diffusion

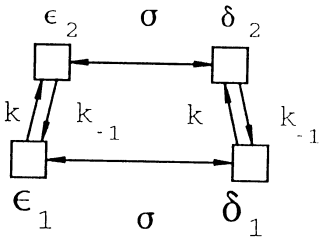


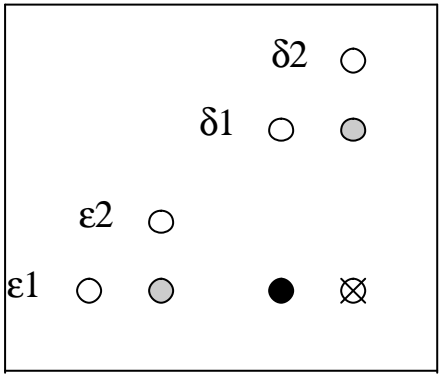
The rotation of Tyr or Phe aromatic ring of can be as slow as 100 s^{-1} and enable the observation of different resonance frequencies for the ε and δ hydrogens.

For a typical protein with τ_c = 6 ns, $\sigma_{max} \approx 10\ sec^{-1}$ for geminal protons (r = 0.17 nm). ⟹ $k >> \sigma_{max}$

Exchange driven spin diffusion







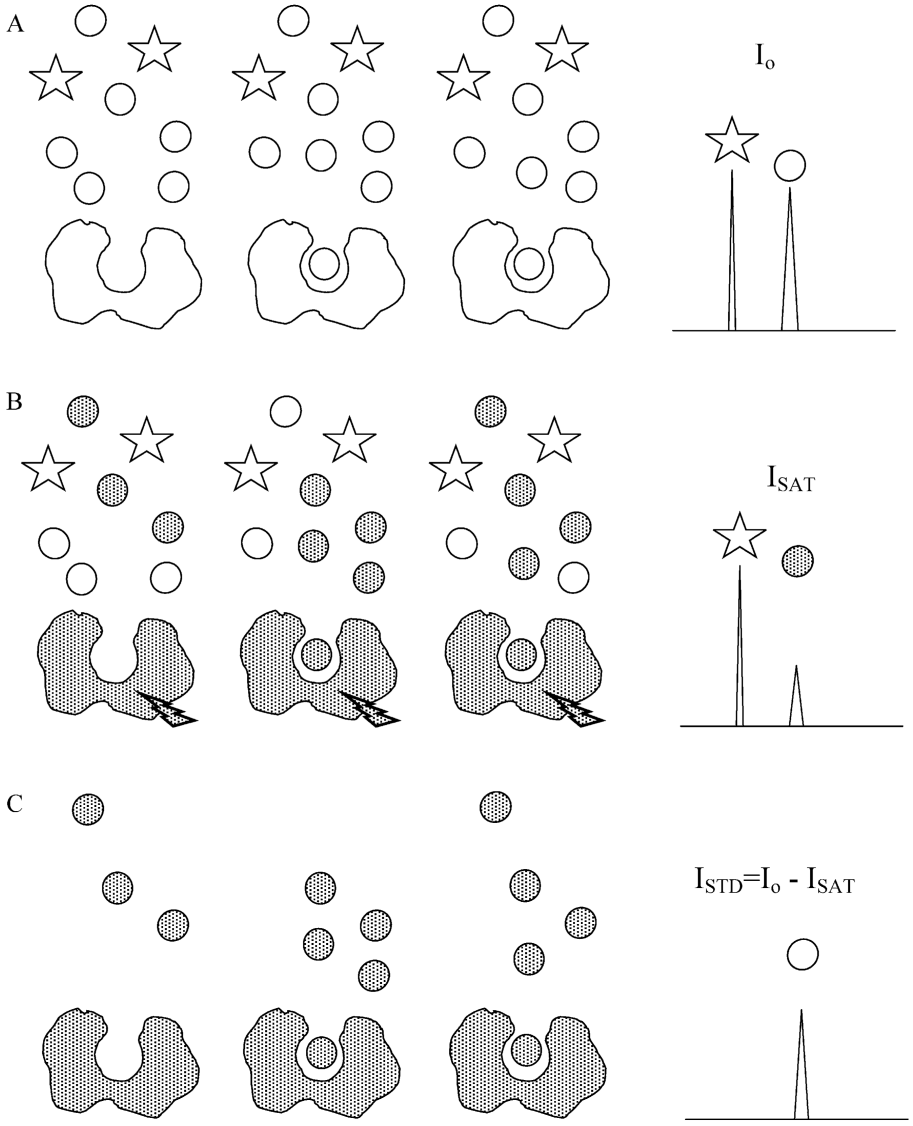
$$\mathbf{L}_{ed} = \begin{pmatrix} r_{e1} & k_1 & s_{1ed} & 0 \\ k_{-1} & r_{e2} & 0 & s_{2ed} \\ s_{1de} & 0 & r_{d1} & k_1 \\ 0 & s_{2de} & k_{-1} & r_{d2} \end{pmatrix}$$

$\varepsilon 1 - \delta 2 = \varepsilon 2 - \delta 1 \approx 0.5$ nm —— $\sigma \approx 0$

Non-zero antidiagonal values build up by spin diffusion.

$L_{14} = 2k_1 + \sigma_2 + \sigma_1$, $L_{23} = 2k_{-1} + \sigma_1 + \sigma_2$, ecc.

Saturation transfer difference



Peng et al., 2004

Very advantageous with large receptor molecules.

$$I_{STD} \propto a_{STD}[EL]$$

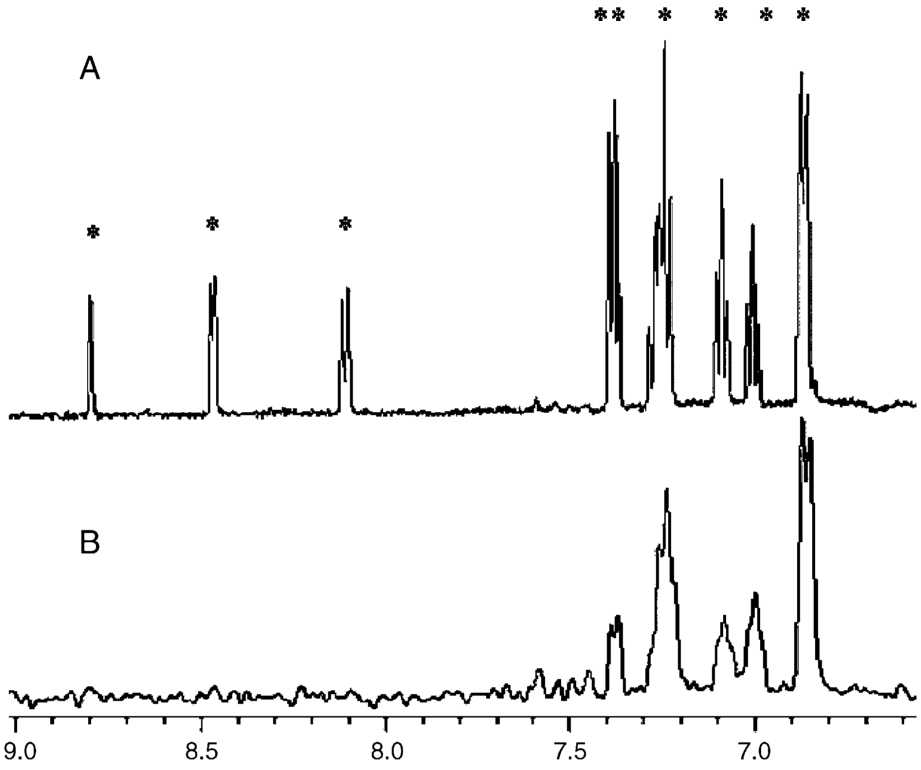
(a_{STD} = amplification factor)

$$I_0 \propto L_T$$

$$\frac{I_{STD}}{I_0} = a_{STD}\frac{[EL]}{[L_T]} = a_{STD}P_B = a_{STD}\frac{P_B^E}{e}$$

$$e\left[\frac{I_{STD}}{I_0}\right] = a_{STD}\frac{[L]}{[L]+K_D}$$

Saturation transfer difference



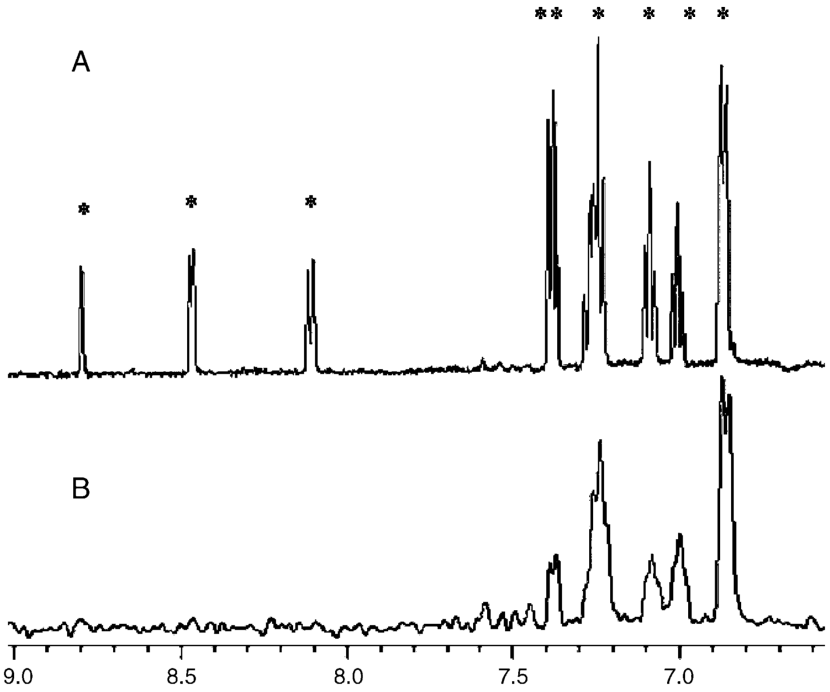
A) Mixture of ligands in the absence of receptor.

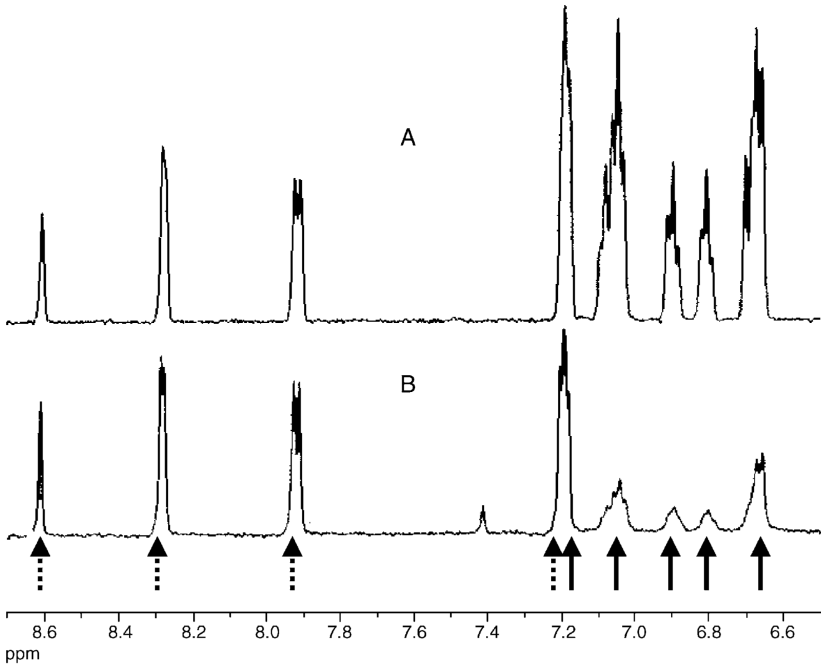
B) STD spectrum of the ligand mixture in the presence of p38 MAP kinase (42 kDa).

Peng et al., 2004

Only the signals of the binding ligand are seen because the receptor is filetered through relaxation filtering.

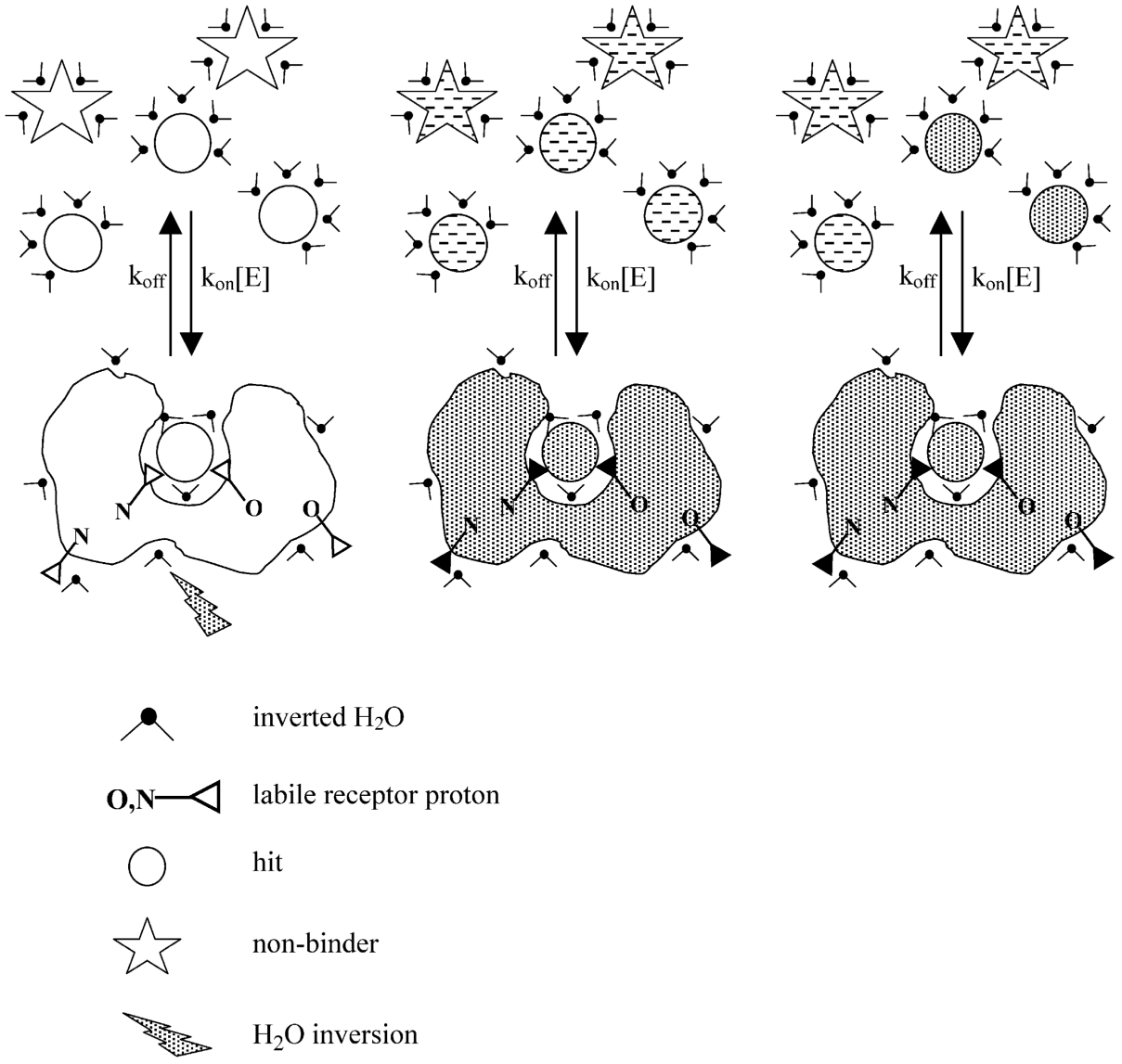
Saturation transfer versus broadening





Peng et al., 2004

Cross-relaxation highlights binding effects more effectively than R_2 increase.



Peng et al., 2004