

# Chemical Exchange and Calcium Signaling



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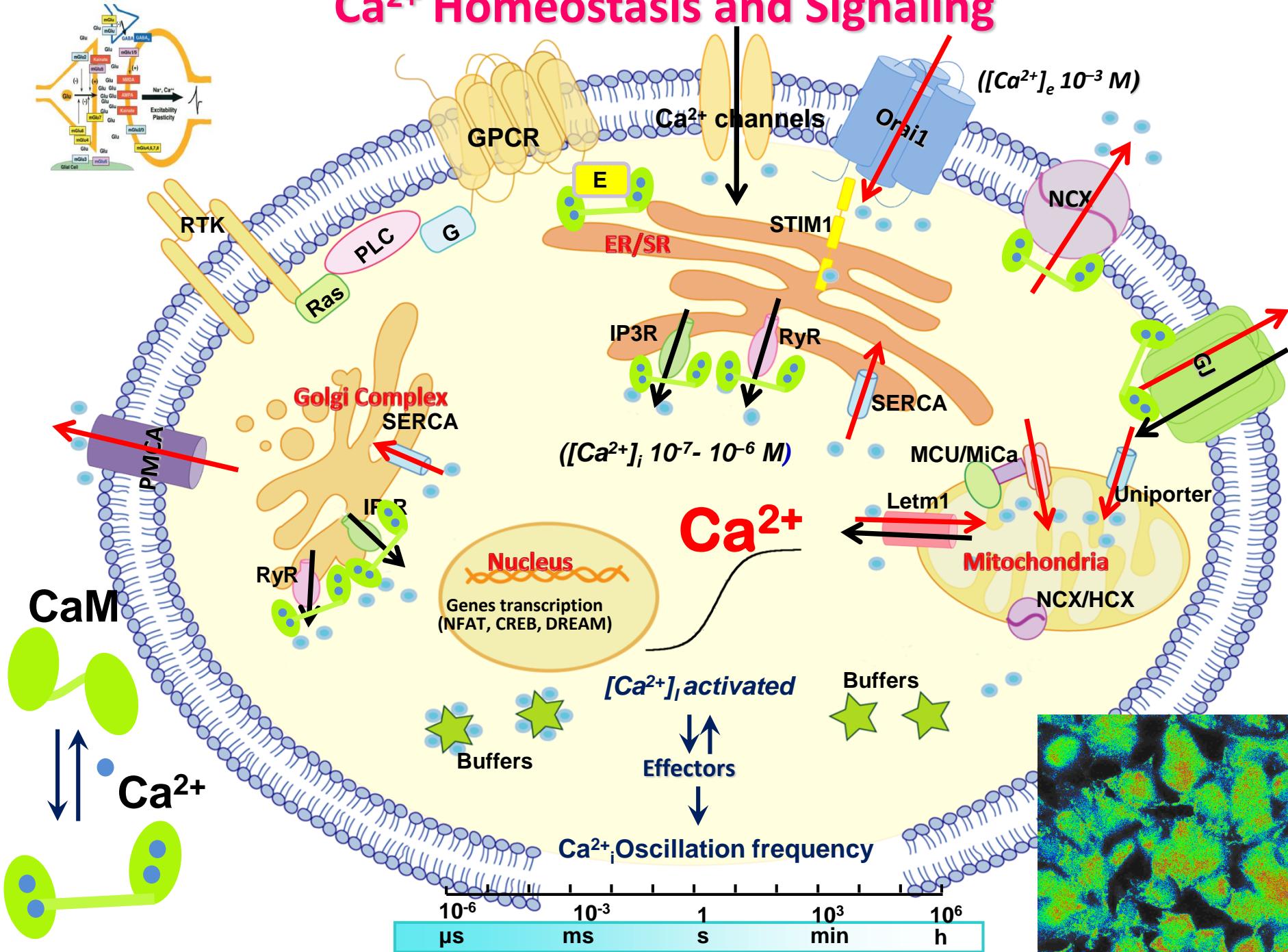
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<http://www.gsuyanglab.com/research>

# Ligand Protein interactions by NMR

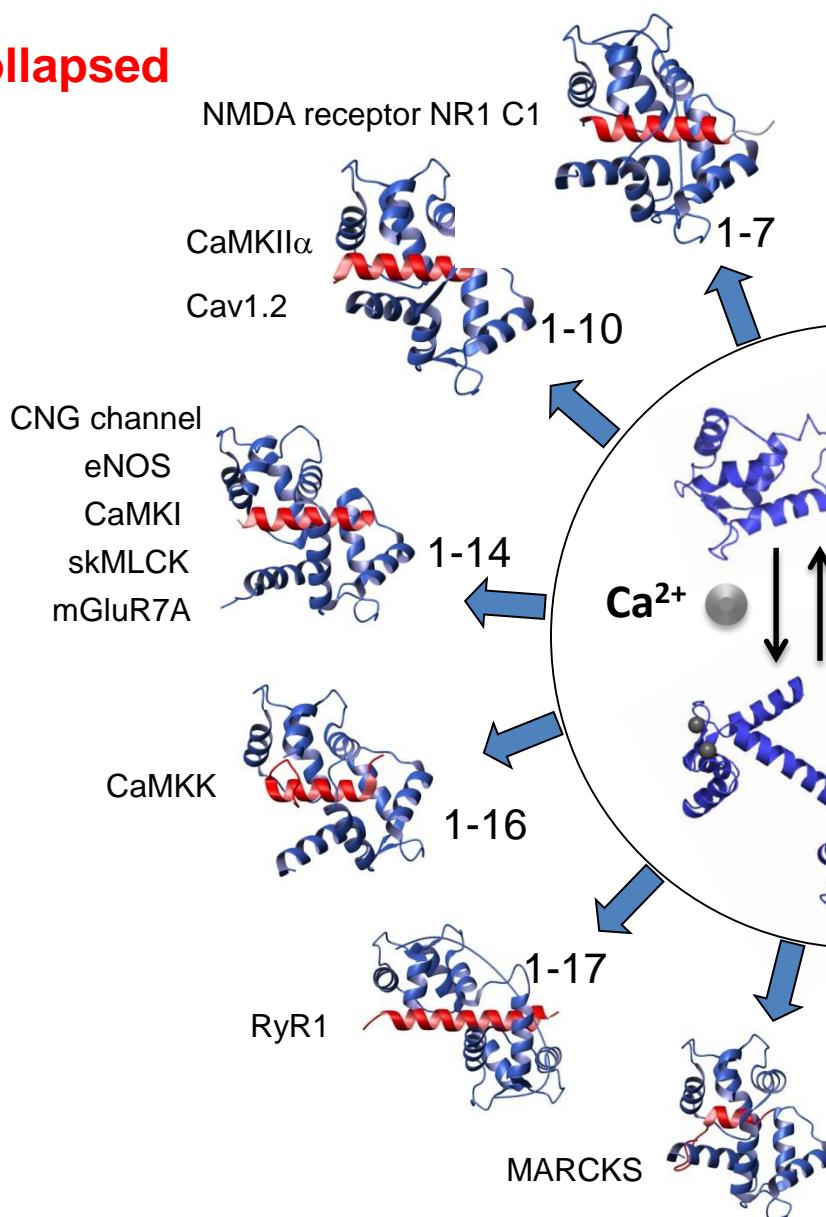
- Molecular Recognition: Calcium signaling
  - CaM regulation of gap junctions
  - Calcium kinetics
- Chemical exchange
  - NMR time scale
  - Fast exchange for binding constants
  - Slow exchange for tight binding
  - Single vs. multiple binding mode
- Applications for monitoring calcium signaling
  - CaM regulation of gap junctions

# $\text{Ca}^{2+}$ Homeostasis and Signaling

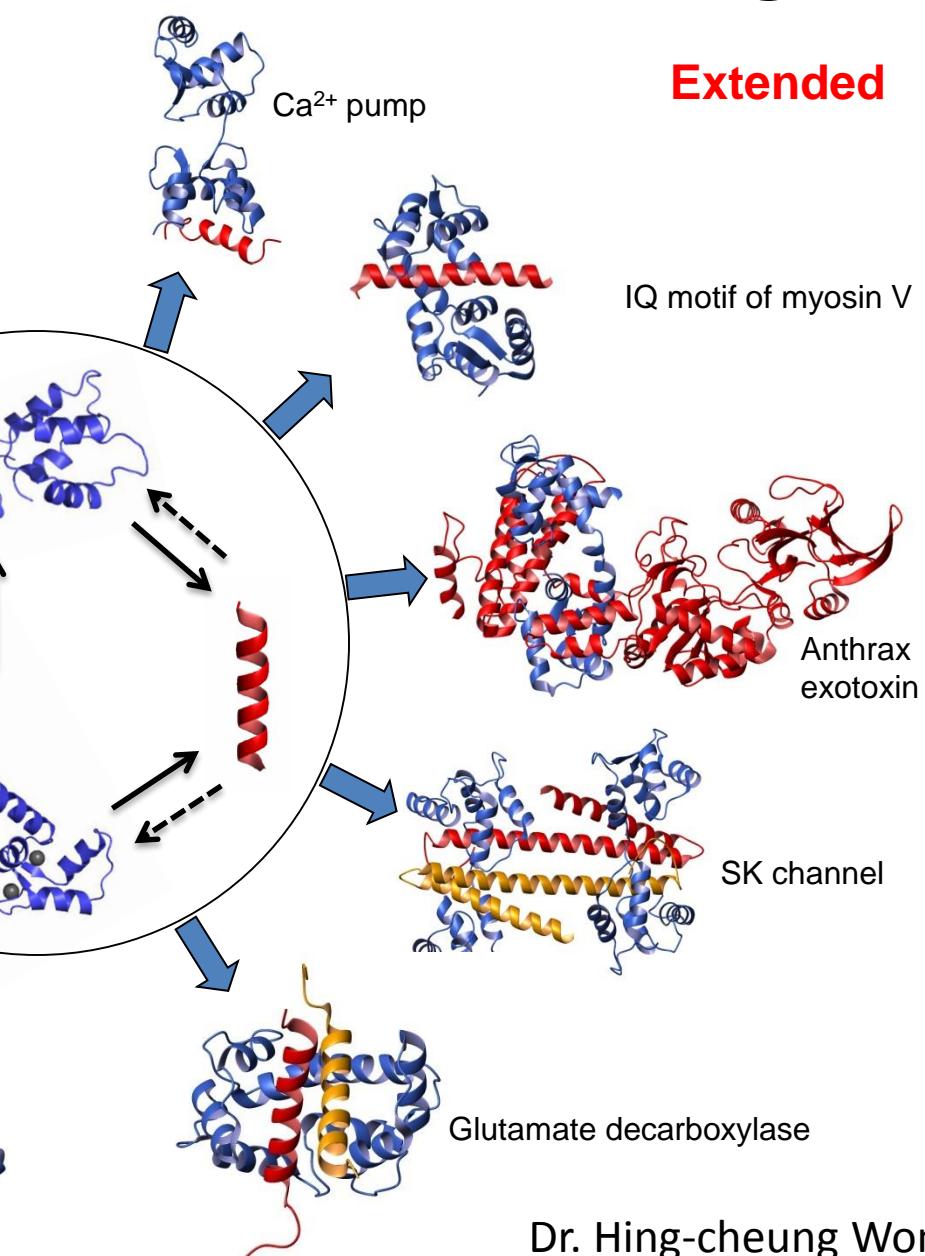


# Binding Modes between CaM and Its Targets

**Collapsed**



**Extended**



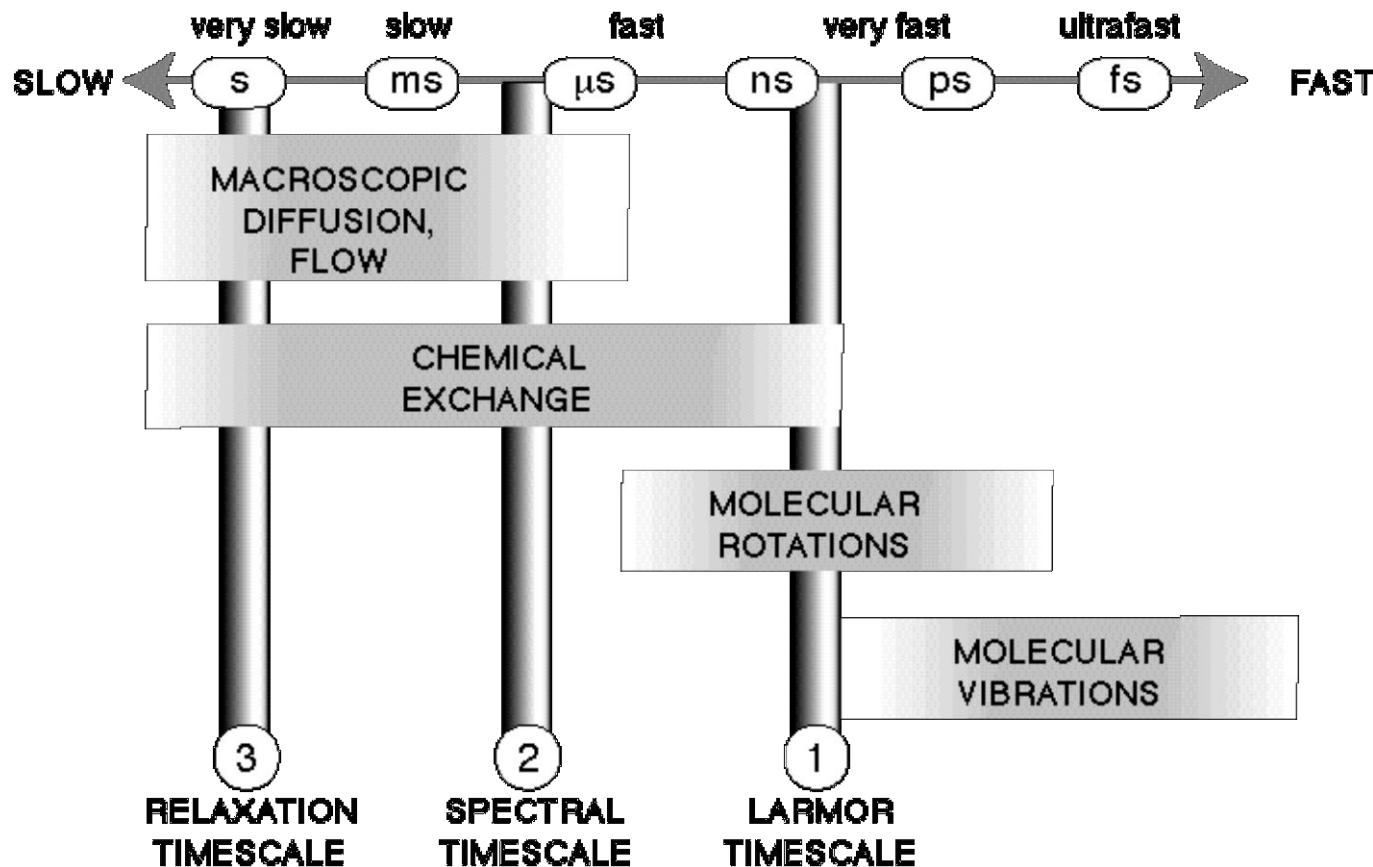
# Questions

How do we know the protein is conformational ensemble?

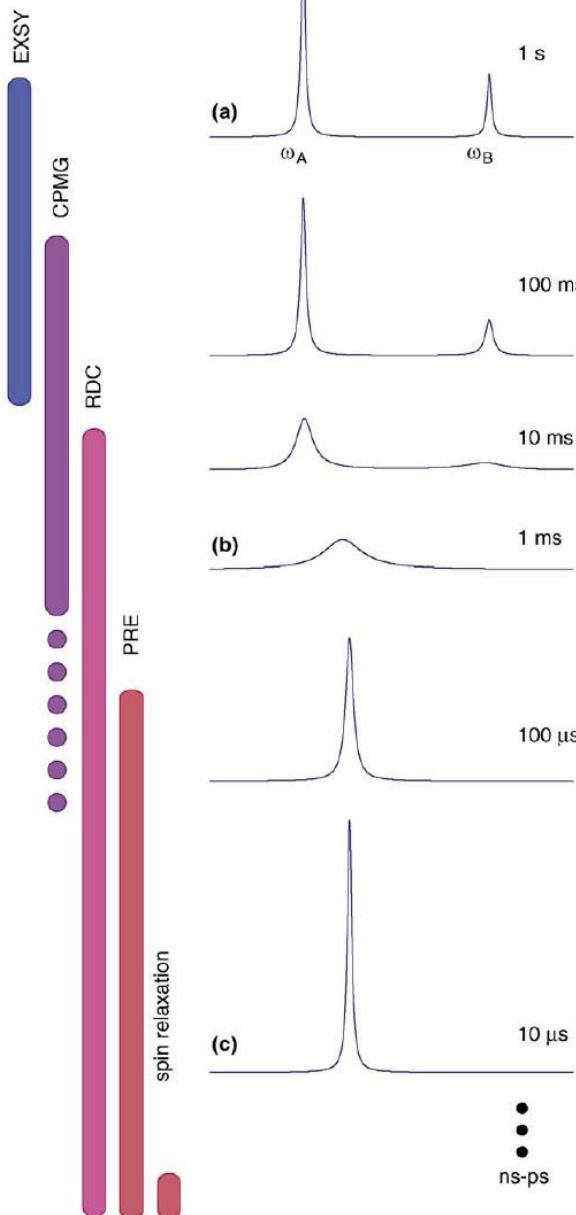
Calcium binding process? Affinity? Multiple binding sites?

Calcium induced conformational changes? Other metal ions such as  $\text{Pb}^{2+}$  have the same effect on affinity and conformational change?  
protein/peptide interaction?

# Typical Motion Time Scale for Physical Processes



# NMR Dynamic Experiments



Initial NMR dynamics experiments in 1970s.

Rapid advancements due to ability to label specific positions in bio-molecules and methodologies development

Magnetization exchange spectroscopy (EXSY)-slow exchange  $0.5 \text{ s}^{-1}$  to over  $50 \text{ s}^{-1}$

CPMG relaxation dispersion: chemical shifts  $100\text{--}2000 \text{s}^{-1}$ ,

$R_1$ rho can extend to more rapid exchange (dot)

Residual dipolar coupling (RDC) and PRE

Spin relaxation for ns-ps

CPMG and PRE are sensitive to low-lying excited states with populations  $> 0.5\%$

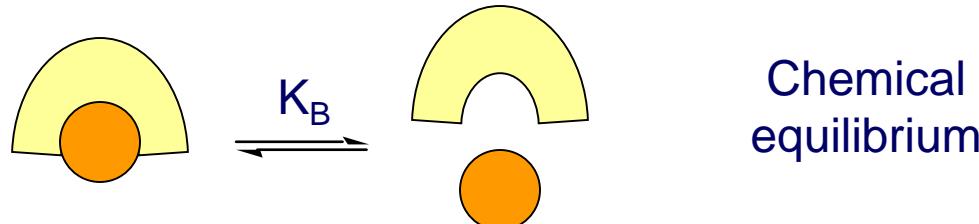
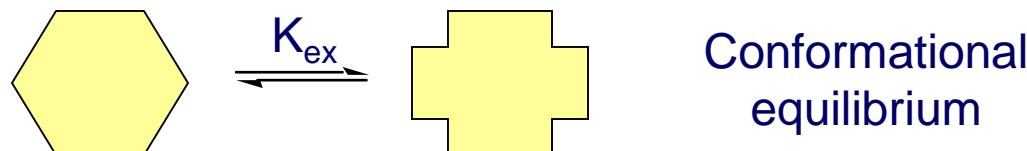
H/D exchange can detect high energy excited states with much lower population

- A. Mittermaier, L. Kay (2009) *Trends Biochem. Sci.* **34**, 601.  
A. Mittermaier, L. Kay (2006) *Science*. **312**, 224  
K. Wuthrich, G Wagner,(1978) *Trends Biochem. Sci.* **3**, 227

# Effects of Chemical Exchange on NMR Spectra

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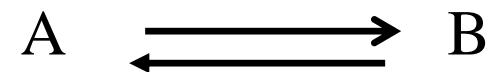
- Chemical exchange refers to any process in which a nucleus exchanges between two or more environments in which its NMR parameters (e.g. chemical shift, scalar coupling, or relaxation) differ.
- DNMR deals with the effects in a broad sense of chemical exchange processes on NMR spectra; and conversely with the information about the changes in the environment of magnetic nuclei that can be derived from observation of NMR spectra.



# Types of Chemical Exchange

## Intramolecular exchange

- Motions of sidechains in proteins
- Helix-coil transitions of nucleic acids
- Unfolding of proteins
- Conformational equilibria



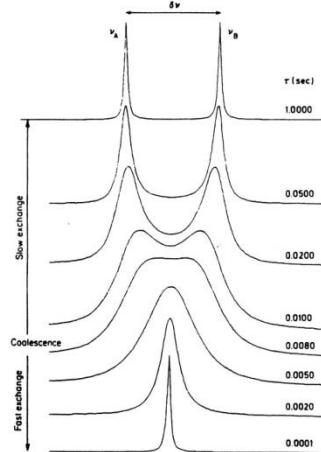
## Intermolecular exchange

- Binding of small molecules to macromolecules
- Protonation/deprotonation equilibria
- Isotope exchange processes
- Enzyme catalyzed reactions



Because NMR detects the molecular motion itself, rather than the numbers of molecules in different states, NMR is able to detect chemical exchange even when the system is in equilibrium

# NMR Time Scale

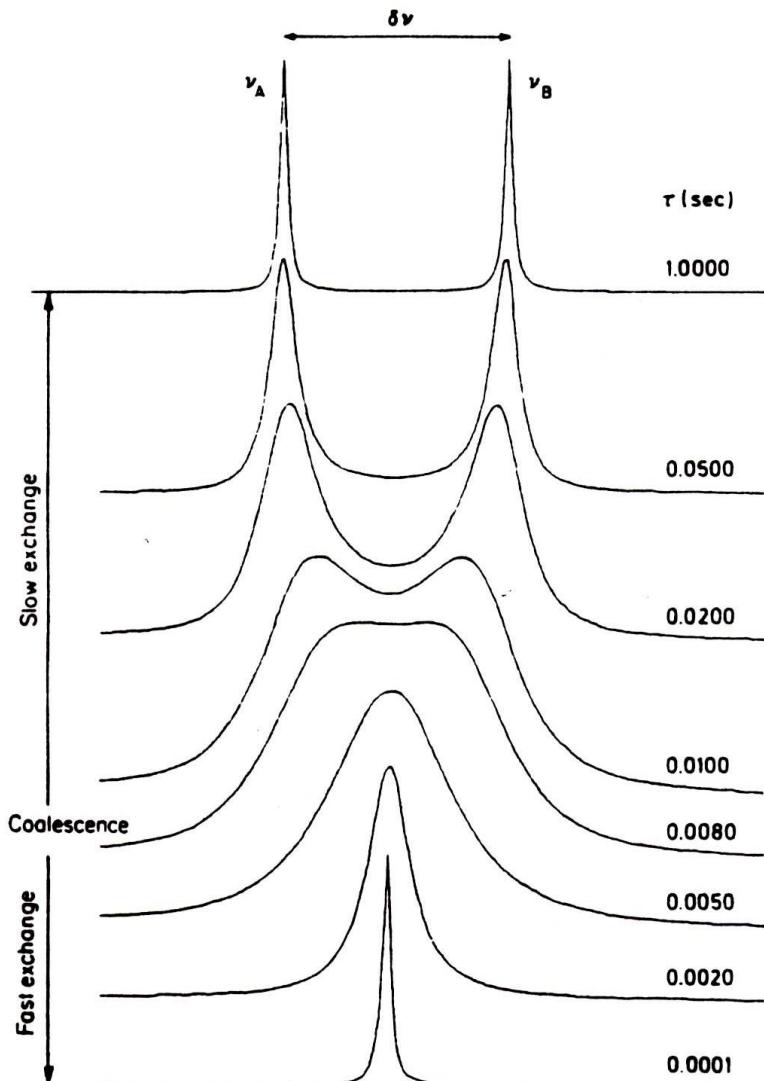
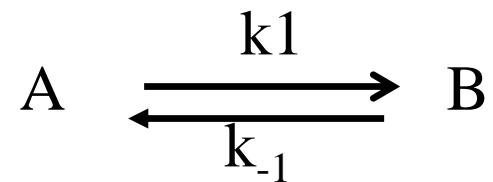


Time Scale   Chem. Shift,  $\delta$    Coupling Const.,  $J$    T2 relaxation

Slow	$k \ll \delta_A - \delta_B$	$k \ll J_A - J_B$	$k \ll 1/T_{2,A} - 1/T_{2,B}$
Intermediate	$k = \delta_A - \delta_B$	$k = J_A - J_B$	$k = 1/T_{2,A} - 1/T_{2,B}$
Fast	$k \gg \delta_A - \delta_B$	$k \gg J_A - J_B$	$k \gg 1/T_{2,A} - 1/T_{2,B}$
Sec <sup>-1</sup>	0 – 1000	0 – 12	1 - 20

- NMR time-scale refers to the chemical shift timescale.
- The range of the rate can be studied  $0.05\text{-}5000\text{ s}^{-1}$  for H can be extended to faster rate using  $^{19}\text{F}$ ,  $^{13}\text{C}$  and etc.

## 2-state First Order Exchange



Lifetime of state A:

$$\tau_A = 1/k_{+1}$$

Lifetime of state B:

$$\tau_B = 1/k_{-1}$$

Use a single lifetime

$$\begin{aligned} 1/\tau &= 1/\tau_A + 1/\tau_B \\ &= k_{+1} + k_{-1} \end{aligned}$$

# Rationale for Chemical Exchange

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For slow exchange



For fast exchange



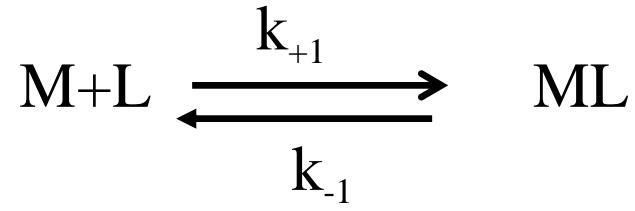
Bloch equation approach:

$$dM_{AX}/dt = -(\Delta\omega_A)M_{AY} - M_{AX}/\tau_A + M_{BX}/\tau_B$$

$$dM_{BX}/dt = -(\Delta\omega_B)M_{BY} - M_{BX}/\tau_B + M_{AX}/\tau_A$$

•  
•  
•

## 2-state 2nd Order Exchange



$$K_d = [M][L]/[ML] = k_{-1}/k_{+1}$$

$$K_d = 10^{-3} - 10^{-9} M$$

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

$$k_{-1} \sim 10^{-1} - 10^{-5} s^{-1}$$

$$\begin{aligned}\text{Lifetime } 1/\tau &= 1/\tau_{ML} + 1/\tau_1 \\ &= k_{-1} (1+f_{ML}/f_L)\end{aligned}$$

$f_{ML}$  and  $f_L$  are the mole fractions of bound and free ligand, respectively

# Slow Exchange $k \ll \delta_A - \delta_B$

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- Separate lines are observed for each state.
- The exchange rate can be readily measured from the line widths of the resonances
- Like the apparent spin-spin relaxation rates,

$$1/T_{2i,obs}$$

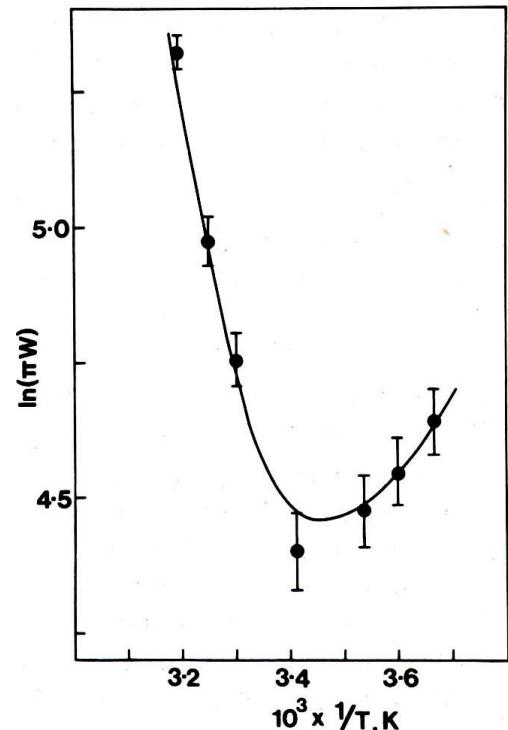
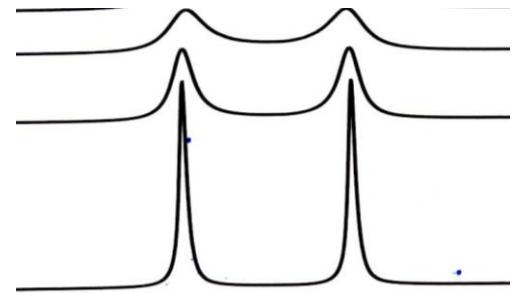
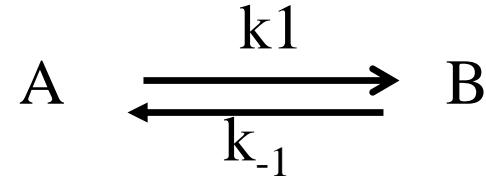
$$1/T_{2A,obs} = 1/T_{2A} + 1/\tau_A = 1/T_{2A} + 1/k_1$$

$$1/T_{2B,obs} = 1/T_{2B} + 1/\tau_B = 1/T_{2B} + 1/k_{-1}$$

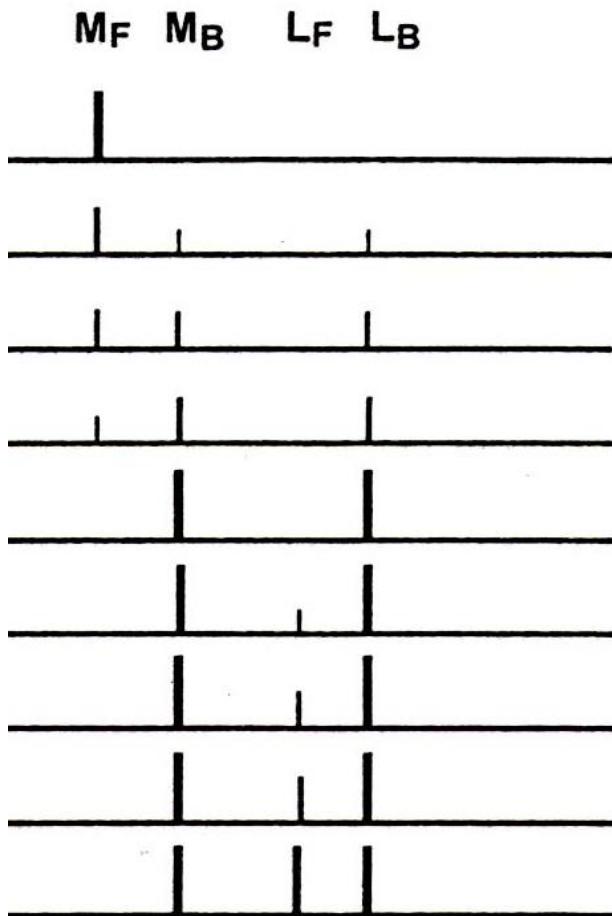
$$\text{line width } Lw = 1/\pi T_2 = 1/\pi T_2 + k_1/\pi$$

Each resonance is broadened by  $\Delta Lw = k/\pi$

Increasing temperature increases  $k$ , line width increases



# Slow Exchange for M+L $\rightleftharpoons$ ML

 $k_1$  $k_{-1}$ 

- Separate resonances potentially are observable for both the free and bound states  $M_F$ ,  $M_B$ ,  $L_F$ , and  $L_B$
- The addition of a ligand to a solution of a protein can be used to determine the stoichiometry of the complex.
- Once a stoichiometric mole ratio is achieved, peaks from free ligand appear with increasing intensity as the excess of free ligand increases.
- Obtain spectra over a range of  $[L]/[M]$  ratios from 1 to 10

# Slow Exchange for M+L

$$\text{M} + \text{L} \rightleftharpoons \text{ML}$$

- ~~For free form~~

$$1/T_{2L,obs} = 1/T_{2L} + 1/\tau_L = 1/T_{2L} + k_{-1} f_{ML}/f_L$$

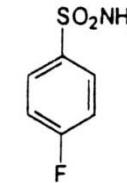
$$1/T_{2M,obs} = 1/T_{2M} + 1/\tau_M = 1/T_{2M} + k_{-1} f_{ML}/f_M$$

For complex form

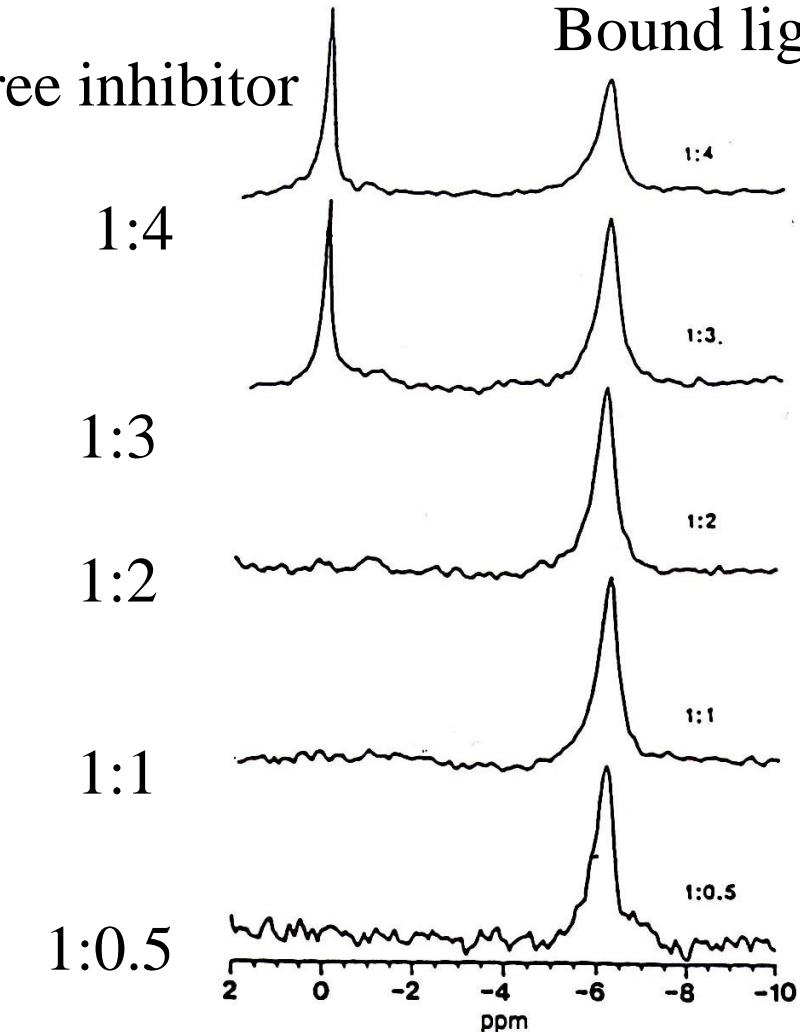
$$1/T_{2ML,obs} = 1/T_{2ML} + 1/\tau_{ML} = 1/T_{2ML} + k_{-1}$$

Measurements of line width during a titration can be used to derive  $k_{-1}$  ( $k_{off}$ ).

# $^{19}\text{F}$ spectra of the enzyme-inhibitor complex at various mole ratio of carbonic anhydrase:inhibitor



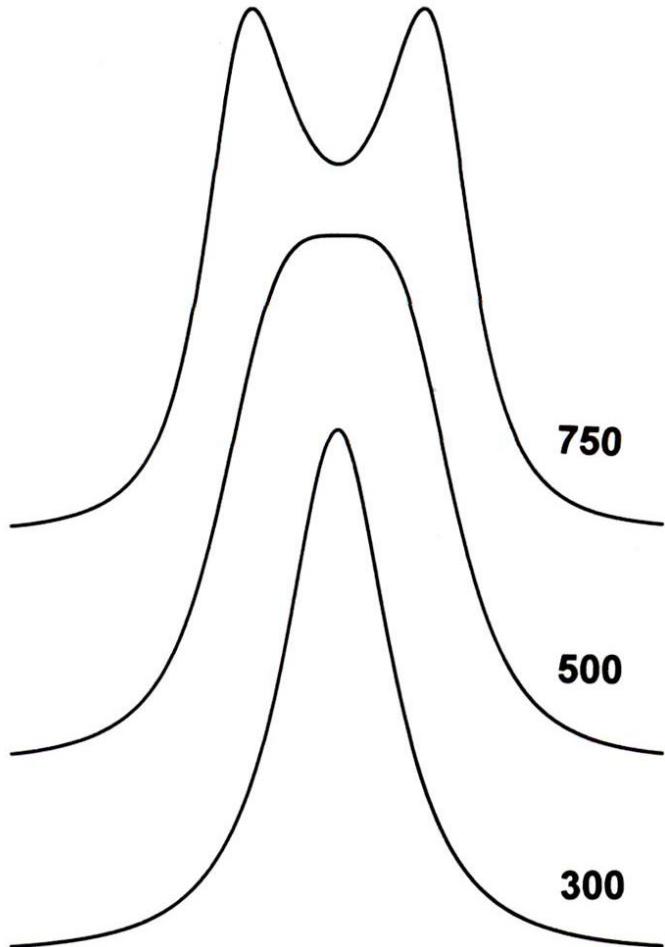
4-fluorobenzenesulfonamide.



- At -6 ppm the broadened peak for the bound ligand is in slow exchange with the peak from free ligand at 0 ppm.
- The stoichiometry of the complex is 2:1. No signal from the free ligand is visible until more than 2 moles of inhibitor are present.

# Coalescence Rate

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- For  $A \leftrightarrow B$  equal concentrations, there will be a rate of interchange where the separate lines for two species are no longer discernible
- The coalescence rate

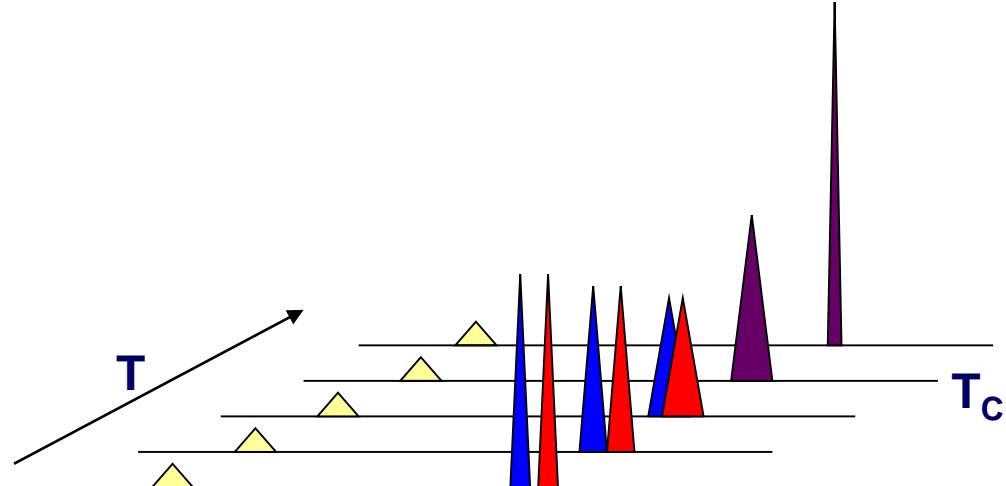
$$k_c = \pi \Delta\delta / \sqrt{2} = 2.22 \Delta\delta$$

$\Delta\delta$  is the chemical shift difference between the two signals in the unit of Hz.

$\Delta\delta$  depends on the magnetic field

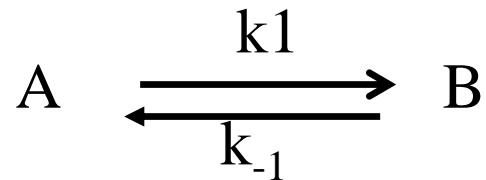
# Coalescence Temperature

- Since the rate depends on the  $\Delta G$  of the inversion, and the  $\Delta G$  is affected by T, higher temperature will make things go faster.
- $T_c$  is the temperature at which fast and slow exchange meet.
- $T > T_c$ , fast exchange
- $T < T_c$ , slow exchange



we can calculate the  $\Delta G^\ddagger$  of the process

$$\Delta G^\ddagger = R * T_c * [ 22.96 + \ln( T_c / \Delta \delta ) ]$$



## Fast Exchange $k \gg \delta_A - \delta_B$

A single resonance is observed, whose chemical shift is the weight average of the chemical shifts of the two individual states

$$\delta_{\text{obs}} = f_A \delta_A + f_B \delta_B, \quad f_A + f_B = 1$$

**For very fast limit**

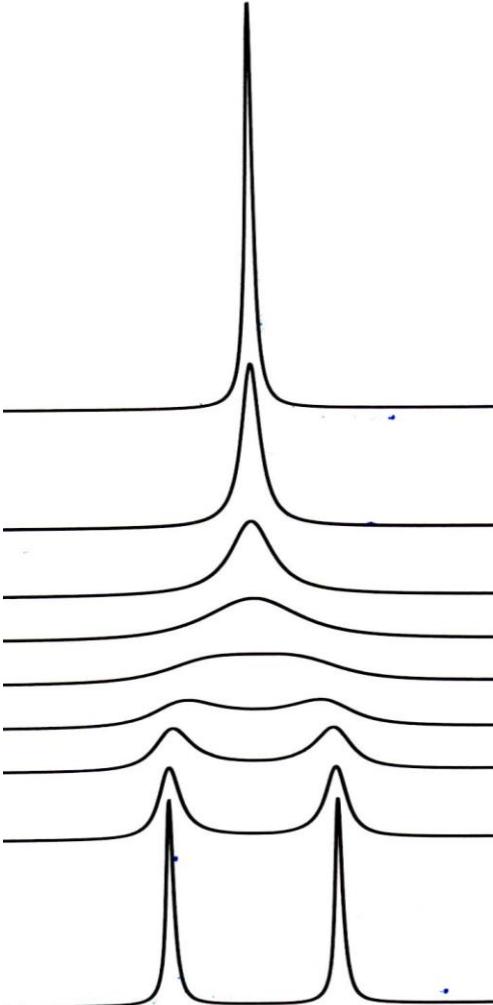
$$1/T_{2,\text{obs}} = f_A/T_{2A} + f_B/T_{2B}$$

**For moderately fast**

$$1/T_{2,\text{obs}} = f_A/T_{2A} + f_B/T_{2B} + f_A f_B^2 4\pi (\Delta\delta_{AB})^2 / k_{-1}$$

Maximal line broadening is observed when

$$f_A = f_B = 0.5$$



# Line Shape Simulation



## Exchange: Chemical Exchange Lineshapes

This application calculates the NMR line shapes for chemical exchange for two equivalent sites. You can also input your experimental data, so that you can graphically compare with the simulated lineshape, but the experimental data is not needed. An [example](#) may help.

### Simulation constants:

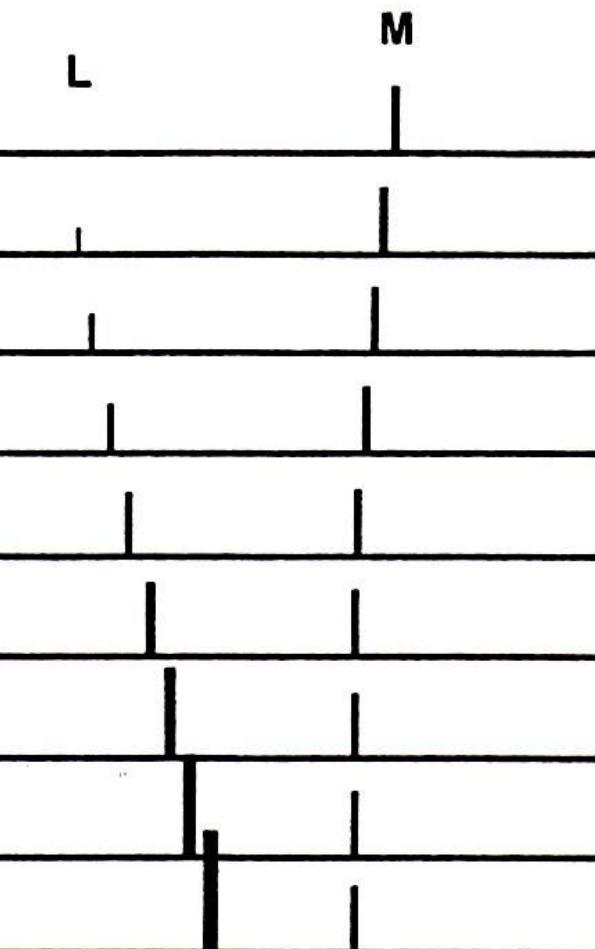
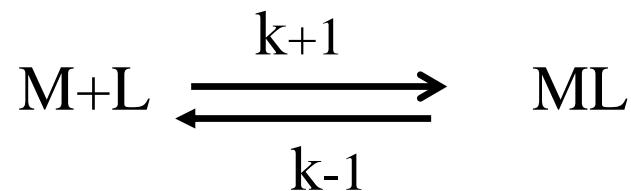
Line separation with no exchange:  Hz

$T_2$ , relaxation time with no exchange:  sec.

Enter one, but not both of the following parameters for the exchange:

Tau, exchange lifetime:  sec. or k, exchange rate:  sec<sup>-1</sup>

# Fast Exchange $k \gg \delta_A - \delta_B$



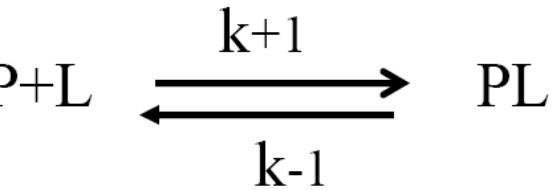
$$\text{For } M \quad \delta_{M,\text{obs}} = f_M \delta_M + f_{ML} \delta_{ML}$$

$$\text{For } L \quad \delta_{L,\text{obs}} = f_L \delta_L + f_{ML} \delta_{ML}$$

$$1/T_{2,\text{obs}} = f_{ML}/T_{2,ML} + f_L/T_{2,L} + f_{ML}f_L^2 4\pi (\delta_{ML} - \delta_L)^2 / k_{-1}$$

- A maximum in the line broadening of ligand or protein resonances occurs during the titration at a mole ratio of approx. ligand:protein 1:3
- The dissociation constant for the complex can be obtained by measuring the chemical shift of the ligand resonance at a series of [L].

# Measuring Binding Constant



$$\delta_{obs} = X_L \delta_L + X_{PL} \delta_{PL}$$

$$\delta_{obs} = X_L \delta_L + (1 - X_L) \delta_{PL}$$

$$\delta_L - \delta_{obs} = ([PL]/[L]_0)(\delta_L - \delta_{PL})$$

For the formation of a 1:1 complex,

$$[P] + [PL] = [P]_0$$

$$[L] + [PL] = [L]_0$$

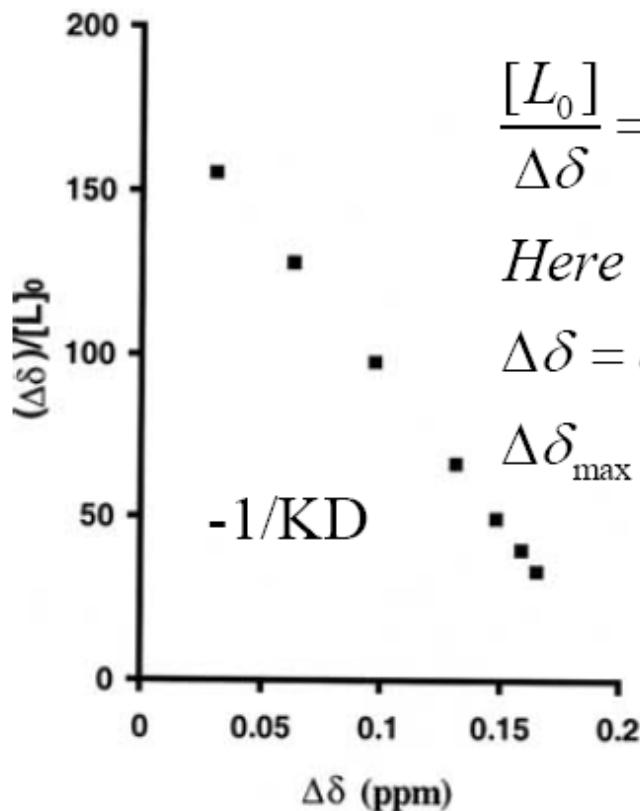
$$K_D = \frac{[PL]}{[P][L]} = \frac{[PL]}{([P]_0 - [PL])([L]_0 - [PL])} \approx \frac{[PL]}{([P]_0 - [PL])([L]_0)}$$

If the ligand concentration is in large excess,  $[L] \approx [L]_0$

$$\frac{(\delta_L - \delta_{obs})}{[L]_0} = -\frac{(\delta_L - \delta_{obs})}{K_D} + \frac{(\delta_L - \delta_{PL})}{K_D}$$

# Plot of NMR data

## Scatchard Plot



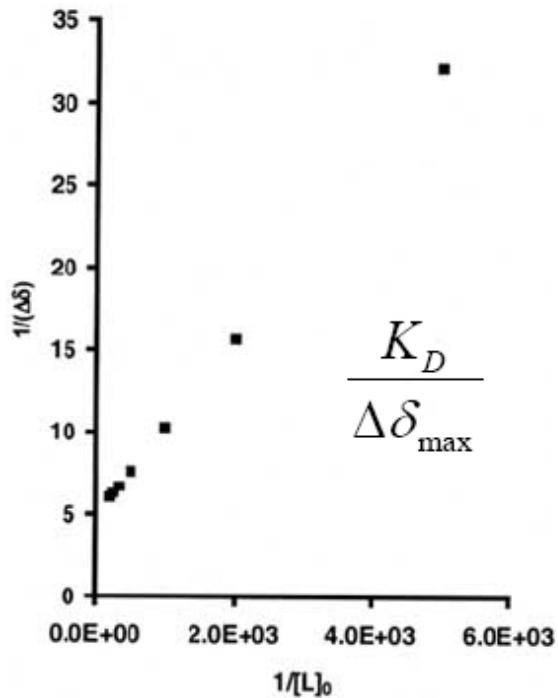
$$\frac{[L_0]}{\Delta\delta} = \frac{K_D}{\Delta\delta_{\max}} + \frac{[L_0]}{\Delta\delta_{\max}}$$

Here

$$\Delta\delta = \delta_L - \delta_{obs}$$

$$\Delta\delta_{\max} = \delta_L - \delta_{PL}$$

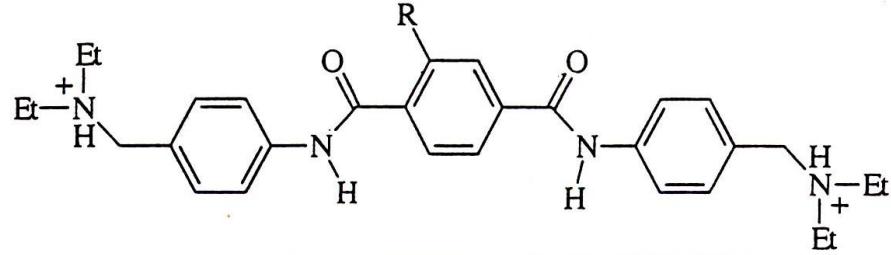
## Benesi-Hildebrand Plot



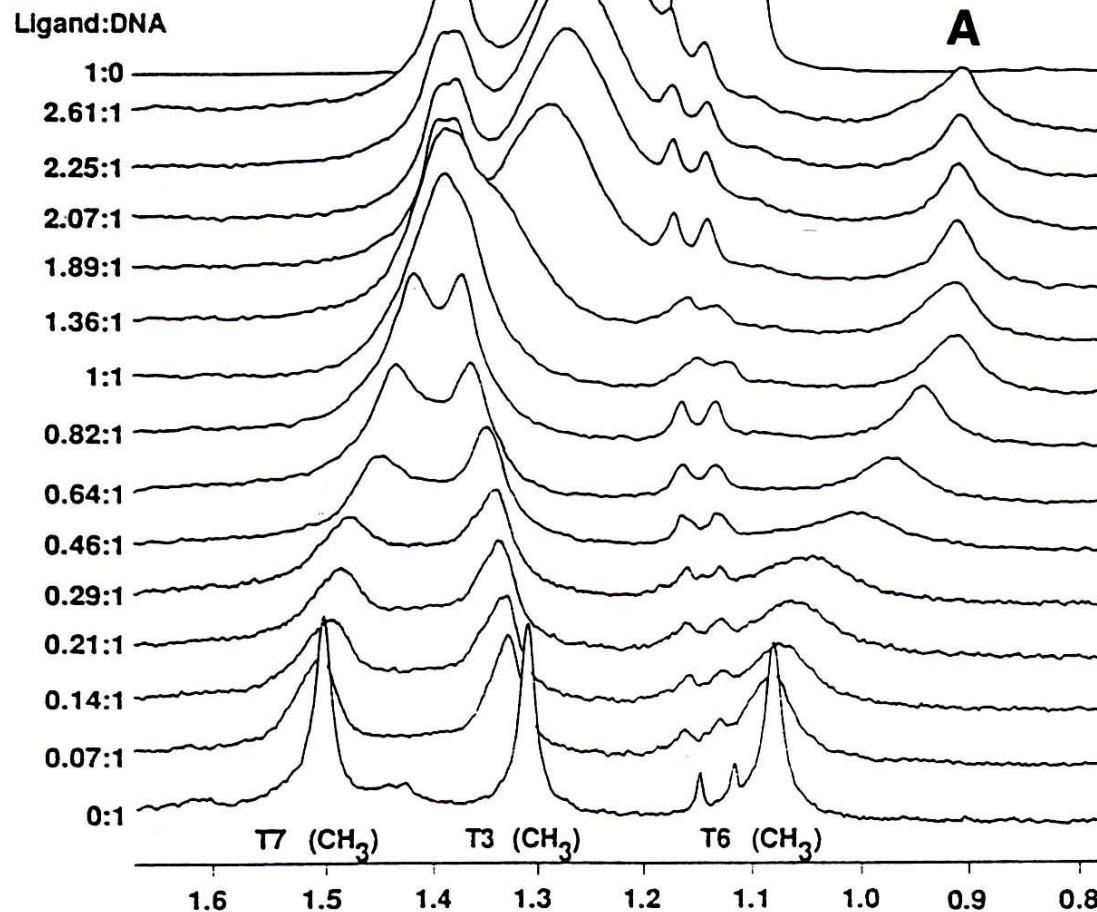
Plot  $\frac{(\delta_L - \delta_{obs})}{[L_0]}$  against  $(\delta_L - \delta_{obs})$

Plot of  $\frac{1}{\Delta\delta}$  against  $1/[L_0]$

# NMR Titration



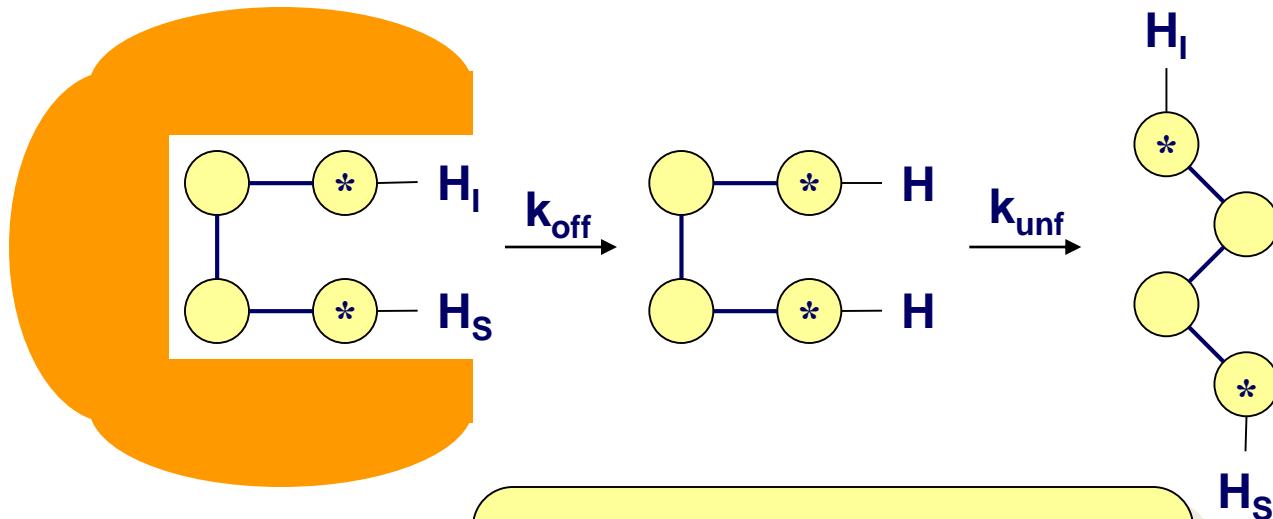
Compound	R
$\text{L}(\text{NO}_2)$	$\text{NO}_2$
$\text{L}(\text{NH}_2)$	$\text{NH}_2$
$\text{L}(\text{Gly})$	$\text{NHCOCH}_2\text{NH}_2$



Spectra of the terephthalamide derivatives I./NH.

- Both T6( $\text{CH}_3$ ) at 1.1 ppm from DNA (A) and L( $\text{CNH}_2$ ) methyl from ligand (B) are in fast exchange.
- Expanded regions from 300 MHz  $^1\text{H}$ NMR spectra from complexes between L( $\text{NH}_2$ ) and d(GGTAAATACC)<sub>2</sub> recorded at 10 °C (Craik)

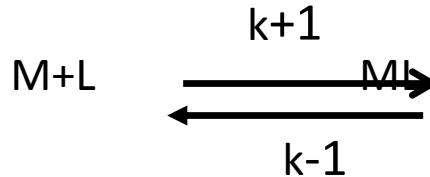
# Binding of Inhibitor to Enzyme



$$K_d = \frac{k_{off}}{k_{on}} = \frac{[protein][ligand]}{[protein-ligand]}$$

- NMR studies are done at mM, making it difficult to determine  $K_d$  with any accuracy if the binding is very tight (nM  $K_d$ ).

# Measuring Binding Constant



$$\delta_{M,obs} = f_M \delta_M + f_{ML} \delta_{ML},$$

The total change in  $\delta$  of M

$$\Delta\delta_{Mo} = \delta_{ML} - \delta_M$$

$$\text{At } [L], \Delta\delta_M = \delta_{M,obs} - \delta_M$$

If [M] is fixed,

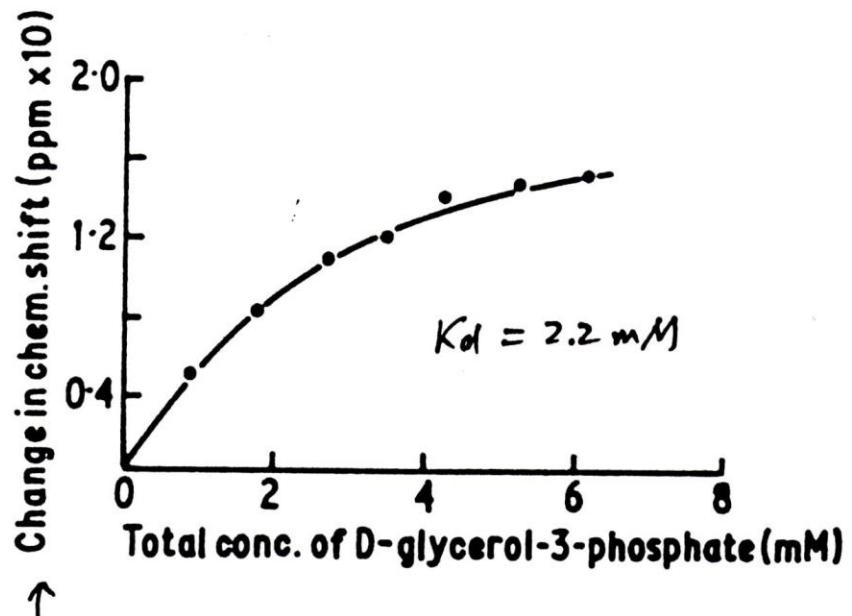
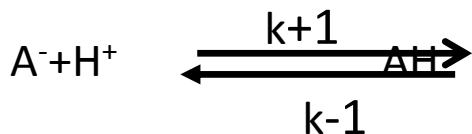
$$\Delta\delta_M = \Delta\delta_{Mo}[L]/([L]+K_d)$$

$$\text{At } 0.5 \Delta\delta_{Mo}, K_d = [L]$$

Similar for

$$H=L, K_d = K_a$$

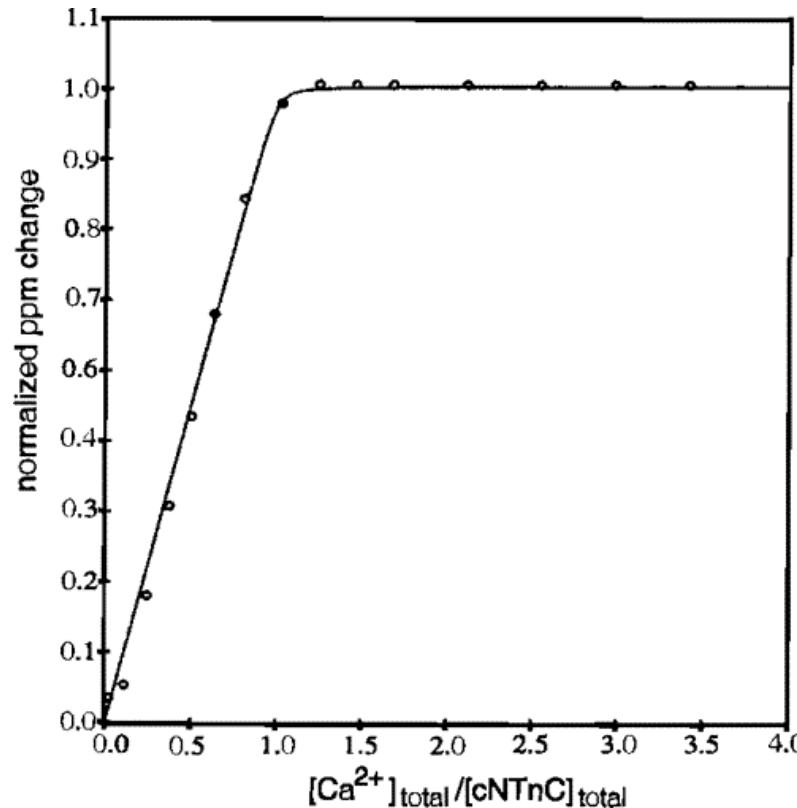
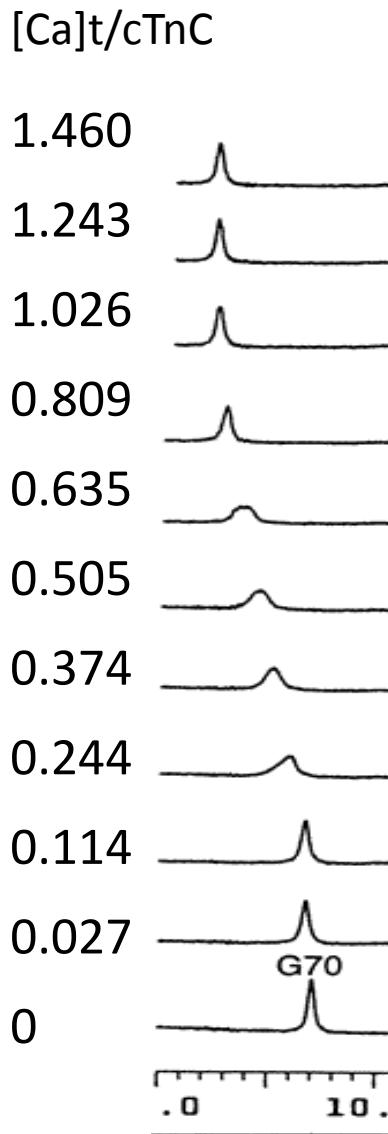
- $\Delta\delta_H = \Delta\delta_{Ho}[H]/([H]+K_a)$



Inhibitor G-3-P binds to  
Triosephosphate Isomerase

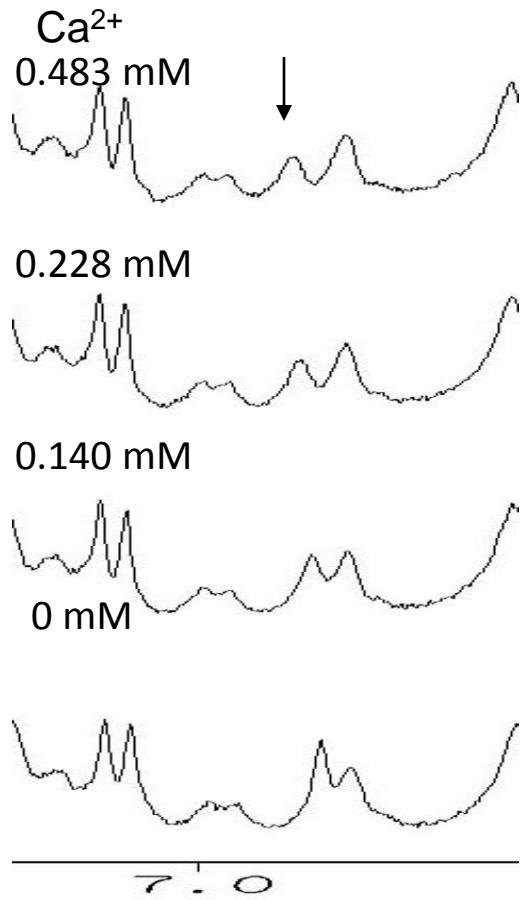
-JMB, 1976 100:319

# NMR studies of Ca(II) Binding to cNTnC

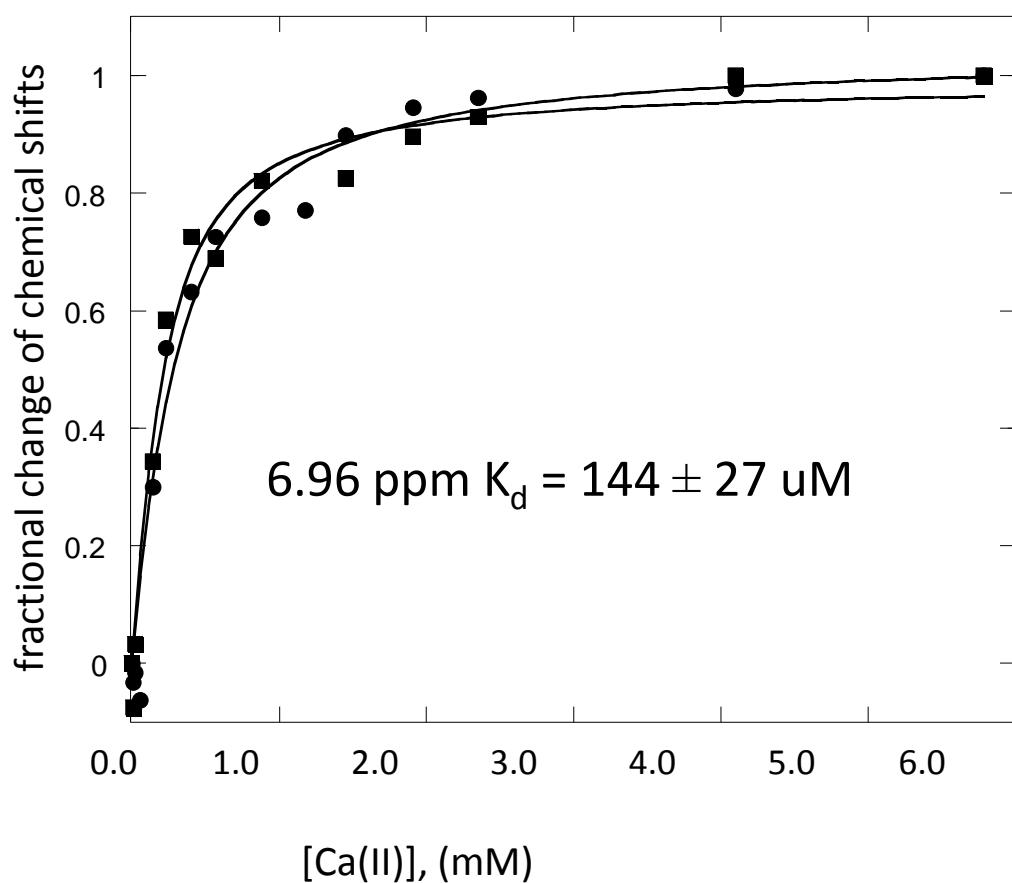


cNTnC is the N-terminal domain of TnC from cardiac muscle with a single calcium binding site II . Peak at 10.3 ppm is G70 at the calcium binding site.  
– Monica et al., 1997

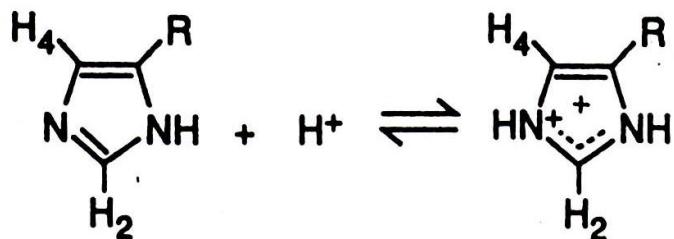
# Monitoring Calcium-binding by NMR



CaM-CD2-III-5G



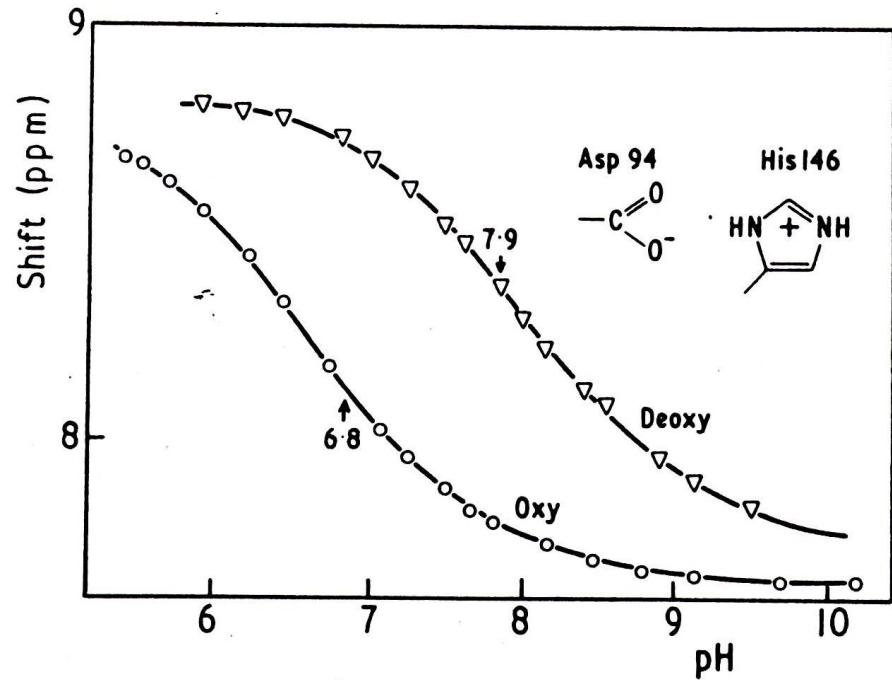
# The Ionization of His in Oxy & DeOxyhemoglobin



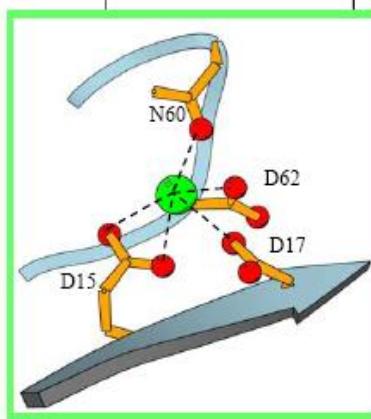
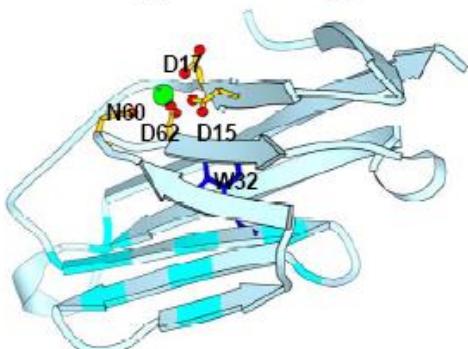
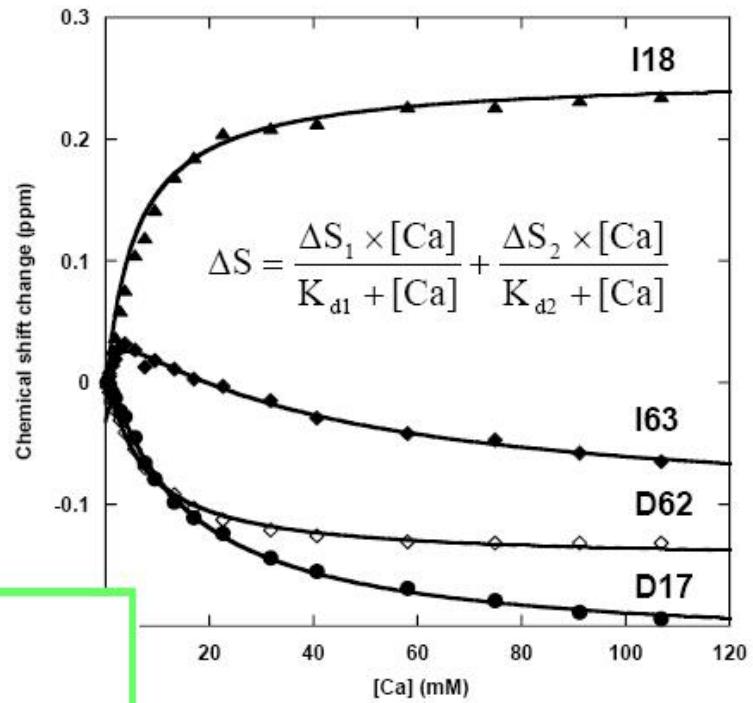
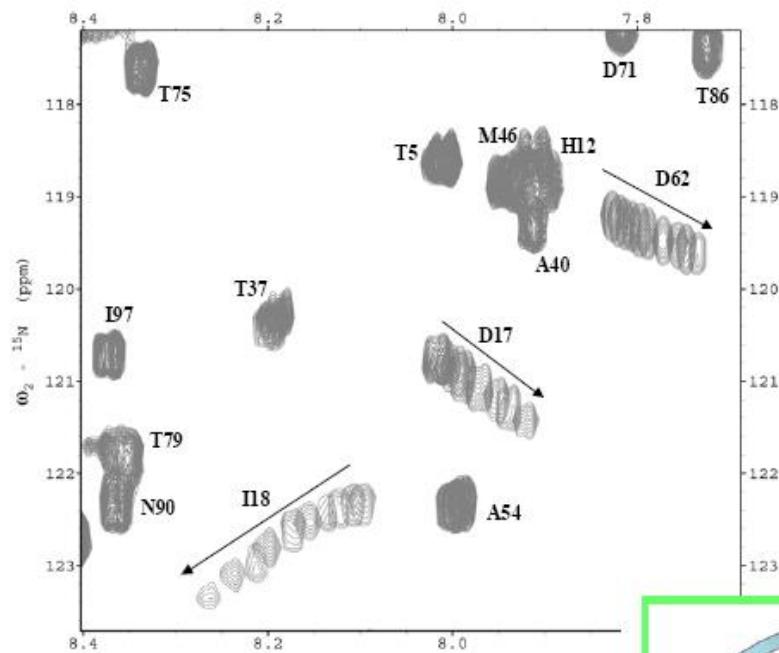
C2H 7.7 ppm    8.7 ppm

C4H 7.0 ppm    7.4 ppm

- Bohr effect: Hb takes up  $\text{H}^+$  on releasing  $\text{O}_2$
- The  $\text{pK}_a$  of His- $\beta$ -146 changes by more than 1pH unit upon ionization due to the stabilization of the charged form by Asp94 in the deoxy structure.

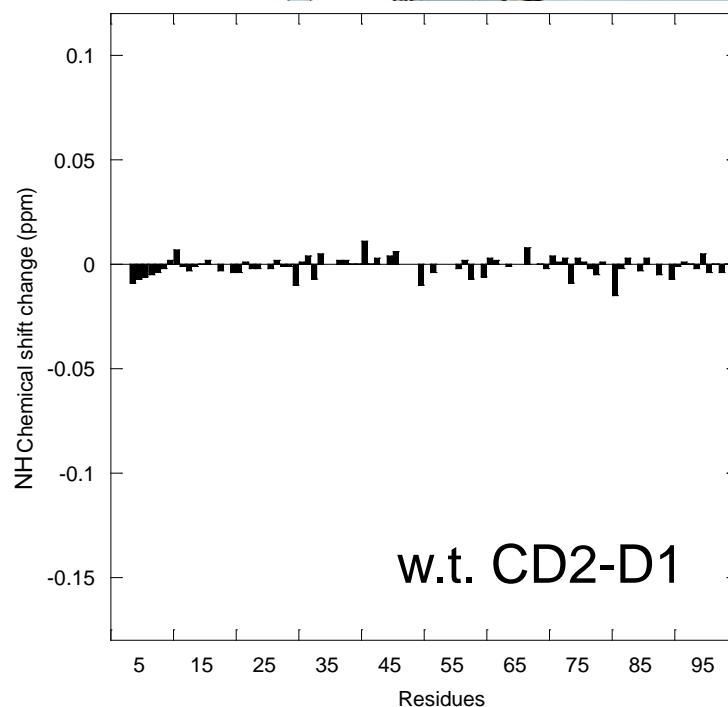
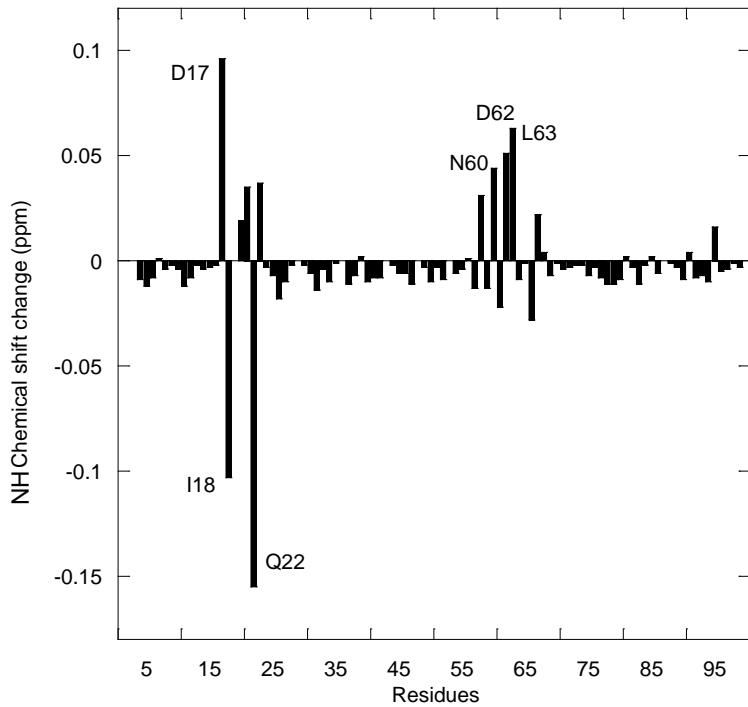
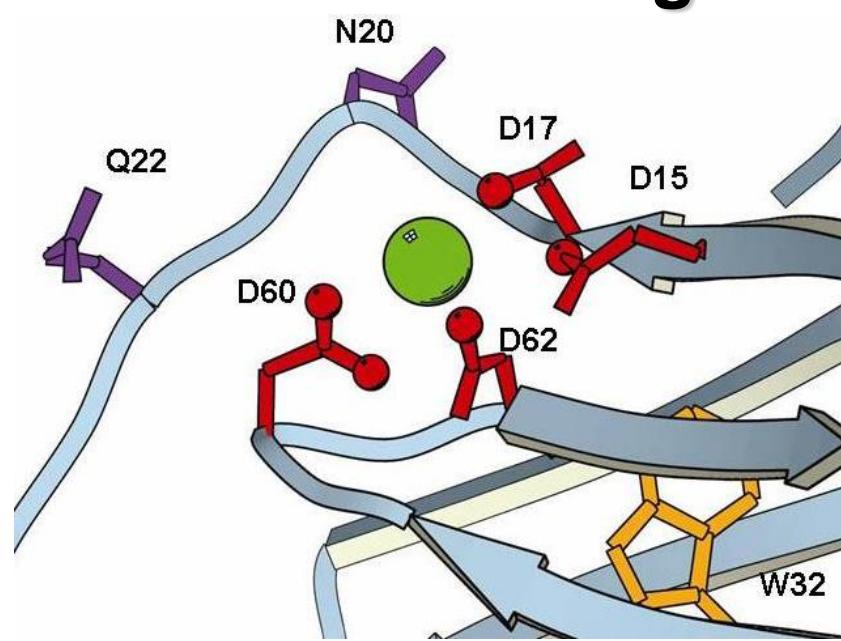
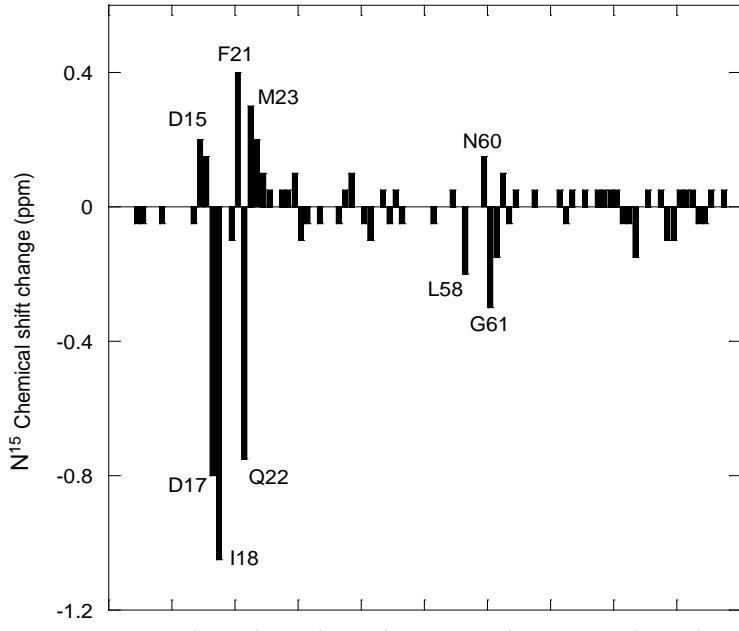


# Calcium Titration of Designed Ca<sup>2+</sup> binding Protein

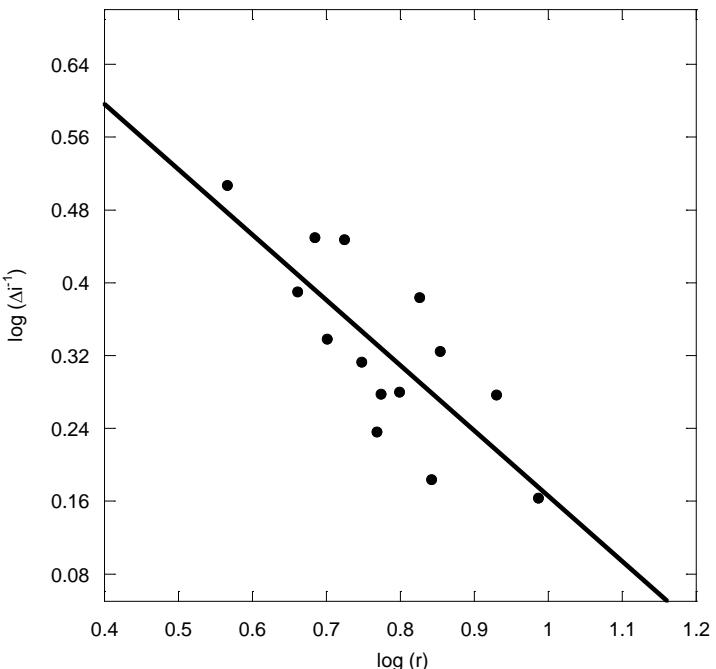
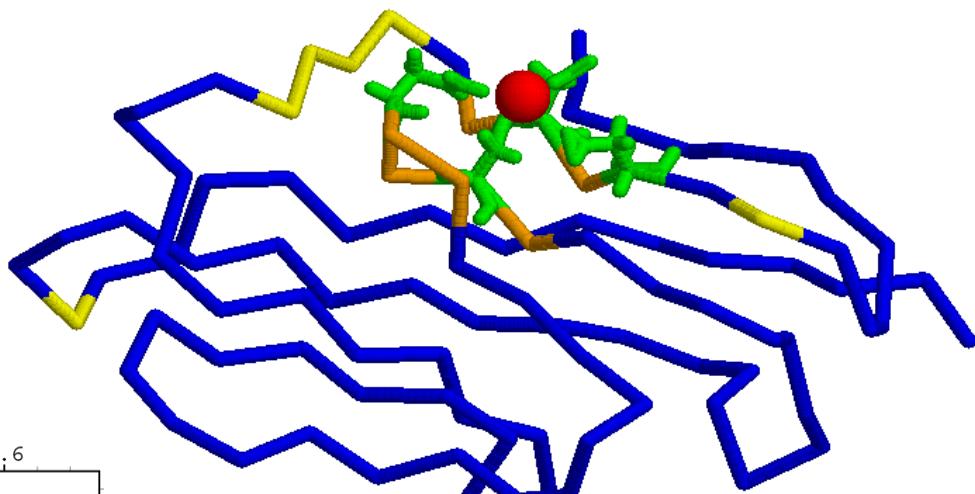
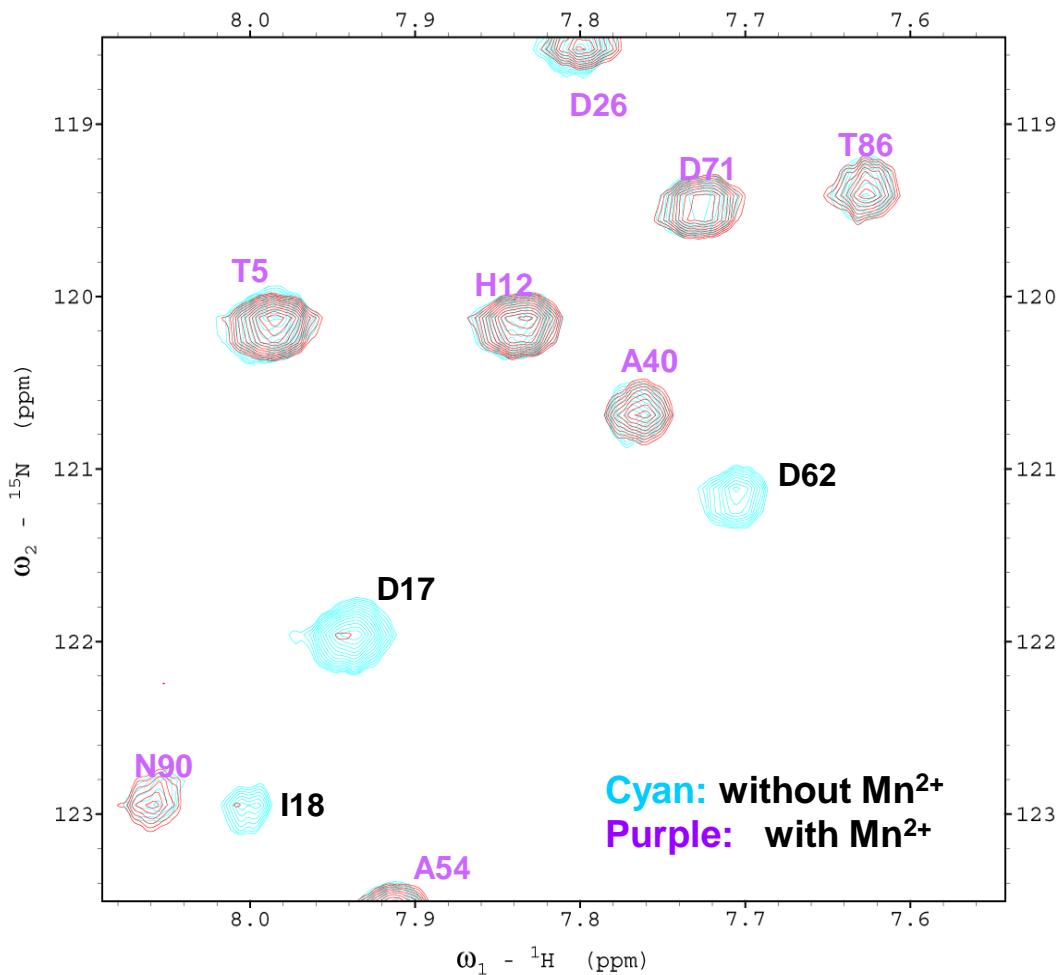


Yang et al. (2005) JACS

# Calcium Induced Chemical Shift Changes



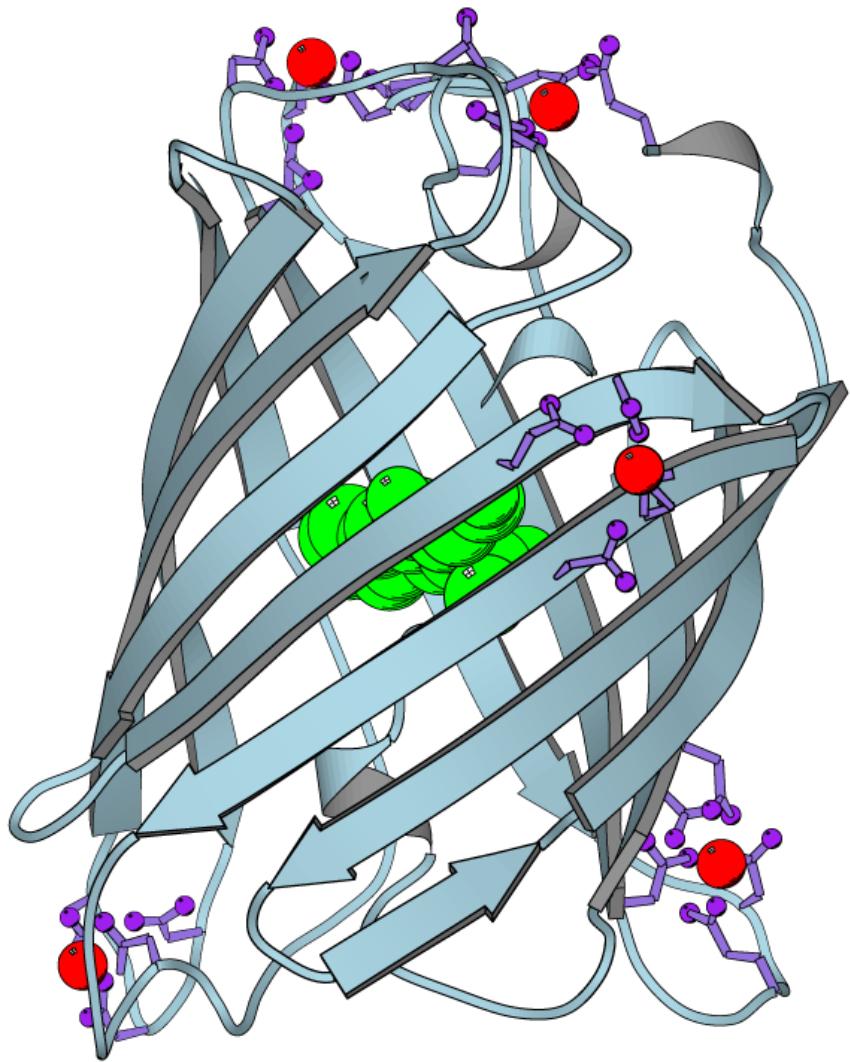
# Manganese Relaxation of CD2-6D15



Yang et al. (2005) JACS, 127, 2085-93

Mildvan, AS, and Cohn M, 1970, Adv. Enzymol. Relat. Areas Mol. Biol., 1970, 33, 1-70

# Developing Calcium Sensors by Design



1. Highly targeting specificity
2. Simple stoichiometric interaction mode to ease calibration
3. Tunable affinities, selectivity & kinetics
4. Minimal perturbation on signaling without using natural calcium binding proteins

JACS, 2002, 2005, 2007 Biochem, 2005, 2006, PEDS, 2007, protein science 2008

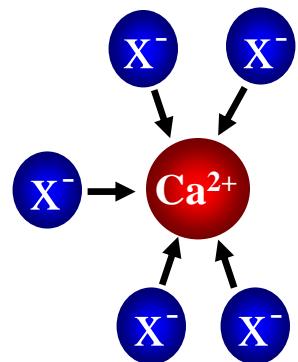
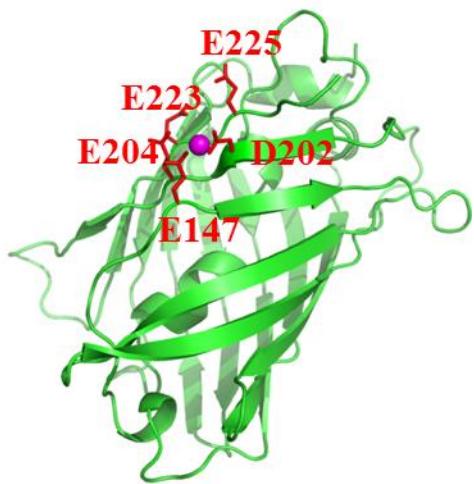
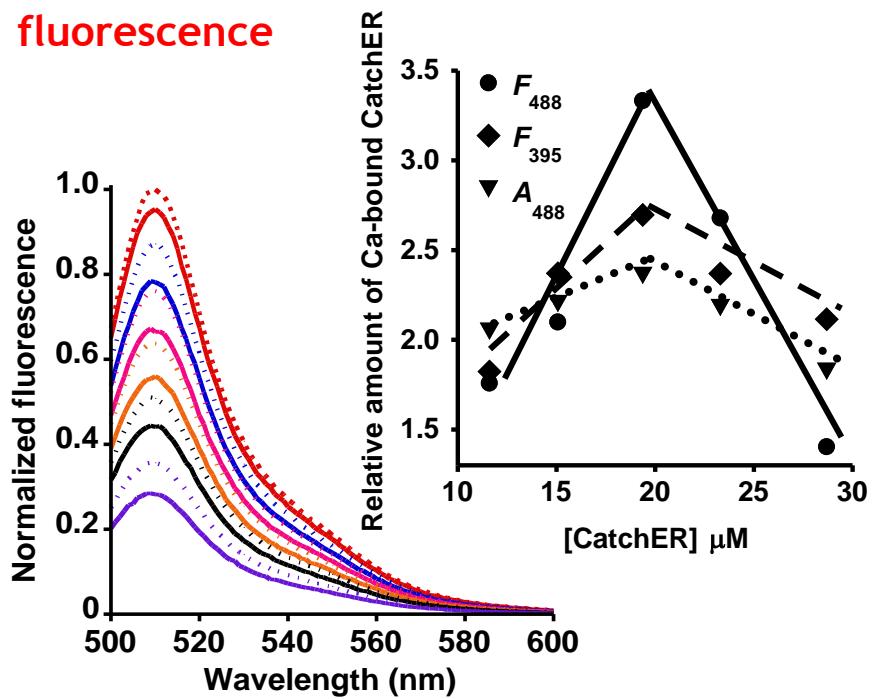
J. Zhou, A. Hofer, J.J. Yang, Biochem, 2007

A. Holder, ... J.J. Yang, Biotech 2009

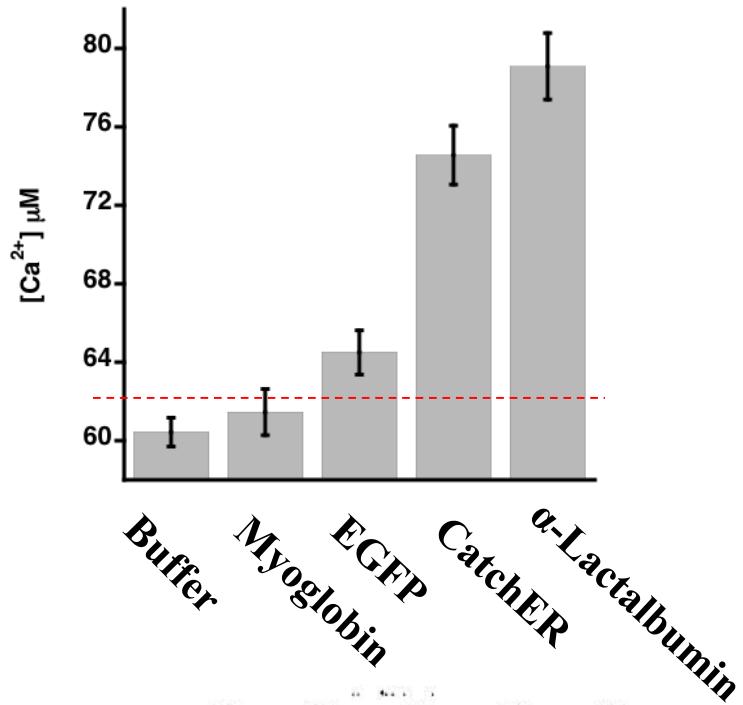
S. Tang,....O. Delbano J.J. Yang, PNAS, 2011

# Catcher: $\text{Ca}^{2+}$ Sensor for Detecting High Concentration

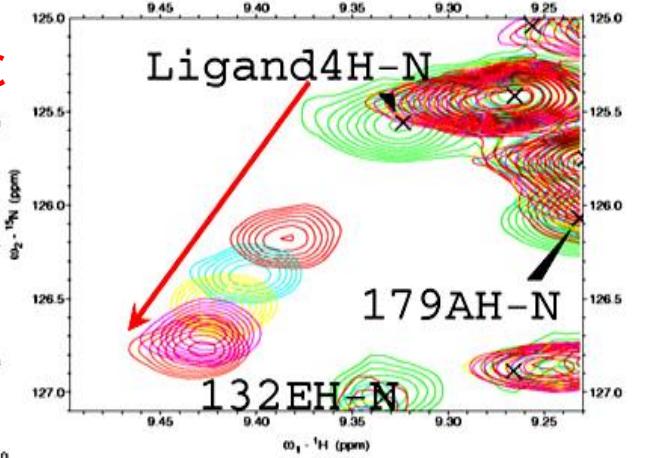
fluorescence



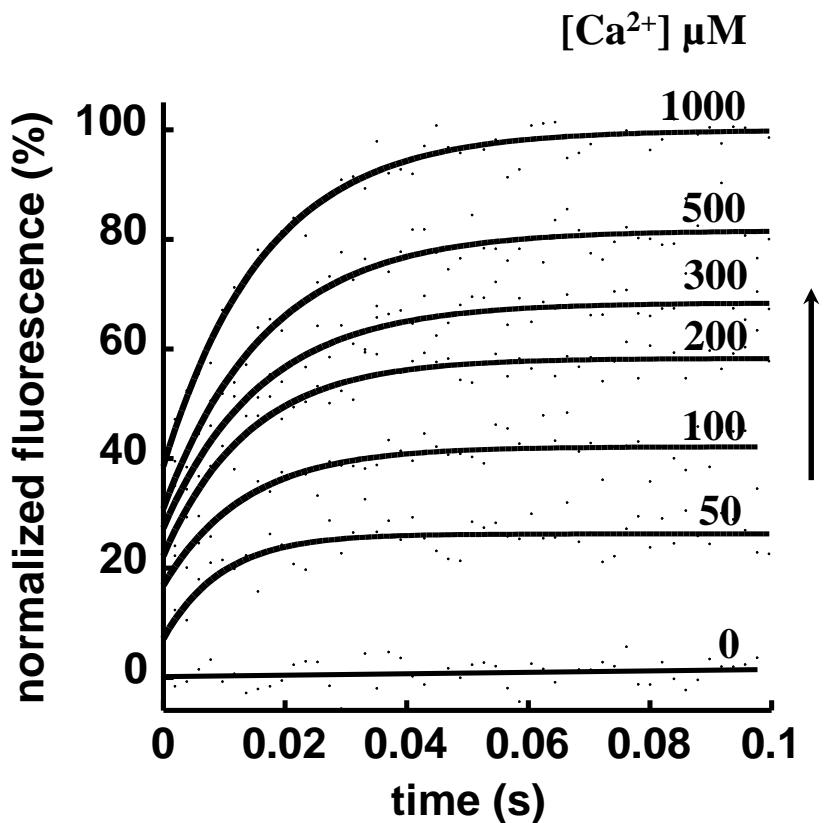
Equilibrium dialysis  
coupled with ICP-OES



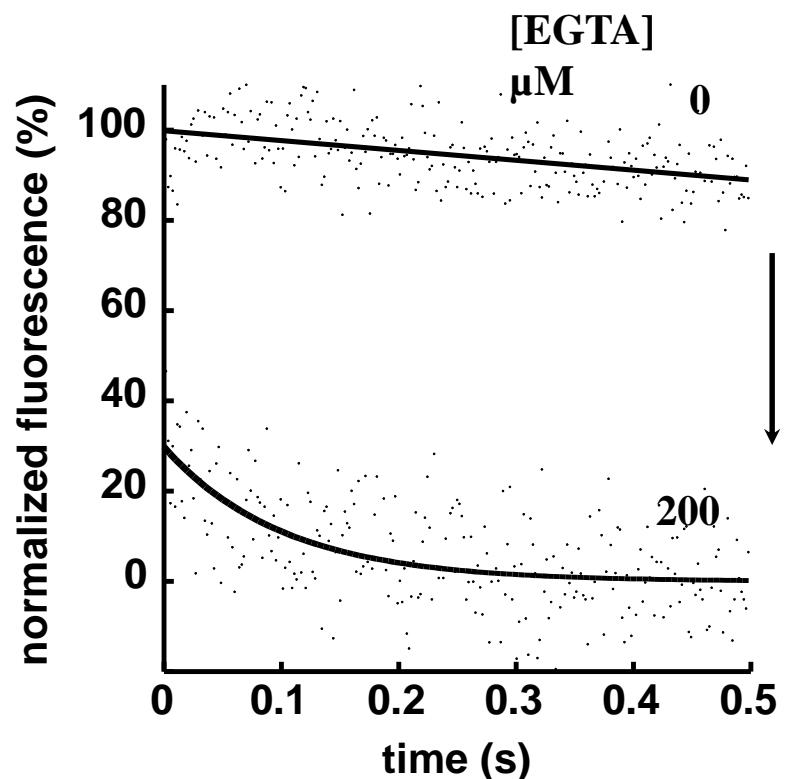
$^1\text{H}, ^{15}\text{N}$ -HSQC



# Fast Kinetics of CatchER

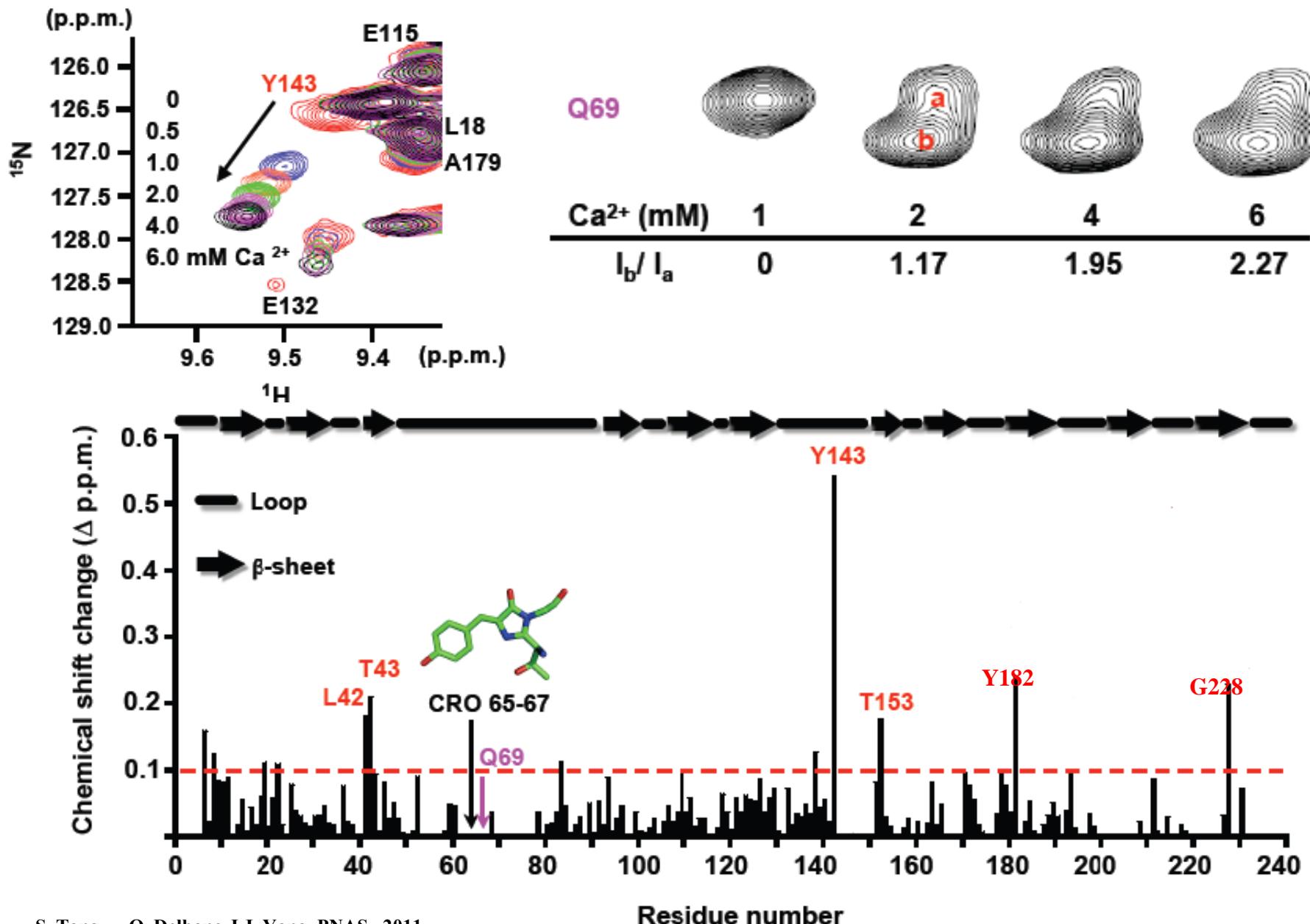


$$k_{\text{on}} = k_{\text{off}}/K_d = 3.9 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$$

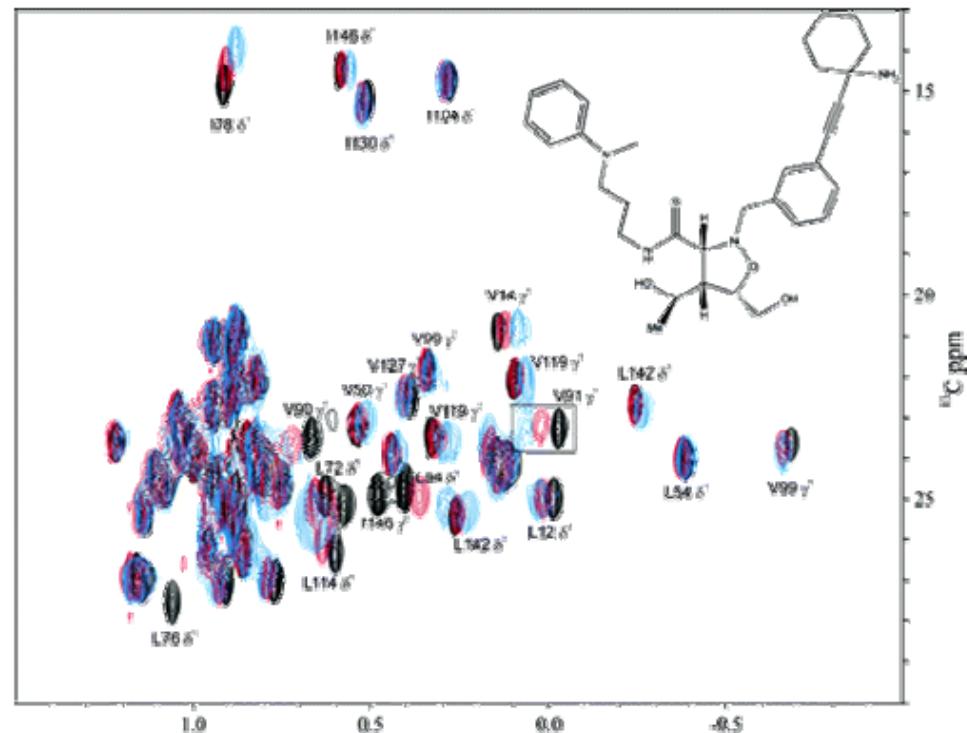
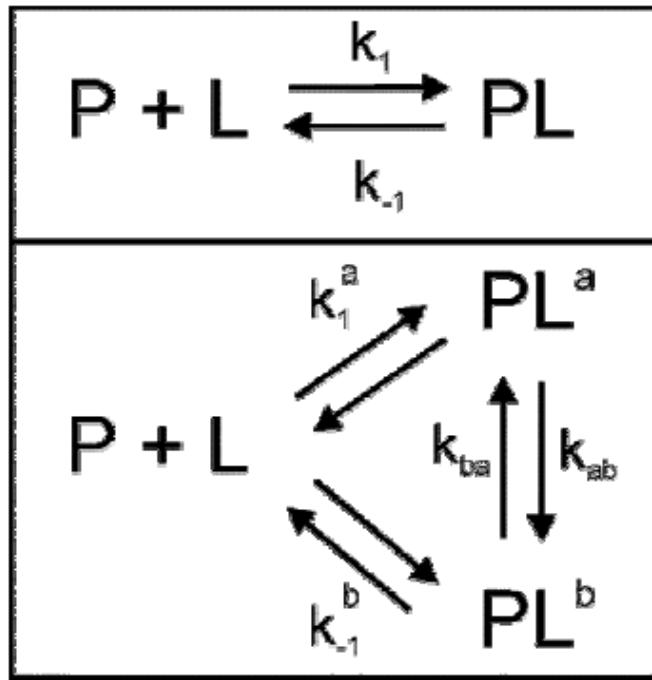


$$\text{Fast Kinetics: } k_{\text{off}} = 700 \text{ s}^{-1}$$

# CatchER's $\text{Ca}^{2+}$ Induced Chemical Shift Changes



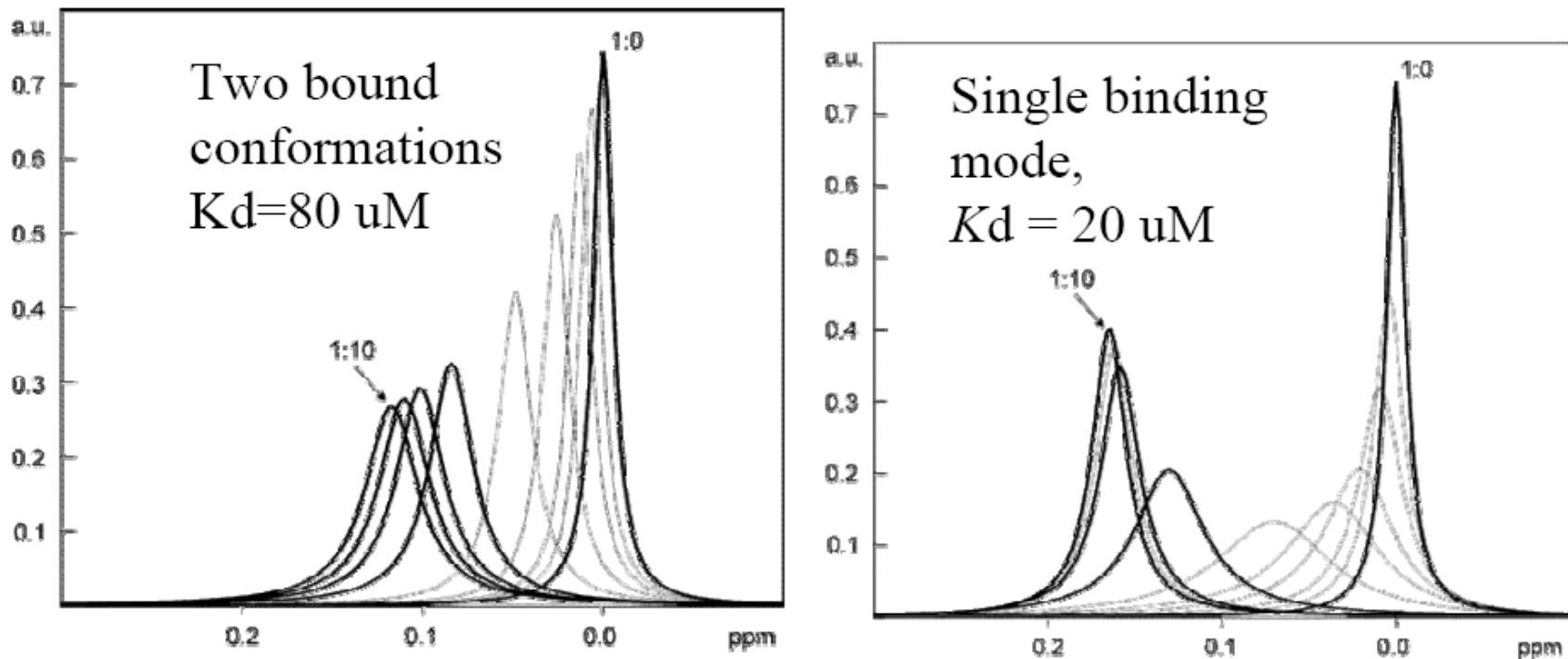
# NMR Distinction of Single- and Multiple-Mode Binding of Small-Molecule Protein Ligands



<sup>1</sup>H-<sup>13</sup>C HSQC spectrum of a 25 μM sample of Bcl-xL that is <sup>13</sup>C-labeled only at the methyl groups of Ile, Leu, and Val. 3,4 Black: no ligand, red 25 μM, and blue 250 μM ligand. The ligand is a first-generation compound from the primary screen; *KD* = 80 μM.

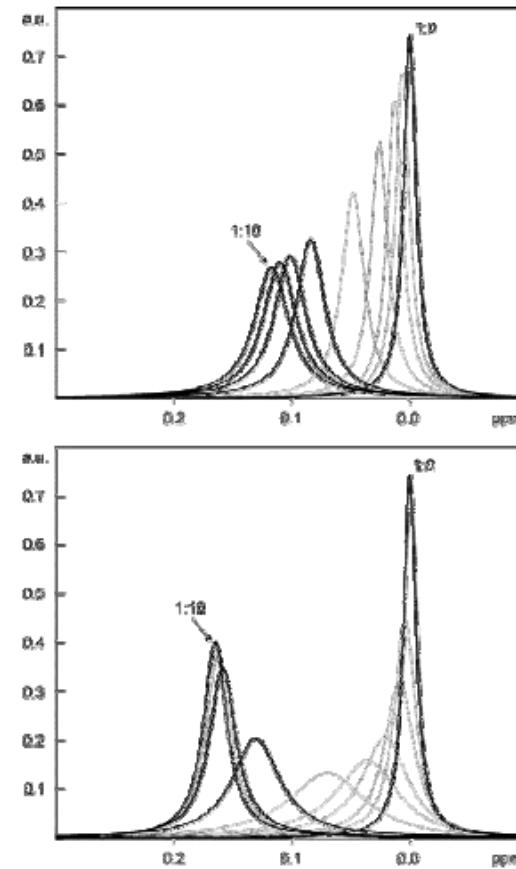
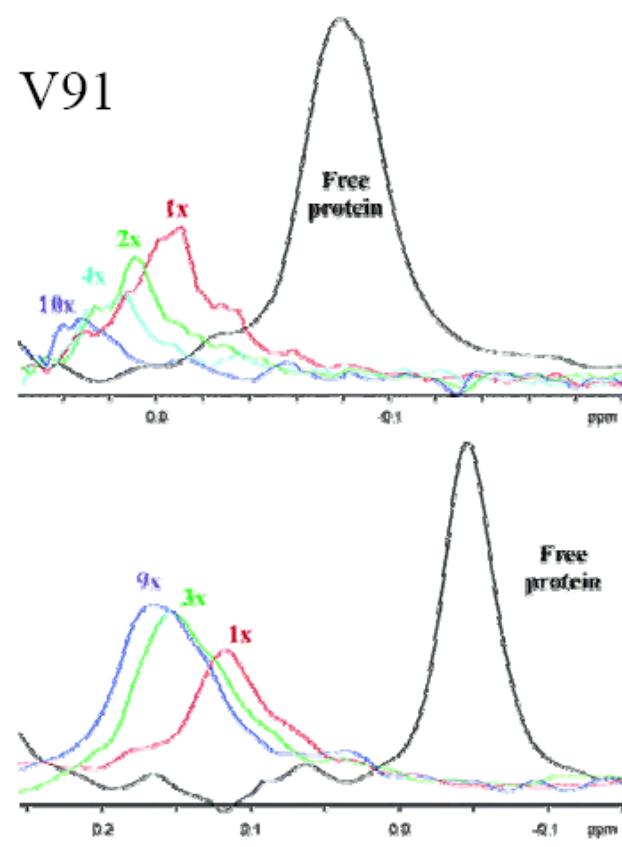
**Reibarkh,M... Wagner, G, JACS, 2006**

# Simulated NMR line shapes in the intermediate exchange regime



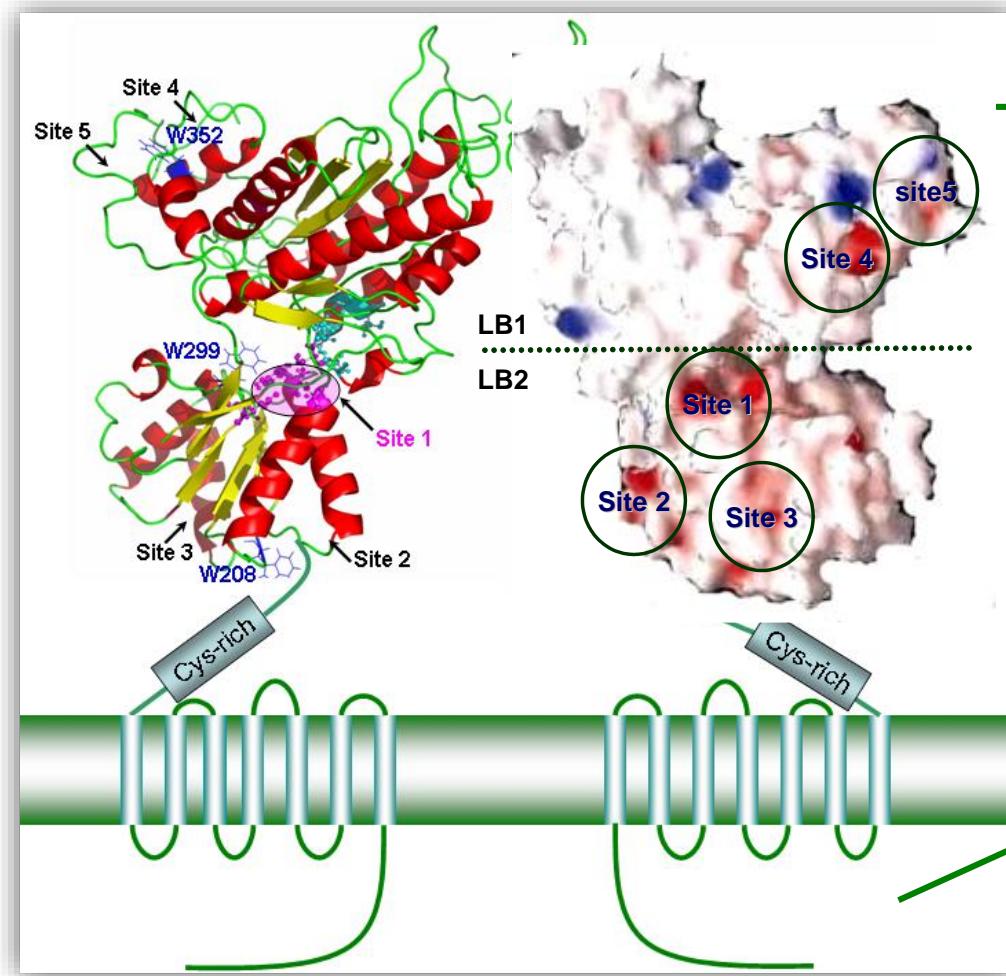
- . The line width in the free protein is 20 Hz,  $\Delta v$  is 100 Hz, the effective line broadening,  $R_a$ , caused by conformational exchange is 80 Hz. The resonance of the free protein is set to 0 ppm at 500 MHz

## Cross-sections of HSQC spectra of <sup>13</sup>C ILV-labeled Bcl-xL



The use of <sup>1</sup>H-<sup>13</sup>C HSQC spectra of methyl-labeled proteins is beneficial compared to <sup>1</sup>H-<sup>15</sup>N labeling: 3-fold higher signal intensity; can be performed in D<sub>2</sub>O; and methyl resonances appear to be less susceptible to line broadening compared to backbone amides. The effect is probably most easily detectable as a gradual loss of peak intensities

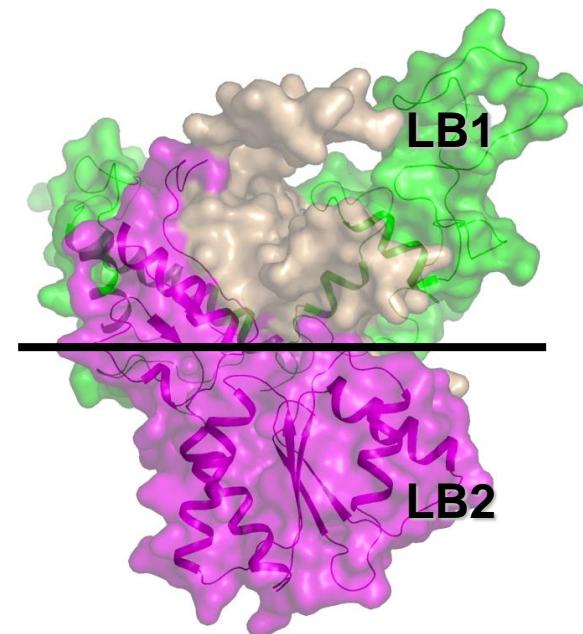
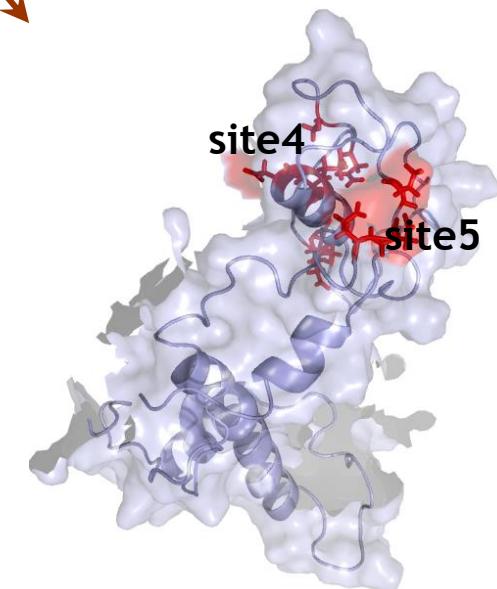
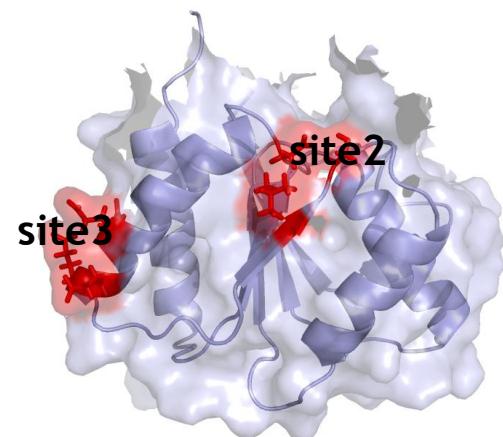
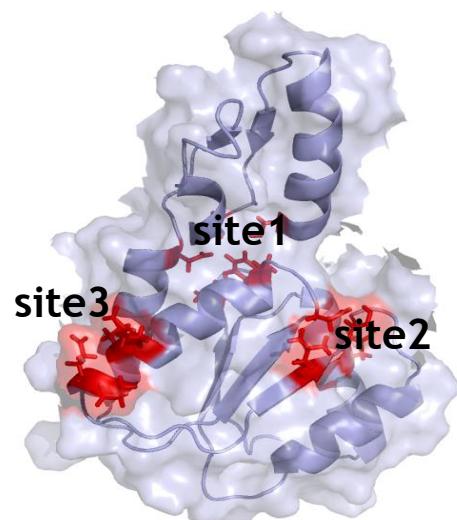
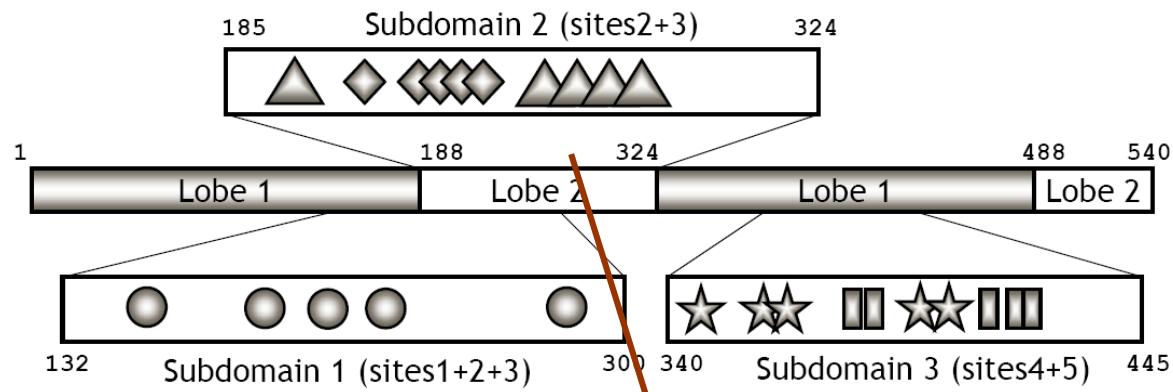
# Integration of Calcium Signaling Via CaSR



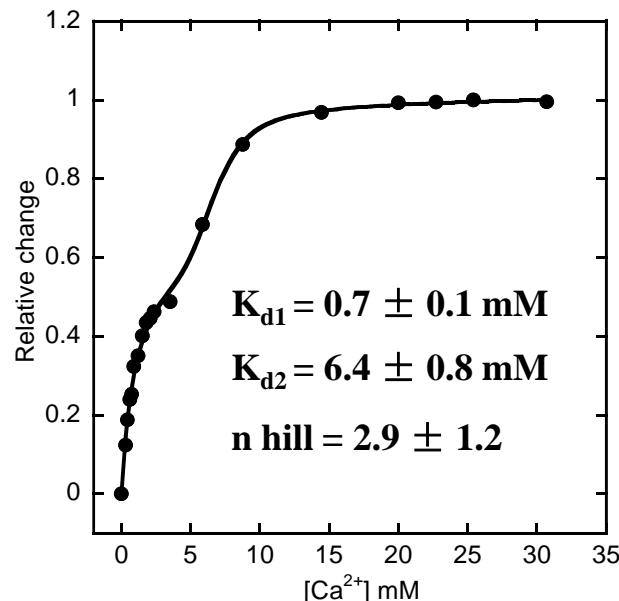
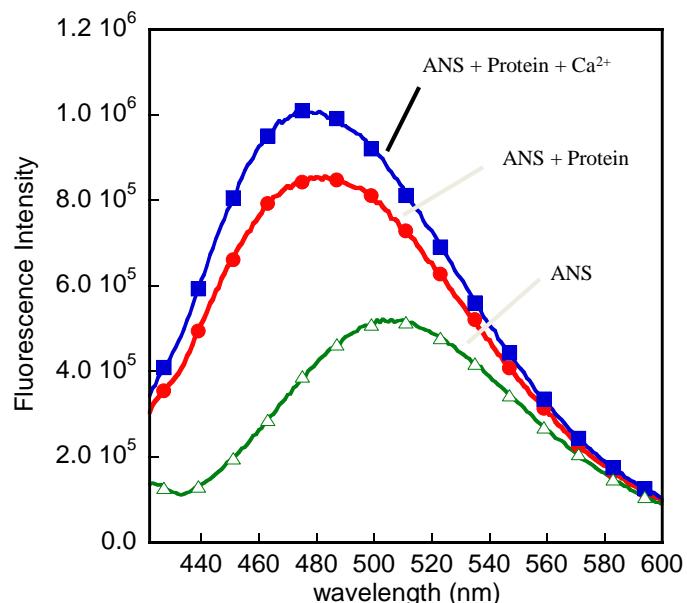
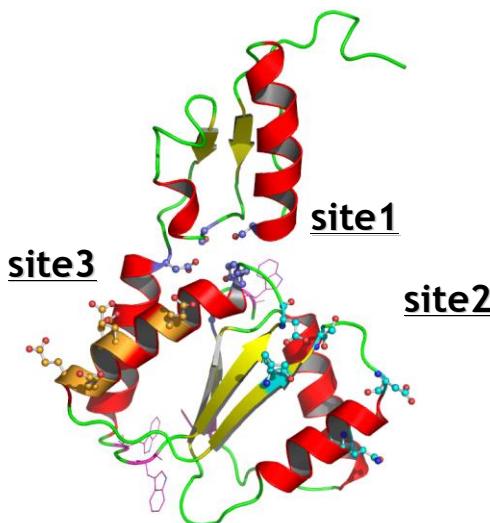
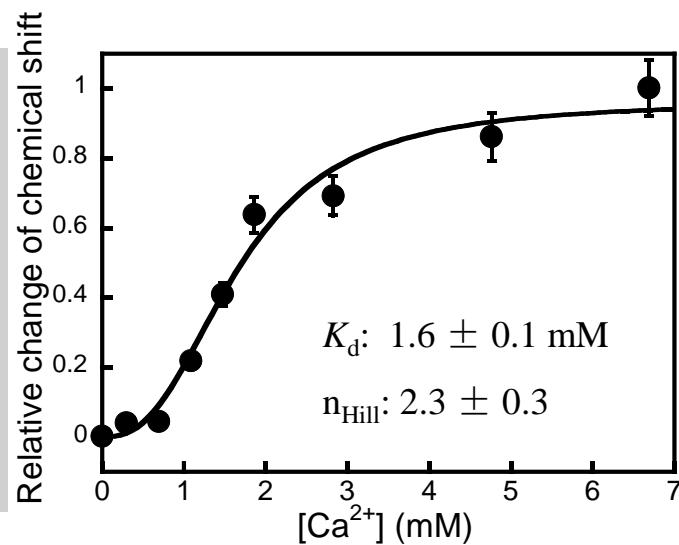
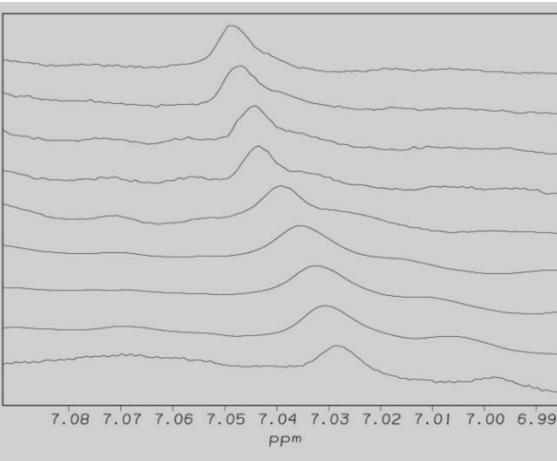
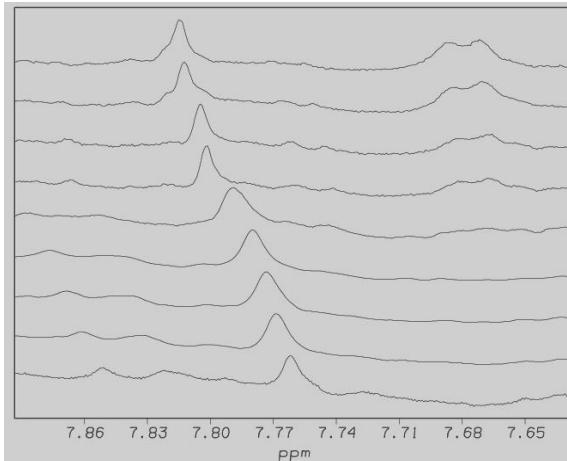
Identification of  $\text{Ca}^{2+}$  binding sites in ECD of CaSR  
How can CaSR sense the change of  $\text{Ca}^{2+}$  within a narrow range?  
(multiple sites? cooperativity?)

Identification of CaM binding region in c-tail of CaSR

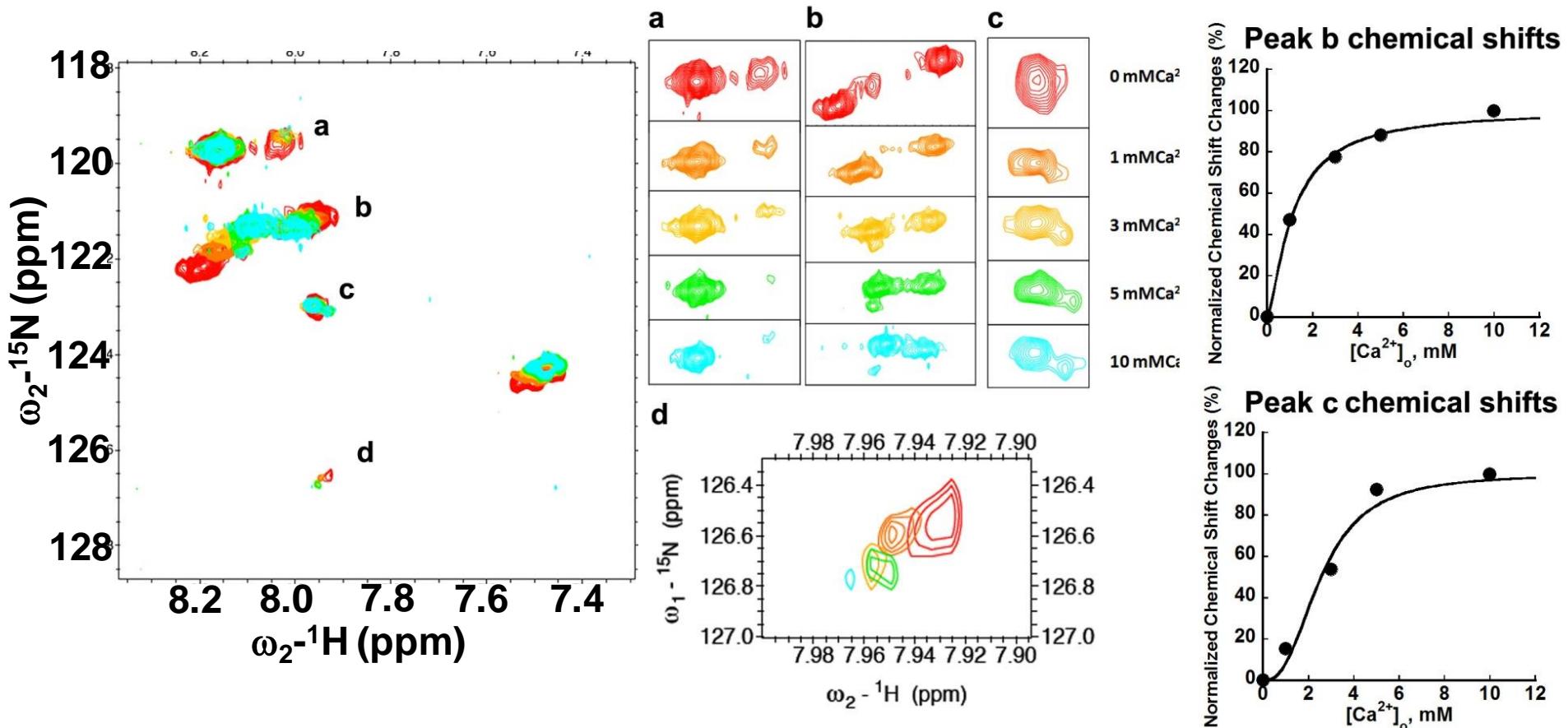
# Subdomain Approach



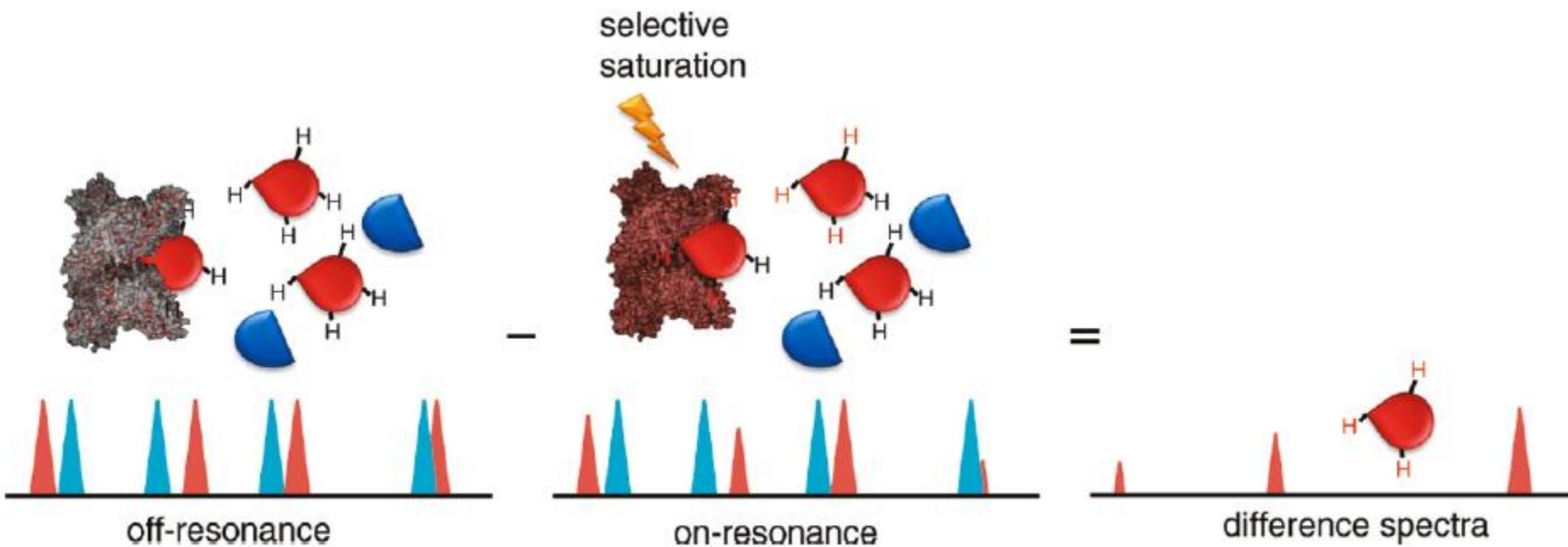
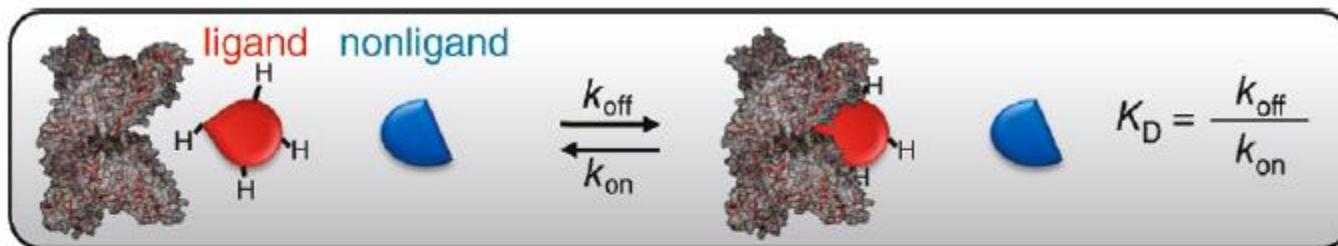
# Two Distinct $\text{Ca}^{2+}$ -Binding Processes Revealed by NMR



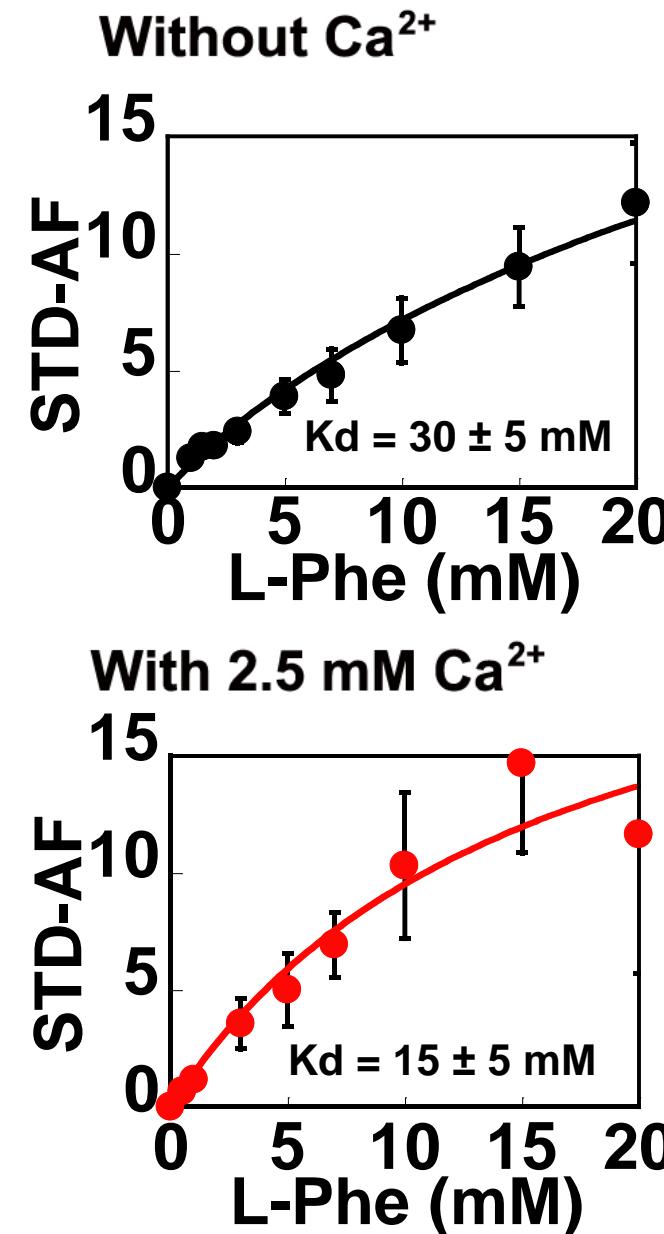
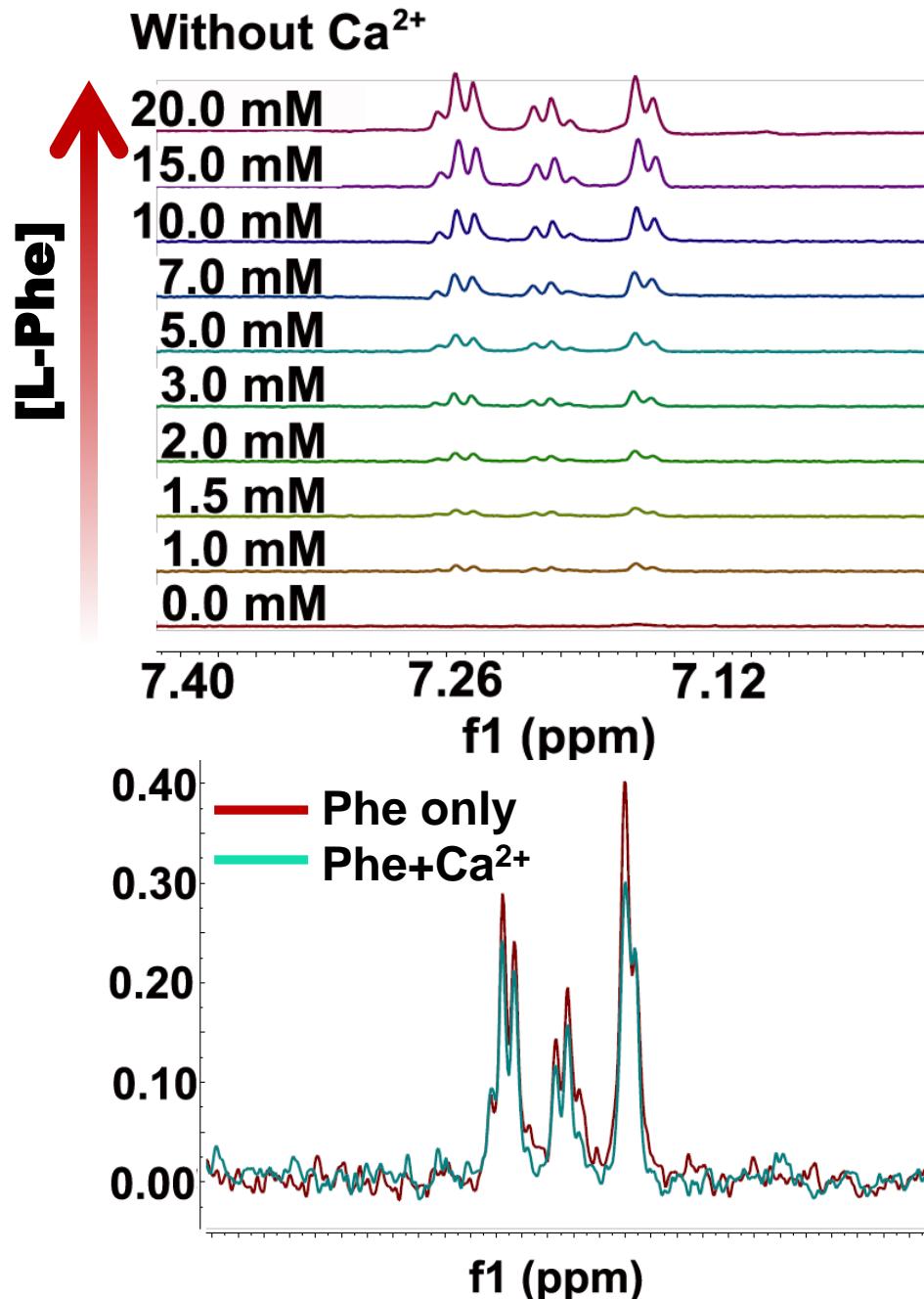
# 2D-NMR Revealed CaSR ECD Binds to $\text{Ca}^{2+}$



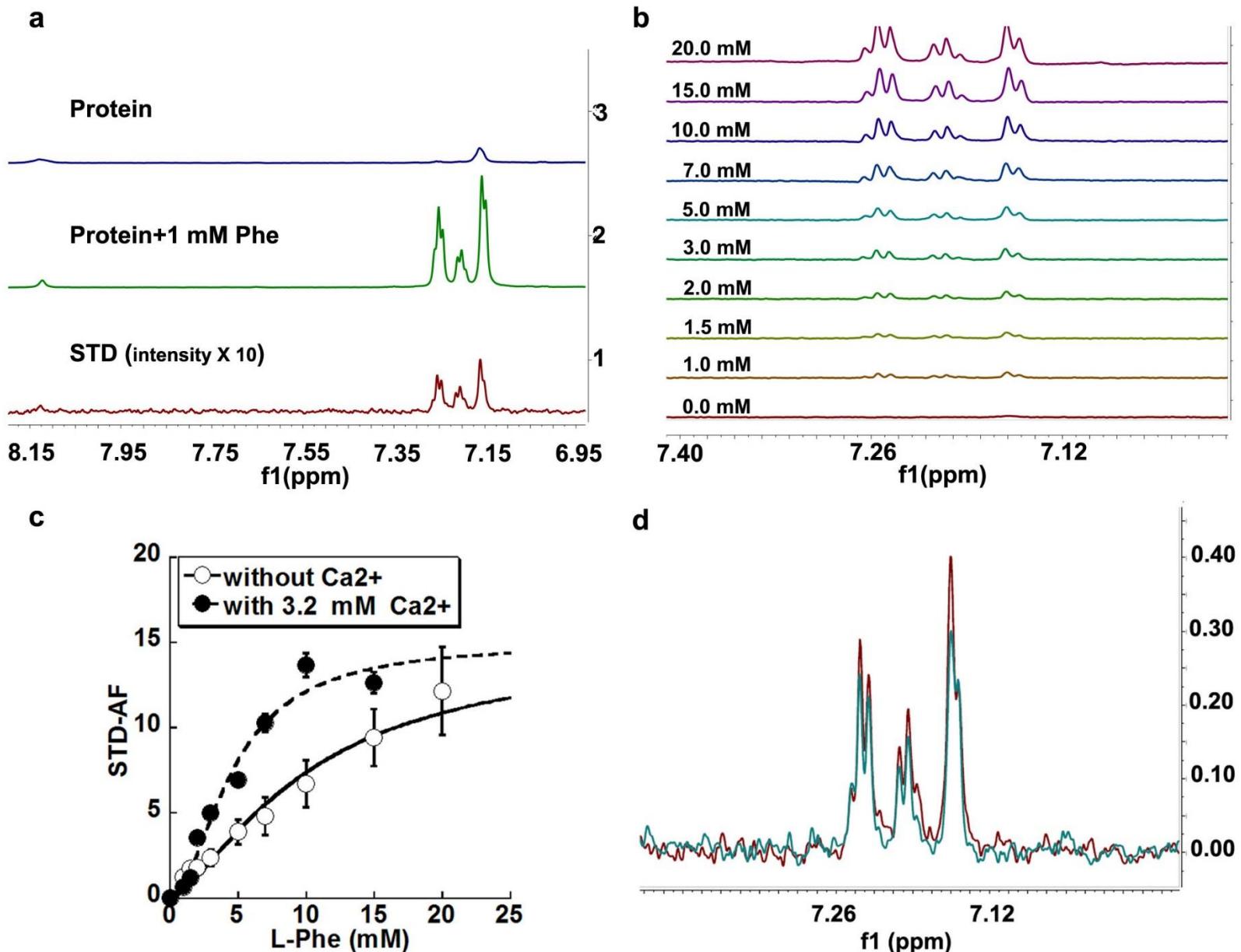
# The Principle of Saturation Transfer Difference (STD)



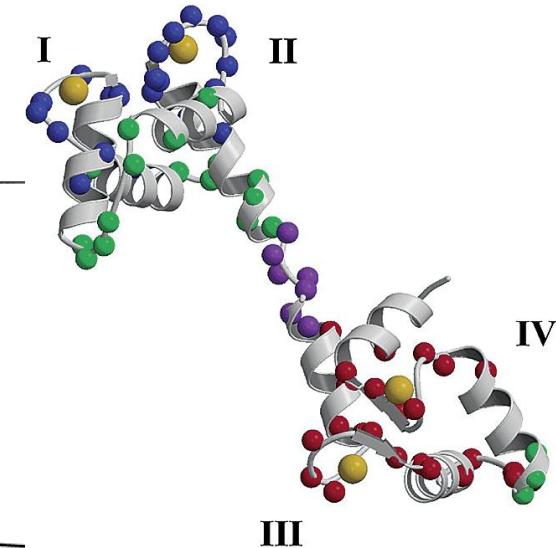
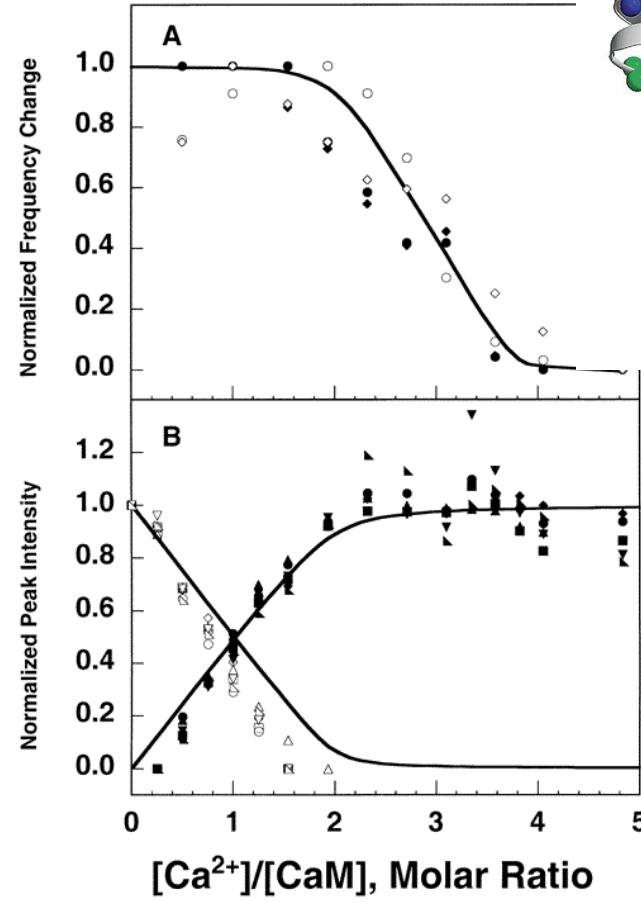
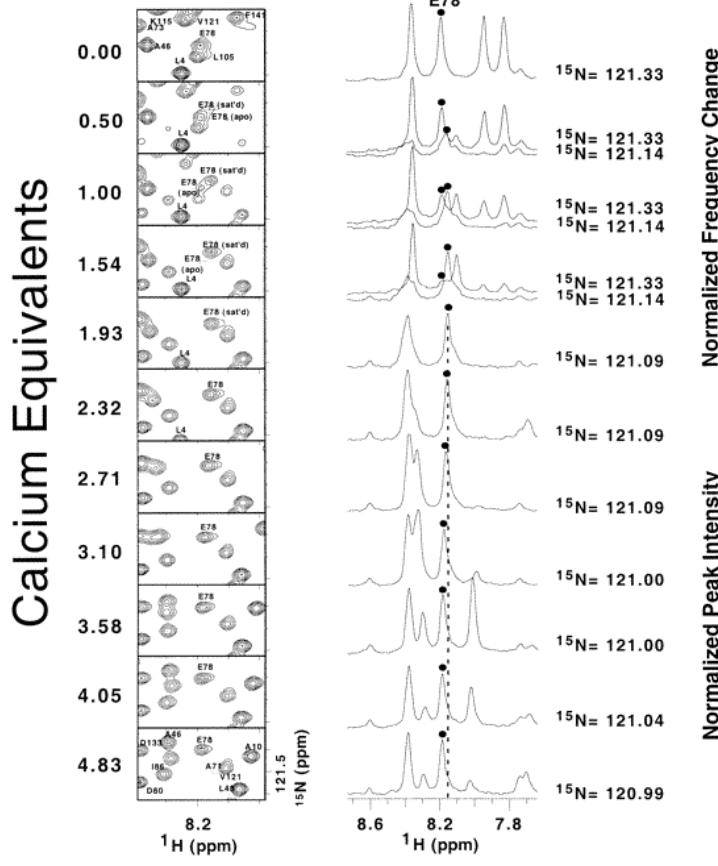
# Monitoring the Ligand-Protein Interaction via STD NMR



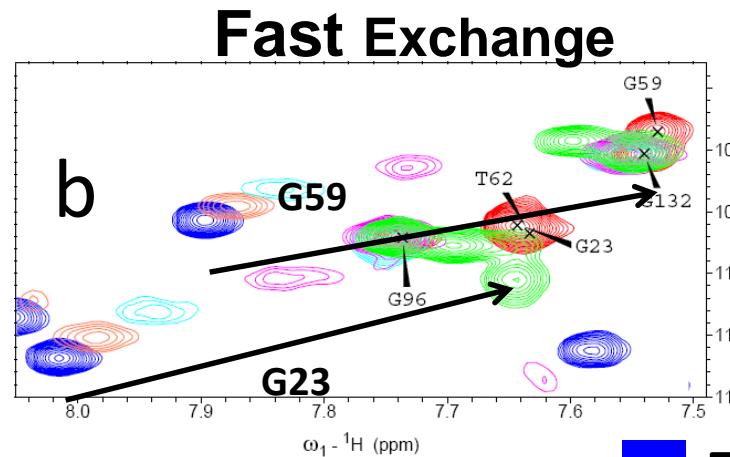
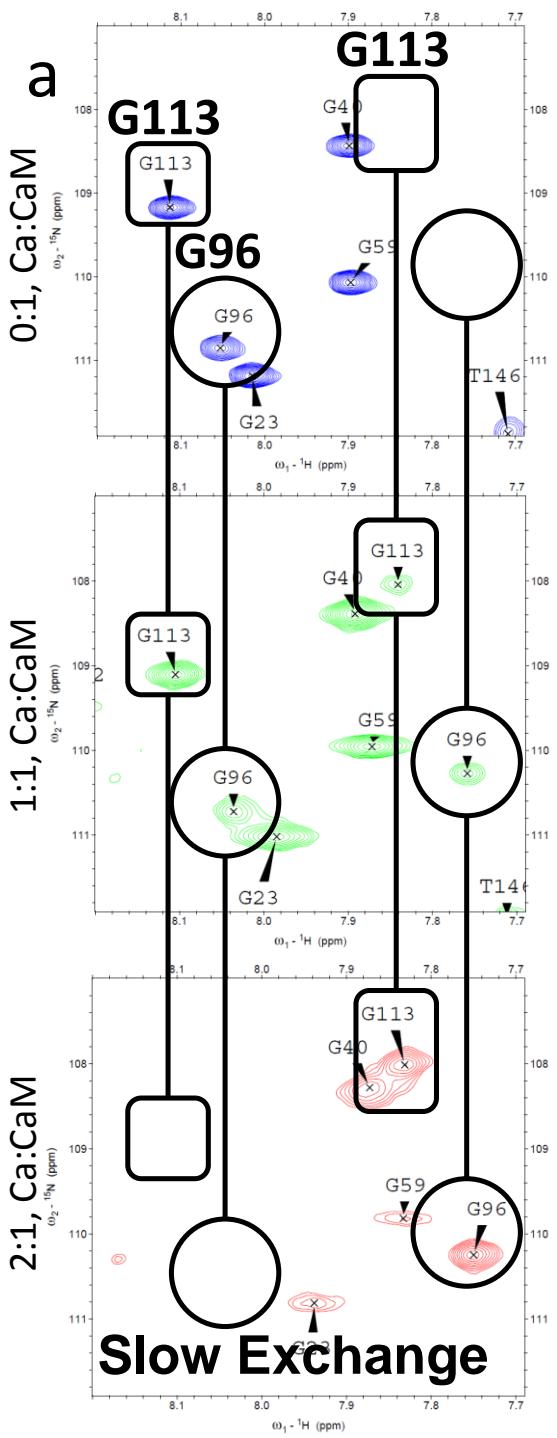
# Monitoring the Ligand-Protein Interaction via STD NMR



# Residues in the linker of Calmodulin underwent sequential phases of conformational change



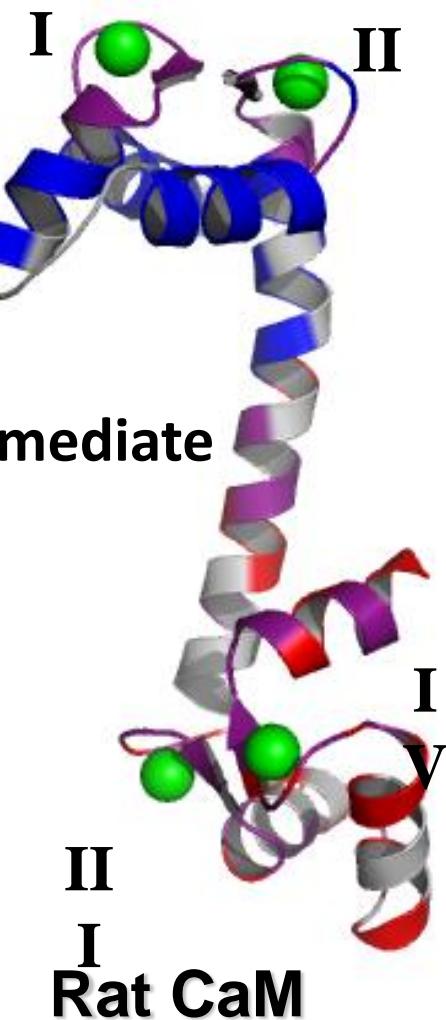
# Chemical Exchange in Rat CaM



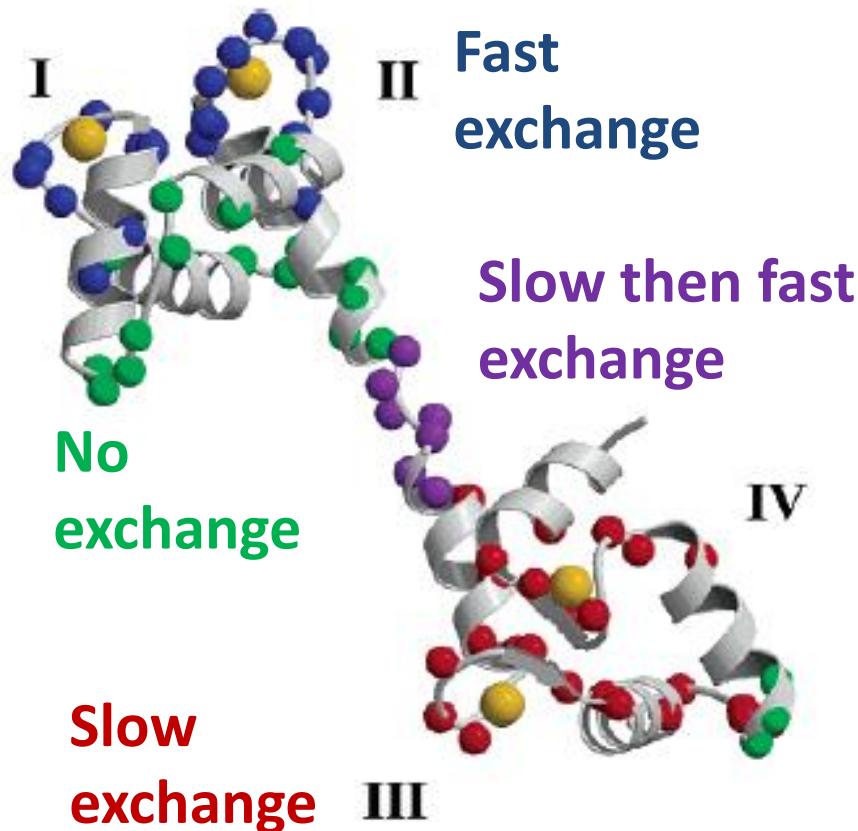
**Indicates:**  
Initial occupancy of C-terminal domain

**Suggests:**  
Temporal occupancy of N-terminal domain at low [Ca] and/or domain coupling

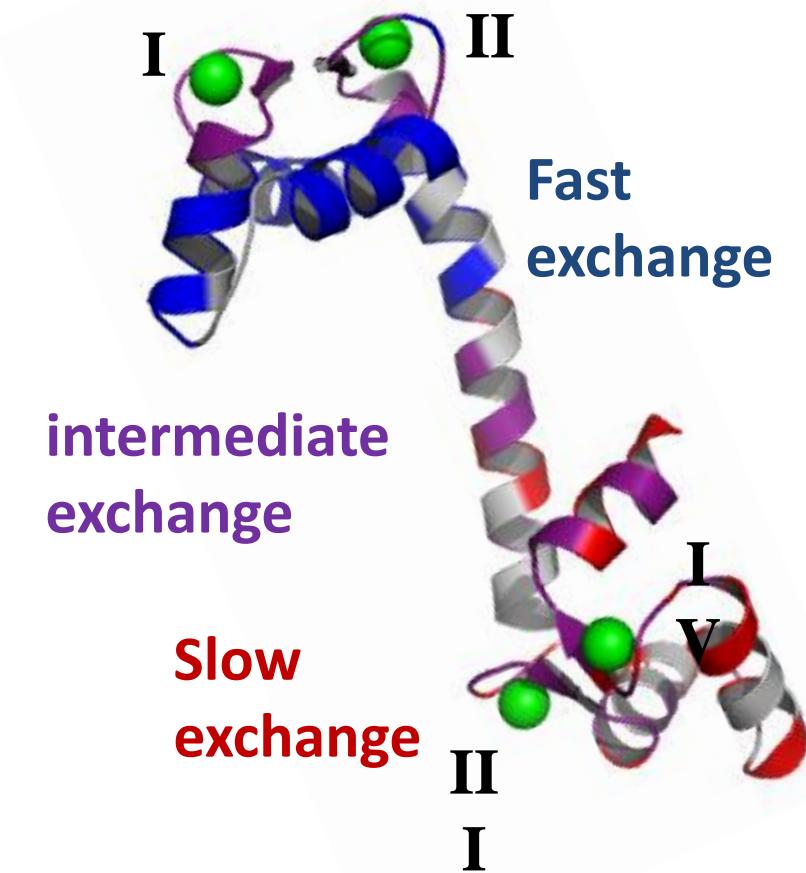
**Fast**  
**Intermediate**  
**Slow**



# Similarities in Chemical Exchange with $\text{Ca}^{2+}$ Titration

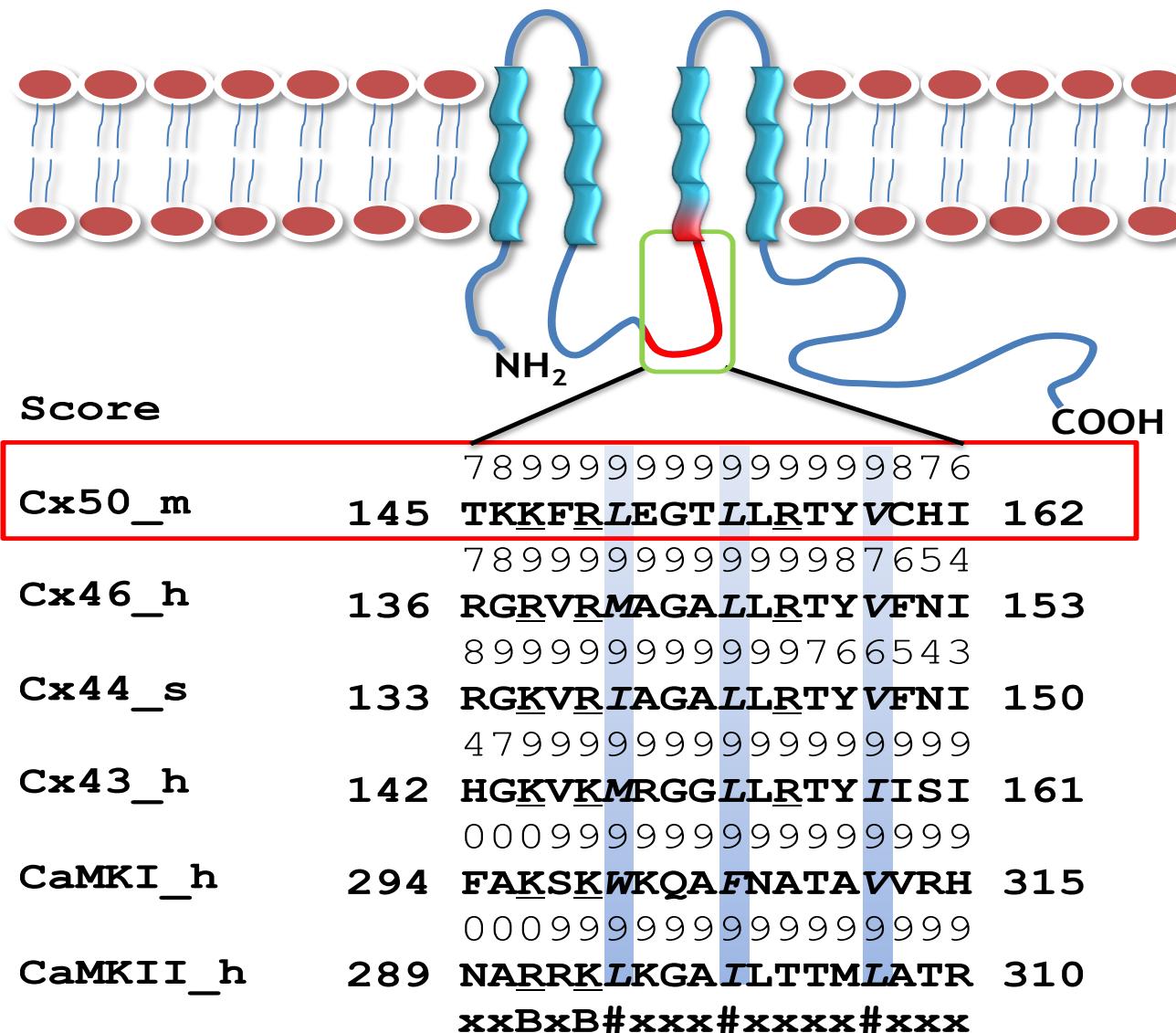


**Paramecium  
CaM**

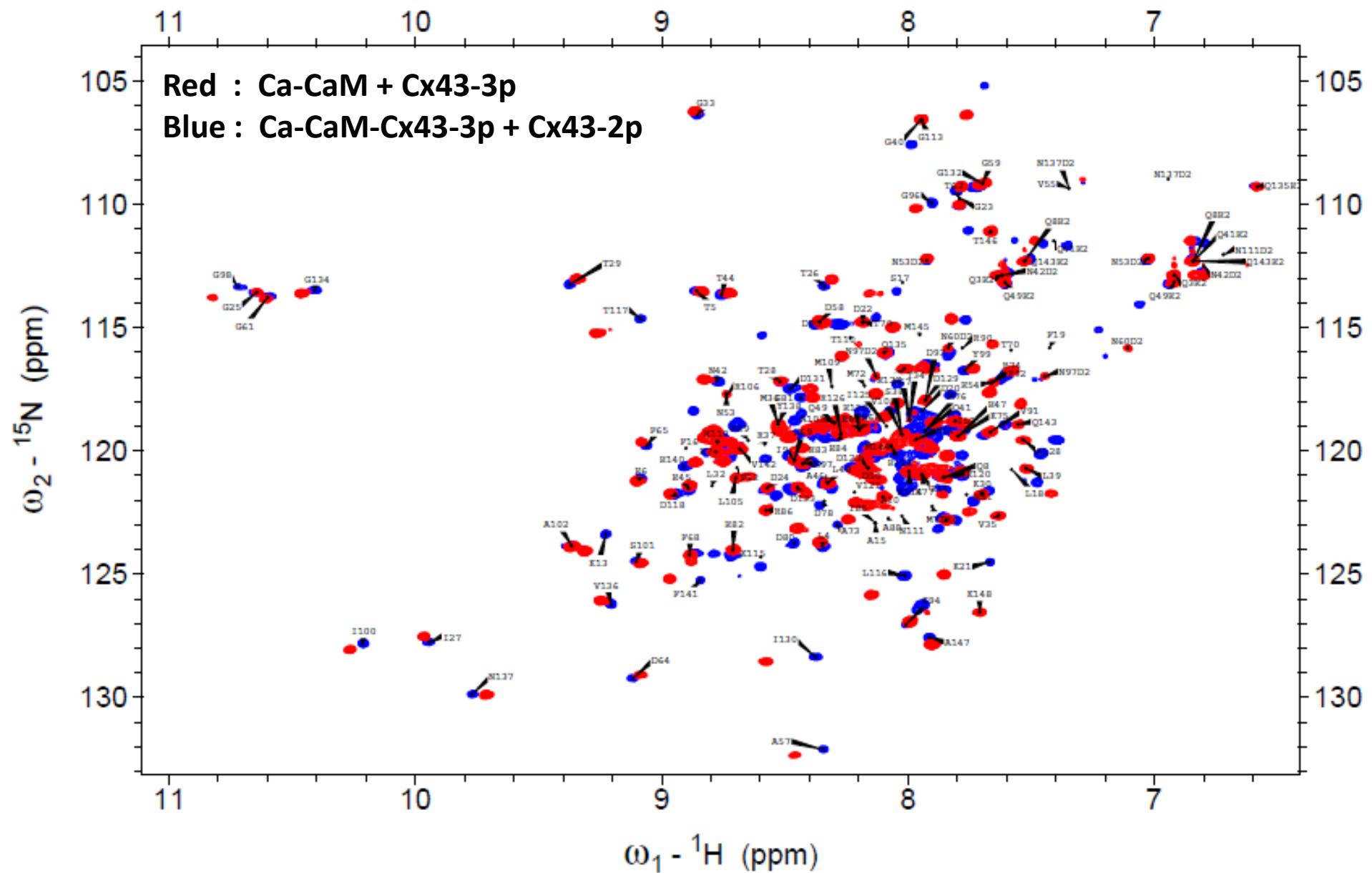


**Rat CaM**  
Kirberger M, Yang JJ, JIBC, 2013.

# Identifying CaM Binding Region in Gap Junction Connexins



# CaM Binding Peptide Fragments of Cx43 Competition Assay



# Monitoring Cx Peptide and Calmodulin

