Estudos de estrutura, dinâmica e função de proteínas (fluídos complexos) por RMN em solução

Roberto K. Salinas

IQ - USP

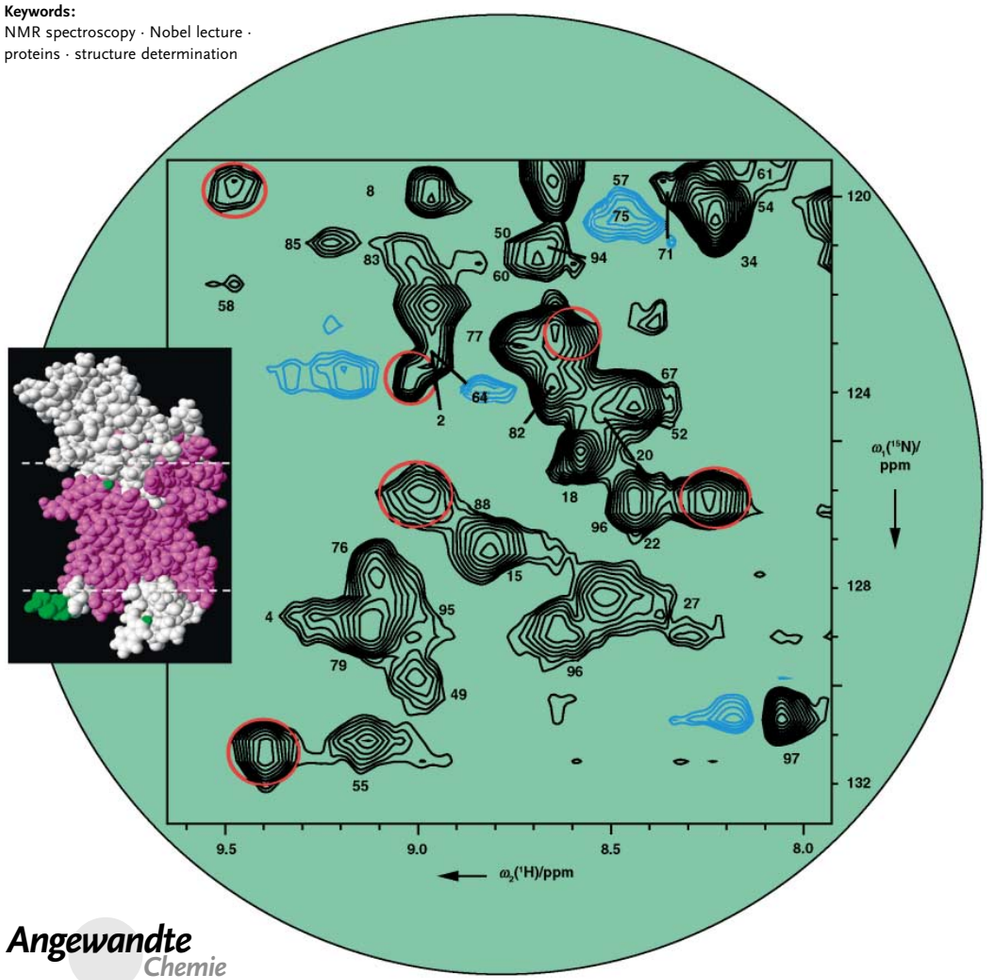
RMN de proteínas é a única técnica capaz de resolver a estrutura de proteínas em solução

NMR of Biomacromolecules

NMR Studies of Structure and Function of Biological Macromolecules (Nobel Lecture)**

*Kurt Wüthrich**

Keywords:
NMR spectroscopy · Nobel lecture · proteins · structure determination



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Angew. Chem. Int. Ed. **2003**, *42*, 3340 – 3363

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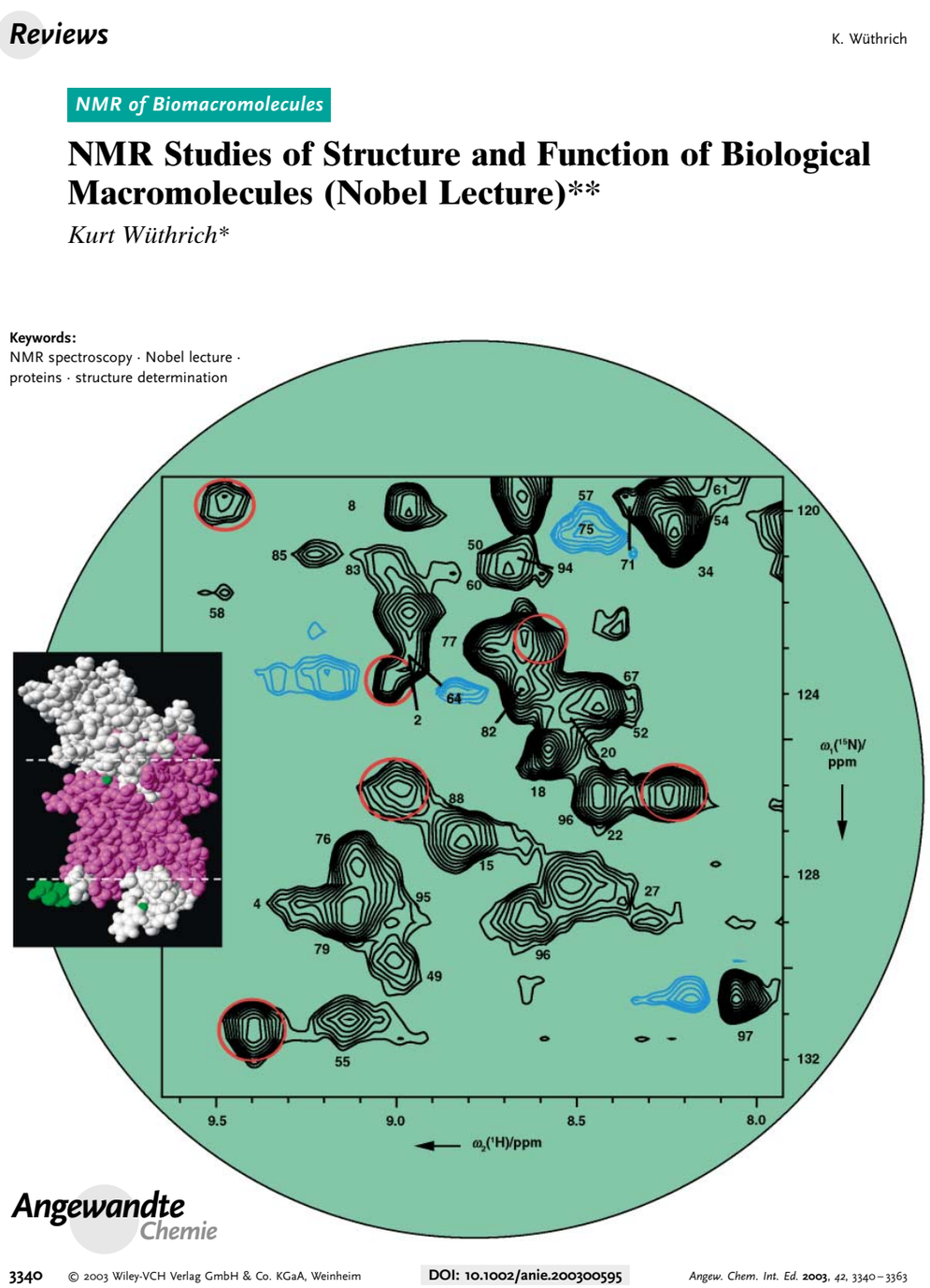

Reviews K. Wüthrich

NMR of Biomacromolecules

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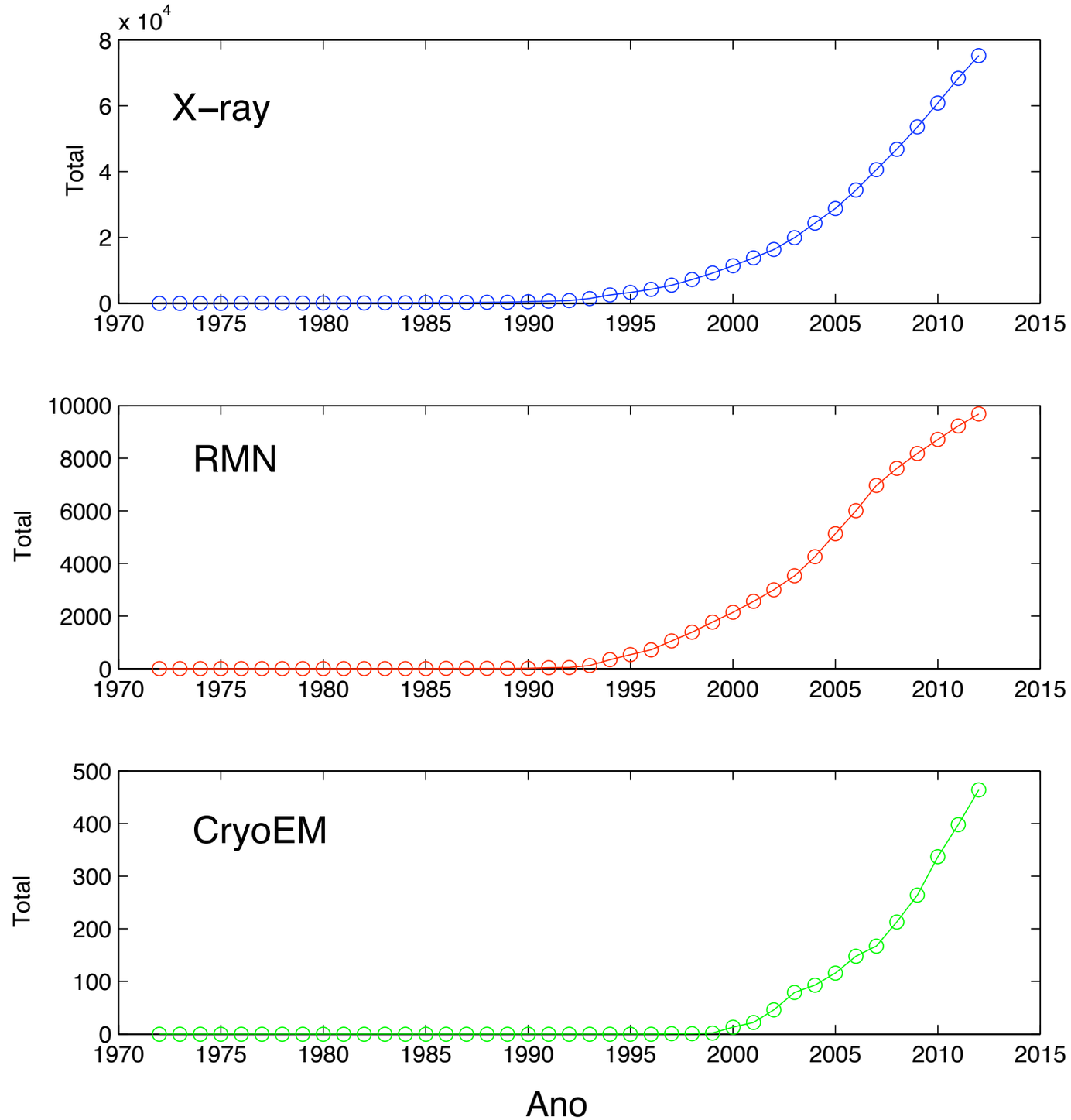
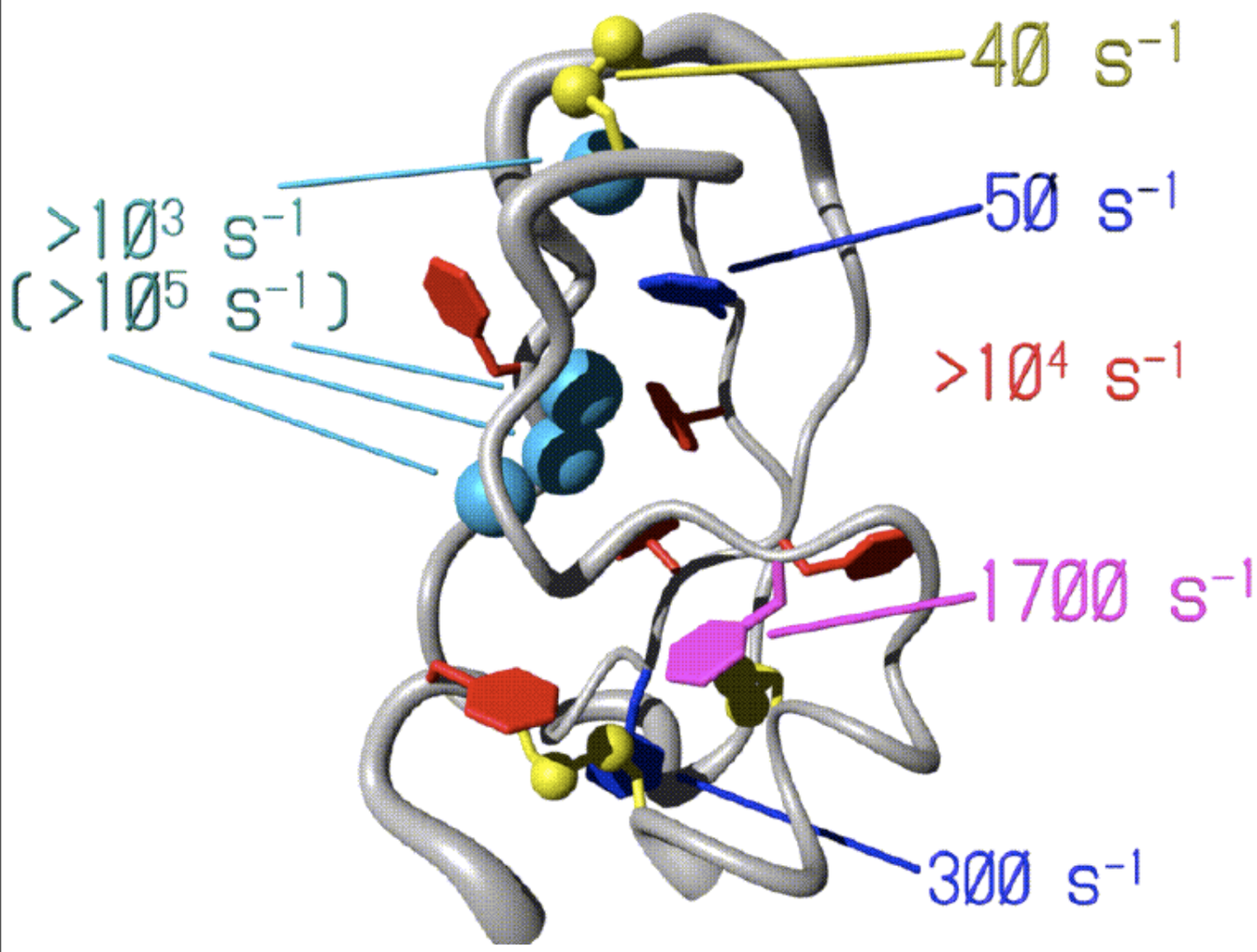
Angewandte Chemie
3340 © 2003 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim DOI: 10.1002/anie.200300595 *Angew. Chem. Int. Ed.* **2003**, *42*, 3340–3363



Número de estruturas depositadas no PDB ao longo dos anos

Proteínas não são estáticas, ao contrário, experimentam várias conformações que interconvertem em diferentes escalas de tempo



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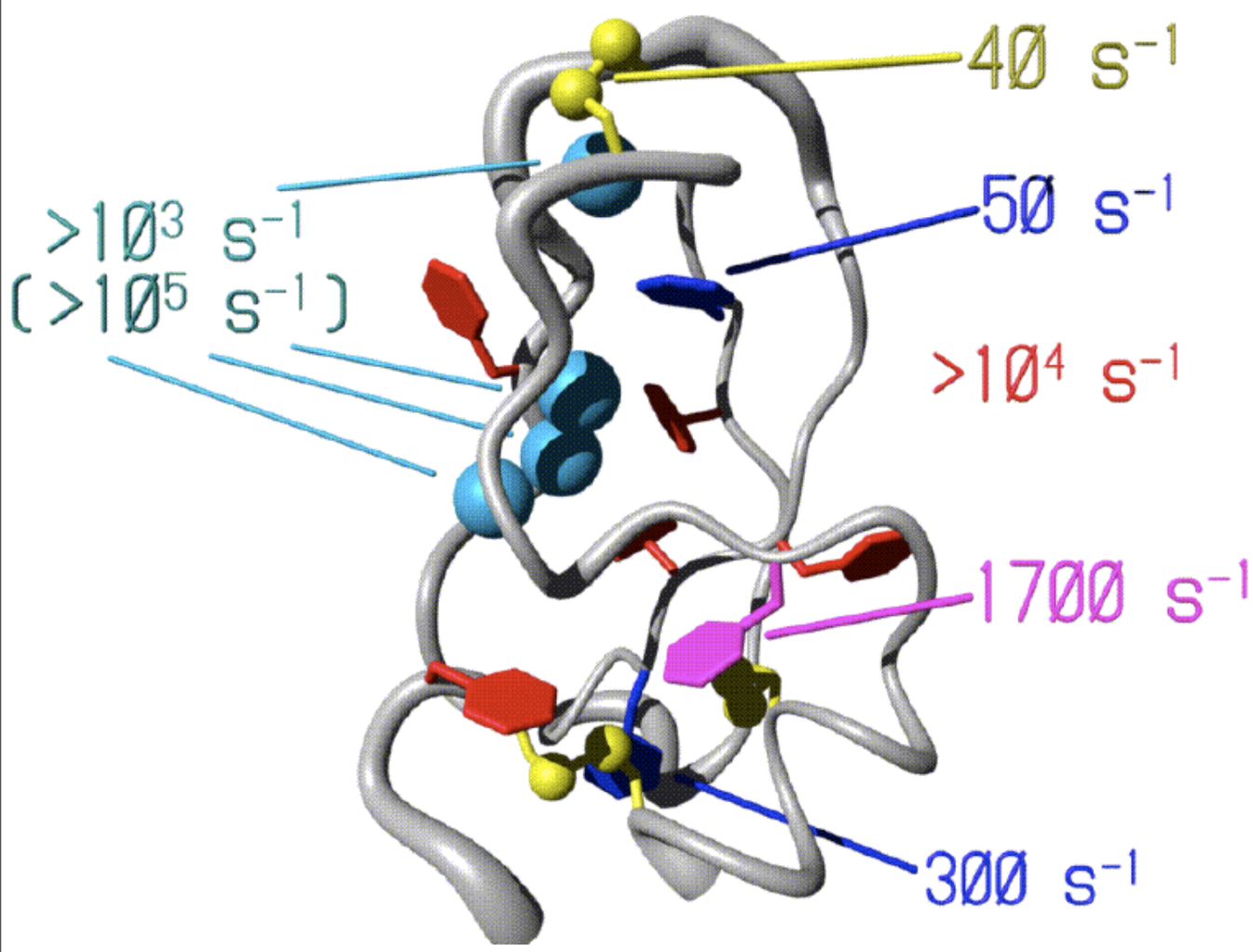

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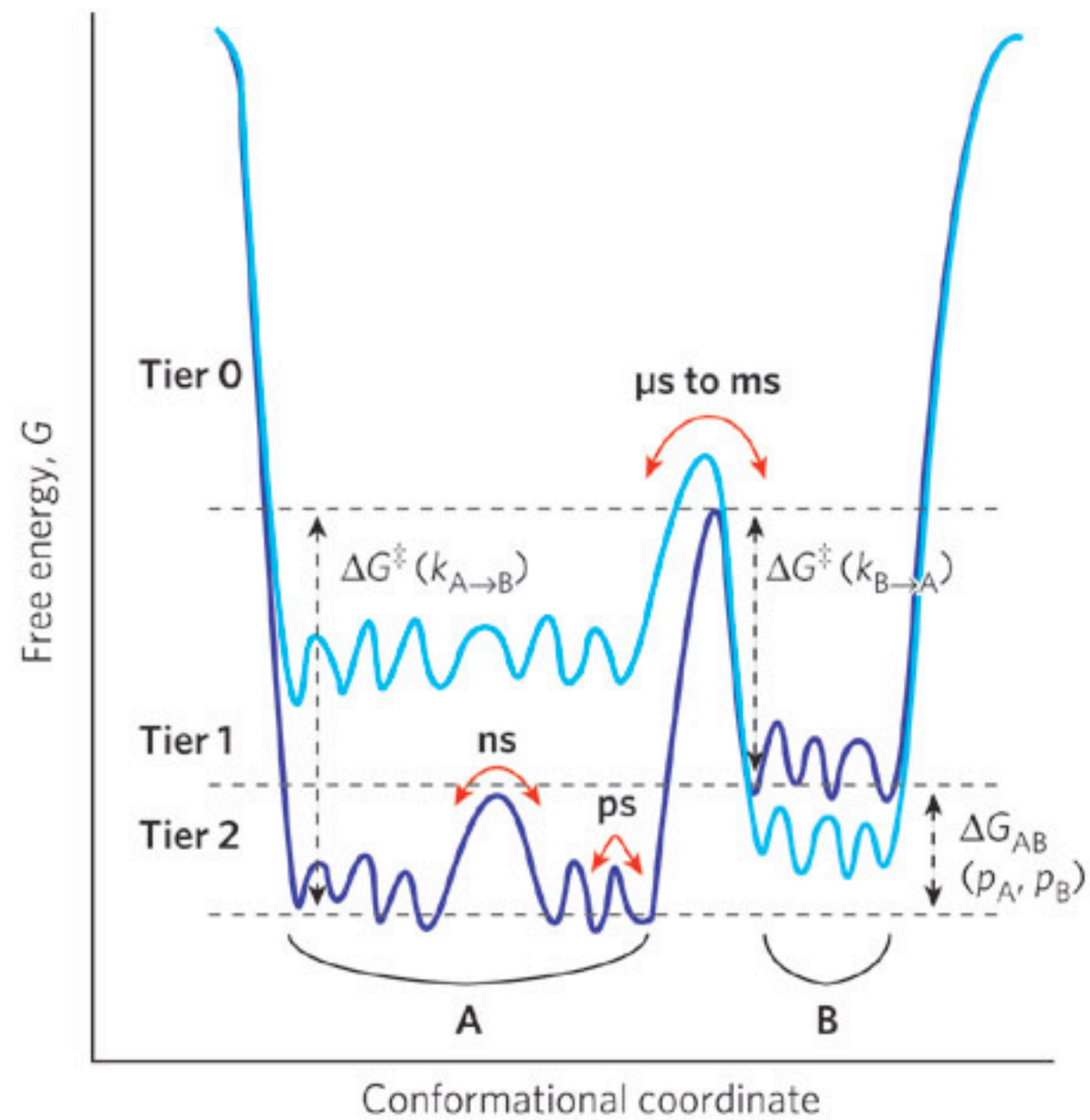
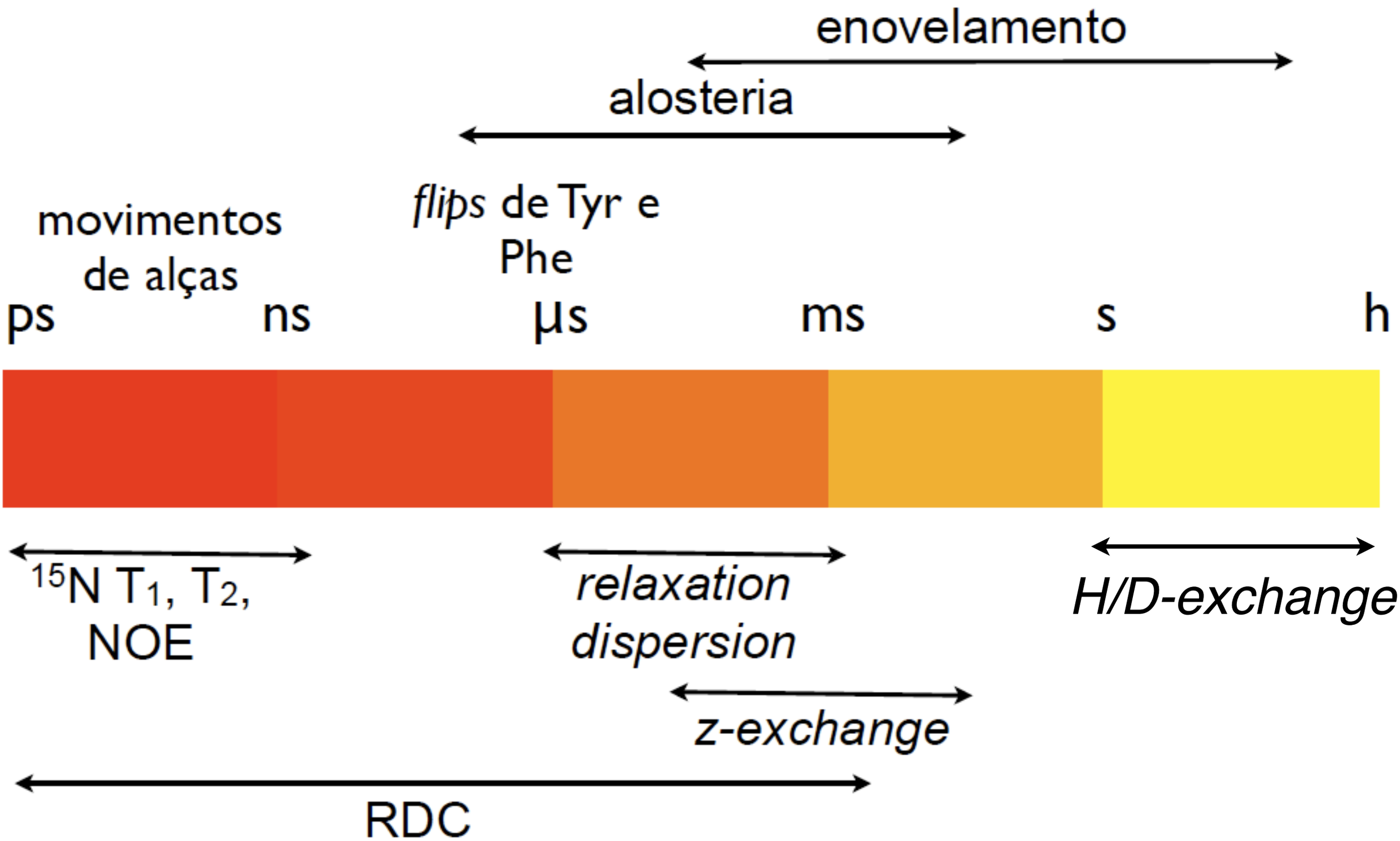


Henzler-Wildman, K. and Kern, D. (2007) *Nature* **450**: 964-972

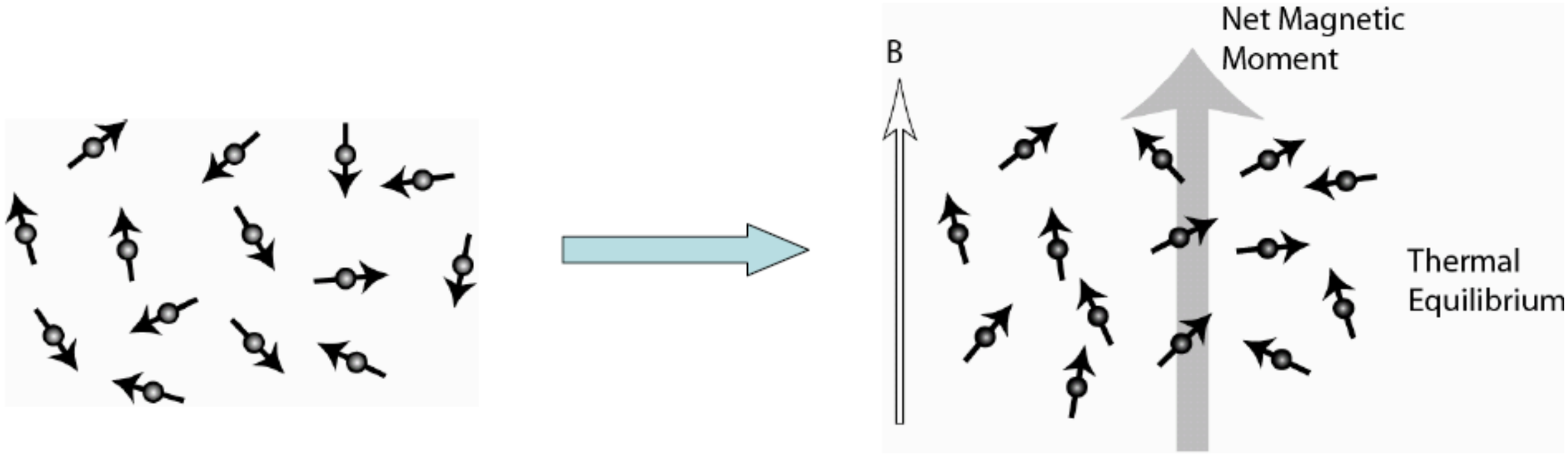
Qual é a natureza e a relevância funcional dos movimentos internos em uma proteína (dinâmica interna)?

- quais tipos de movimento proteínas experimentam?
- quais são as escalas de tempo envolvidas?
- estes movimentos/flexibilidade são importantes para a função biológica?
- como podemos descrever estrutura e dinâmica ao mesmo tempo?

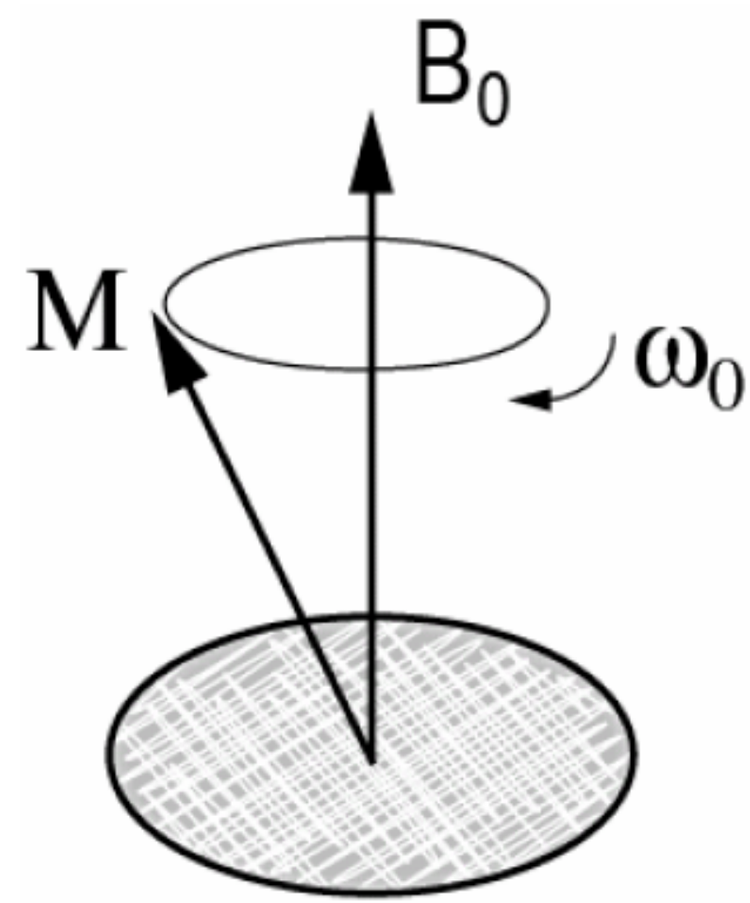
RMN fornece informações sobre dinâmica em diferentes escalas de tempo



Espectroscopia de RMN



O momento magnético macroscópico (**M**) assume um movimento de precessão ao redor do campo magnético externo (B_0), este movimento é caracterizado por uma frequência angular ω_0, característica de cada núcleo



Espectrômetros de RMN

$\omega_0 = -\gamma B_0$

B_0 = 18 Tesla

ω_0 = 800 MHz (^{1}H)

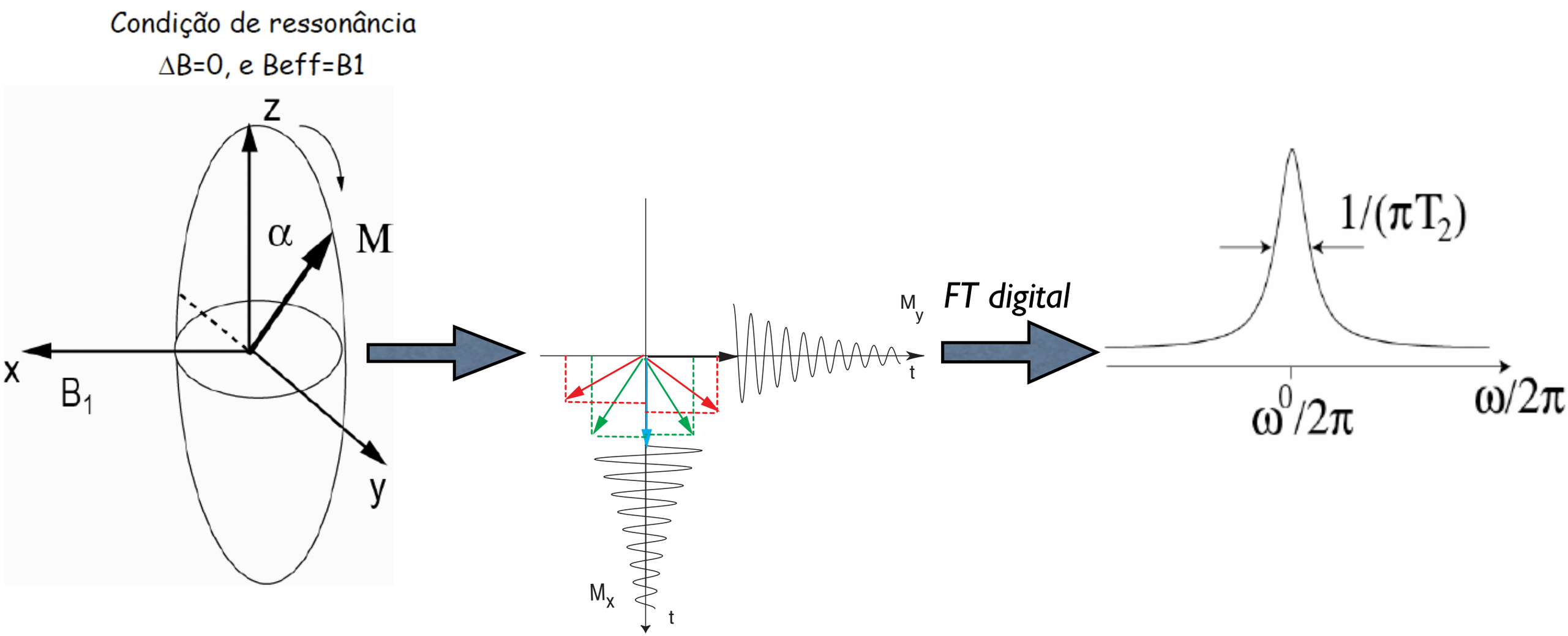
1 ppm = 800 Hz

B_0 = 11 Tesla

ω_0 = 500 MHz (^{1}H)

1 ppm = 500 Hz

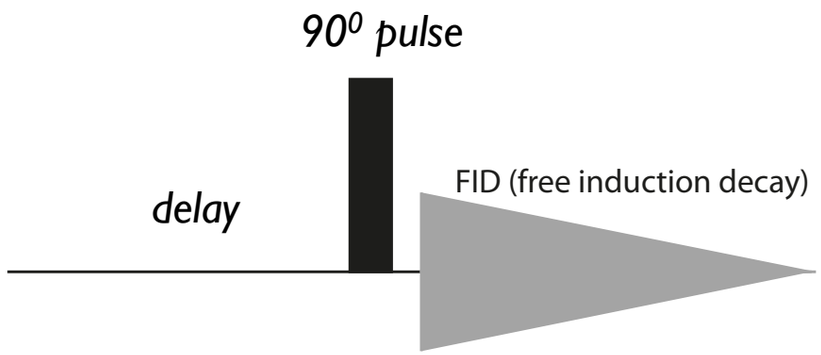
A aplicação de um campo magnético oscilante (**B_1**), perpendicular a **B_0**, e com frequência $\omega_{rf} \sim \omega_0$ (condição de ressonância) desloca **M** da posição de equilíbrio

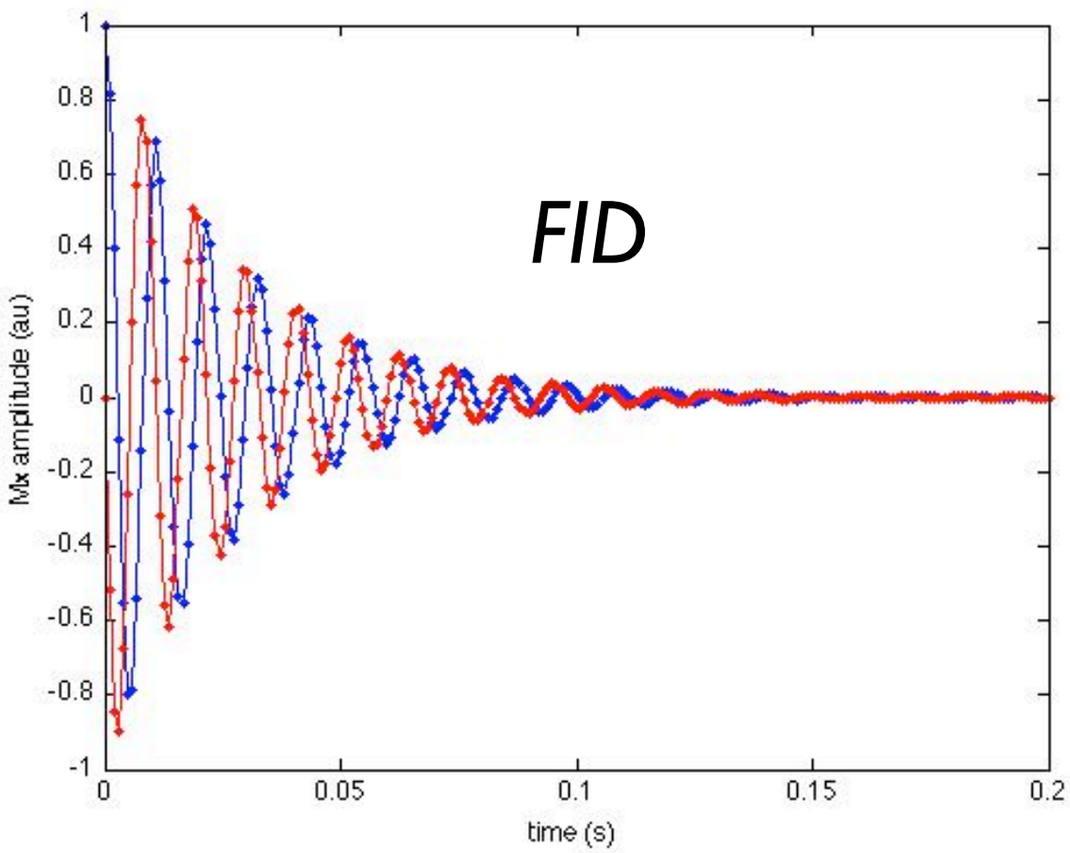


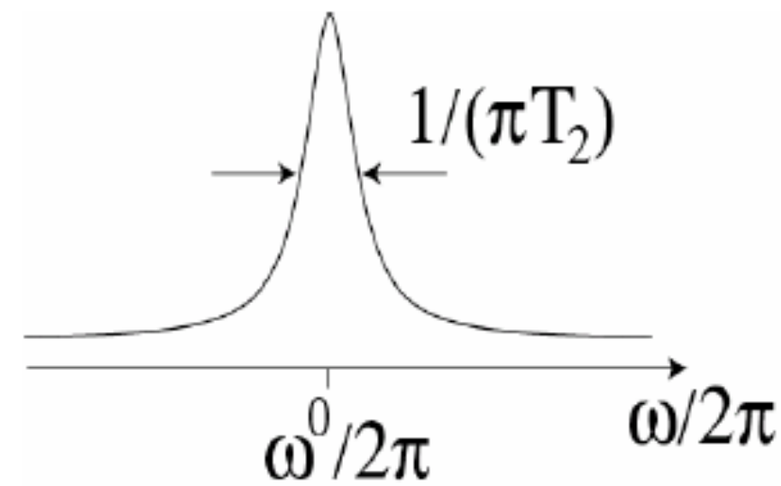
Imediatamente após a aplicação do pulso de 90^0, **M** passa a precessar ao redor de B_0 induzindo a formação de uma corrente na bobina detectora (*Free Induction Decay*)

O experimento de um pulso de 90^0

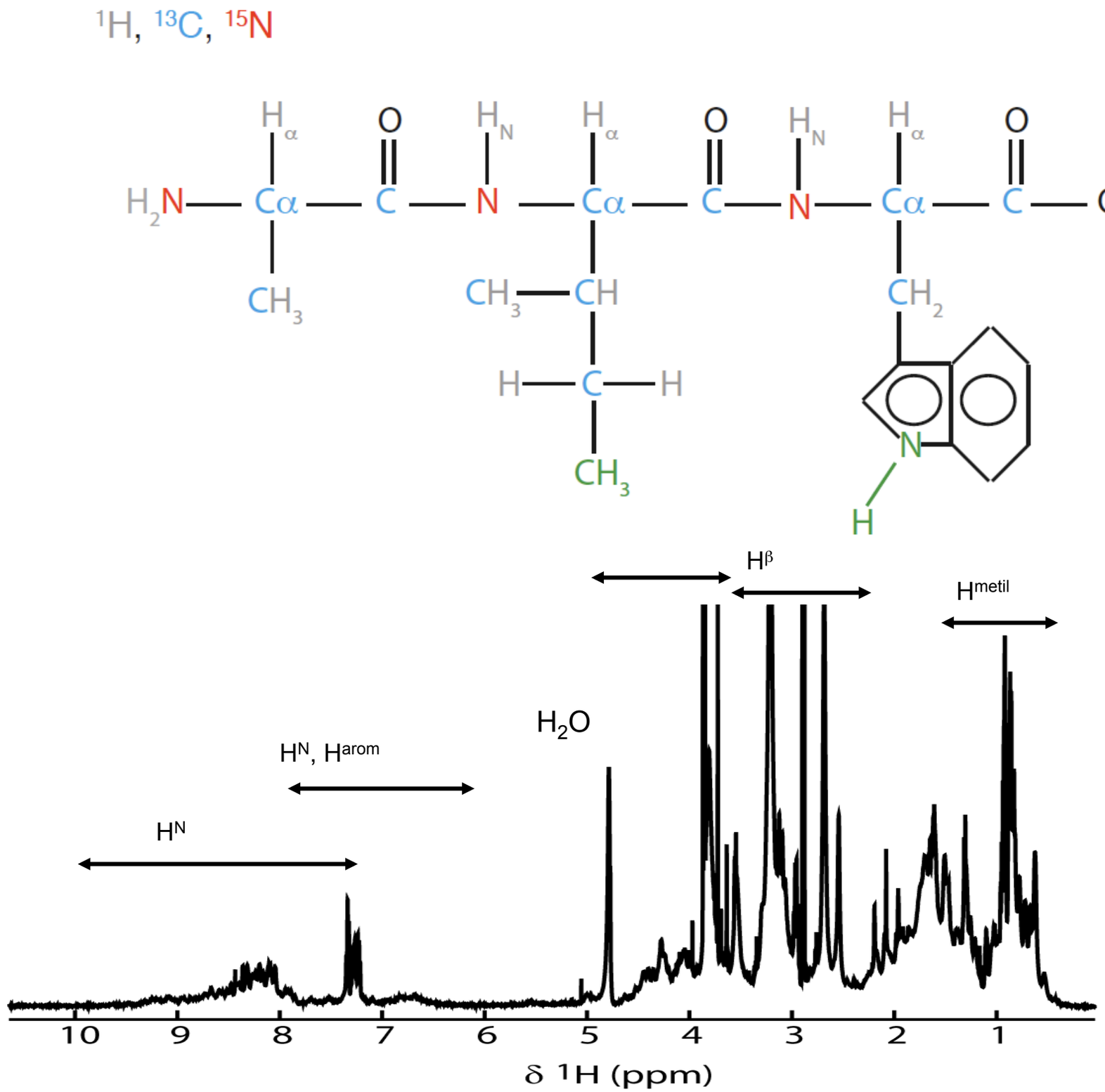
1D 1H





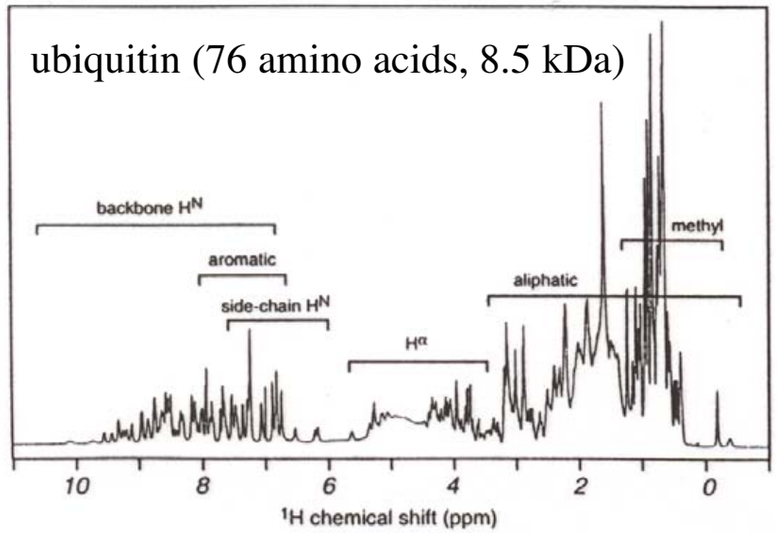


Típico espectro de RMN de uma proteína: a frequência de precessão (δ_0) de cada spin é característica do seu ambiente químico

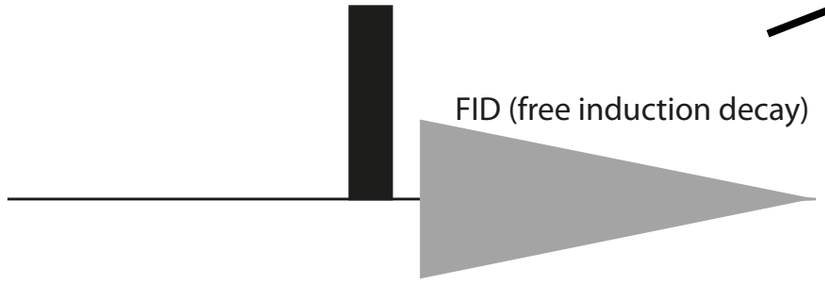


Protein NMR

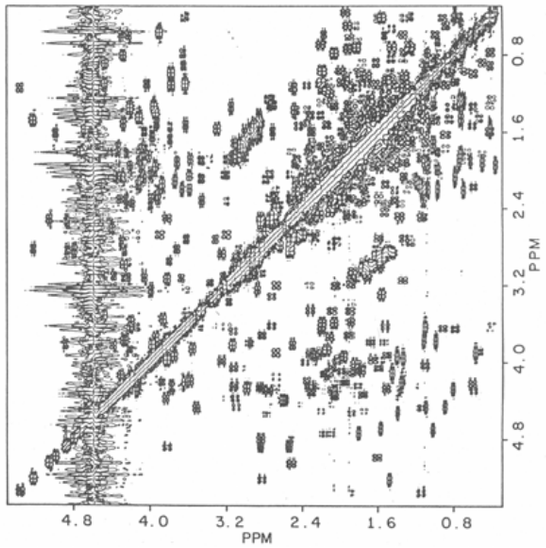
Protein 1D-^{1}H spectra display large spectral overlap; it is possible to obtain a general idea about the degree of folding



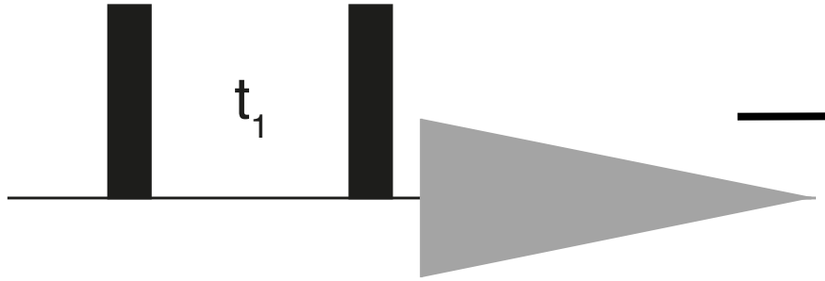
1D 1H



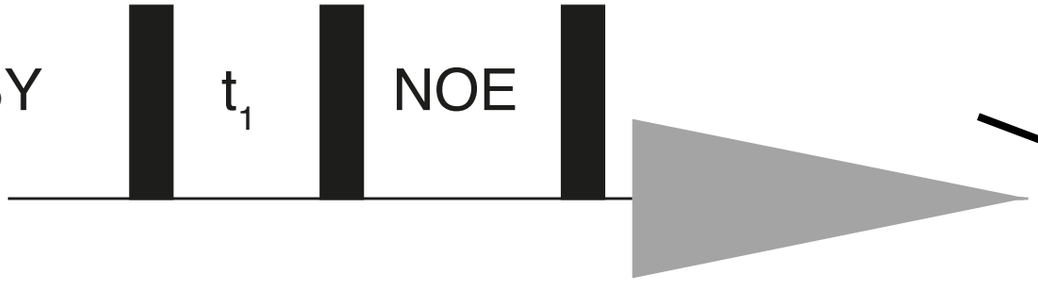
cytochrome *c*, 12.5 kDa

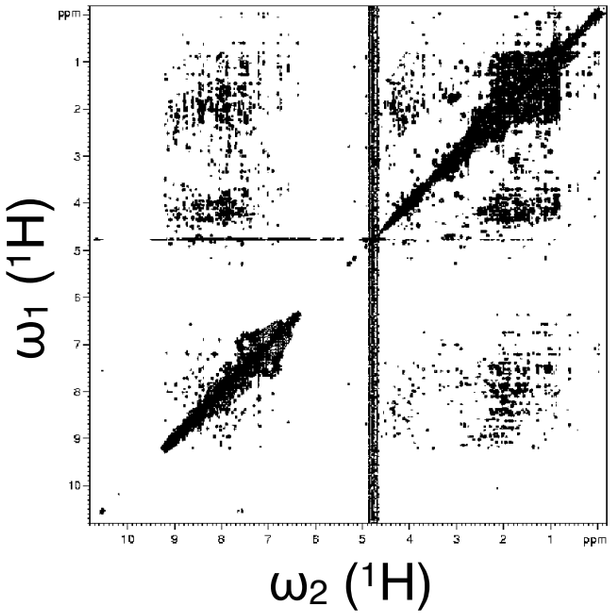


2D COSY



2D NOESY





Addition of a second spectral dimension alleviates the spectral overlap, by spreading the information along an extra dimension, for example COSY or NOE correlations (applicable to small peptides and proteins up to 5-6 kDa)

NOEs de longo alcance são a principal fonte de determinação estrutural

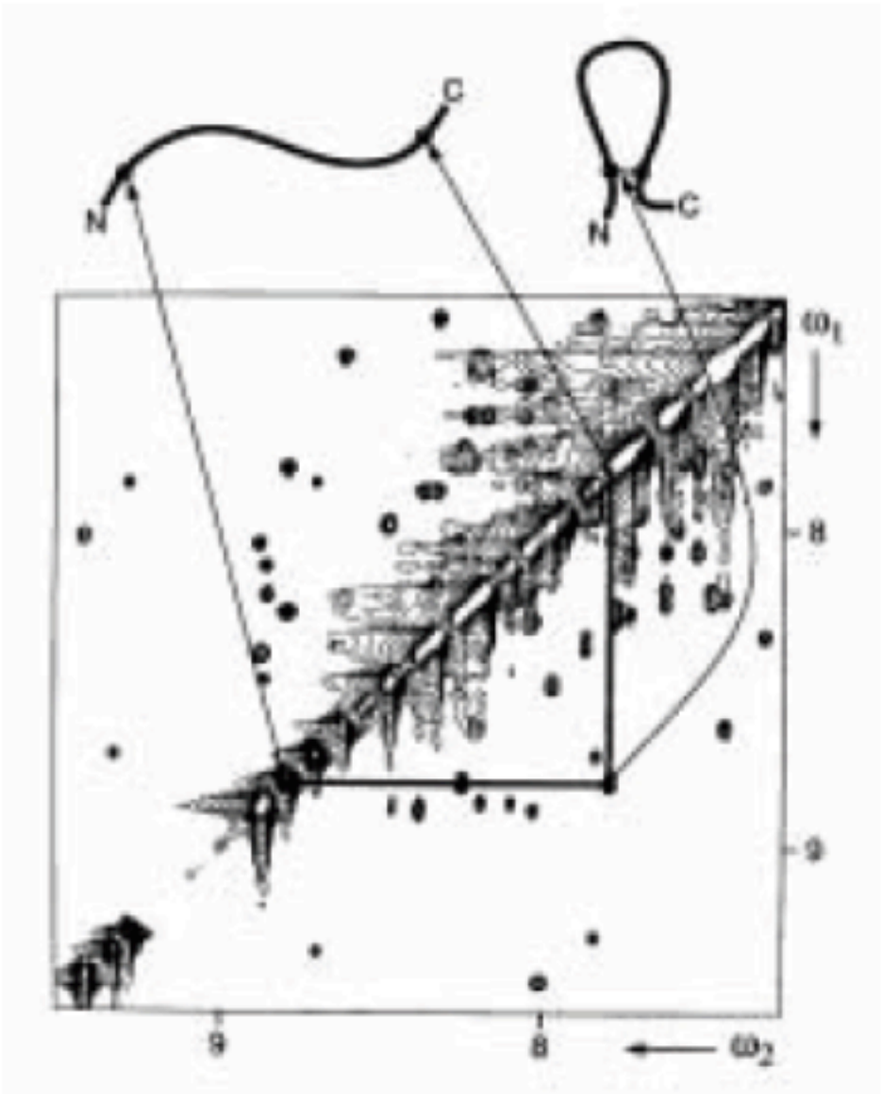
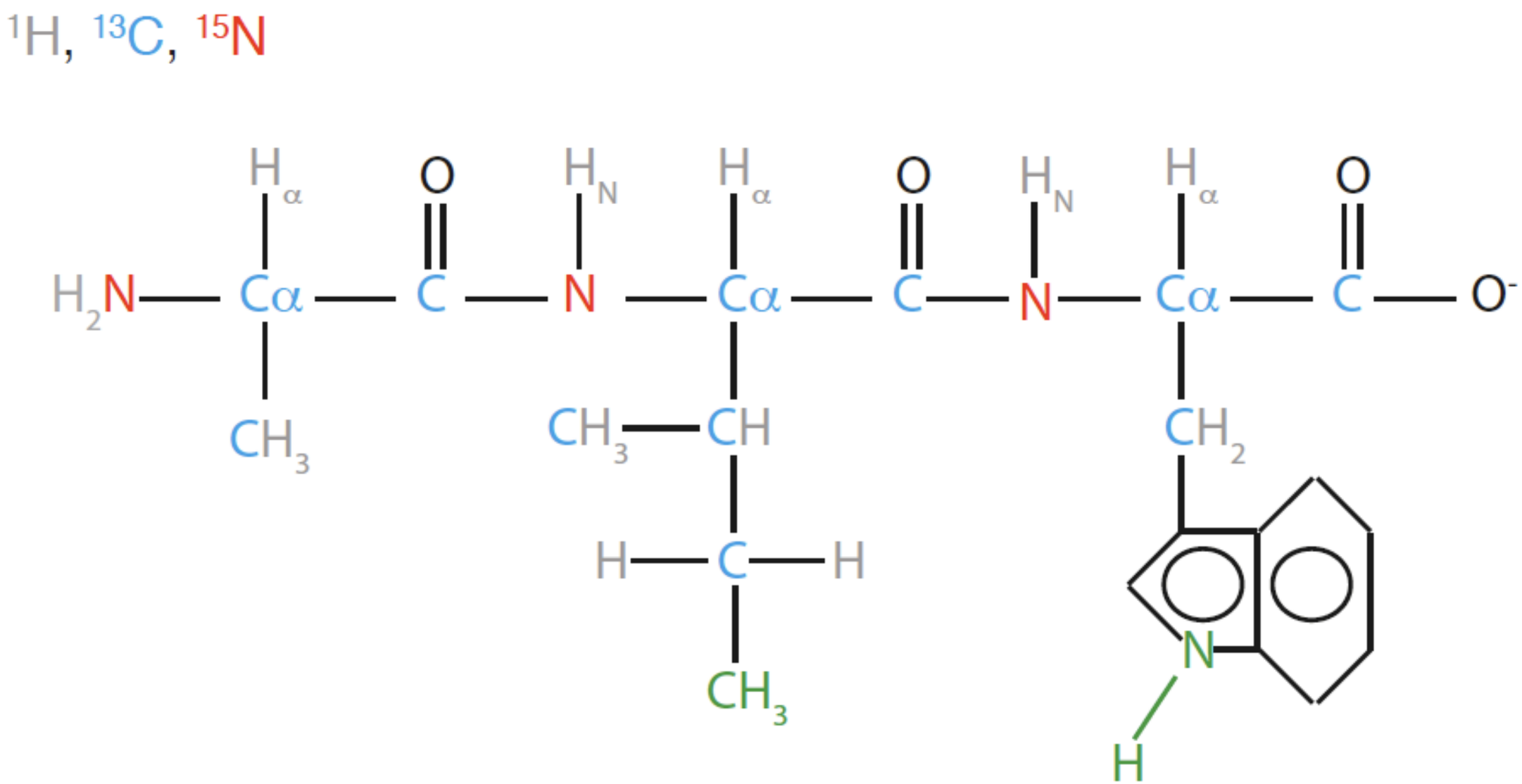


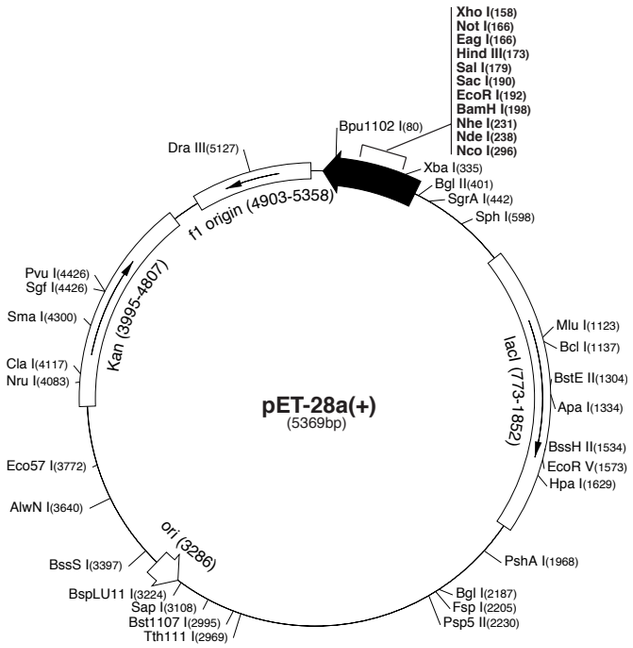
Figure 24. Scheme indicating the relations between an experimental 2D [^{1}H,^{1}H]-NOESY spectrum, a polypeptide with the chain ends indicated by N and C, sequence-specific assignments for two hydrogen atoms in the polypeptide chain indicated by circles, and the NOE upper distance constraint derived from the NOESY cross-peak connecting the chemical shift positions of the two assigned hydrogen atoms (see text).

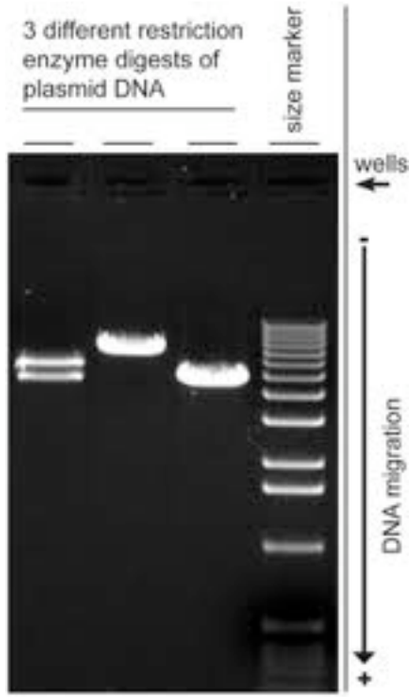
Experimentos com proteínas maiores que 10 kDa requerem marcação isotópica

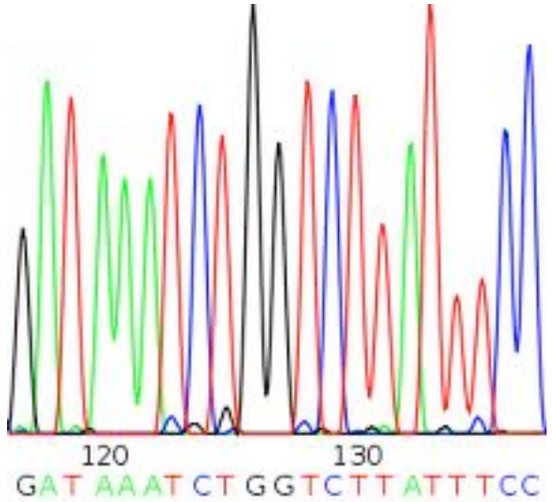


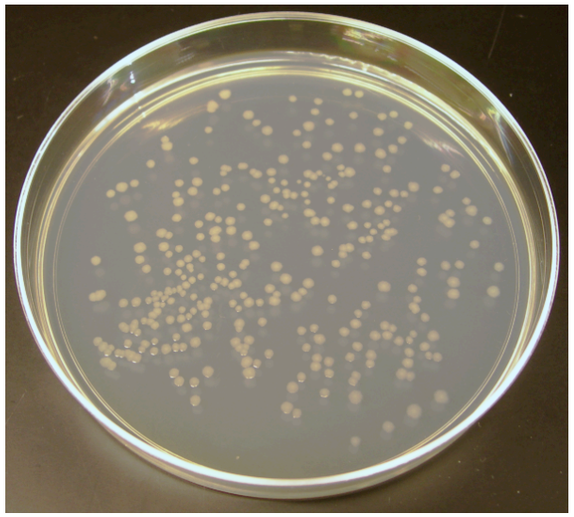
Isotope enrichment (^{15}N and ^{13}C) allows the acquisition of multidimensional heteronuclear experiments, which contribute to further decrease the spectral overlap and increase the molecular weight limit (20-30 kDa)

Produção de proteínas enriquecidas com ^{15}N, ^{13}C ou ^{2}H





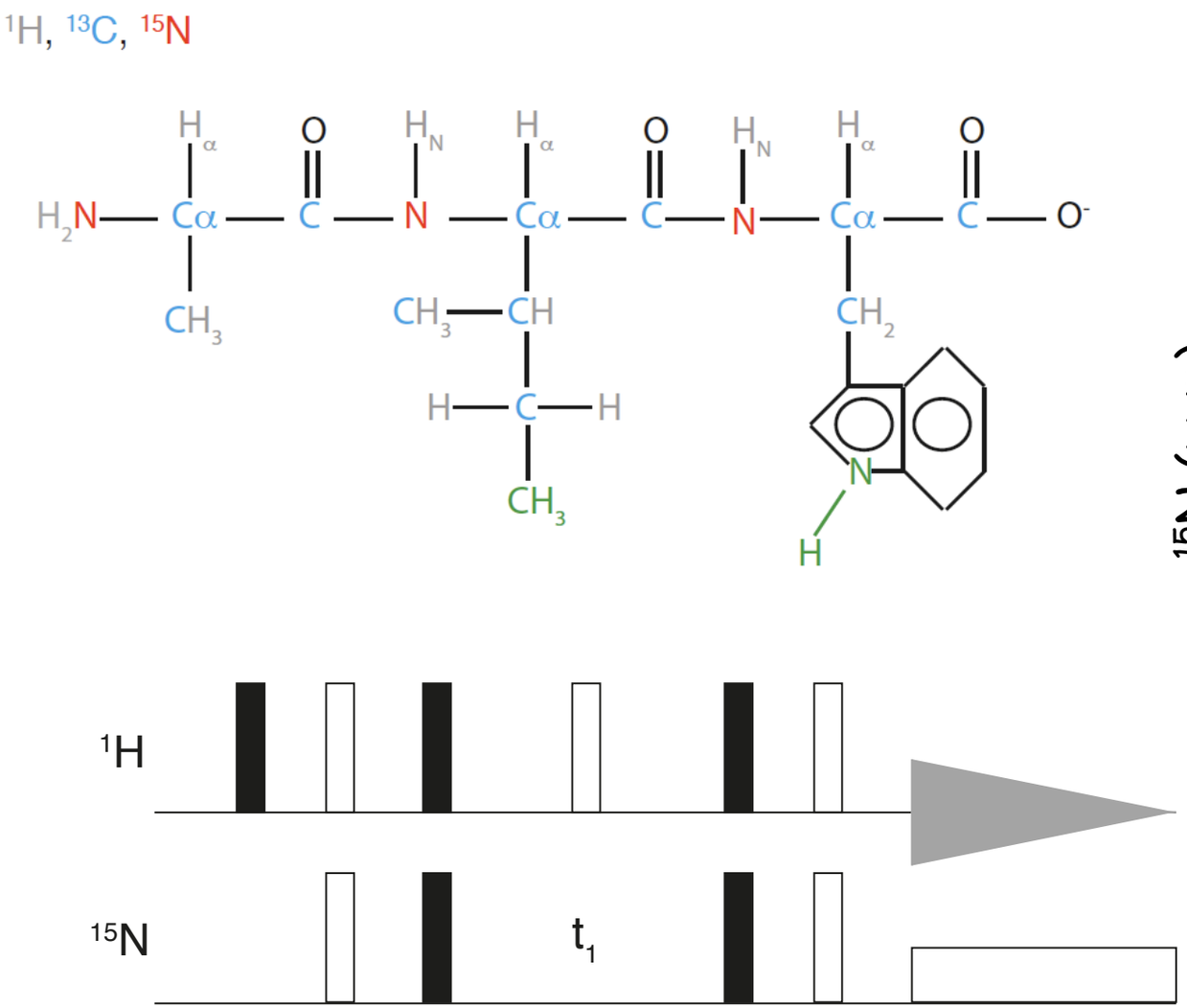


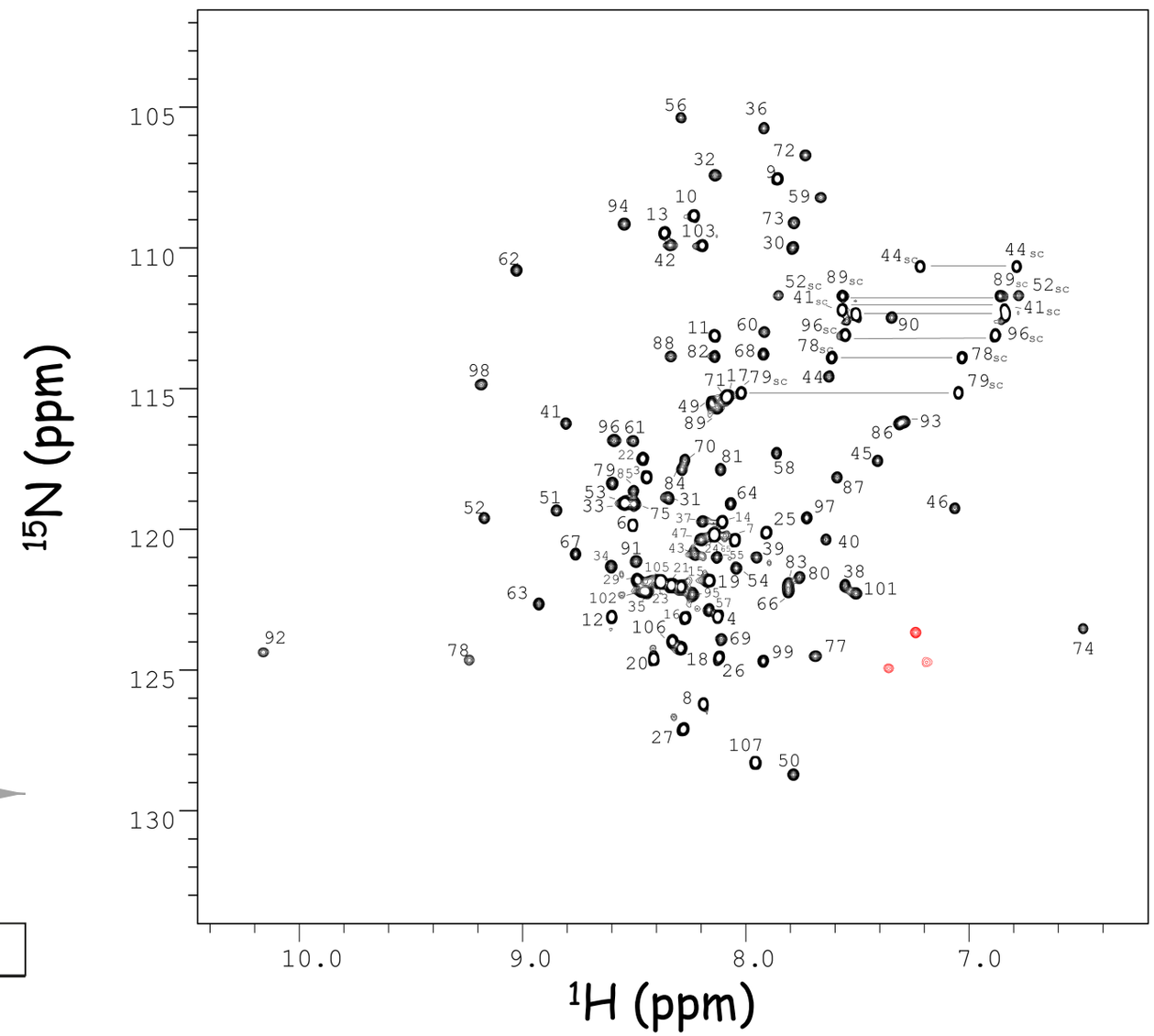


Heteronuclear NMR

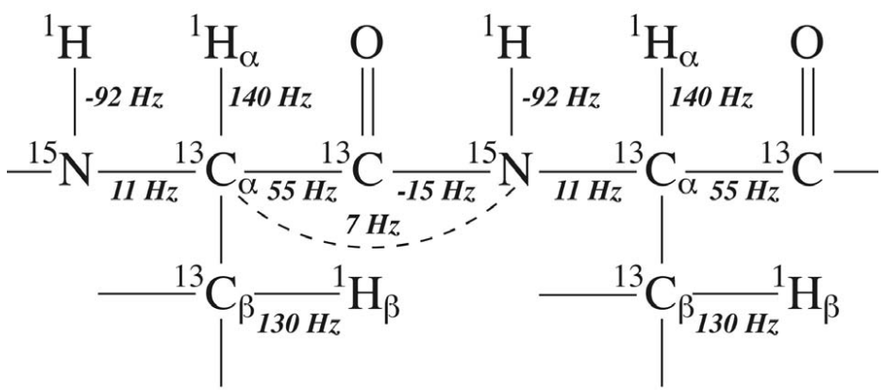
2D ^{1}H-^{15}N (or ^{1}H-^{13}C) HSQC or HMQC experiments filter out signals from protons not attached to ^{15}N (or ^{13}C). Addition of a second ^{15}N or ^{13}C dimension reduces the spectral overlap observed in the 1D-^{1}H spectrum

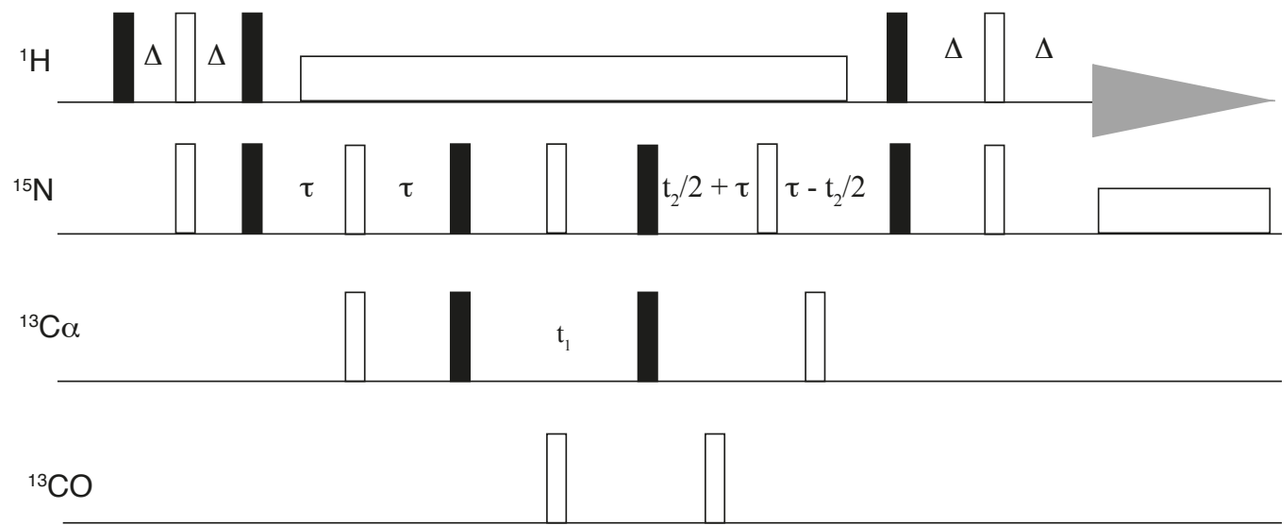
O espectro de HSQC



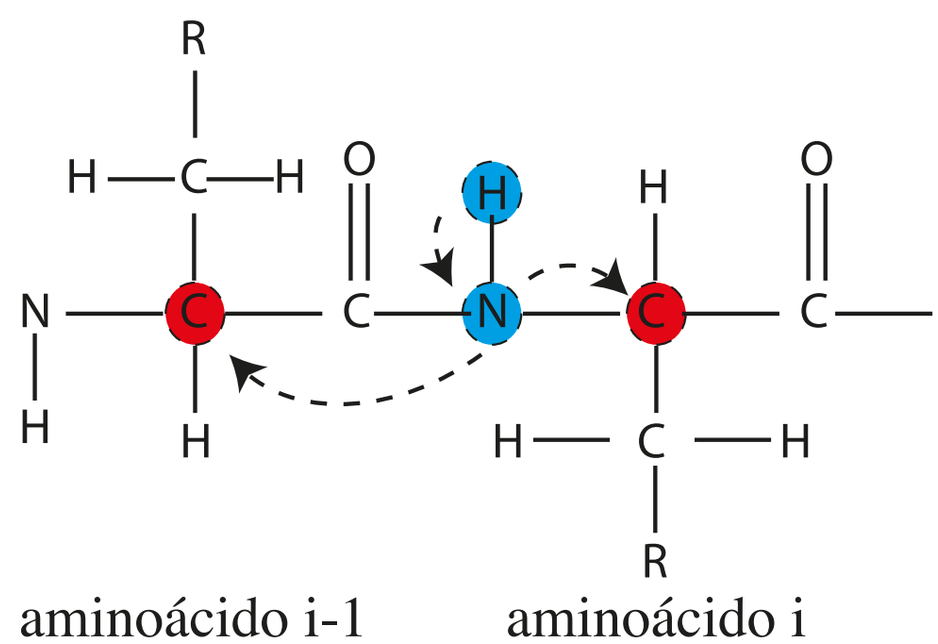


Double labelling with ^{13}C and ^{15}N allows the use of triple resonance experiments to obtain backbone resonance assignments, further expanding the molecular weight limit to 20-30 kDa



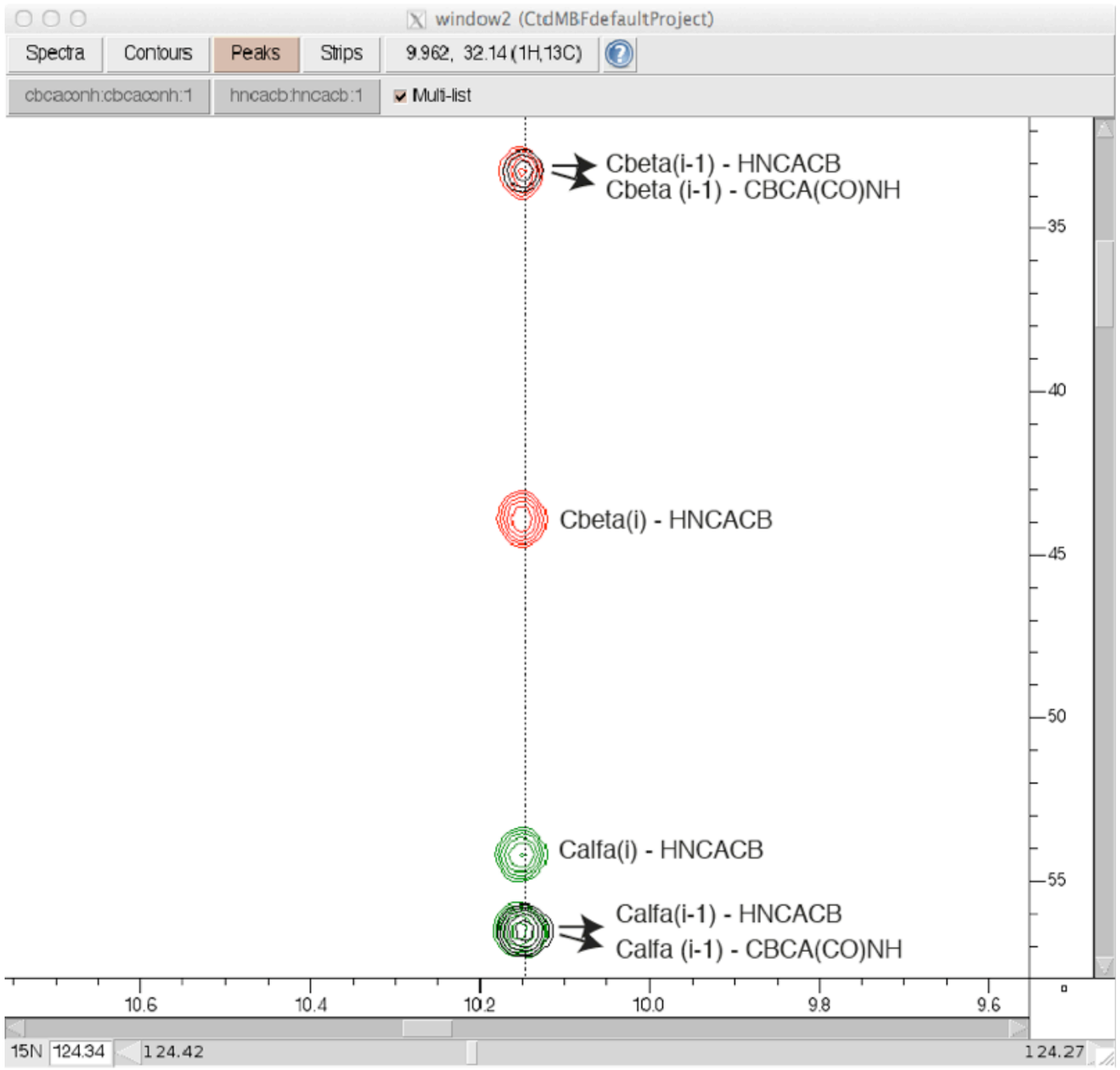


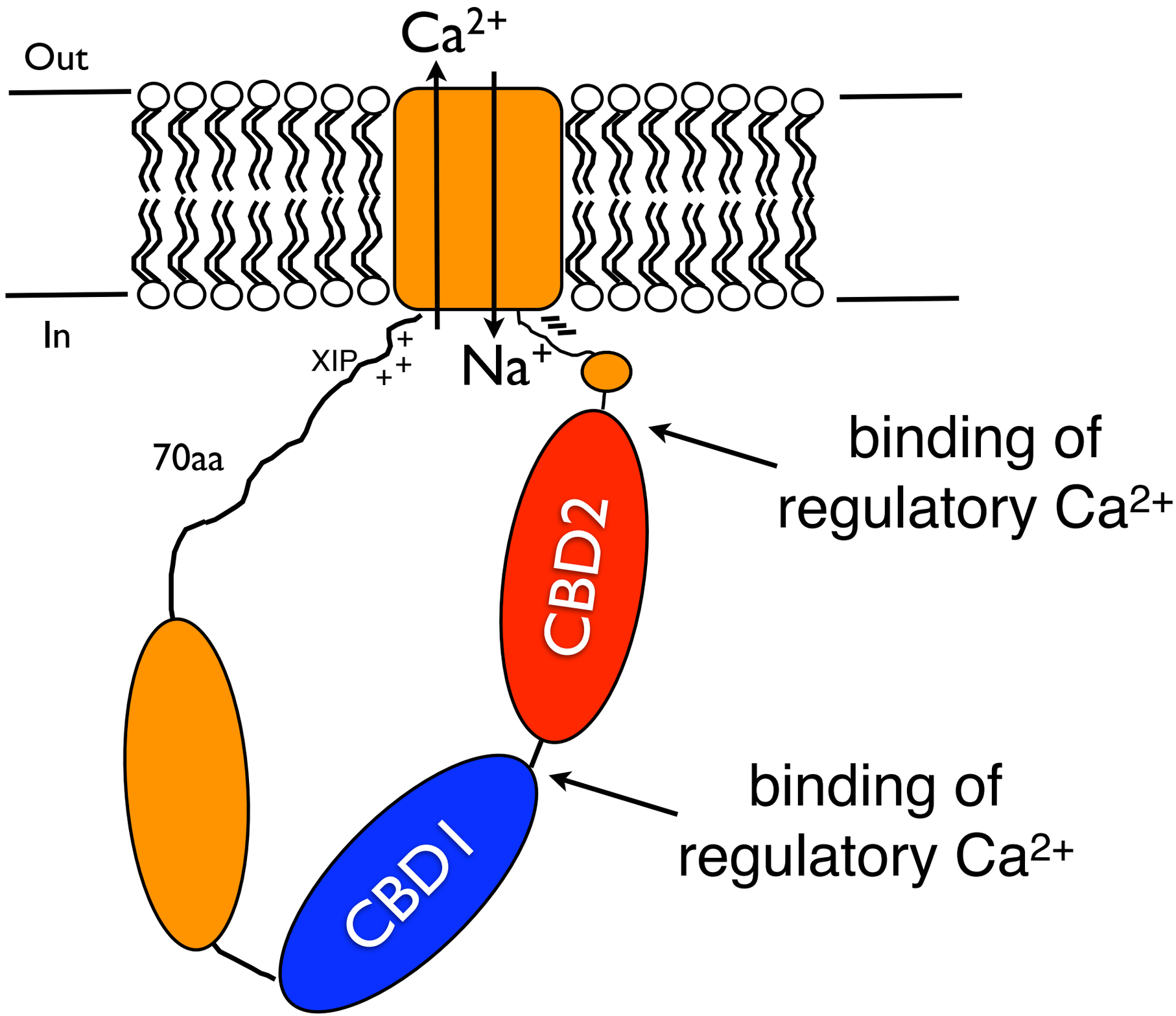
HNCA: N(i)-H(i)-Ca(i)/Ca(i-1)



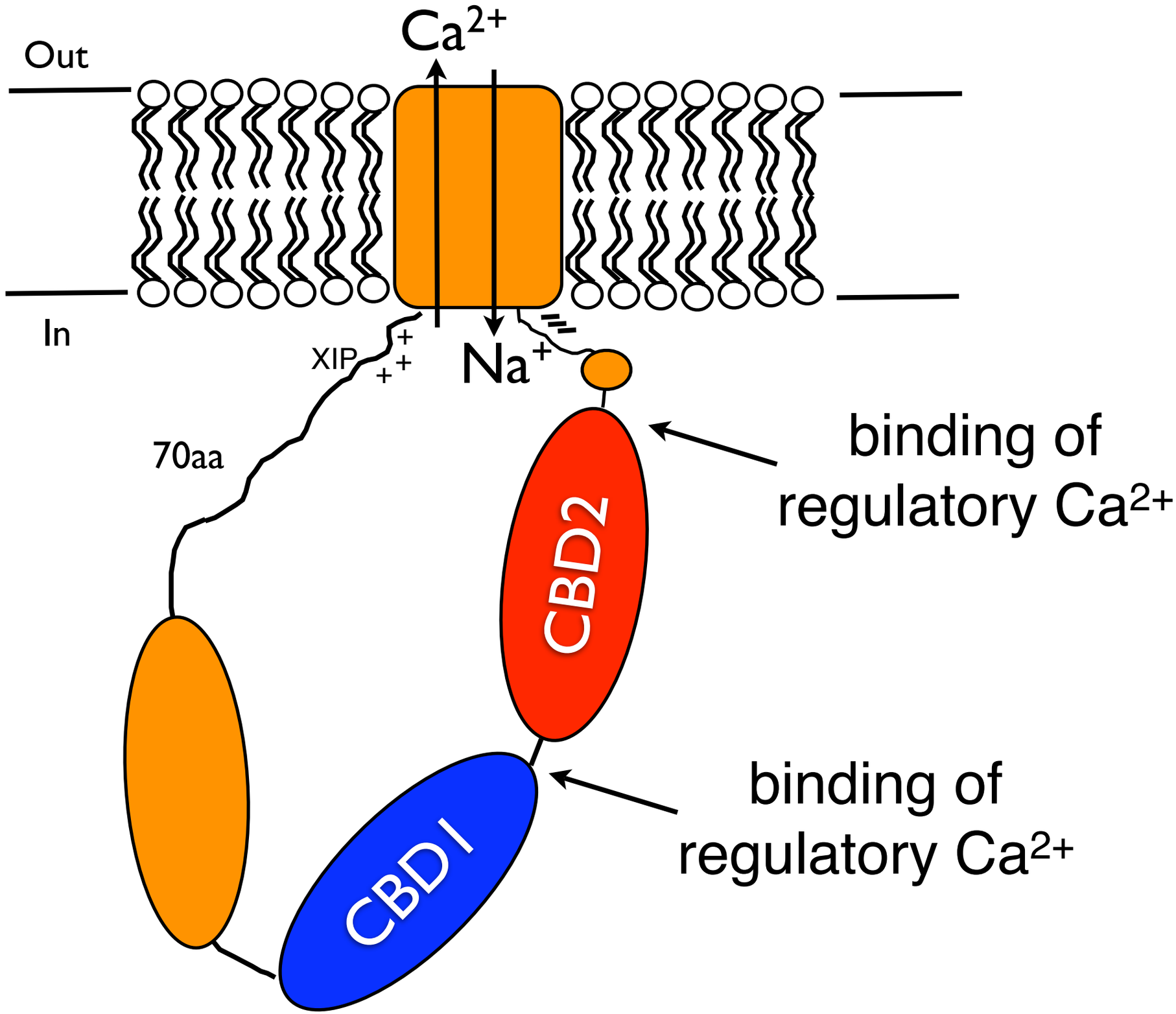
-^{1}J and ^{2}J are uniform along the protein backbone; independent of the polypeptide conformation

Example: HNCACB and CBCA(CO)NH

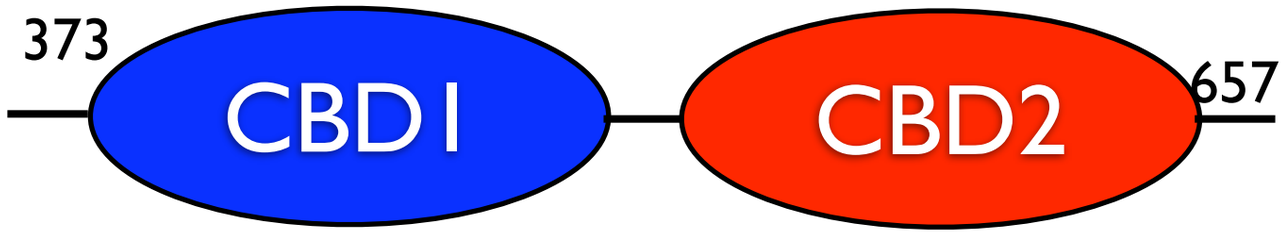



Out
In
Ca^{2+}
Na^+
XIP
70aa
CBD2
CBD1
binding of regulatory Ca^{2+}
binding of regulatory Ca^{2+}

How does the binding of regulatory Ca^{2+} to the cytoplasmic domain regulates the Na^+/Ca^{2+} exchanger?



Goal

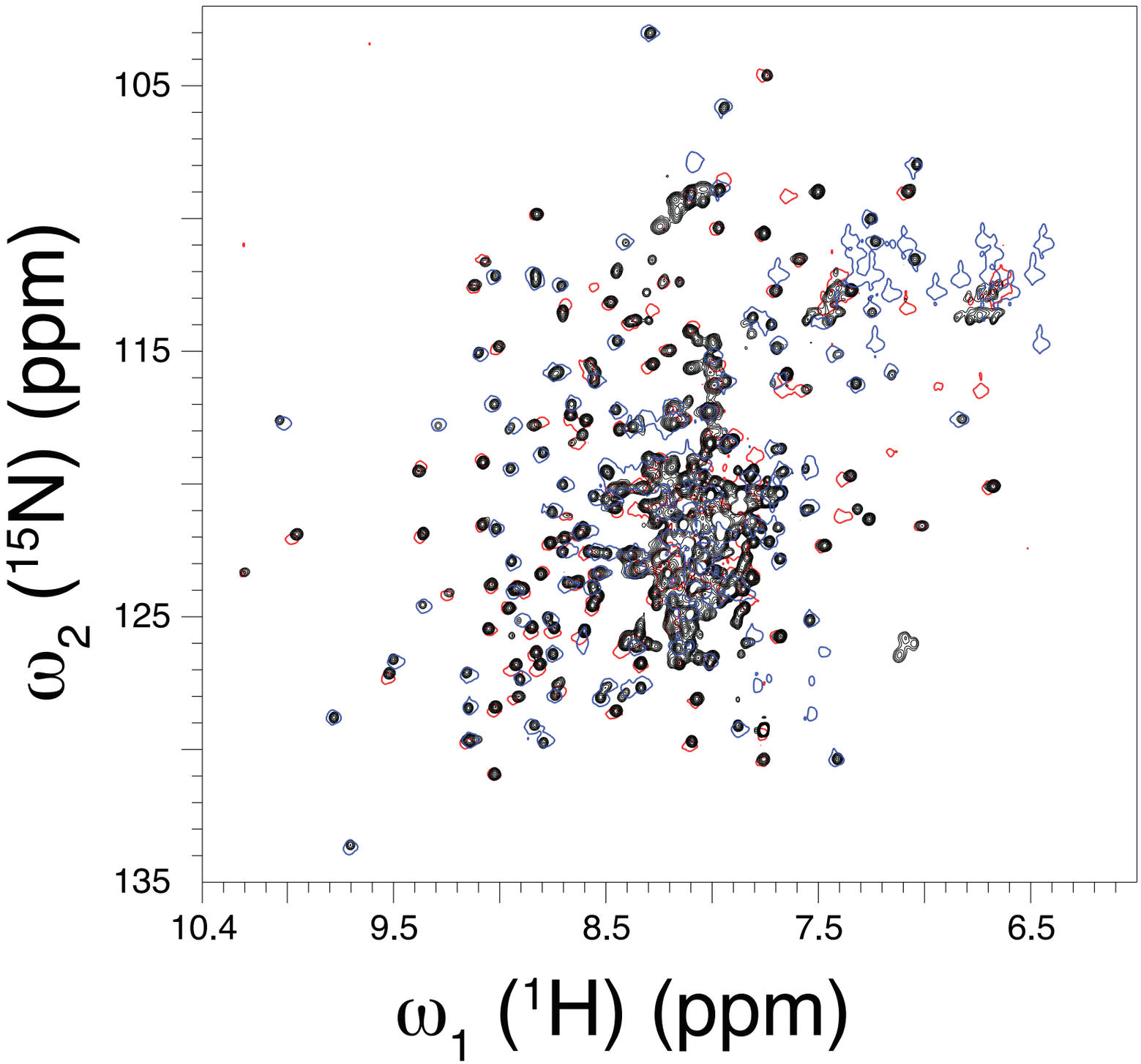


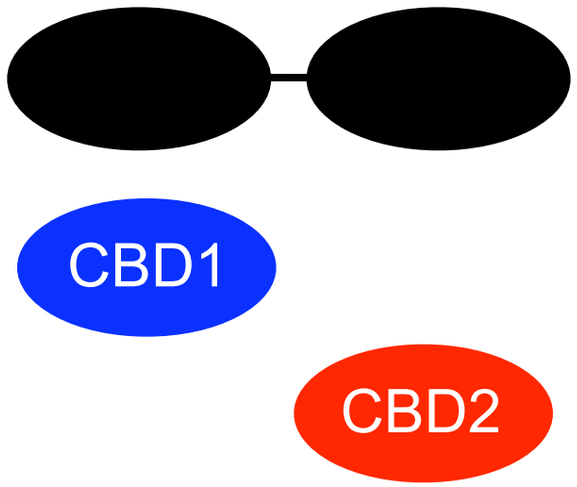
Characterize the inter-domain dynamics of CBD12 using ^{15}N relaxation and RDCs

1. How does CBD1 and CBD2 interact with each other?
2. How does the binding of Ca^{2+} to either one of the two domains modulate this interaction?

Salinas et al. (2011) *J. Biol. Chem.* **286**: 32123-32131

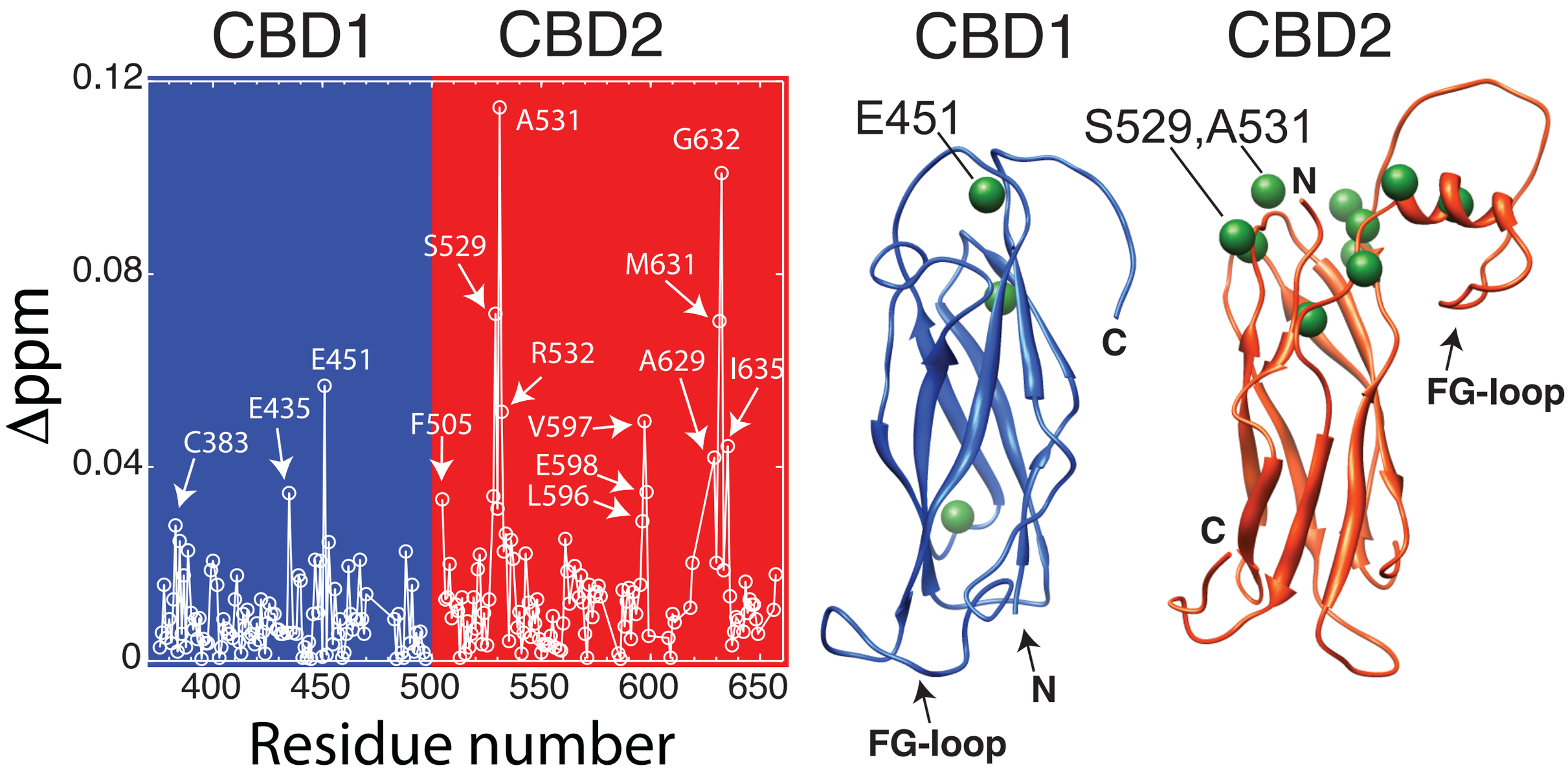
Chemical shift analysis reveals a minimal interaction interface between CBD1 and CBD2



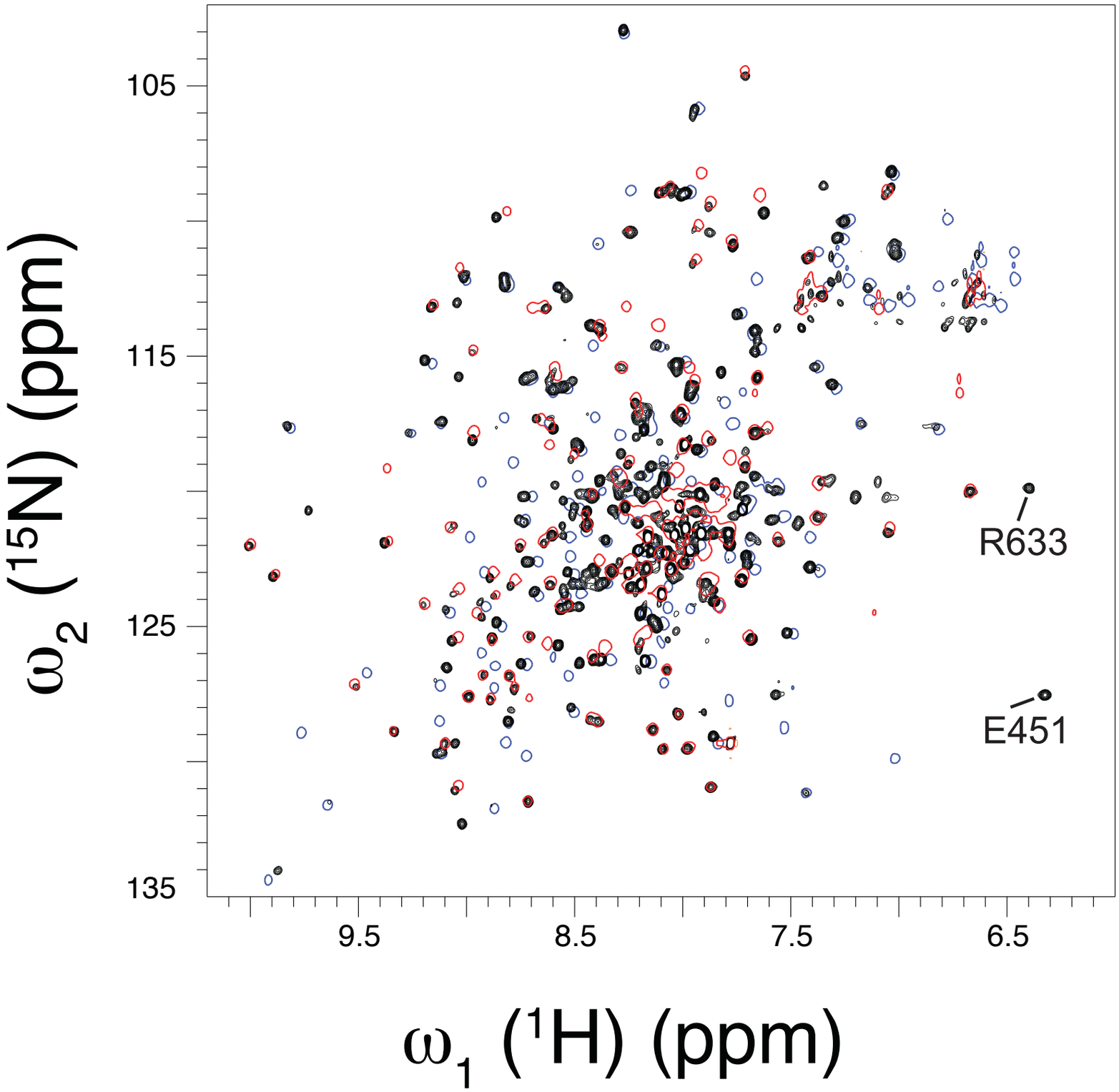


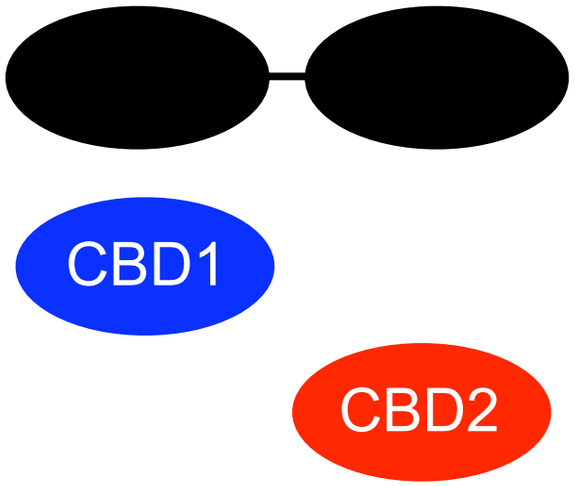
Free state

Free state


CBD1
CBD2
0.12
0.08
0.04
0
Δppm
A531
G632
S529
M631
E451
R532
A629
I635
E435
F505
V597
C383
E598
L596
400
450
500
550
600
650
Residue number
CBD1
CBD2
E451
S529,A531
N
C
FG-loop
C
N
FG-loop

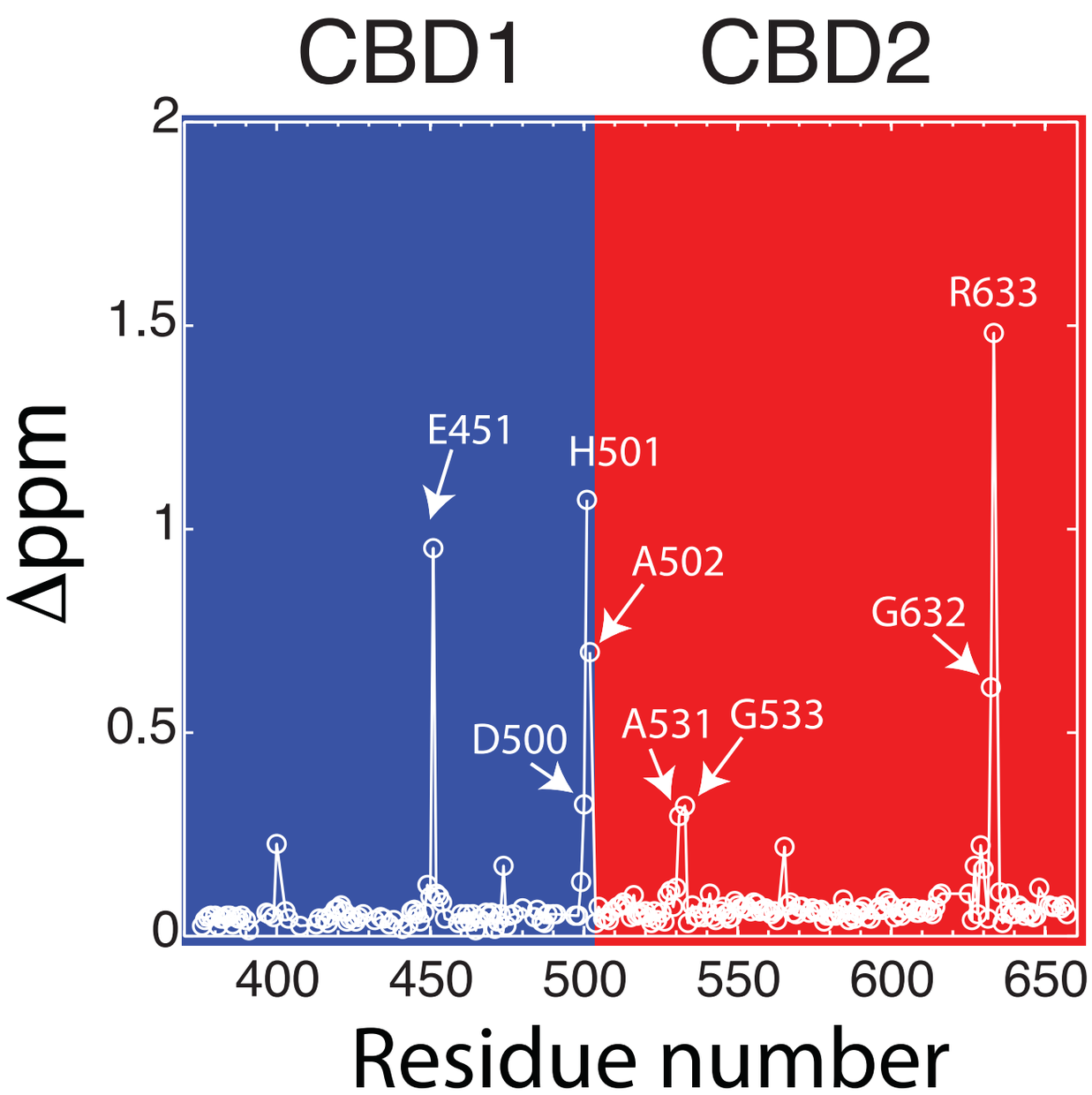
Chemical shift analysis reveals a minimal interaction interface between CBD1 and CBD2

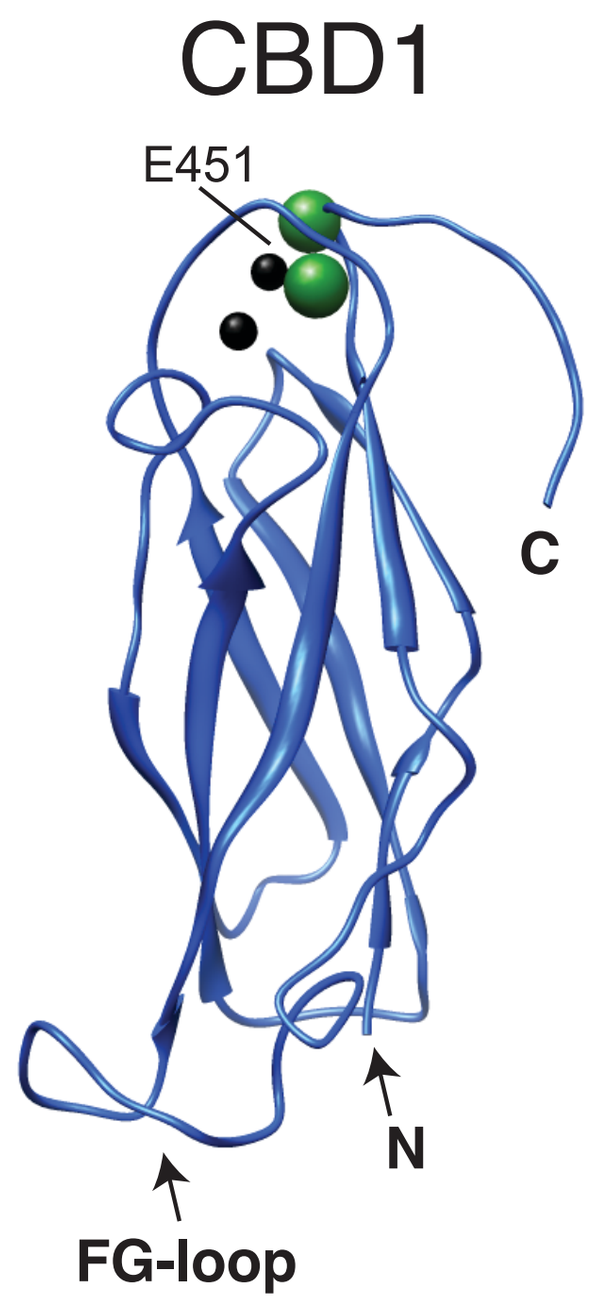


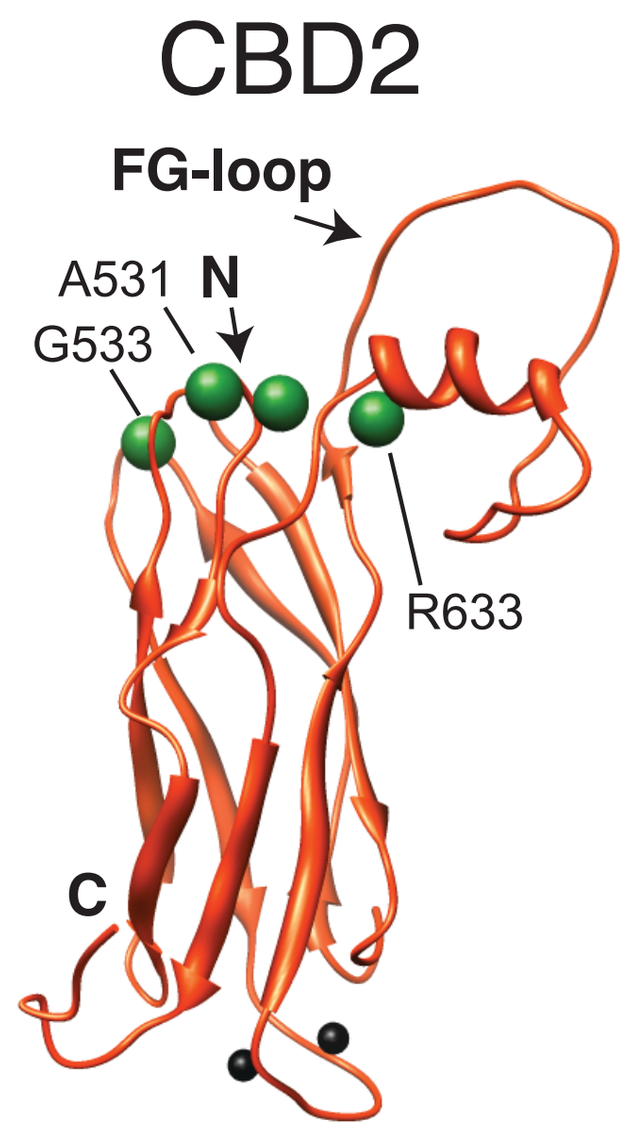


Ca^{2+}-bound state

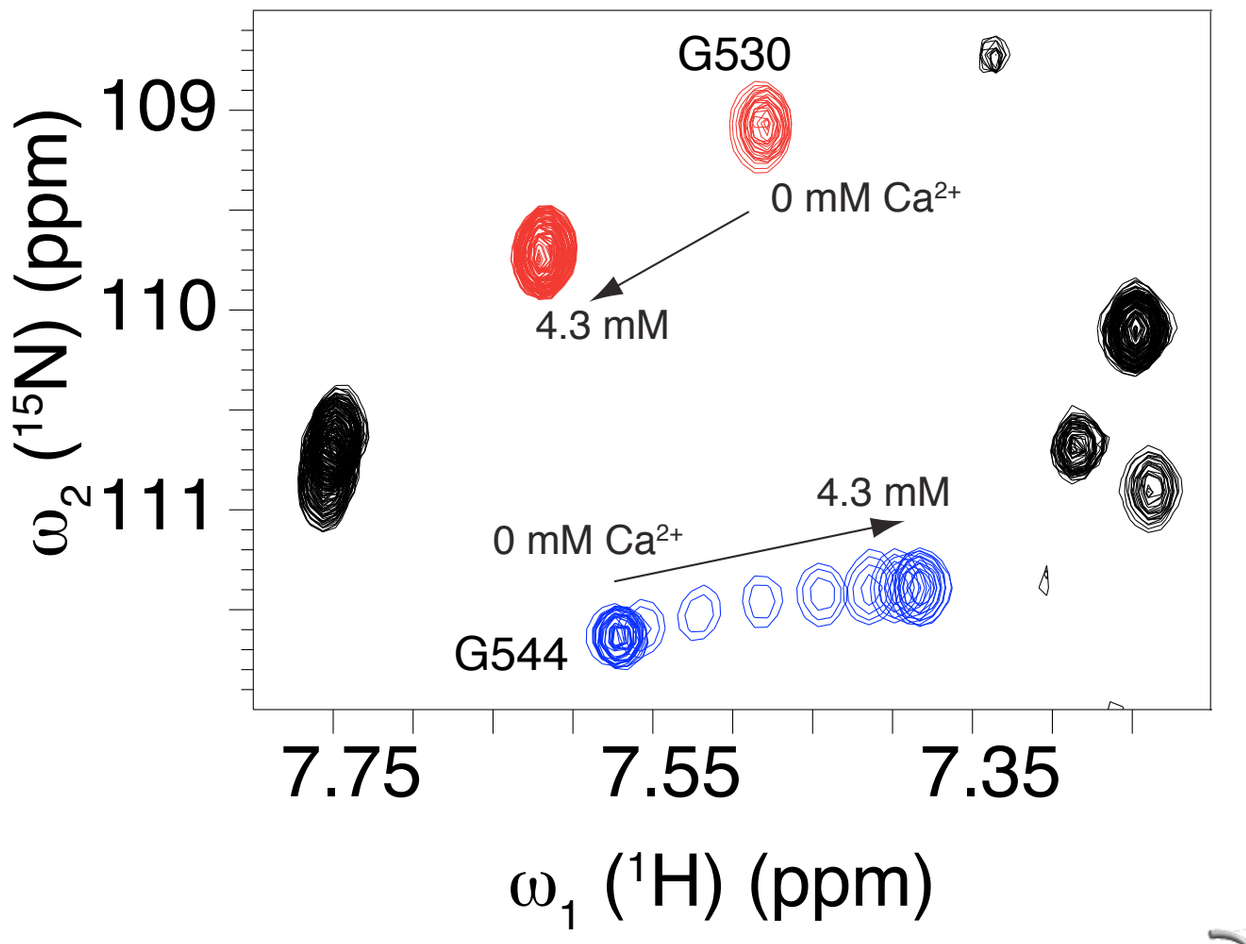
Perturbations are much more pronounced in the Ca^{2+}-bound state than in the free state

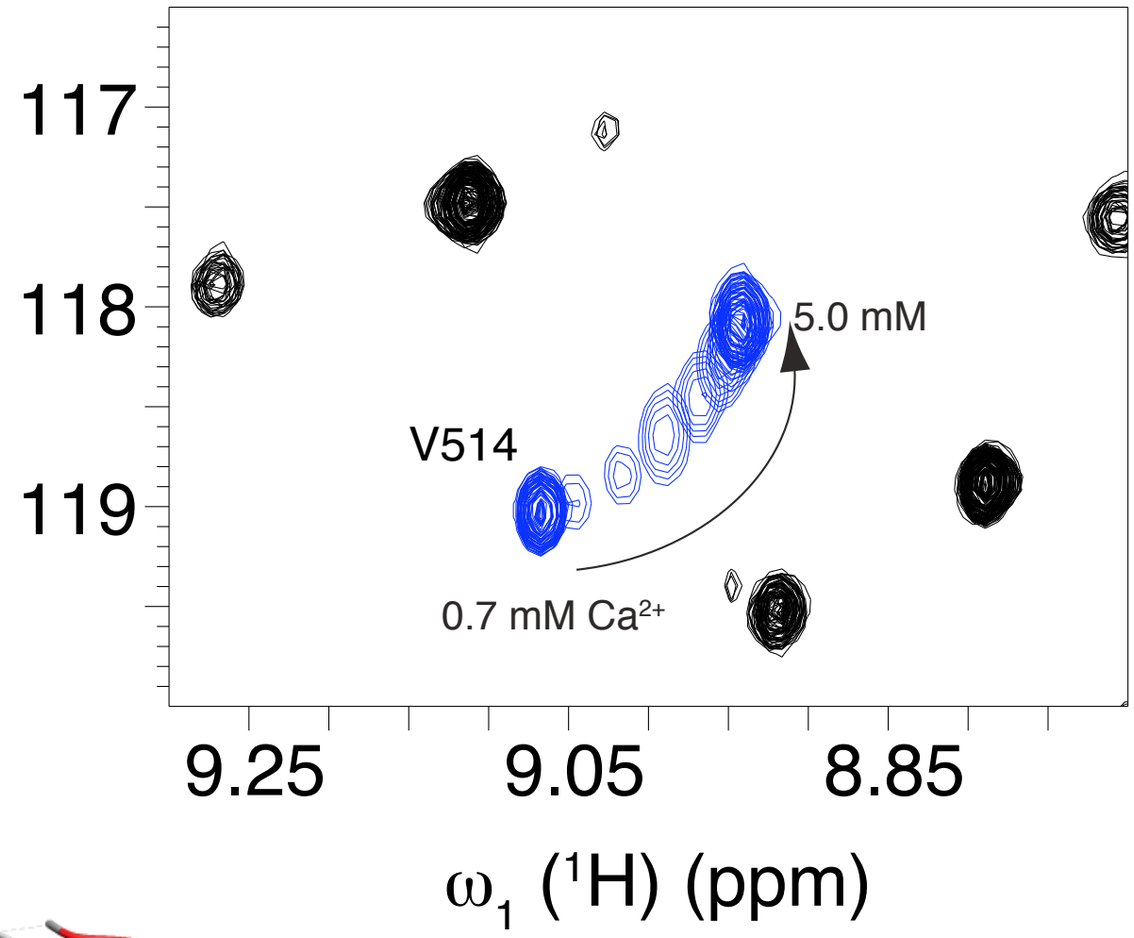


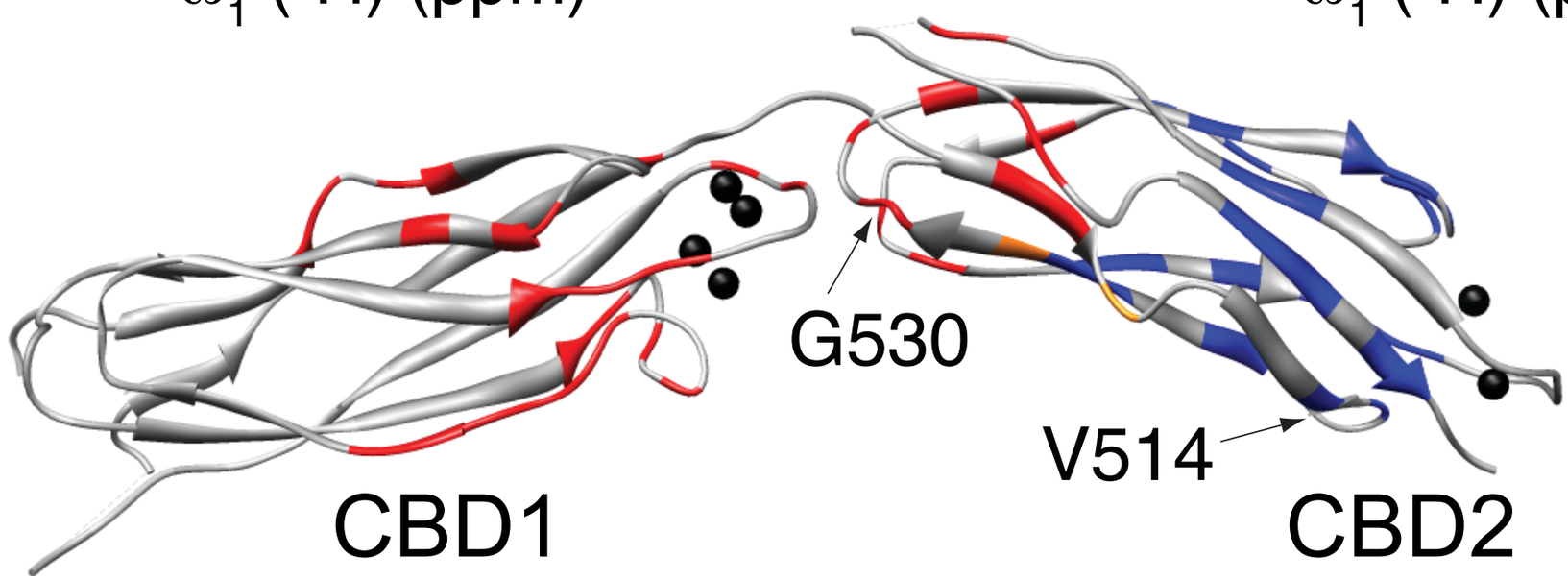




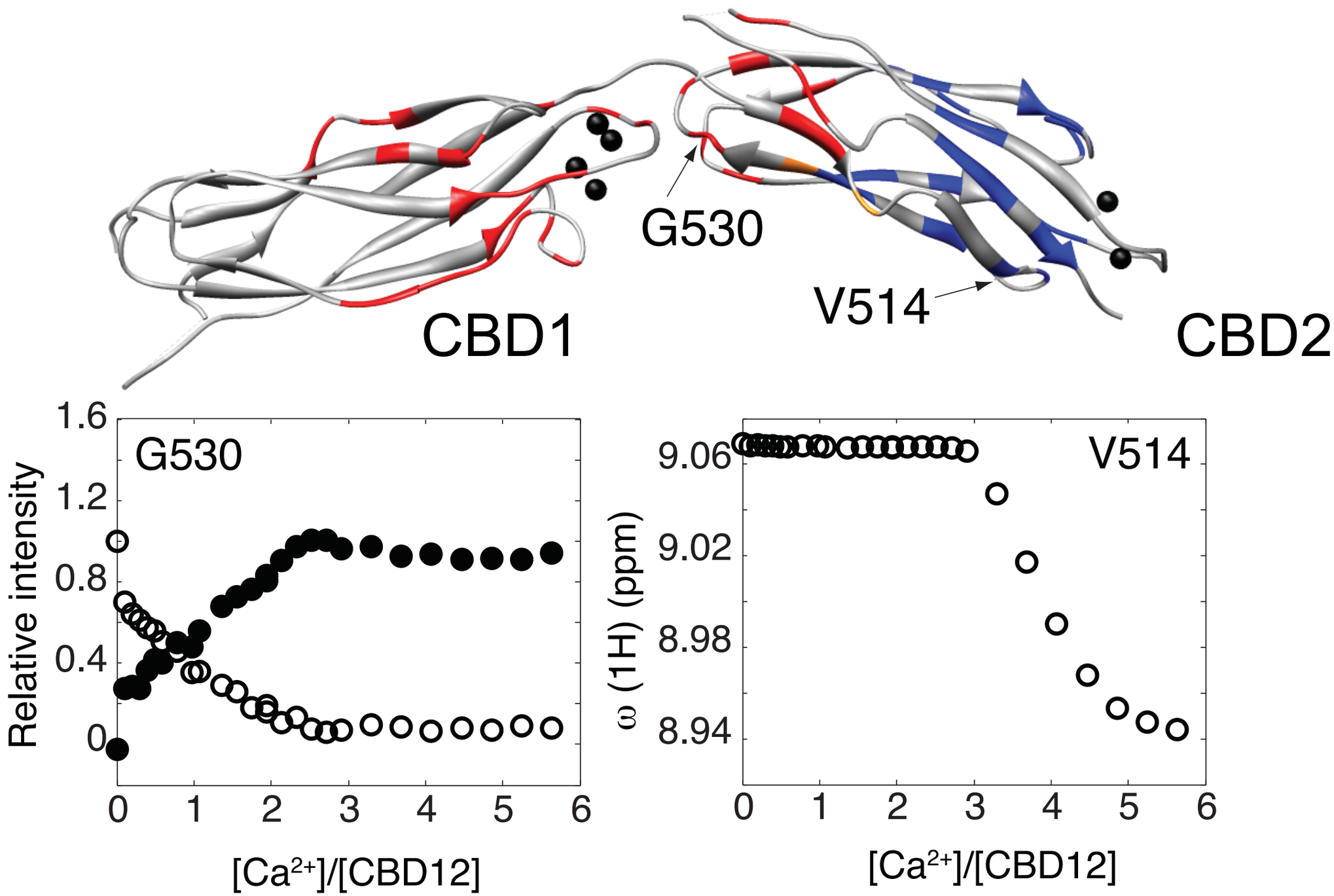
CBD1 and CBD2 are independent Ca^{2+} binding modules despite being tethered by the linker



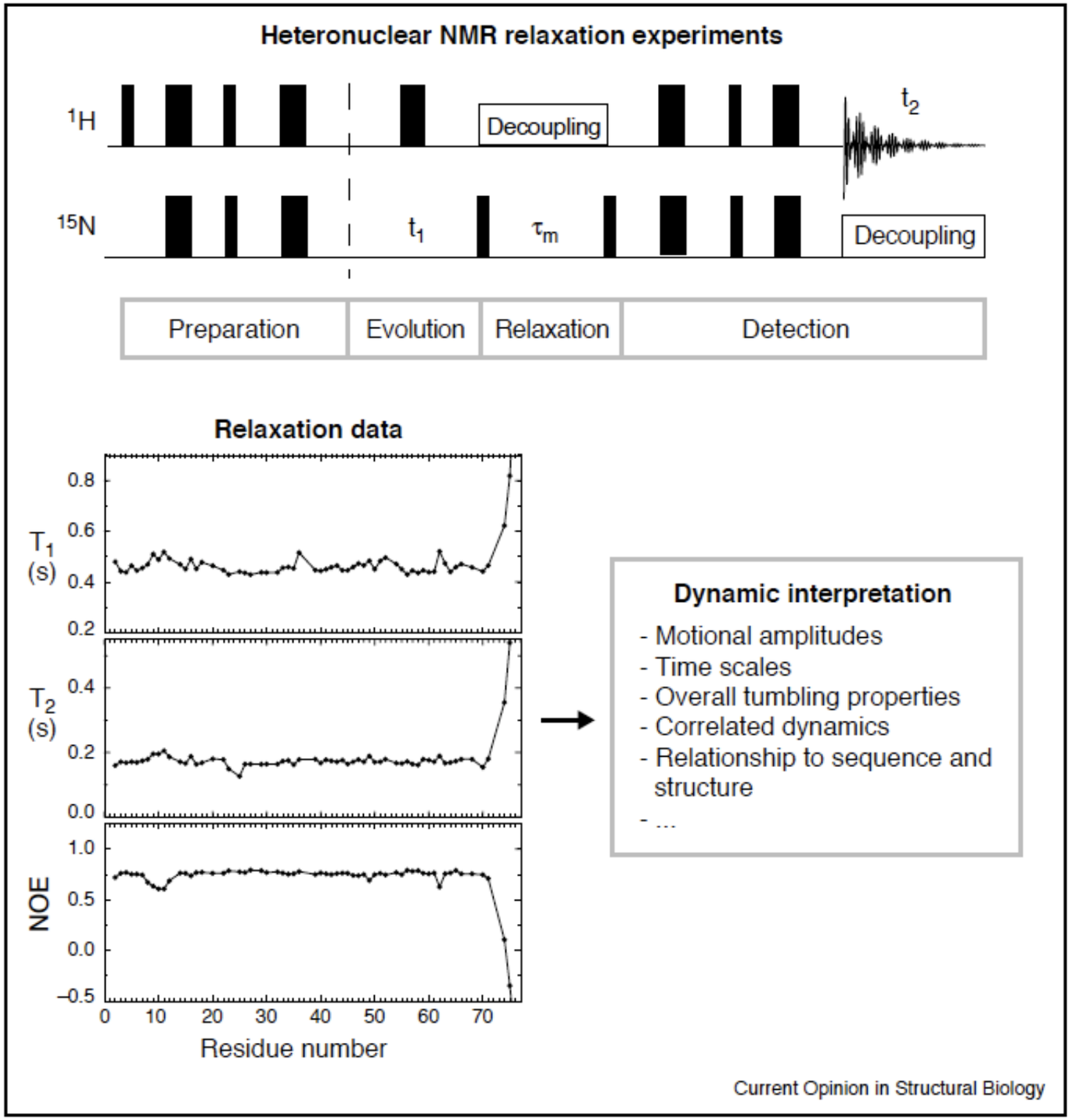




CBD1 and CBD2 are independent Ca^{2+} binding modules despite being tethered by the linker



What is the effect of Ca^{2+}-binding on the internal dynamics of CBD12?



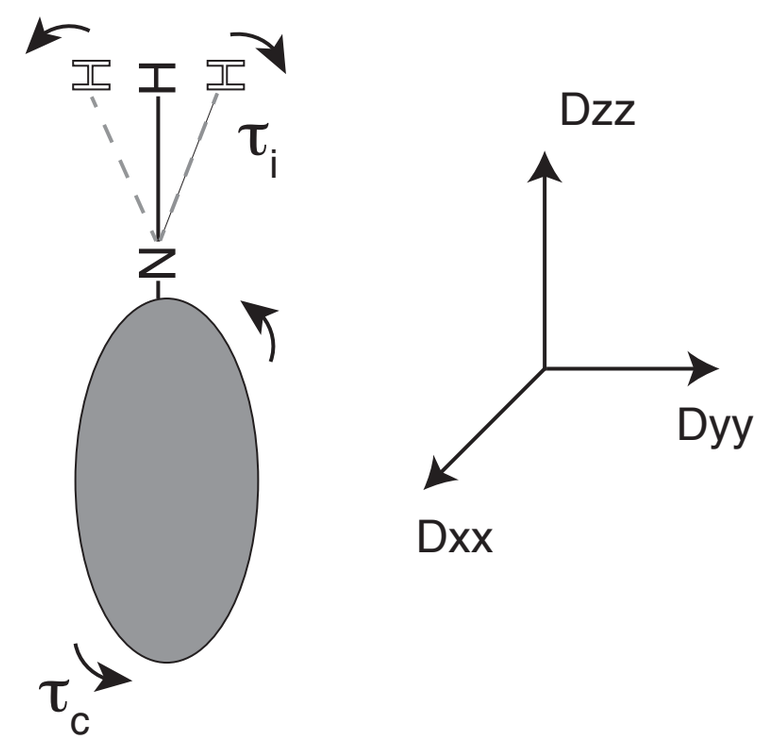
Brüschweiler (2003) *Curr. Opin. Struct. Biol.* 13: 175-183

$$\frac{1}{T_{1,2}} = \sum_i a_i j(w_i, \tau_c)$$

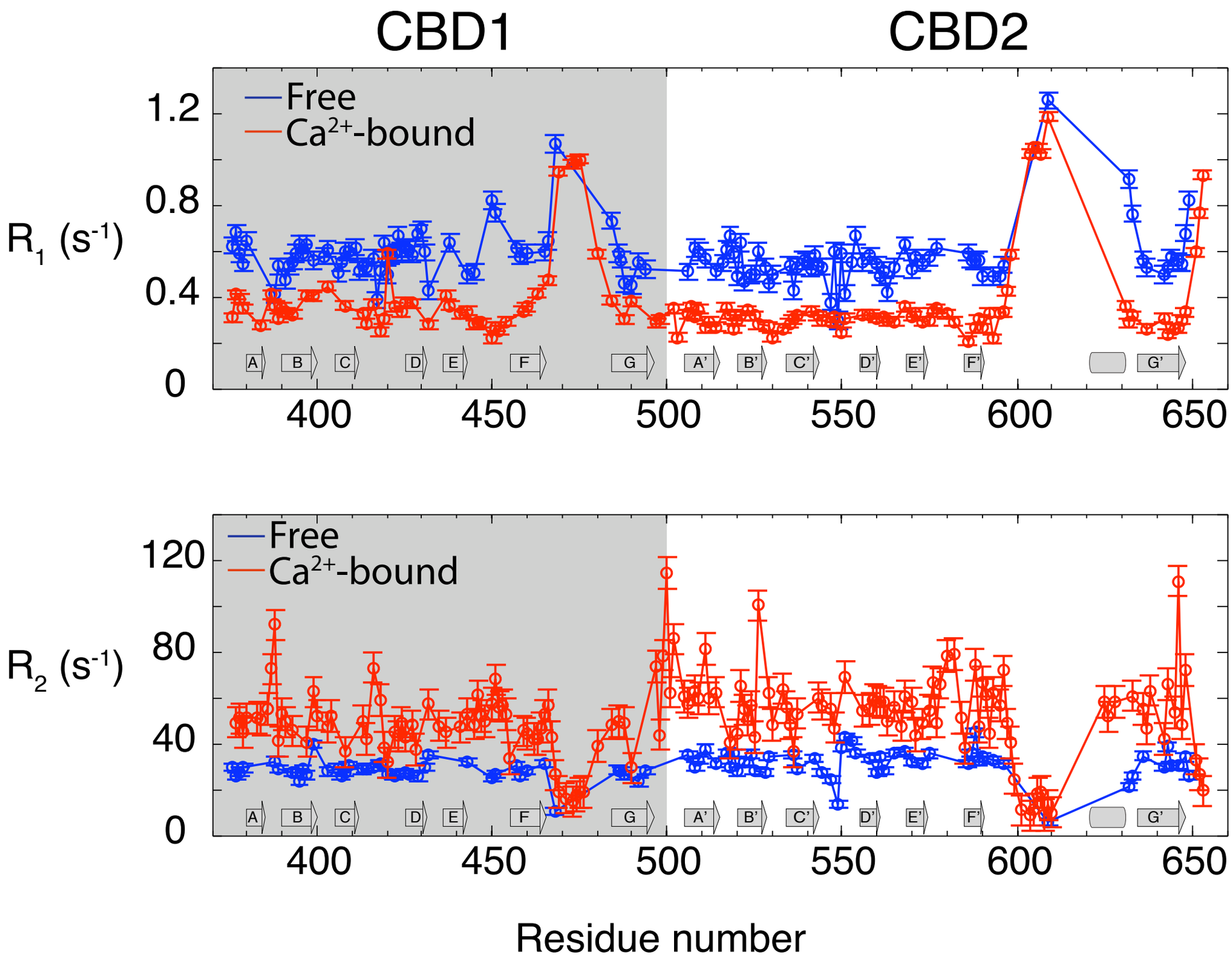
$$J(w, \tau_c) = \frac{\tau_c}{1 + \omega_2 \tau_c^{\,2}}$$

$$J(\omega) = S^2 \frac{2\tau_c}{1 + \omega^2 \tau_c^{\,2}} + \left(1 - S^2\right) \frac{2\tau_{eff}}{1 + \omega^2 \tau_{eff}^{\,2}},$$

$$\frac{1}{\tau_{eff}} = \frac{1}{\tau_c} + \frac{1}{\tau_{\text{int}}}$$



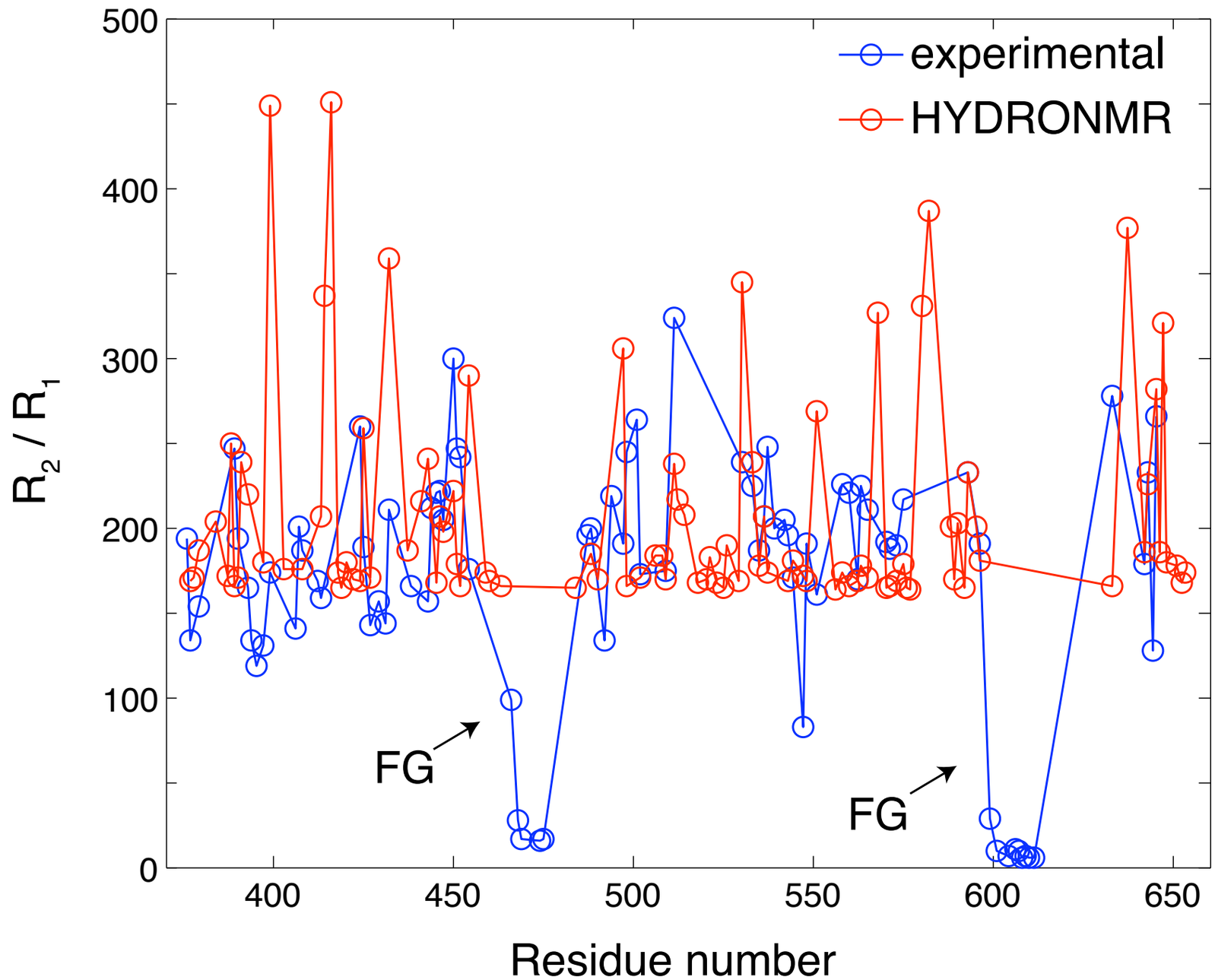
Backbone ^{15}N longitudinal (R_1) and transverse (R_2) relaxation rates of CBD12



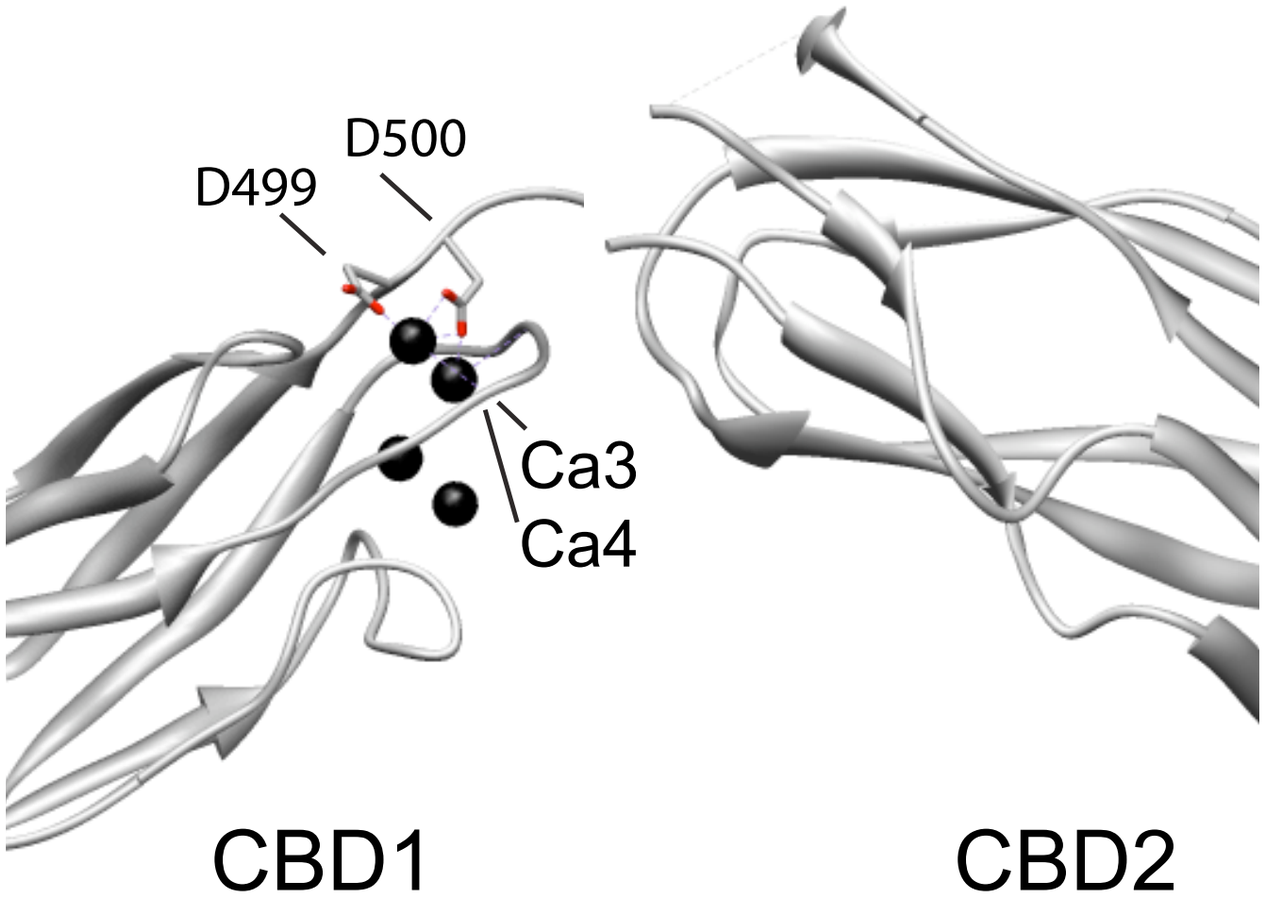
Ca^{2+} binding leads to a large increase of the overall rotational correlation time (ns) of CBD12

	CBD1	CBD2
CBD12 Free	16.92 ± 1.73	18.69 ± 1.38
CBD12 Ca^{2+}-bound	30.18 ± 4.55	33.33 ± 3.82
Isolated domains (Free-state)	11.03 ± 0.02	11.4 ± 2.3

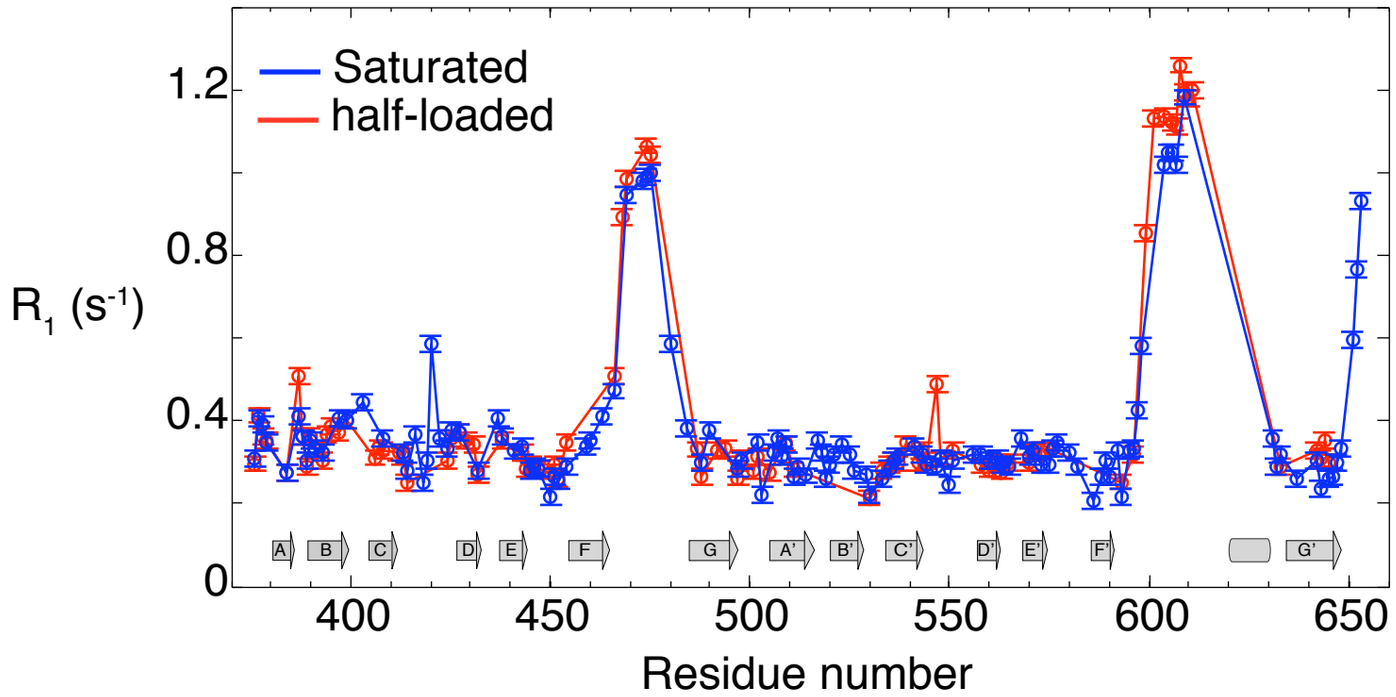
Calculated ^{15}N R_2/R_1 data based on a static model of CBD12 show overall consistency with the experimental data

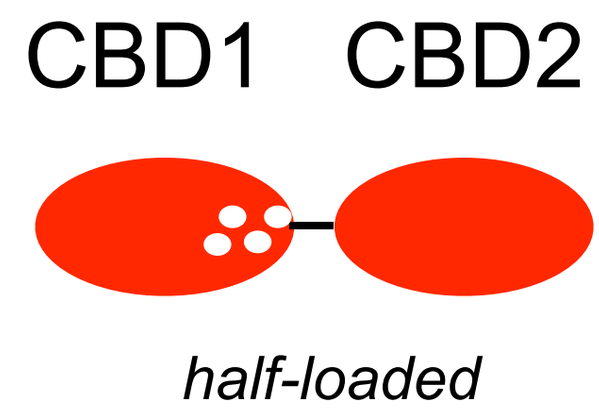


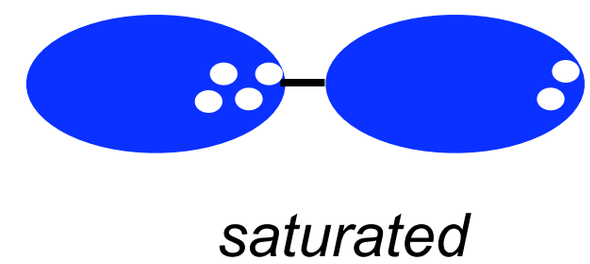
Ca^{2+}-binding rigidifies the linker and increases the dynamic coupling between CBD1 and CBD2

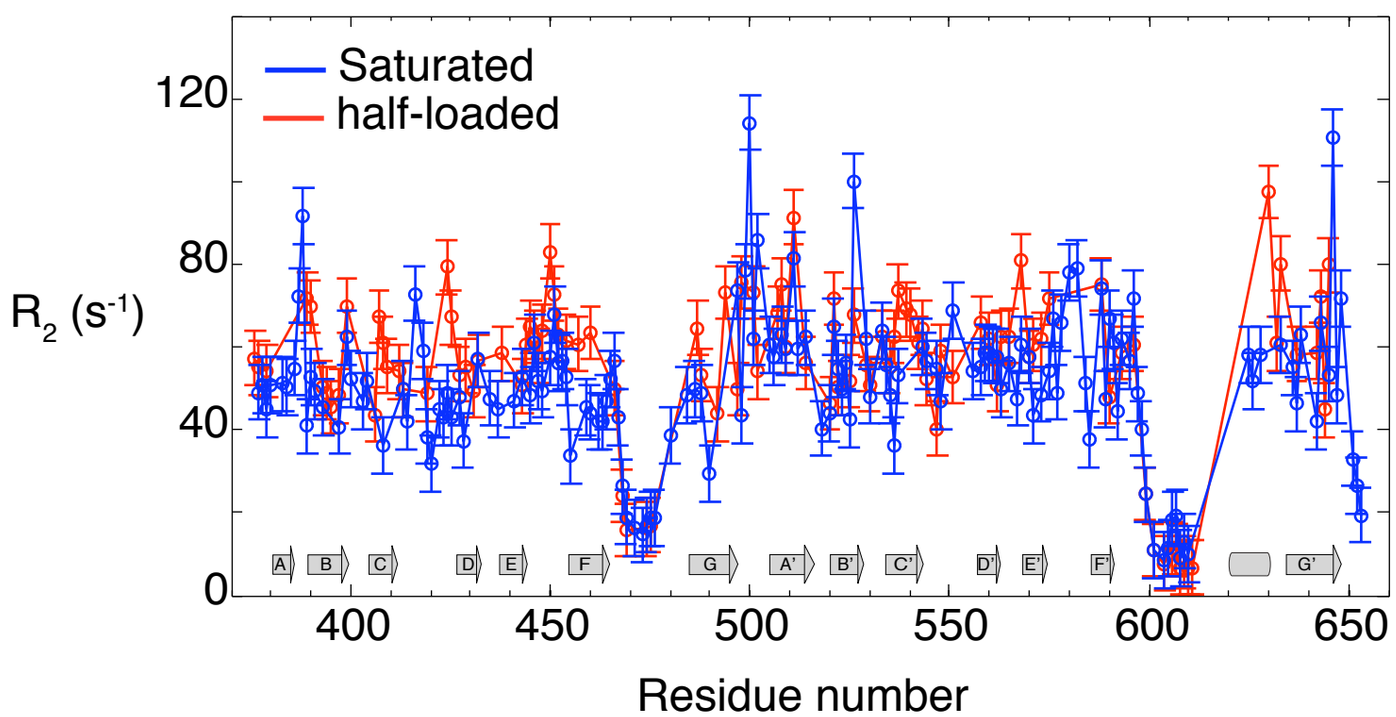


Ca^{2+}-binding to CBD1 alone is sufficient to alter the ^{15}N relaxation rates

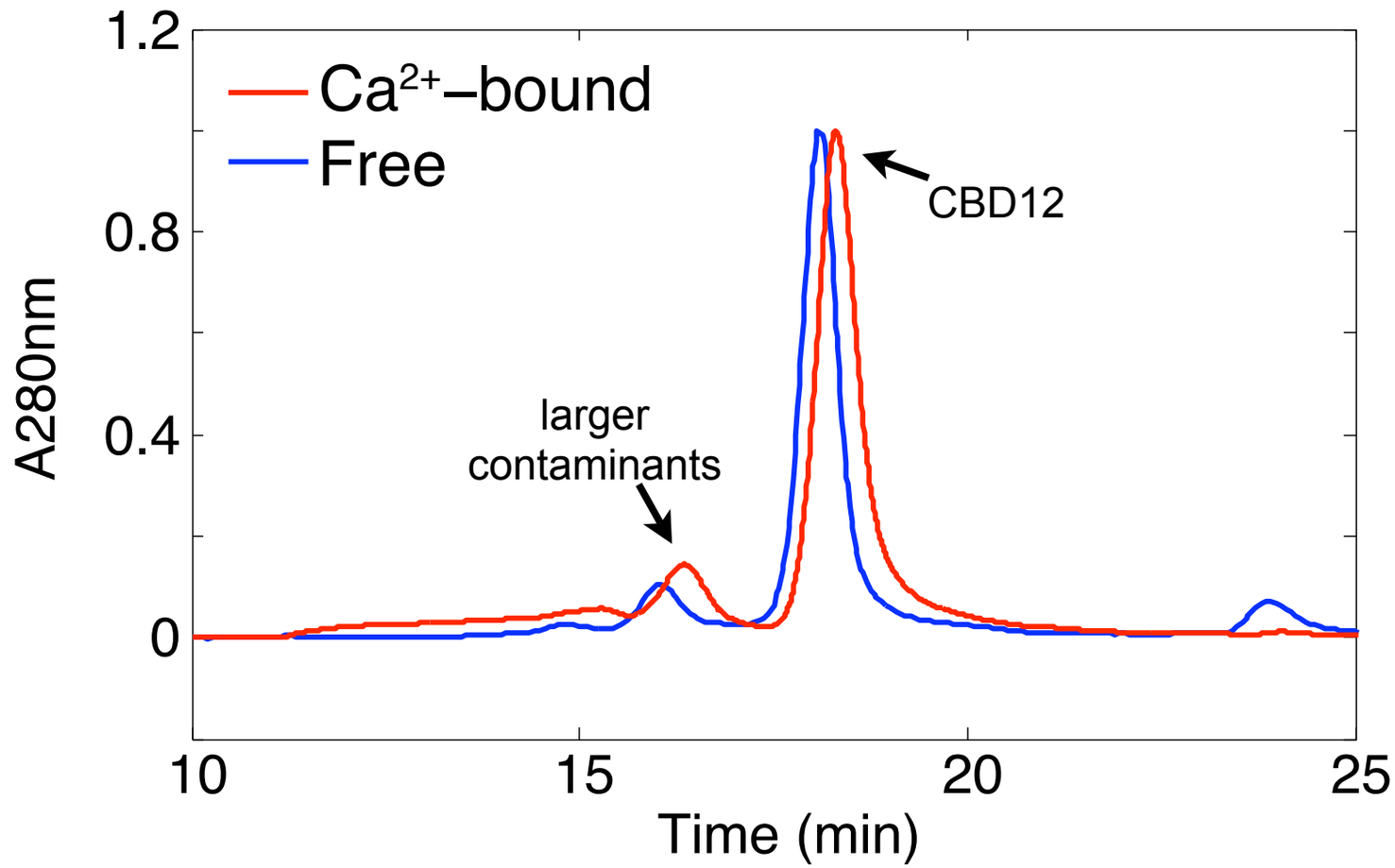




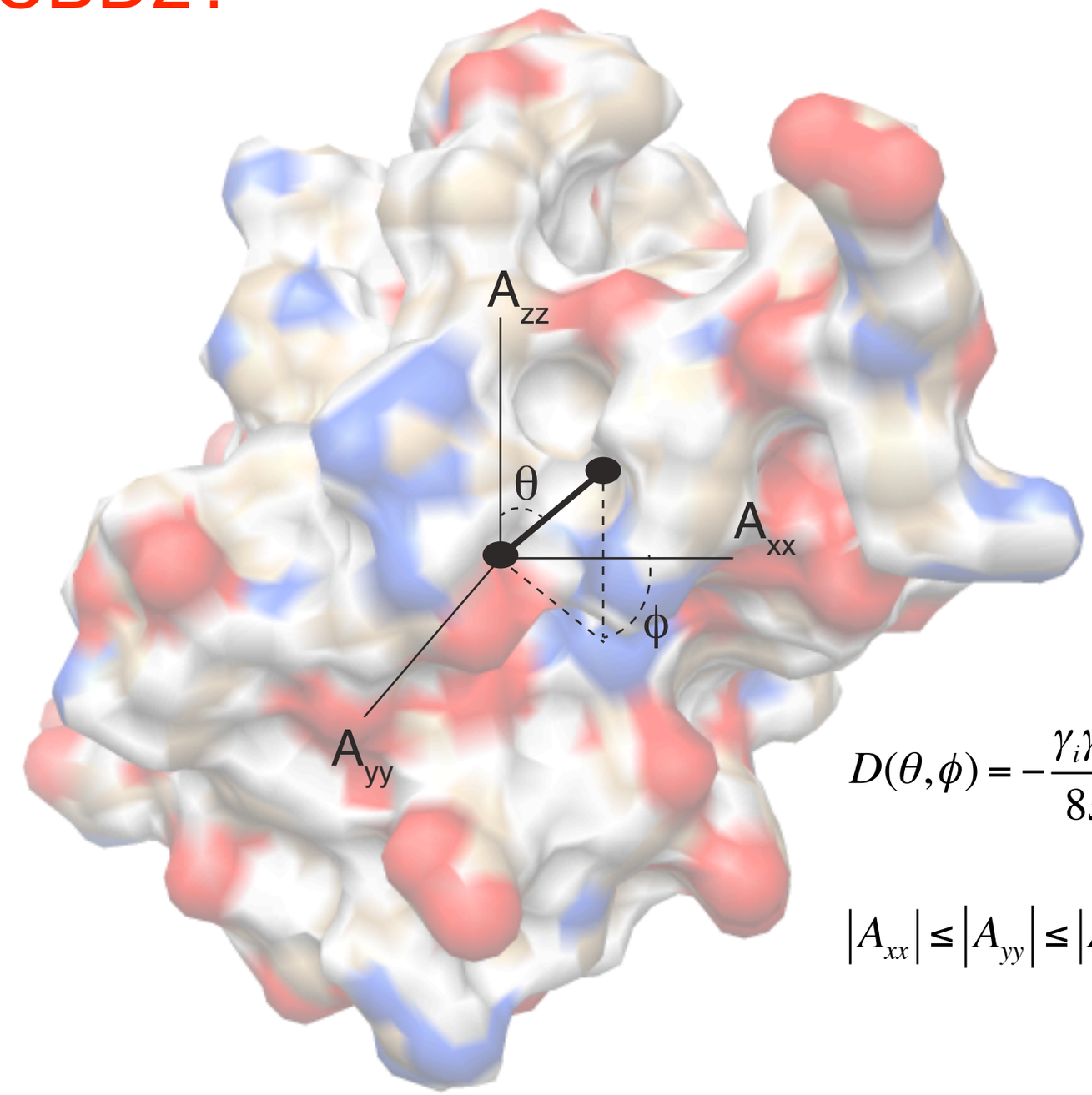




Gel filtration chromatography indicates that oligomerization is not the cause for the slower tumbling rate in the Ca^{2+}-bound state



What is the ensemble average orientation between CBD1 and CBD2?



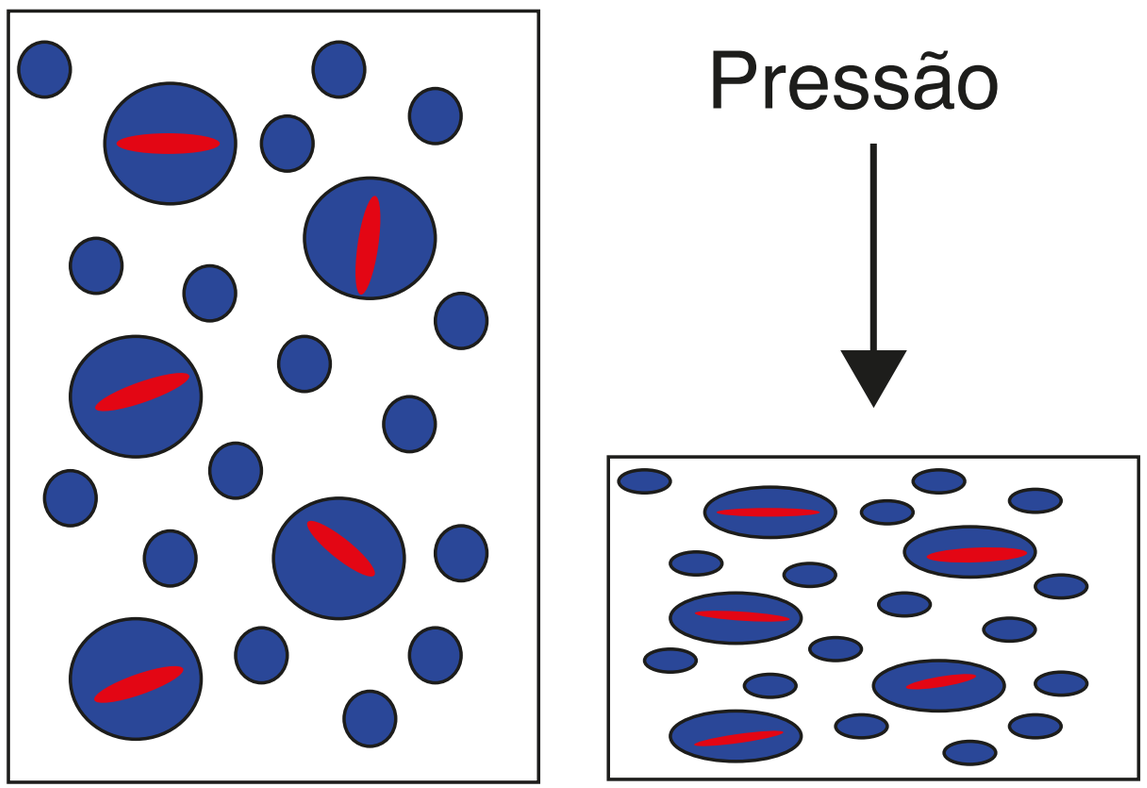
$$D(\theta,\phi) = -\frac{\gamma_i \gamma_j \mu_0 h}{8\pi^3 r_{ij}^3}\left(A_{zz}\cos^2\theta + A_{xx} sen^2\theta \cos^2\phi + A_{yy} sen^2\theta sen^2\phi\right),$$

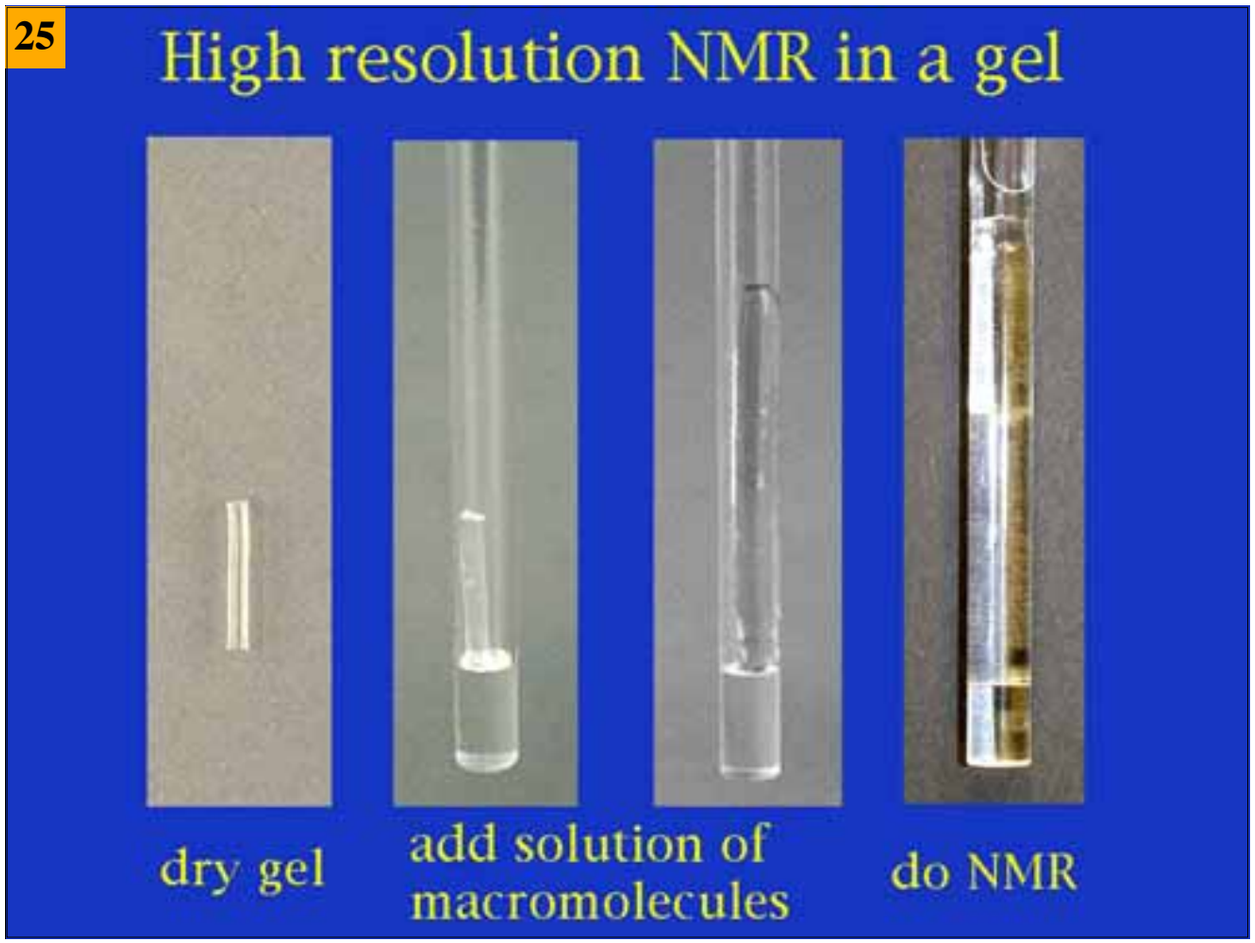
$$|A_{xx}| \le |A_{yy}| \le |A_{zz}|$$

^{1}H-^{15}N dipolar couplings contain information about the orientation of the ^{1}H-^{15}N bond vectors with respect the alignment tensor

CBD12 was partially aligned in compressed polyacrylamide gels

Proteins in a polyacrylamide gel:

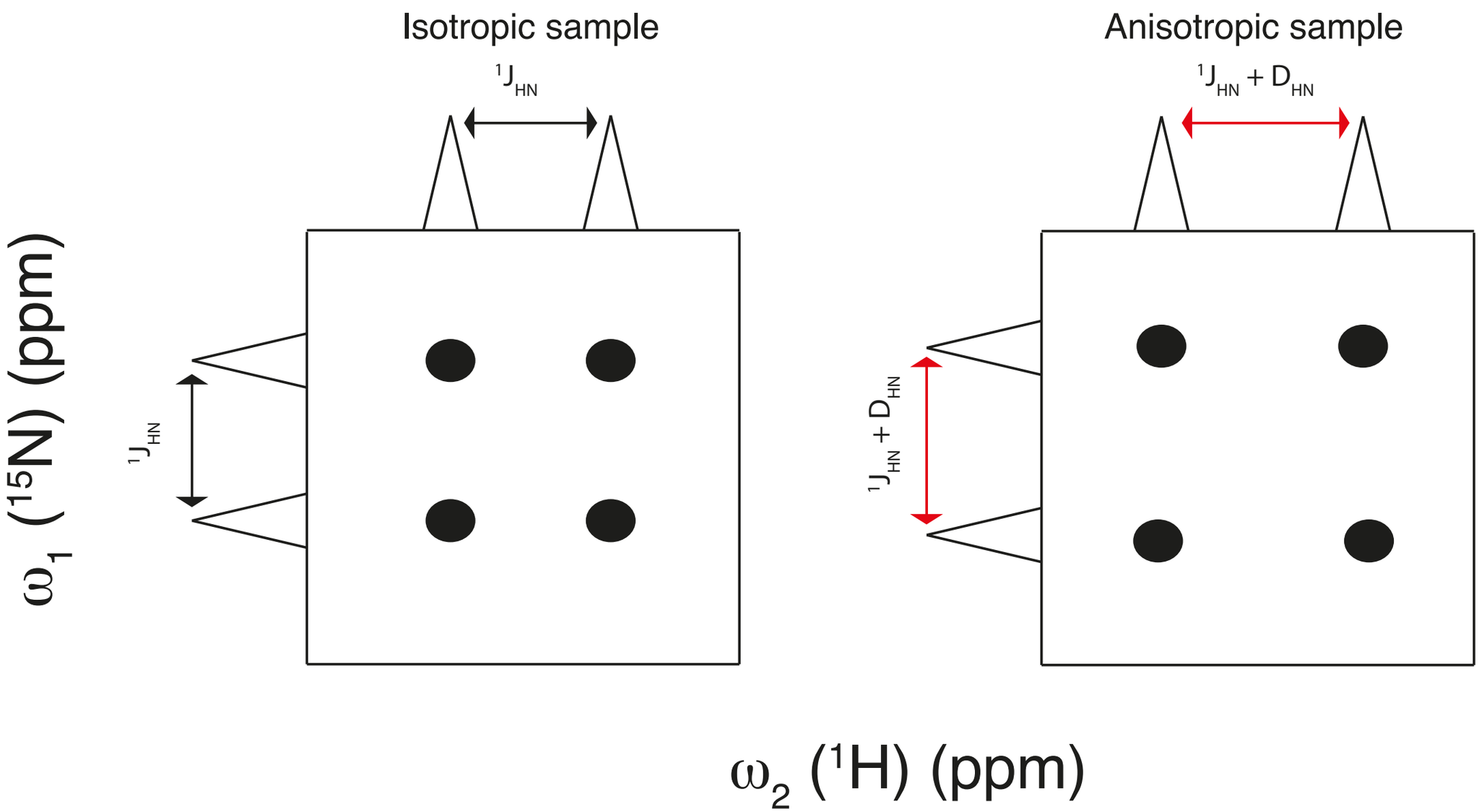




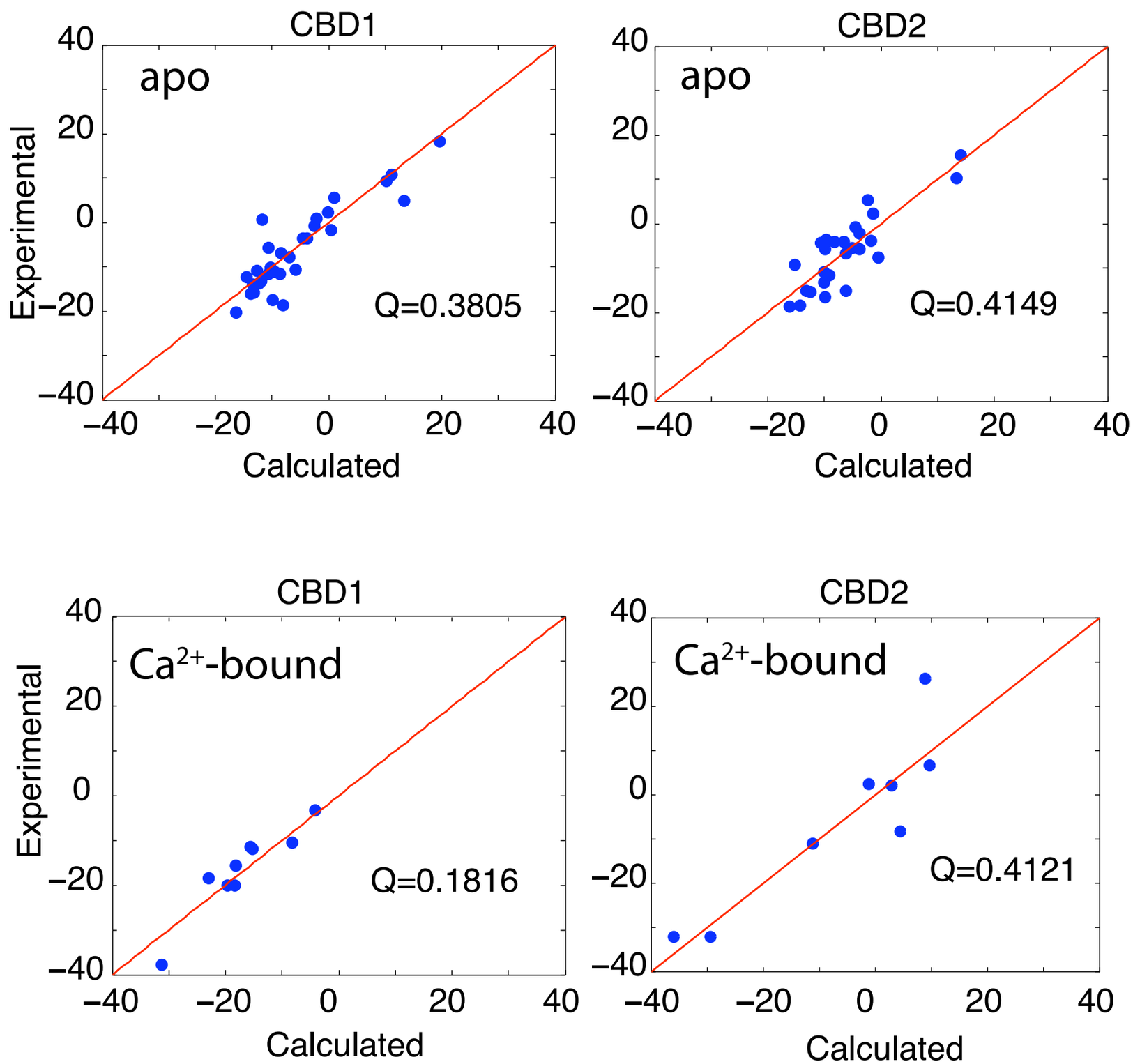
Stephan Grzesiek, EMBO course, 2005

Sass et al. (2000) *J. Biomol. NMR* 18:303-309

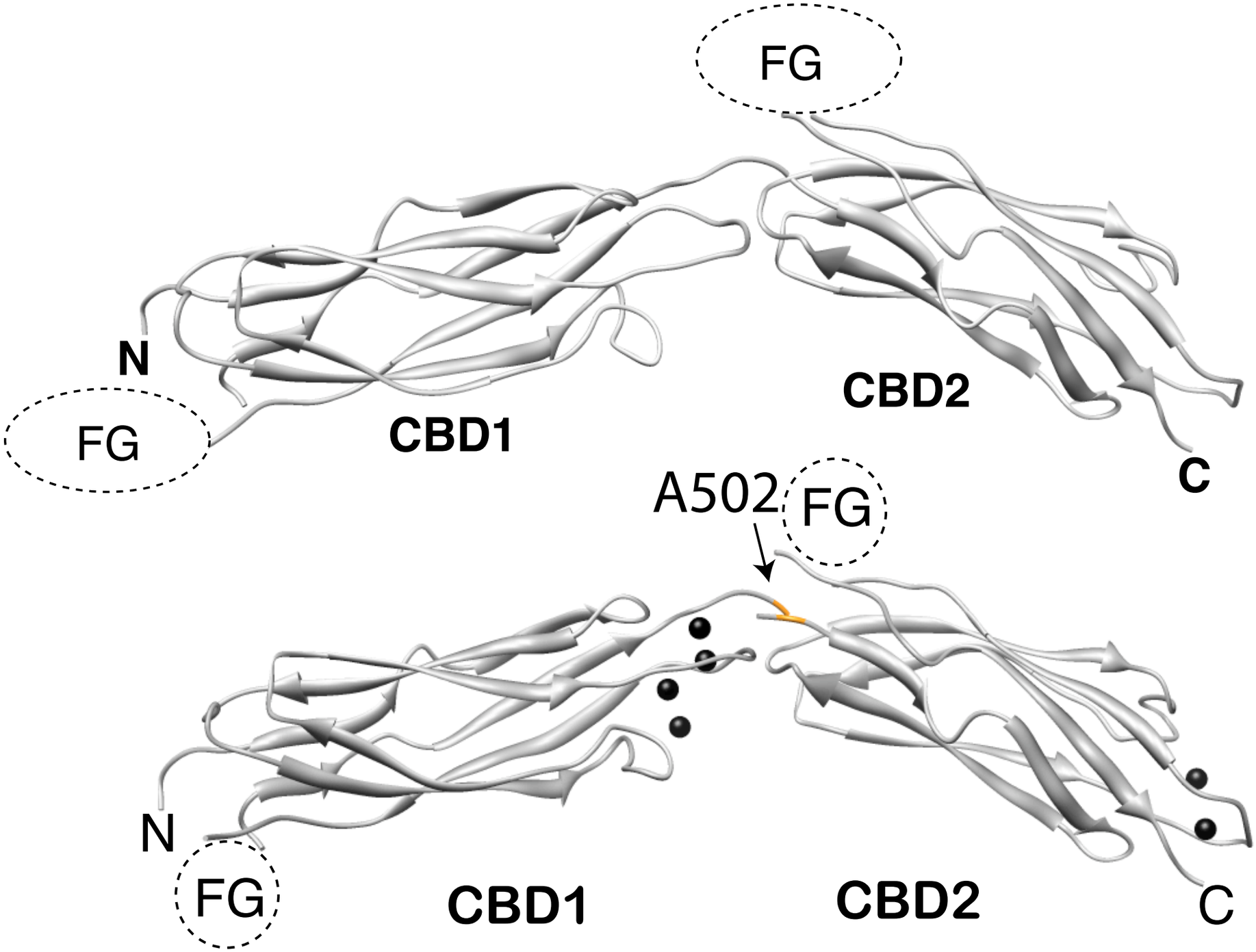
D_{NH} residual dipolar couplings were obtained by measuring the $^1J_{NH}$ splitting for the unaligned sample (isotropic) and the partially aligned sample (anisotropic)



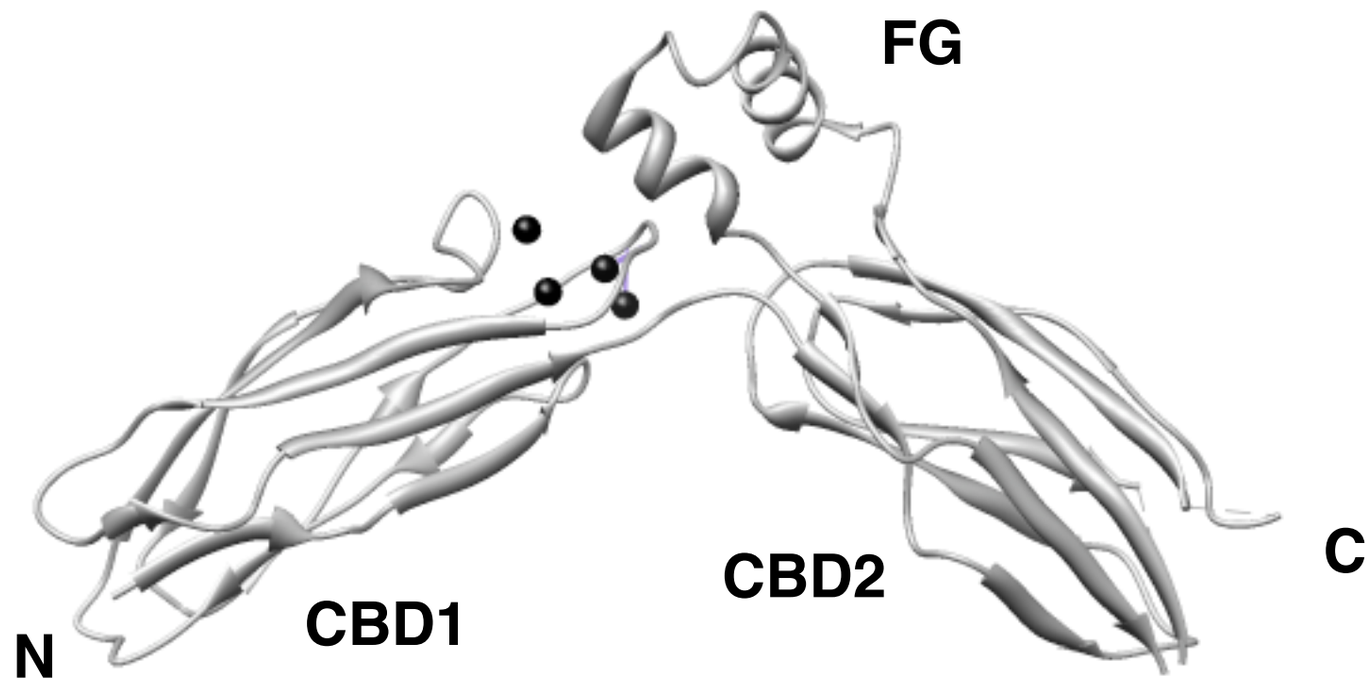
One-bond backbone ^{1}H-^{15}N residual dipolar couplings (RDCs) of CBD12 partially aligned in compressed polyacrylamide gel



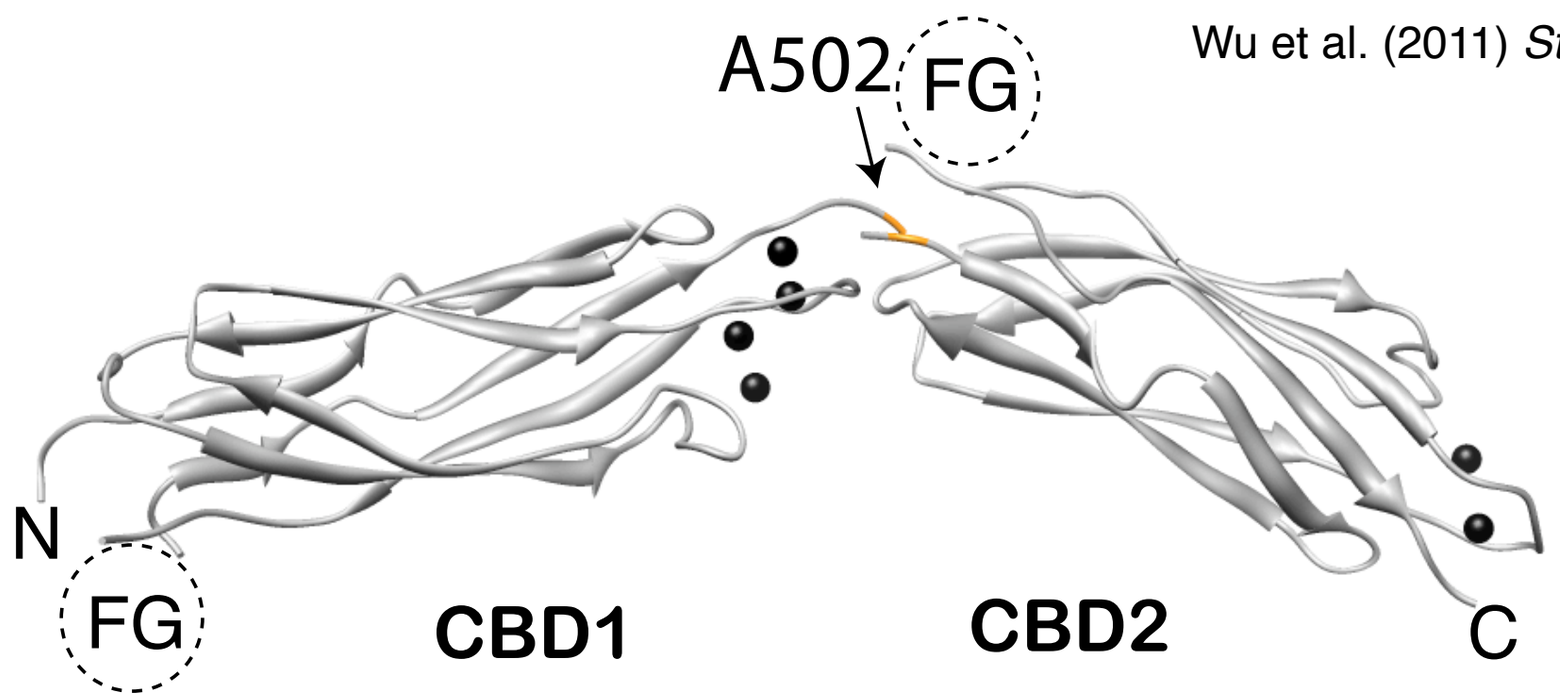
The time and ensemble average orientation between CBD1 and CBD2 is elongated in the absence and presence of Ca^{2+}



The crystal structure of Drosophila's CBD12 in the Ca^{2+}-bound state is consistent with the NMR-derived model



Wu et al. (2011) *Structure* 19:1509-1517



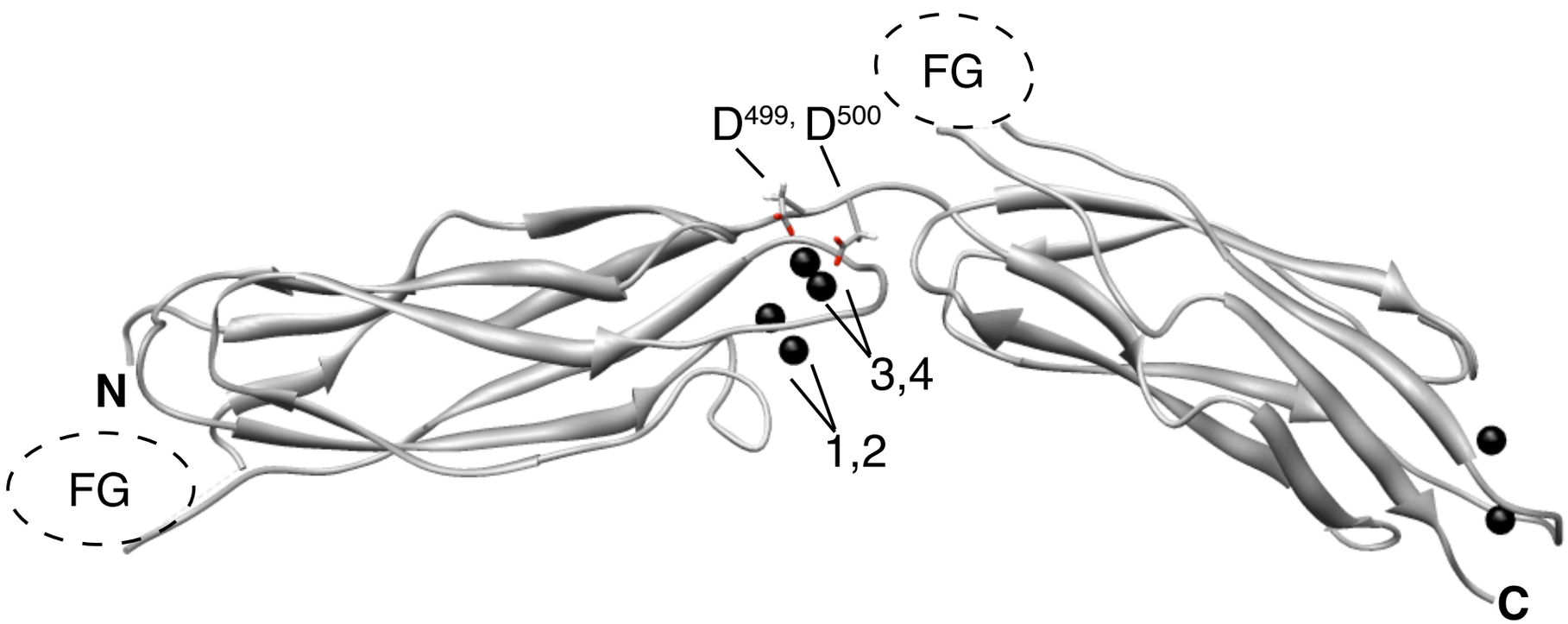
The larger overall alignment observed in the Ca^{2+}-bound state is consistent with more restricted inter-domain flexibility

	CBD1	**CBD2**
CBD12 free state	Q = 0.38 R = 0.23 D_a = 12.23 Hz	Q = 0.41 R = 0.41 D_a = 12.04 Hz
CBD12 Ca^{2+}-bound state	Q = 0.18 R = 0.26 D_a = 24.05 Hz	Q = 0.41 R = 0.39 D_a = 25.29 Hz

Summary

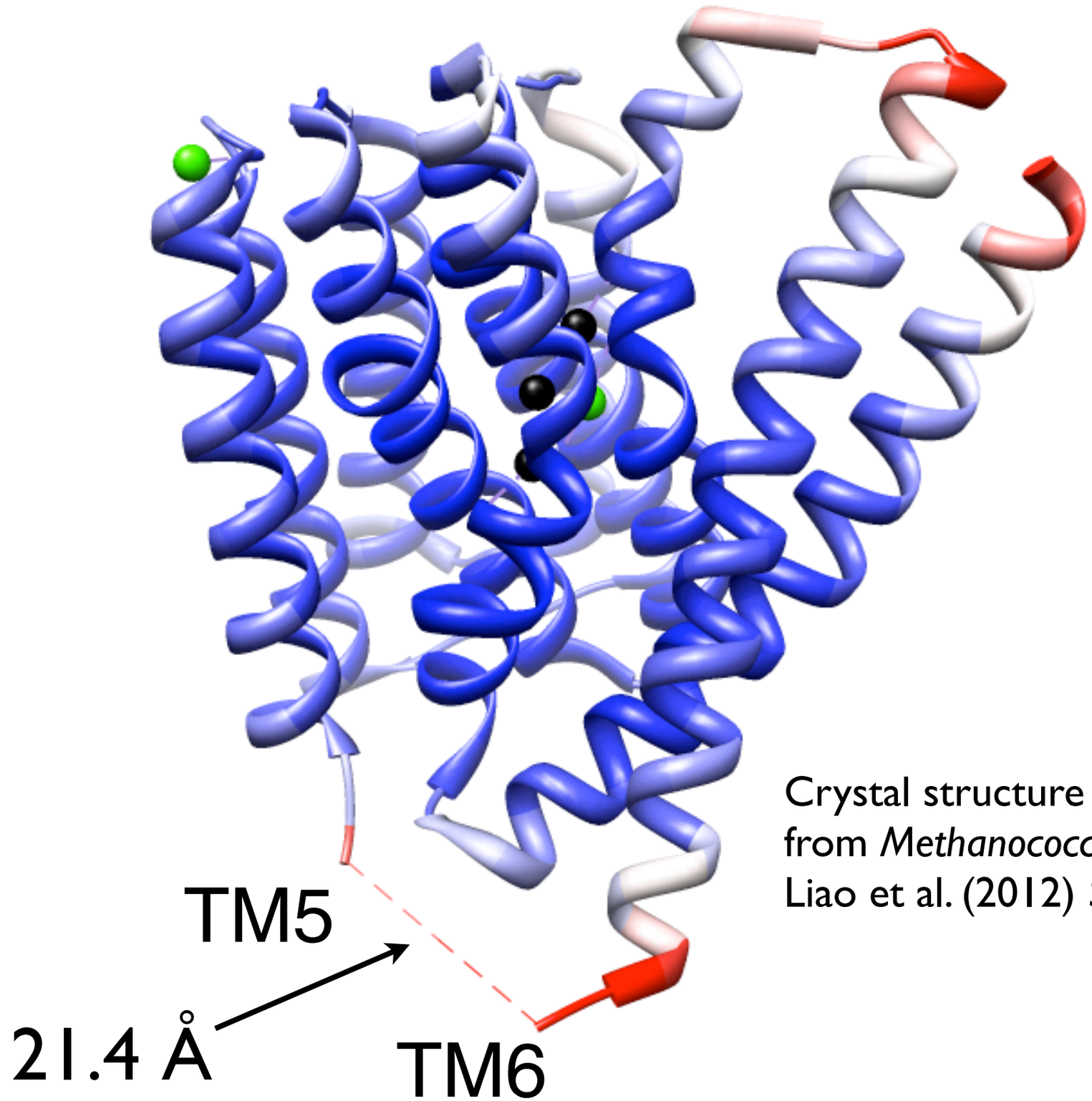
1. CBD1 and CBD2 sample a wide range of relative arrangements on the nanosecond time scale

2. Binding of Ca^{2+} to the linker restricts the inter-domain flexibility in a major way, stabilizing a rigid elongated confomation



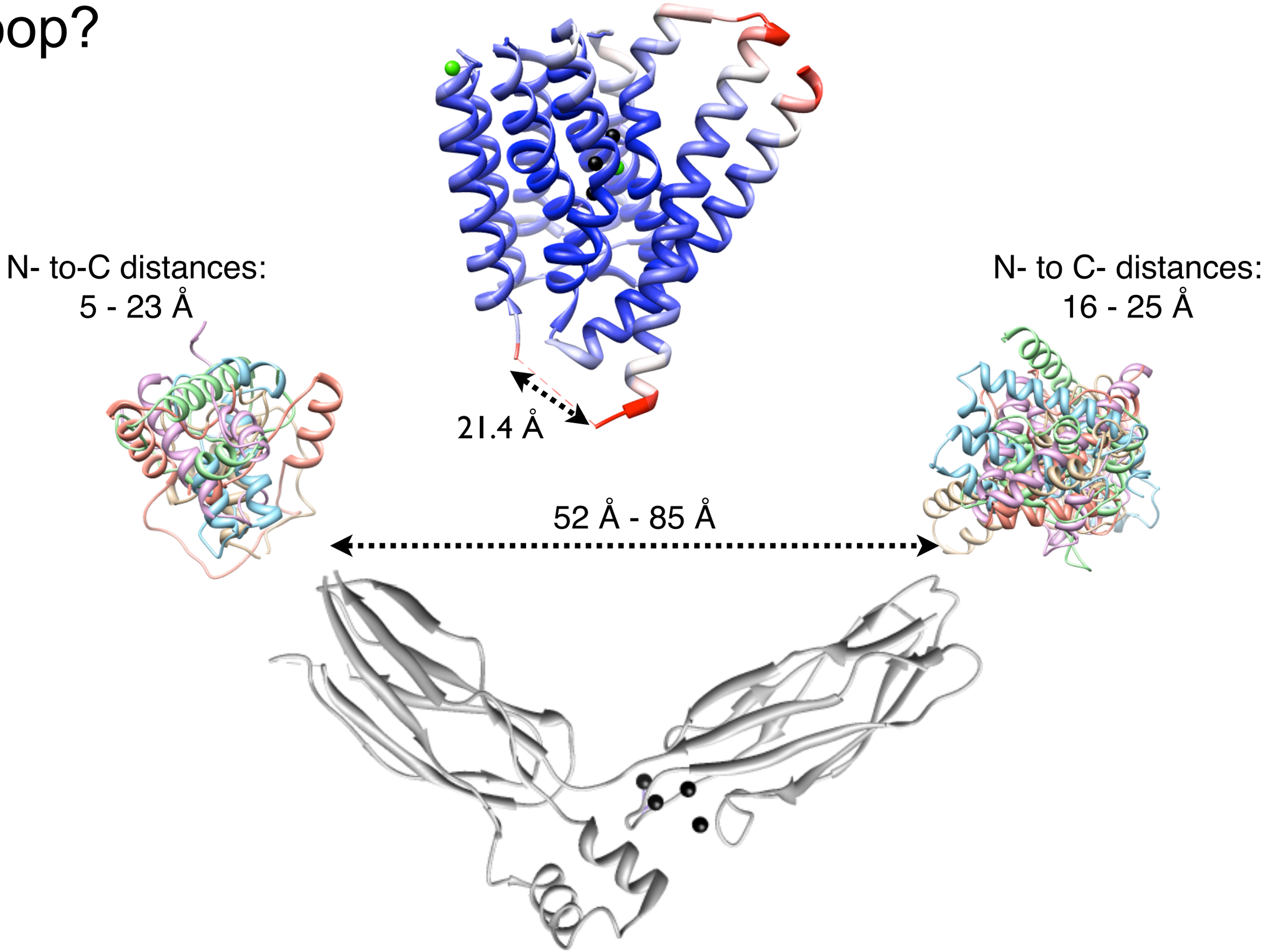
Salinas et al. (2011) *J. Biol. Chem.* **286**: 32123-32131

Can we propose an atomic model for the Ca^{2+}-regulation of the exchanger?

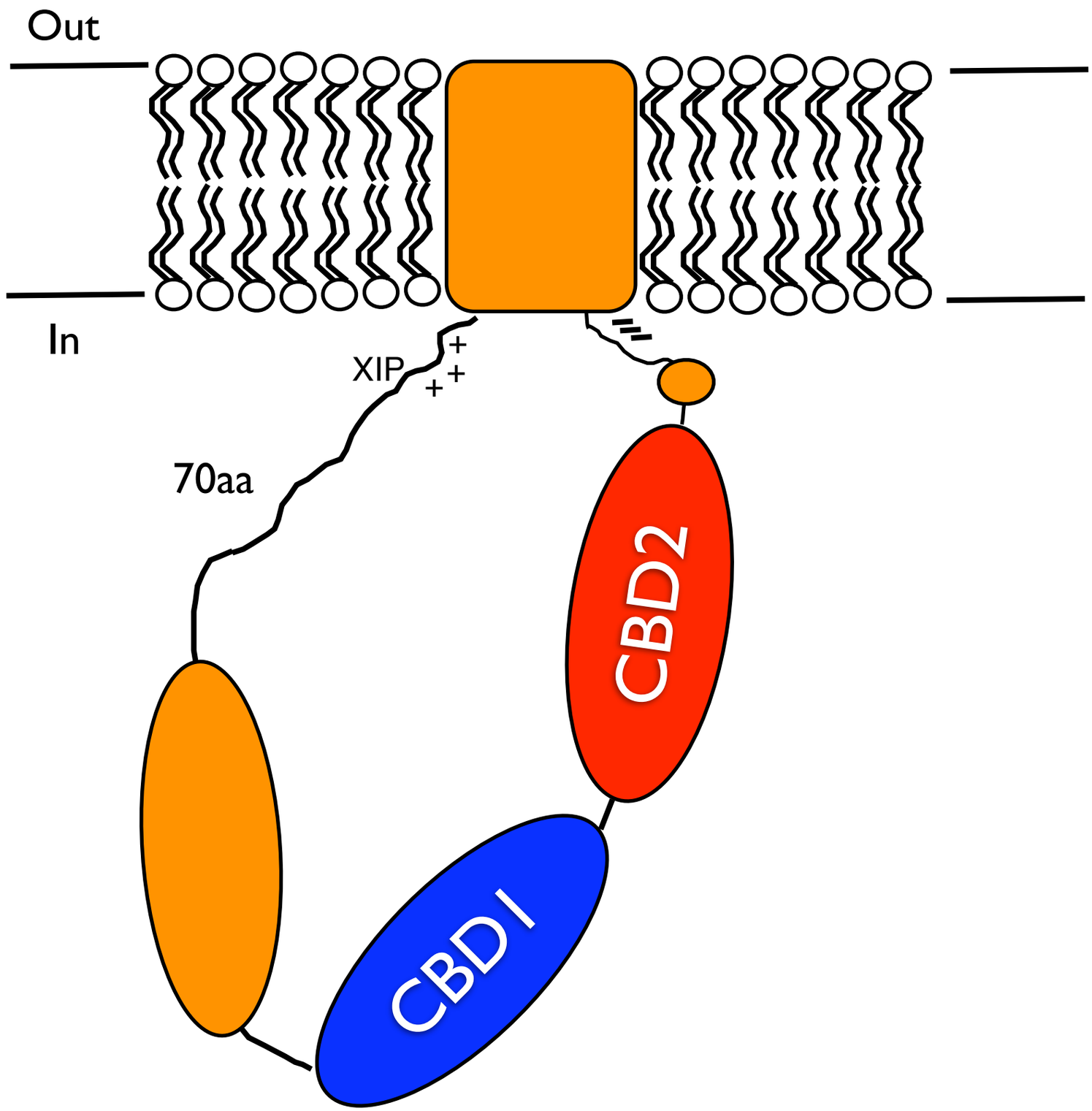


Crystal structure of an NCX homologue from *Methanococcus jannaschii*
Liao et al. (2012) *Science* **335**: 686

How can we build an atomic model of the cytosolic loop?



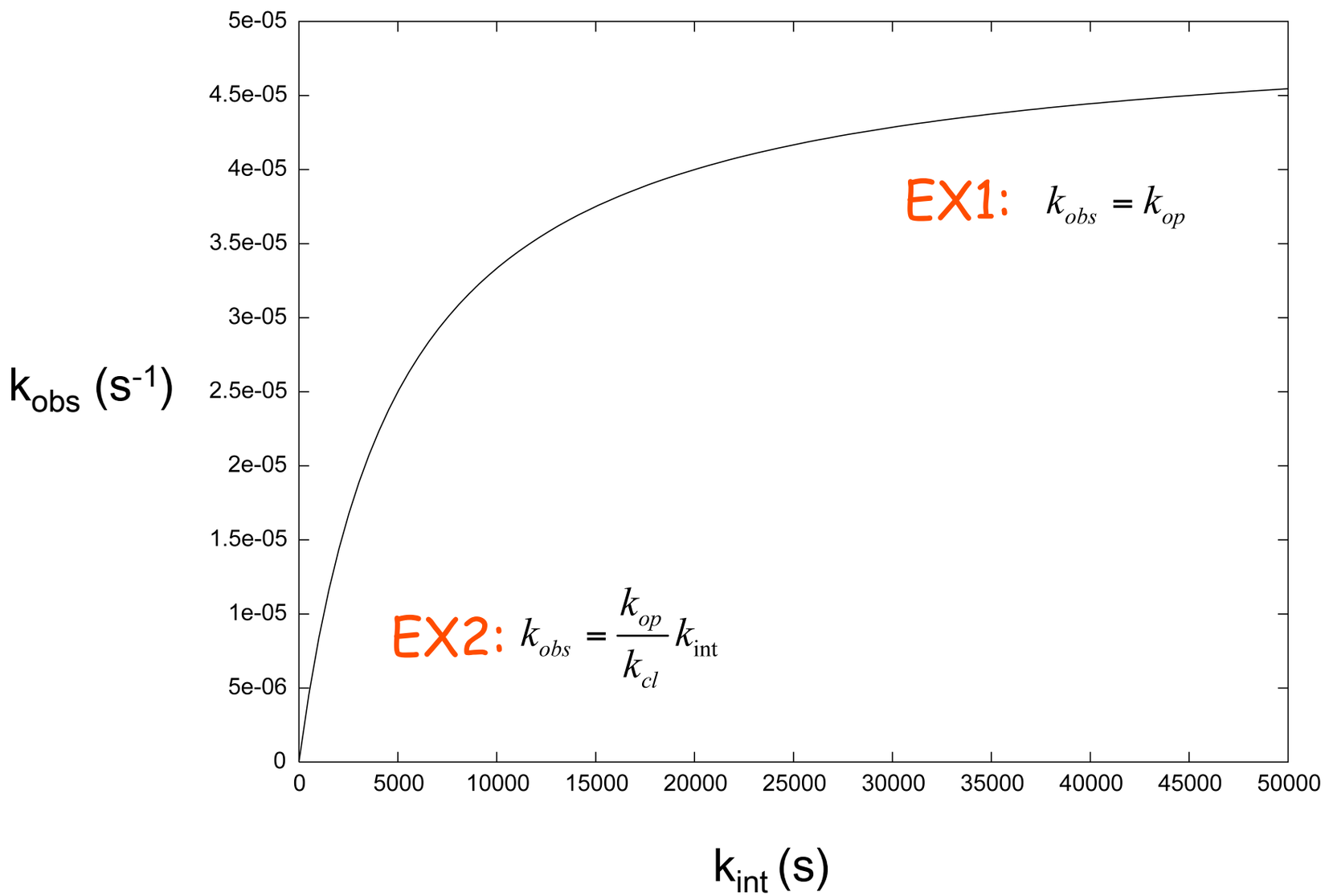
These findings directly point to a functional role of CBD12 in the context of the exchanger



Cooperative α-helix unfolding in a protein-DNA complex from hydrogen-deuterium exchange

Salinas, Diercks, Kaptein e Boelens (2006) *Protein Sci.* **15**: 1752-1759

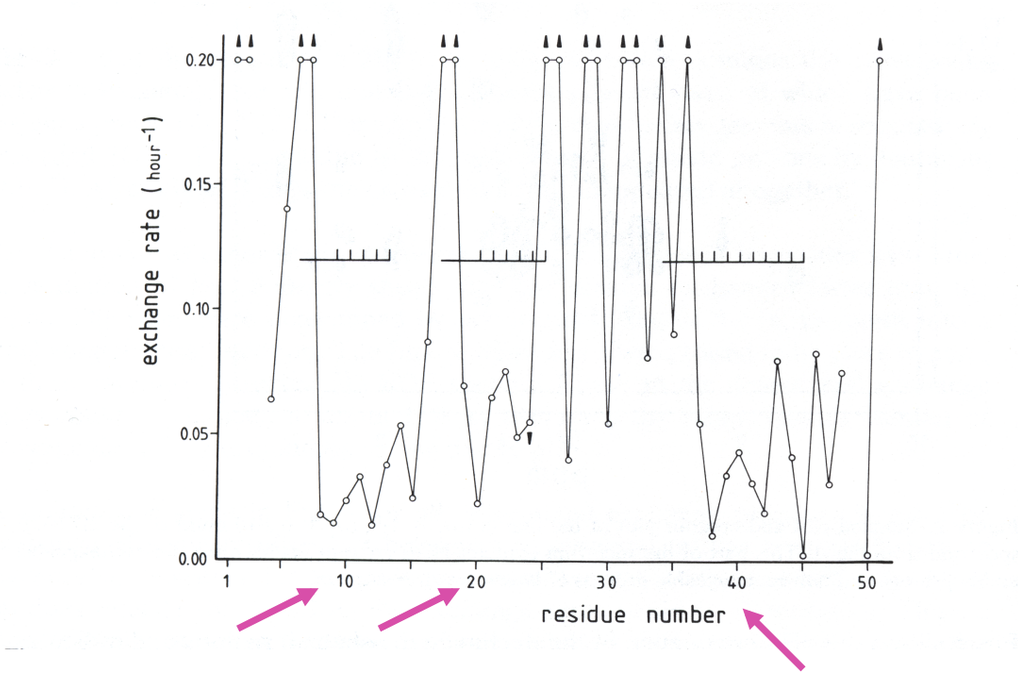
$$NH\ (closed) \underset{kcl}{\overset{kop}{\rightleftarrows}} NH\ (open) \xrightarrow{kint} ND\ (exchanged)$$



NHs localizados no centro das hélices I, II e III do lacHP formam o núcleo menos acessível ao solvente

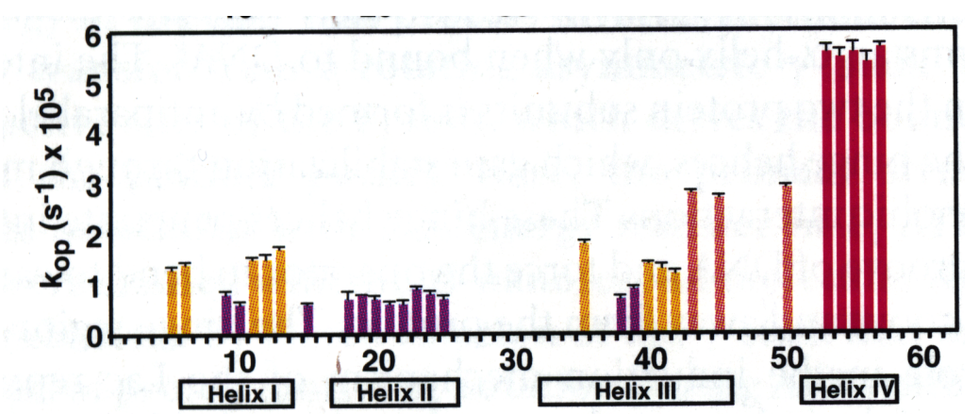
Free lac

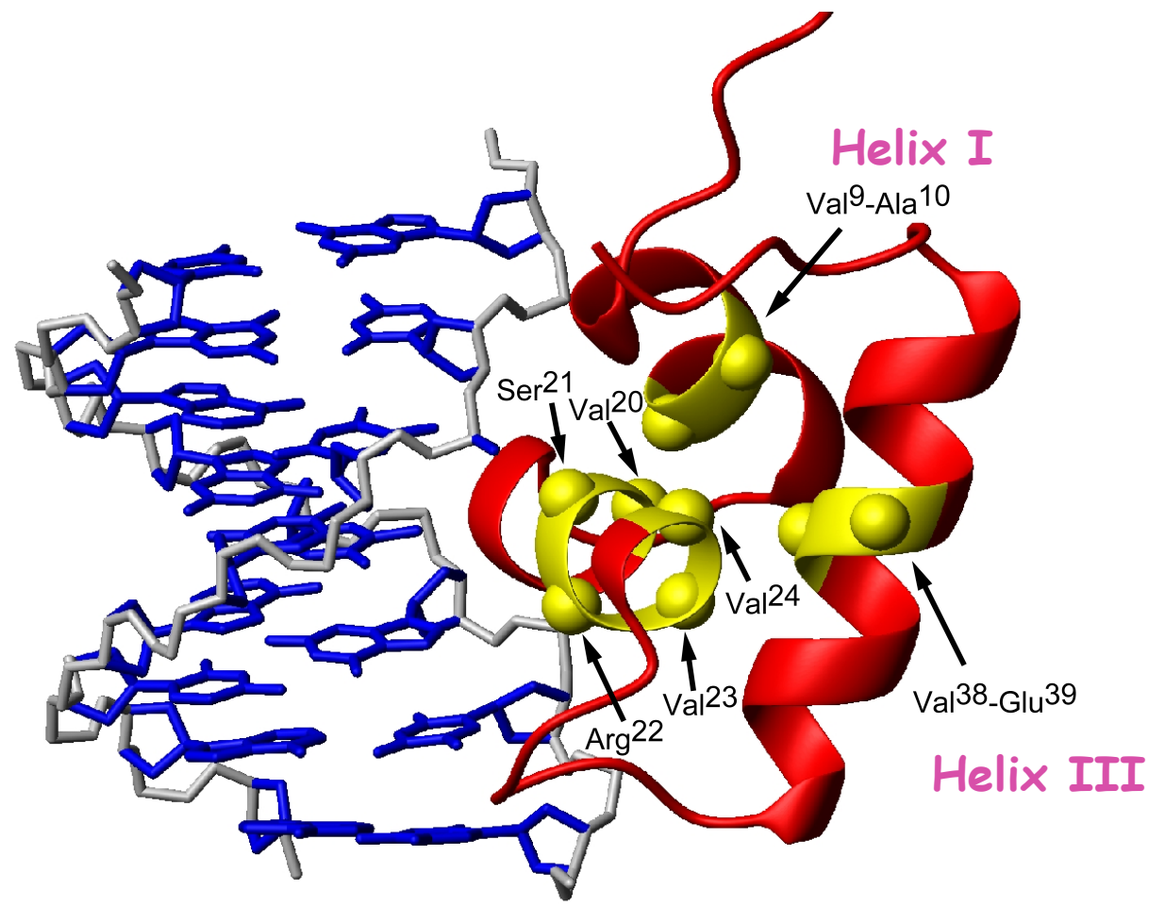
Boelens *et al.* (1985) *J. Biomol. Struct. Dyn.* 3 269-280.



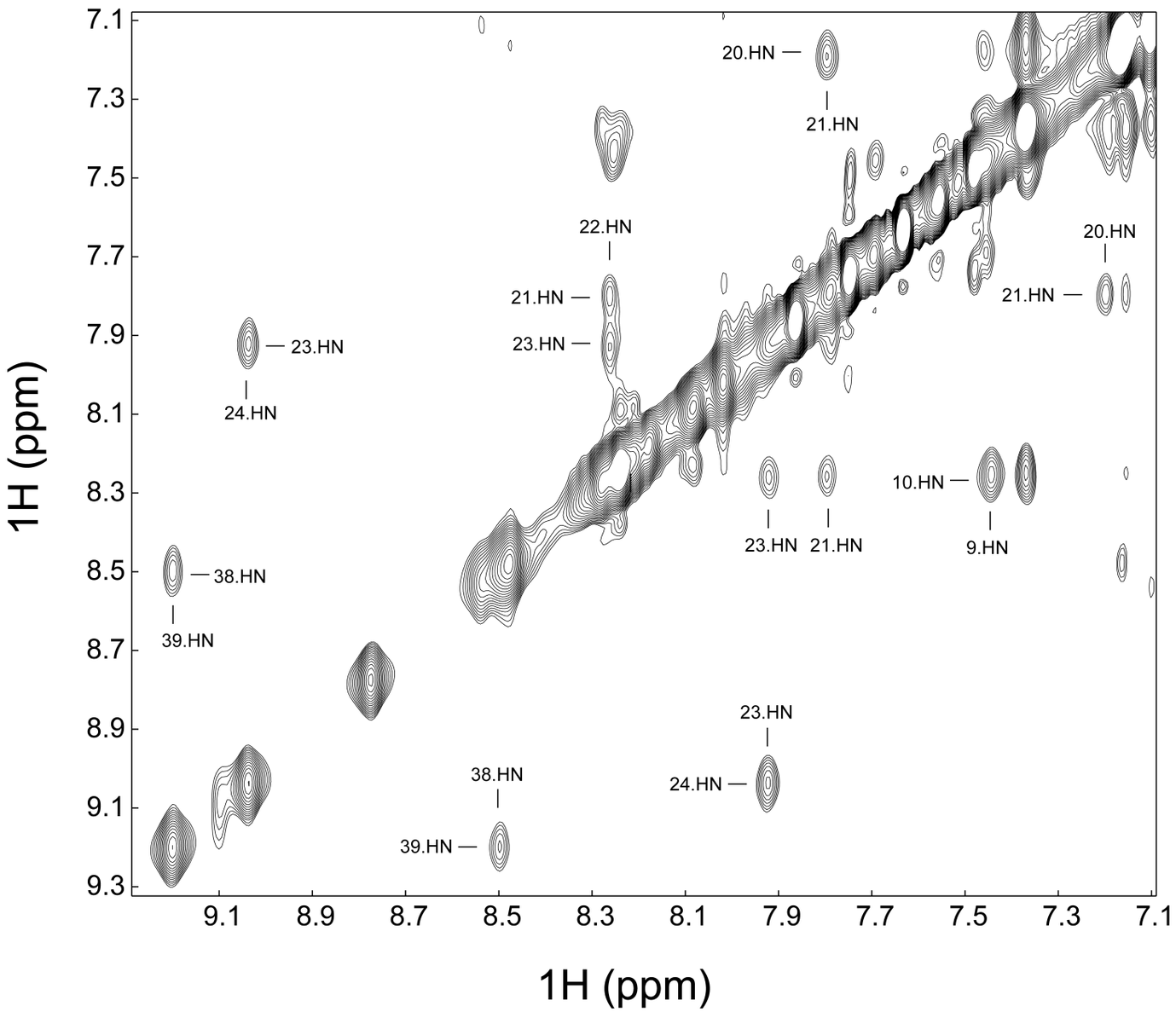
Lac-DNA complex

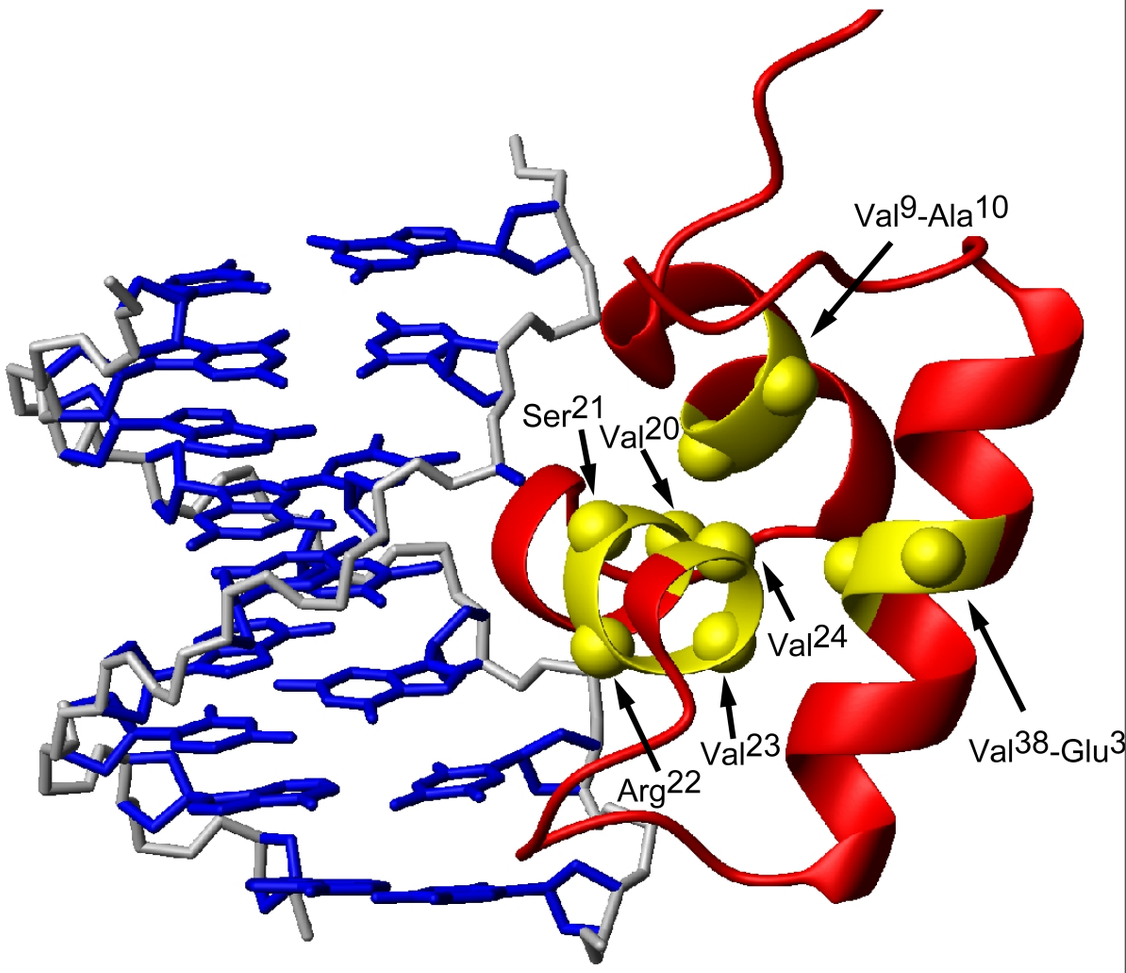
Kalodimos *et al.* (2002) *Nature Struct. Biol.* 9 193-197.



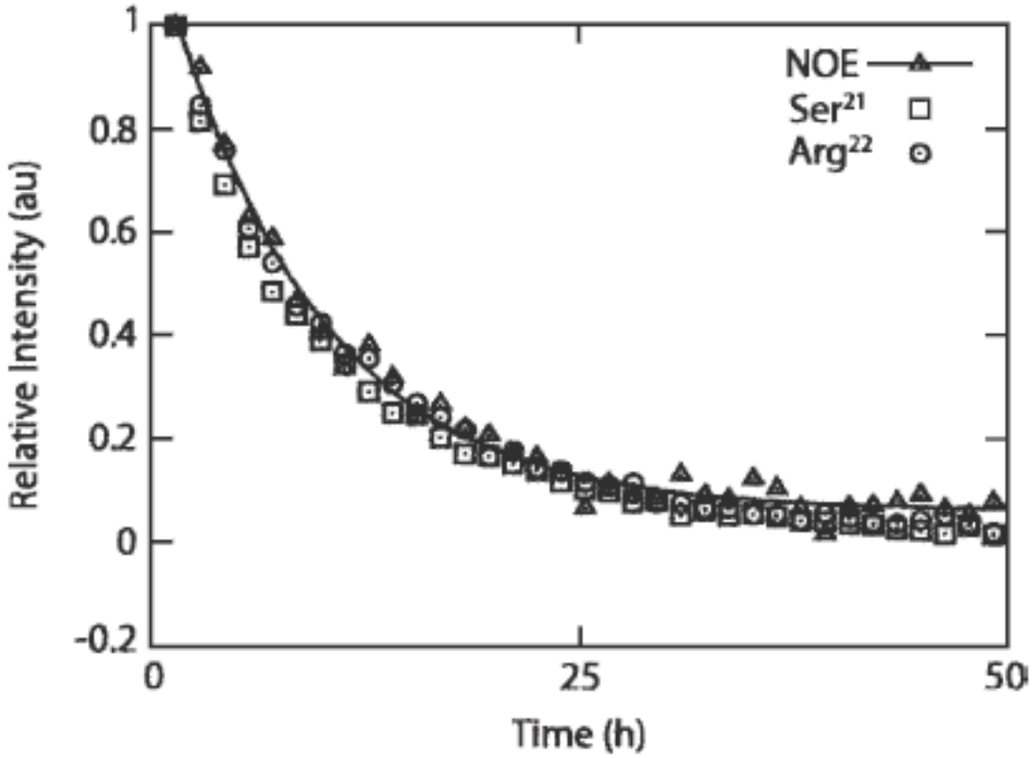


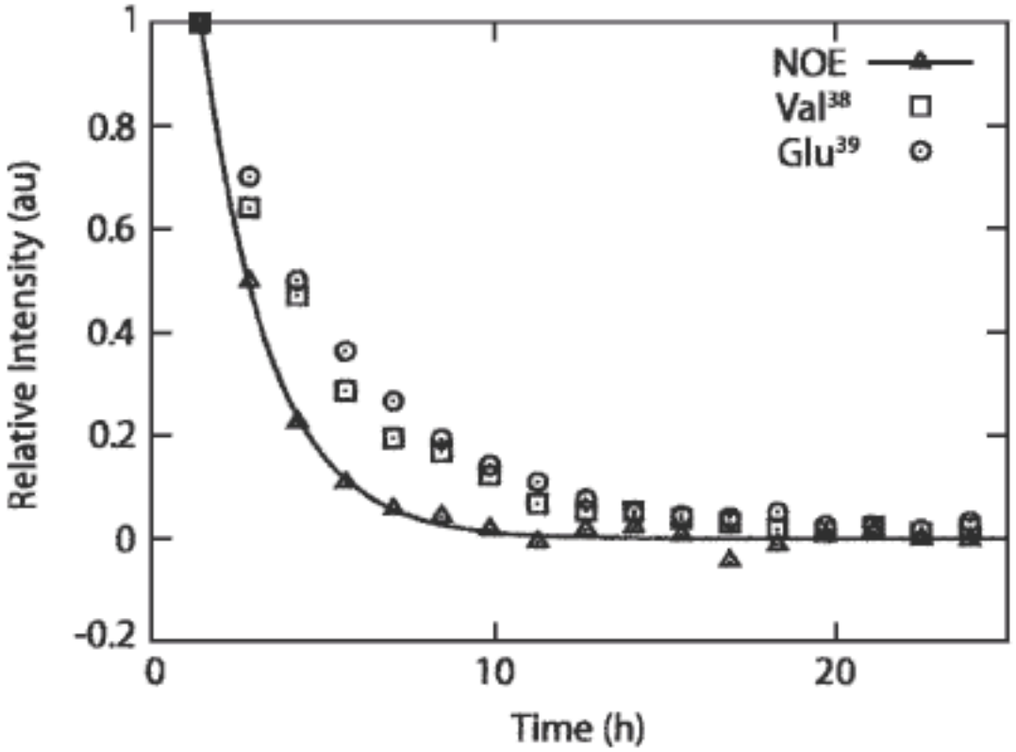
Movimentos correlacionados podem ser detectados através de medidas da velocidade de decaimento de dois NHs e de um NOE entre eles

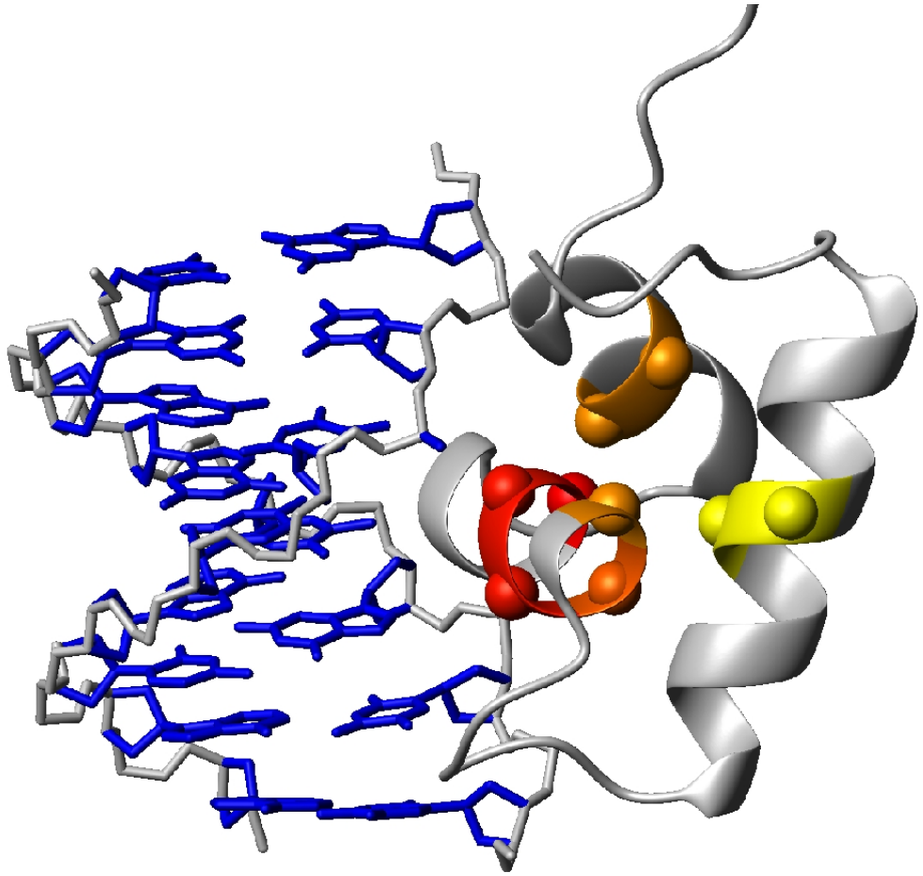


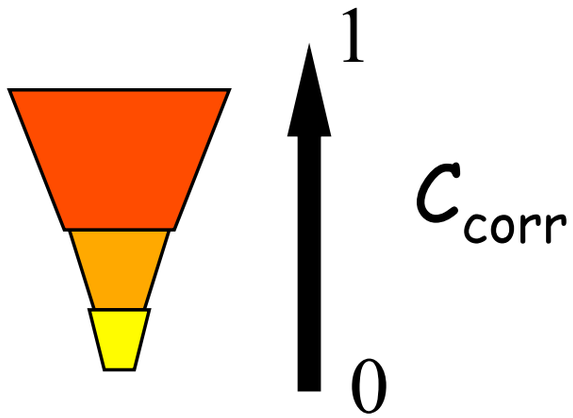


2D NOE em D_2O

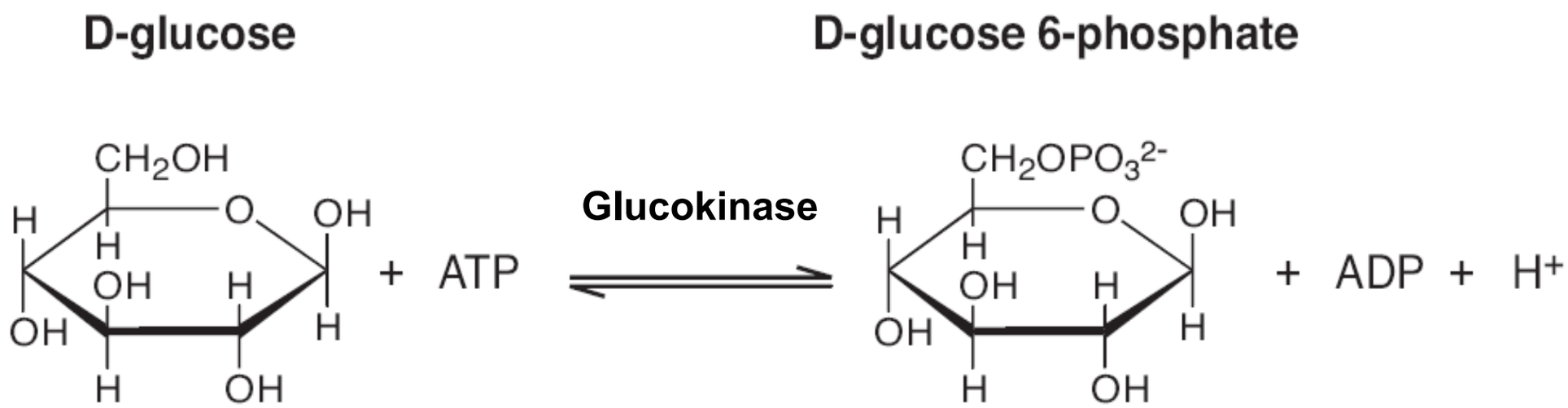








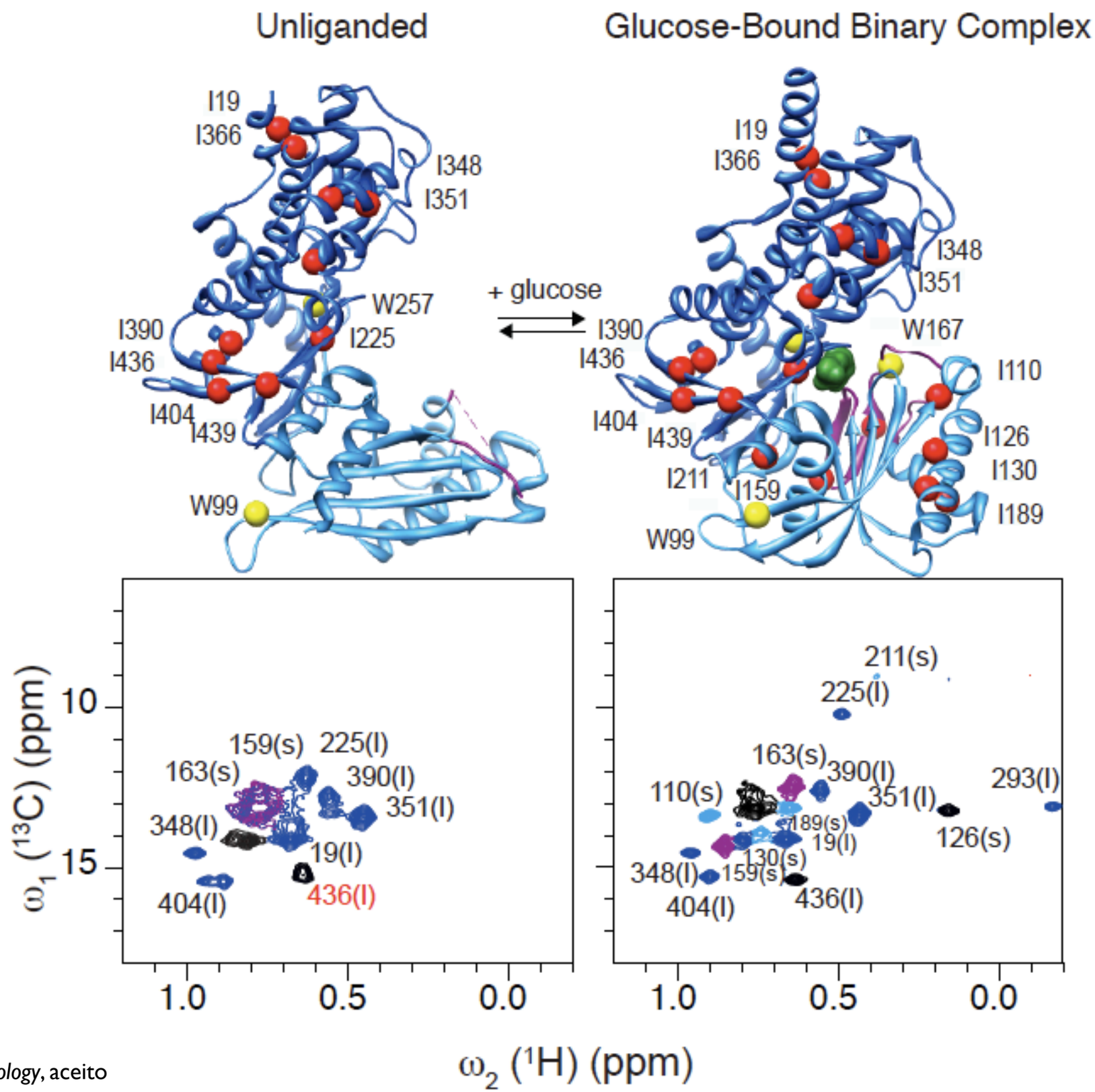
Salinas, Diercks, Kaptein e Boelens (2006) *Protein Sci.* **15**: 1752-1759



Glicoquinase (hexoquinase IV) catalisa a primeira etapa determinante da velocidade da glicólise no pâncreas e no fígado

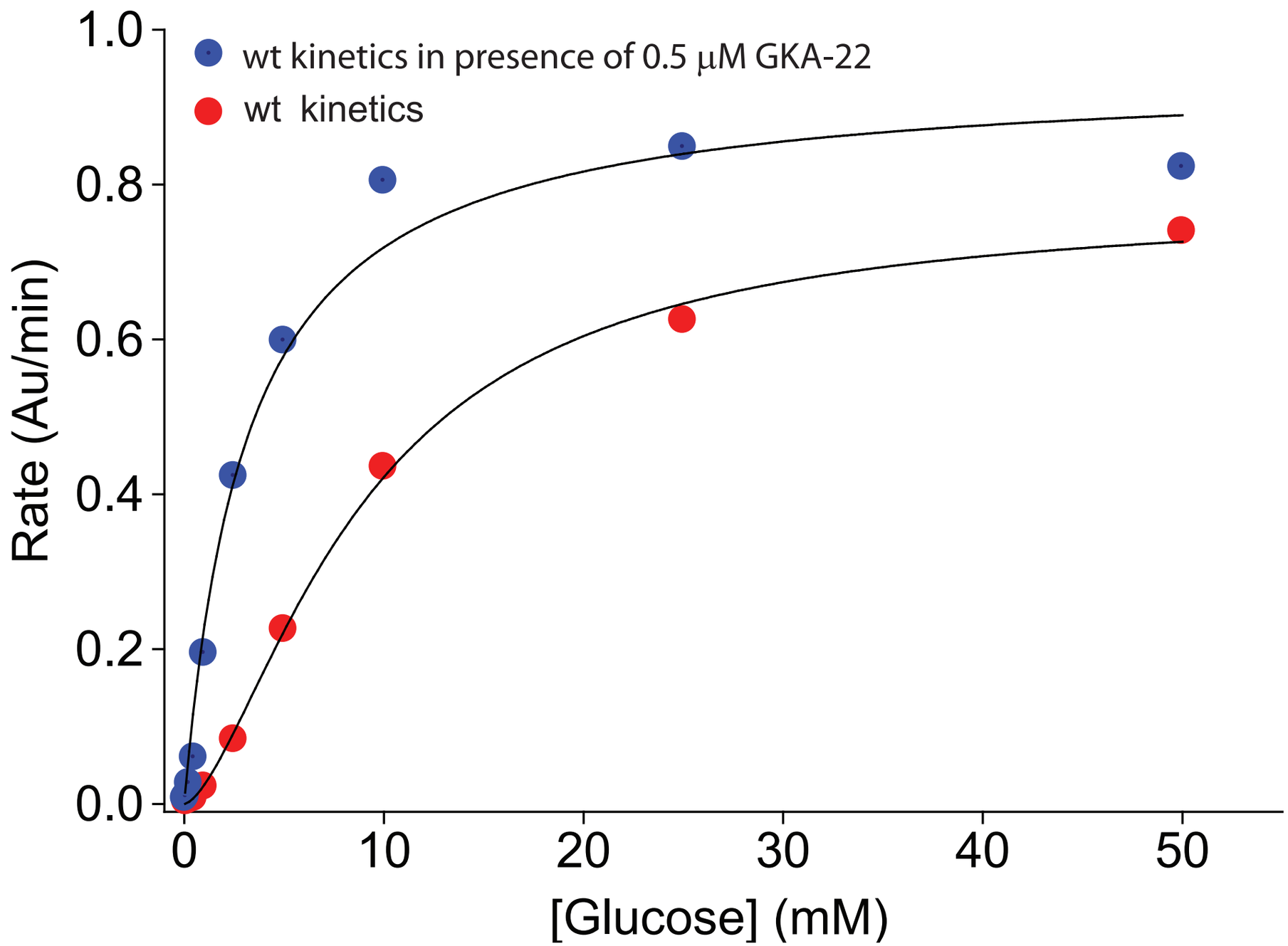
Larion, Salinas, et al. *Plos Biology*, aceito

GK é uma proteína intrínsicamente dinâmica em diferentes escalas de tempo. Esta flexibilidade desaparece no complexo de glicose



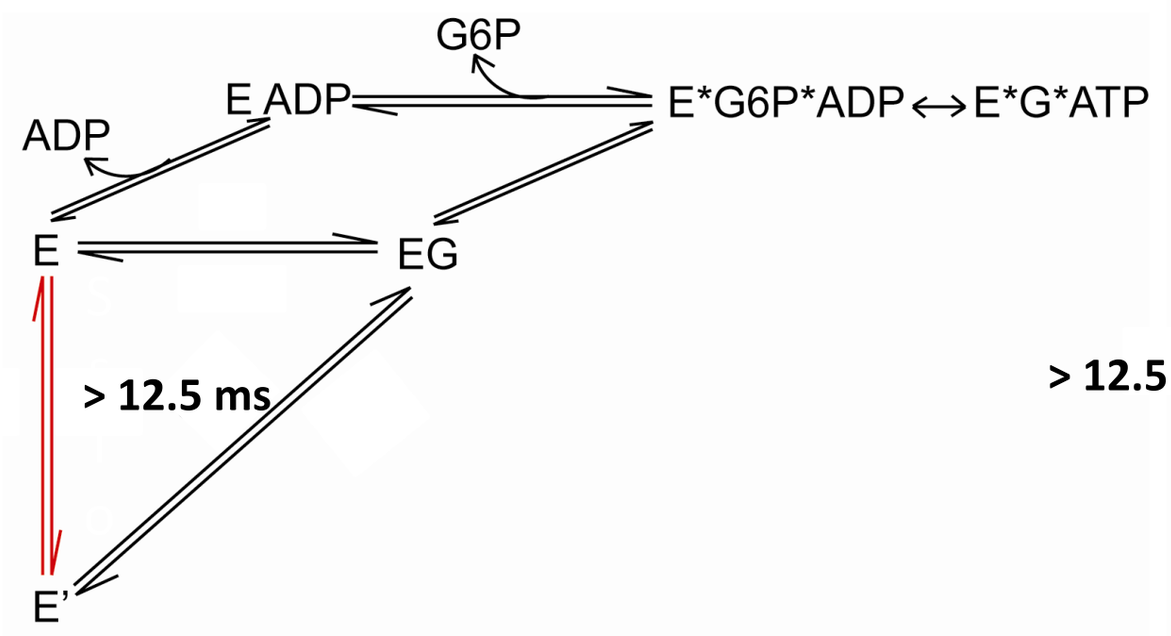
Larion, Salinas, et al. *Plos Biology*, aceito

Glicoquinase é uma enzima monomérica, com apenas um sítio de ligação ao substrato

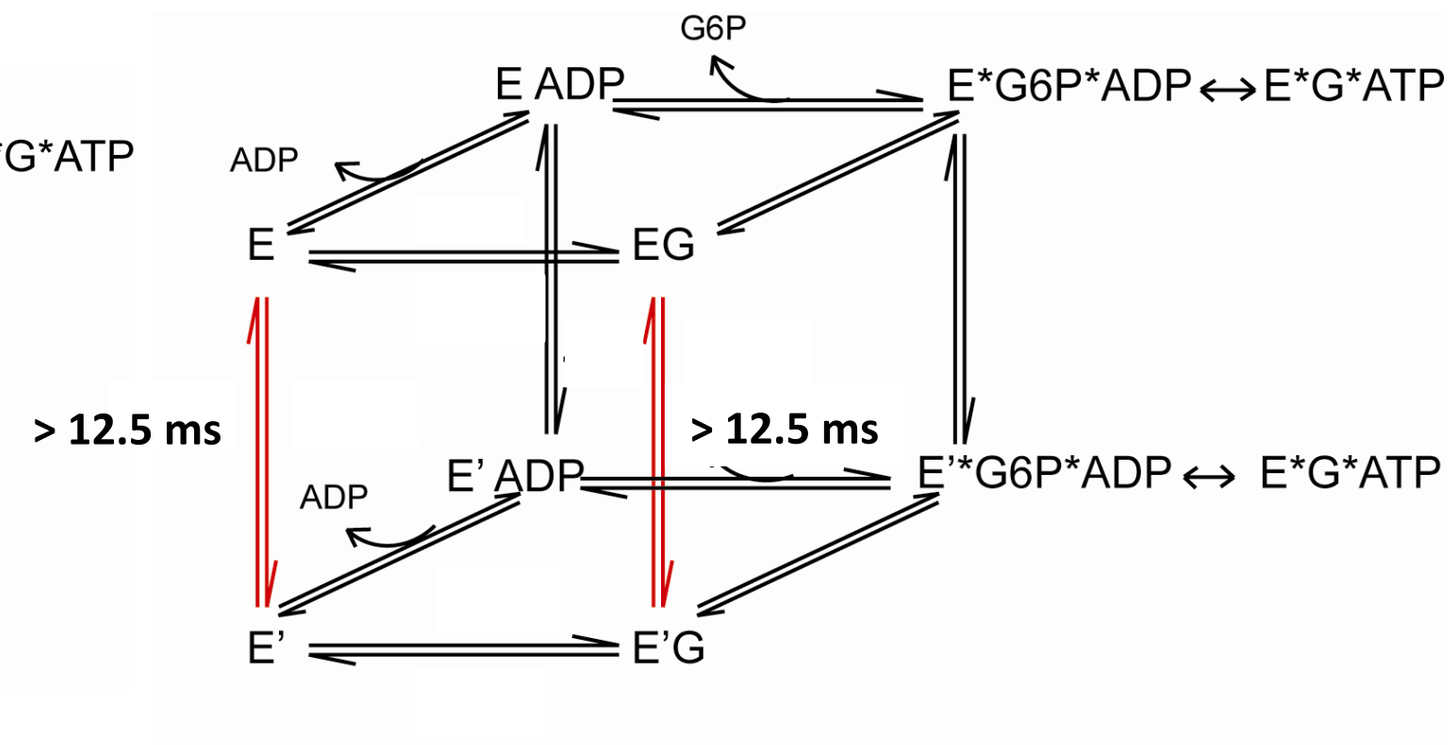


Modelos cinéticos propostos para explicar a cooperatividade postulam a existência de transições conformacionais lentas (~1/k_{cat})

Mnemonic Mechanism



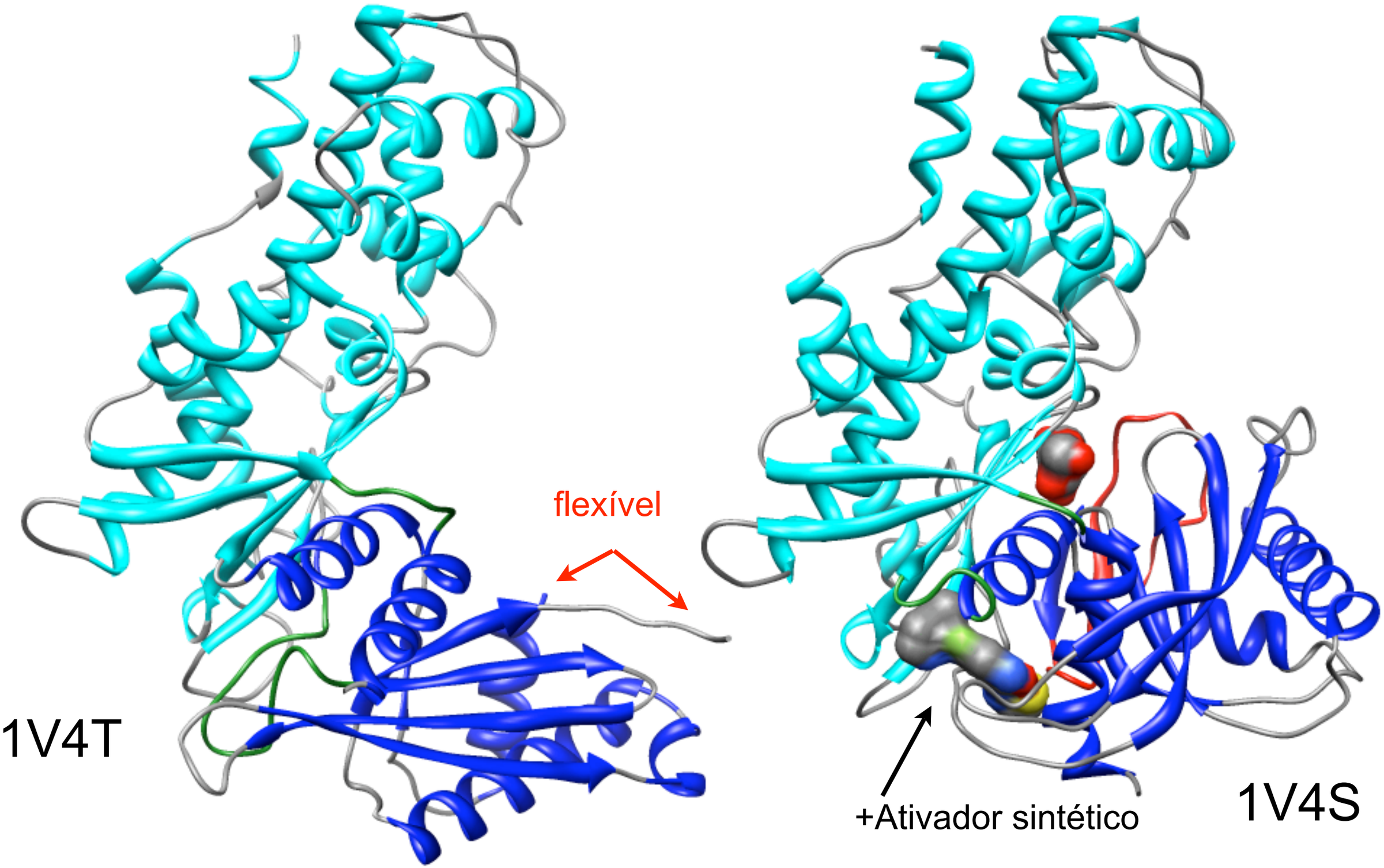
Ligand Induced Slow Transition Model



J Ricard, J.C Meunier, J Buc Eur J. Biochem. 1974, **49** 195

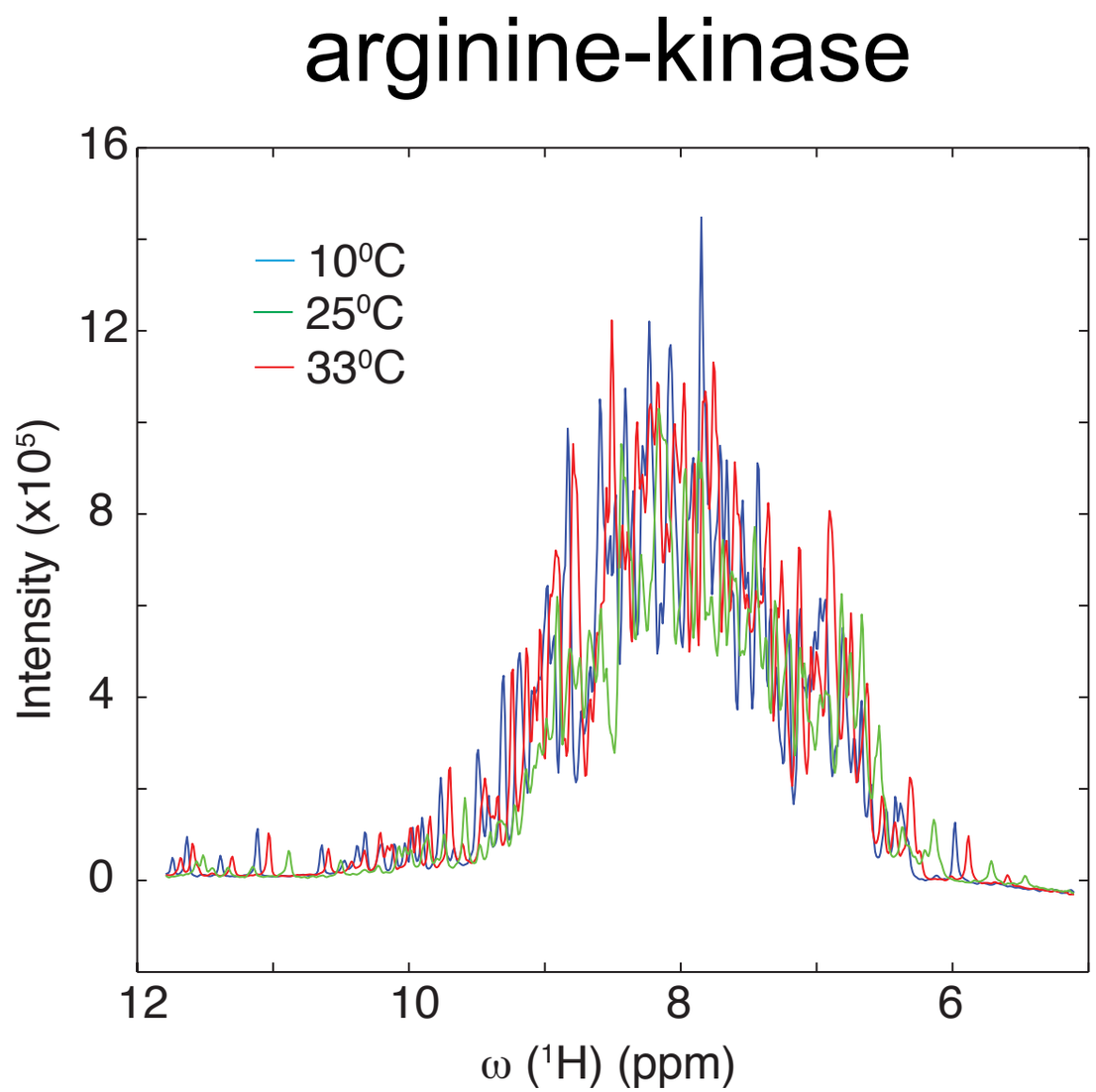
Ainslie et al., J. Biol. Chem. 1972 **247, 7088**

Estruturas cristalográficas mostram que glicoquinase experimenta conformações discretas na ausência e presença de glicose



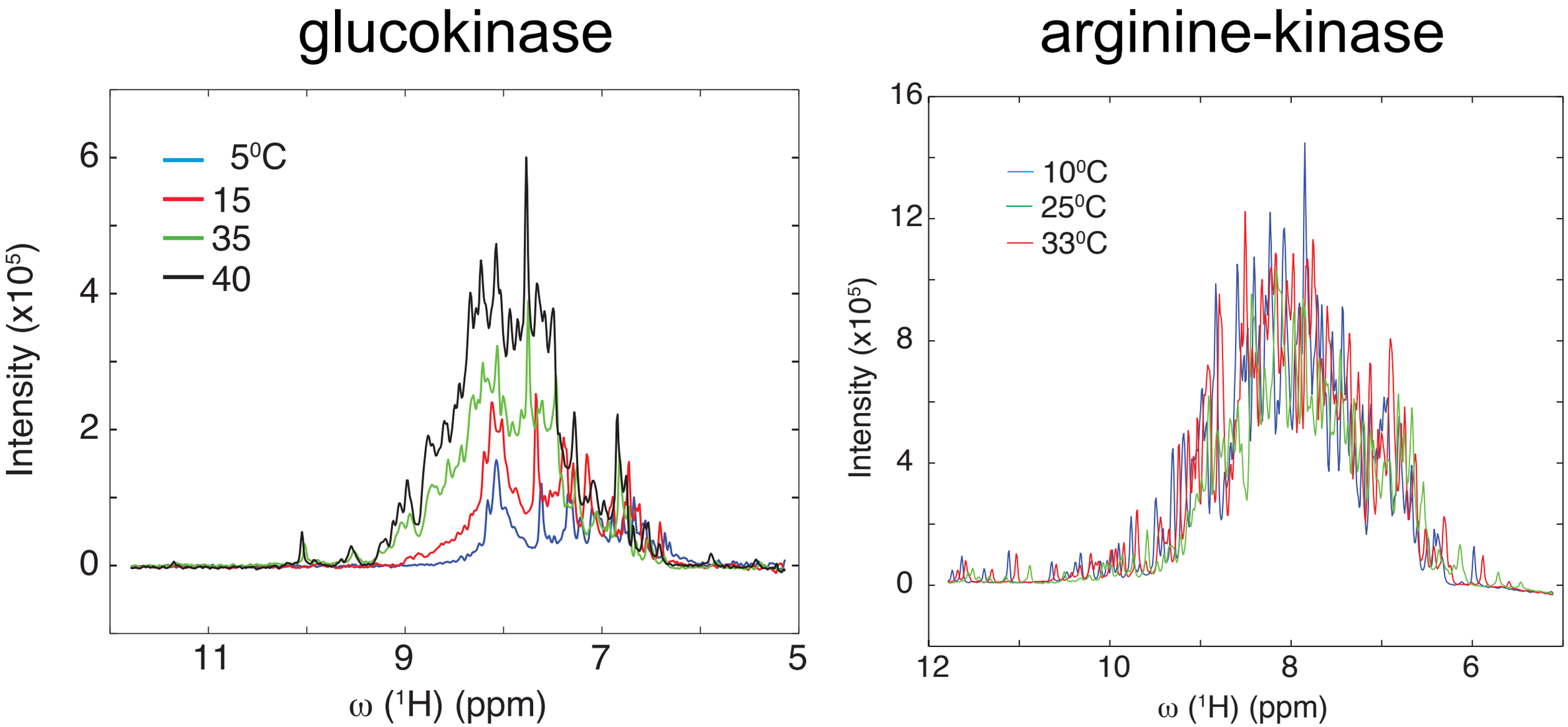
Kamata et al. (2004) *Structure* **12**: 429-438

O efeito da temperatura sobre o espectro de glicoquinase não é explicado apenas pelo tamanho da enzima



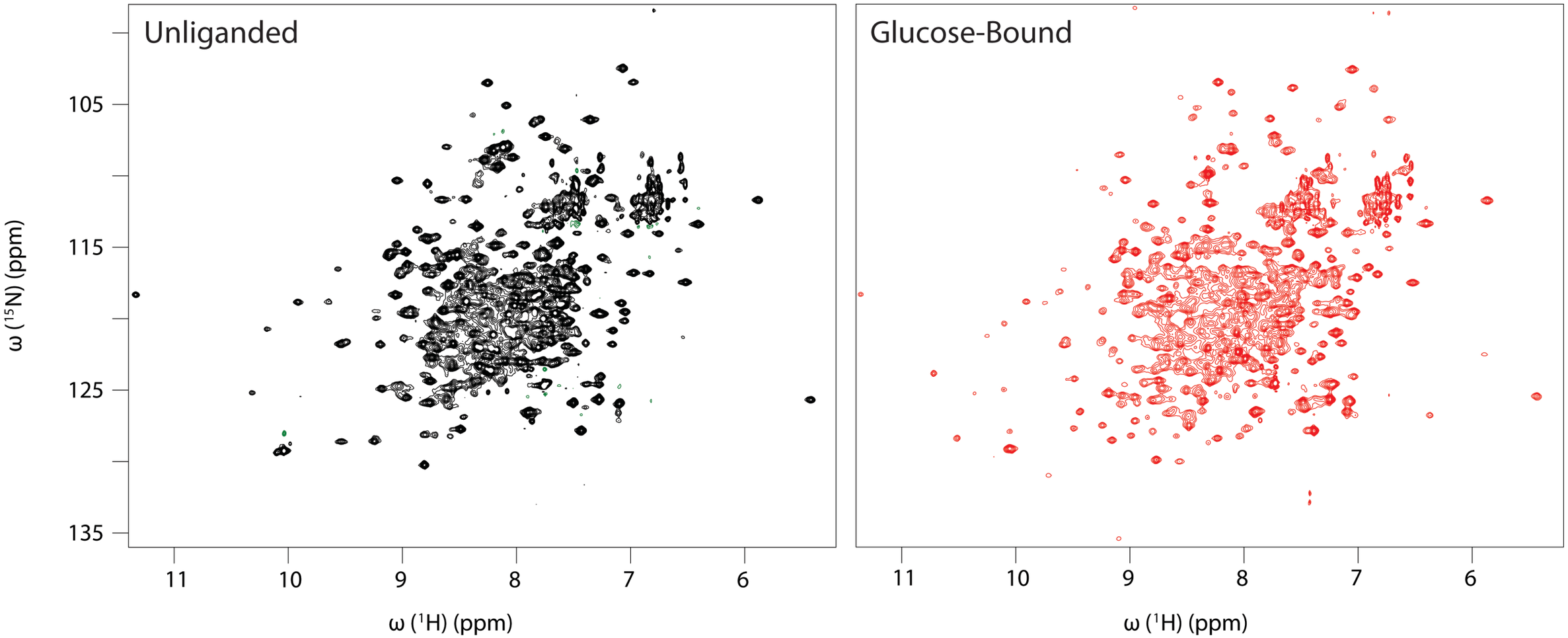
Larion, Salinas, Bruschweiler, Brüschweriler e Miller (2010) *Biochemistry* **49**: 7969

O efeito da temperatura sobre o espectro de glicoquinase não é explicado apenas pelo tamanho da enzima



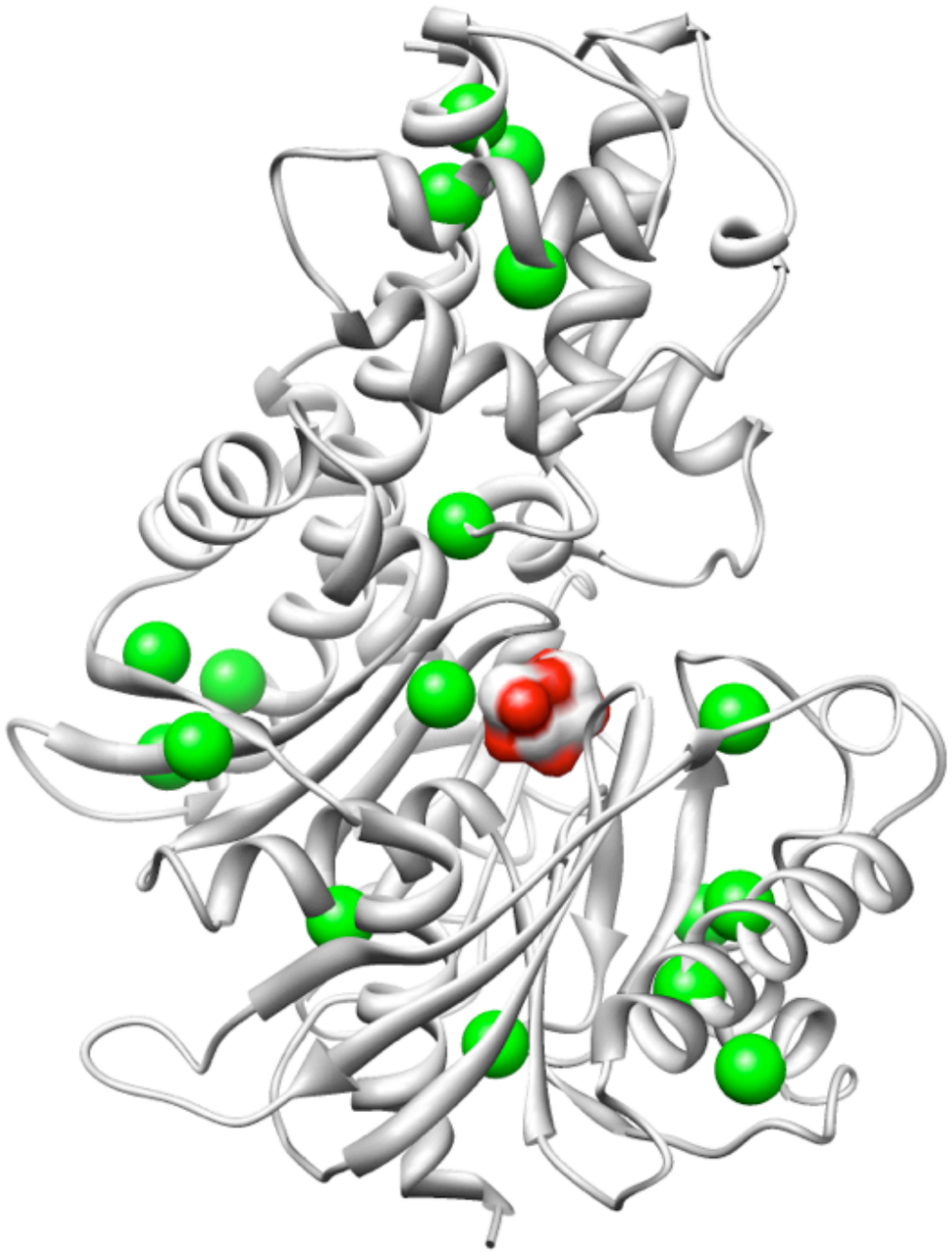
Larion, Salinas, Bruschweiler, Brüschweriler e Miller (2010) *Biochemistry* **49**: 7969

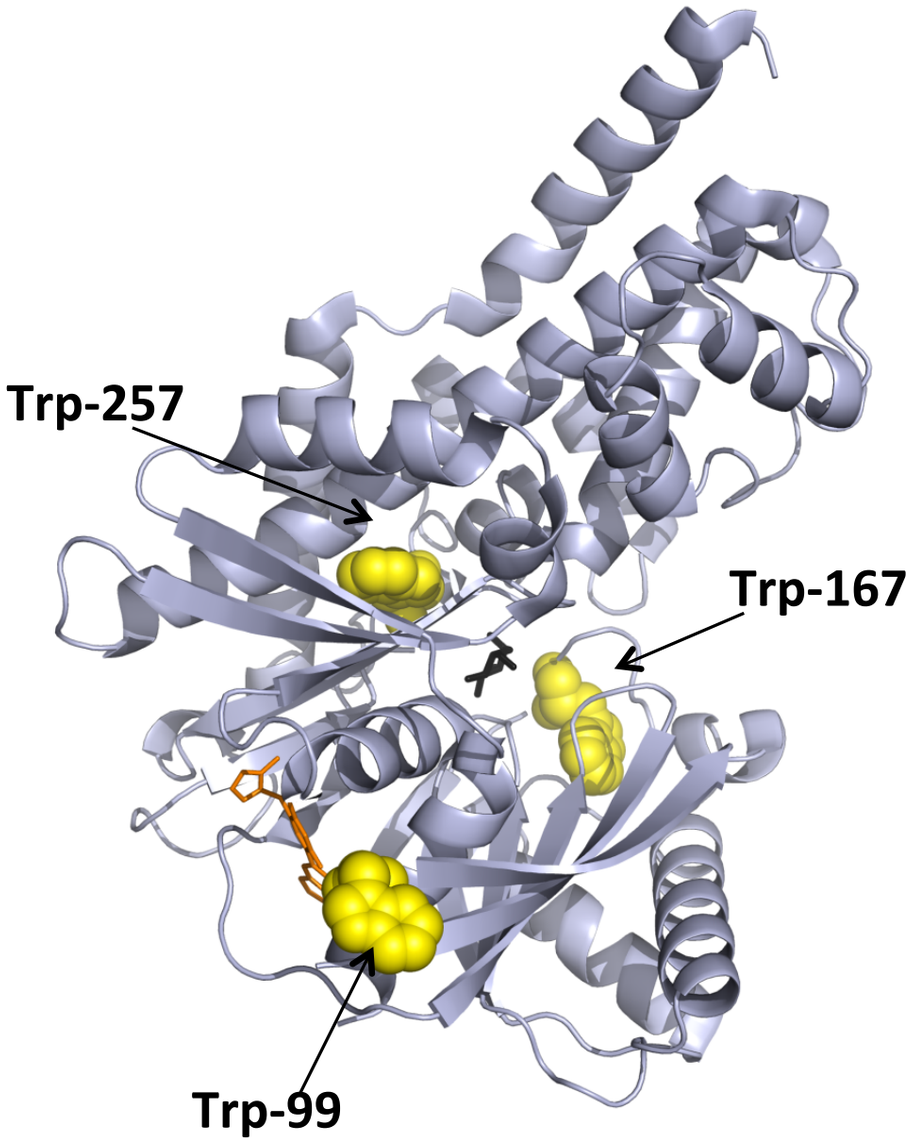
Glucoquinase é um desafio devido ao elevado peso molecular (52 kDa) e à grande sobreposição espectral



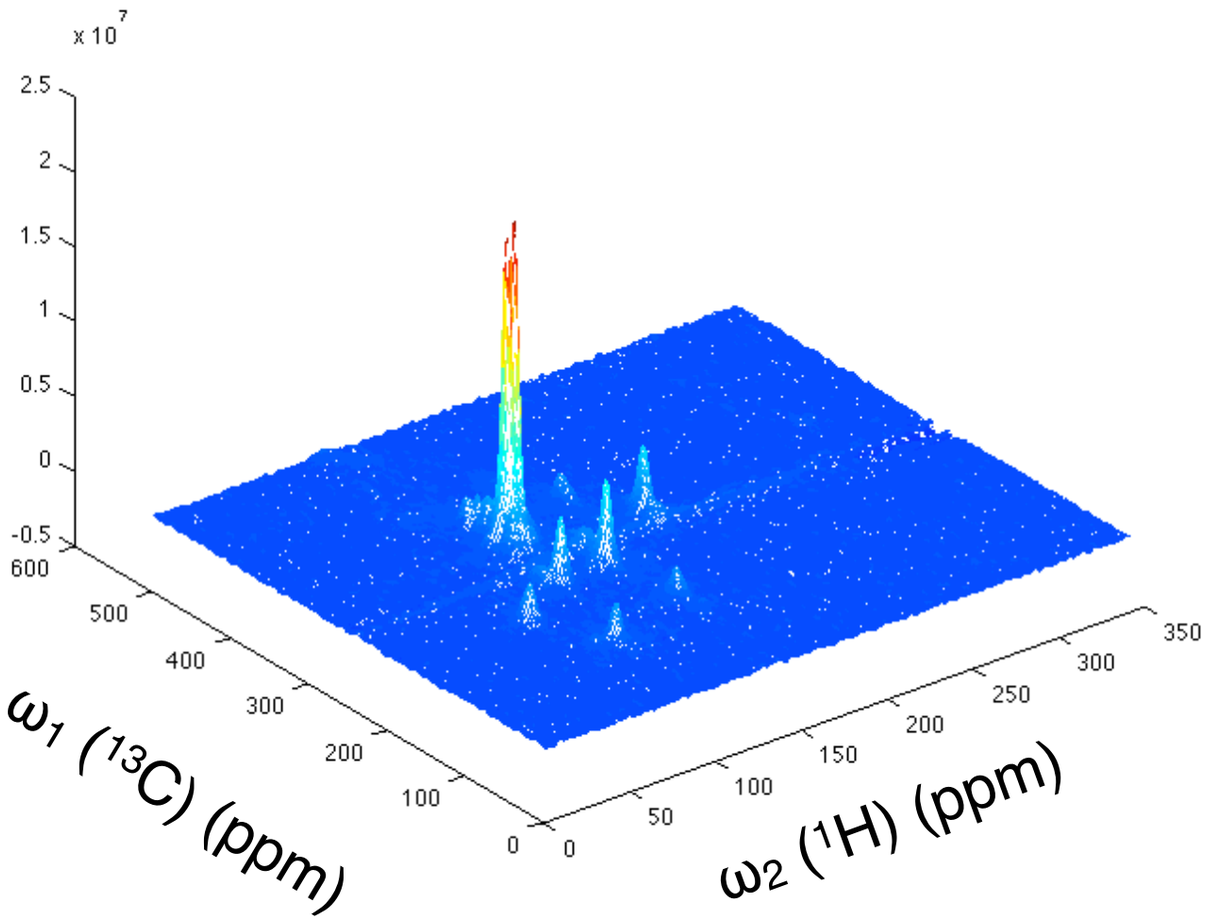
Larion, Salinas, Bruschweiler, Brüschweriler e Miller (2010) *Biochemistry* **49**: 7969

Para diminuir a complexidade espectral nós marcamos seletivamente a cadeia lateral das isoleucinas ($^{13}C^{\delta1}(H^{\delta1})_3$) e triptofanos ($^{15}N^{\varepsilon}H^{\varepsilon}$)



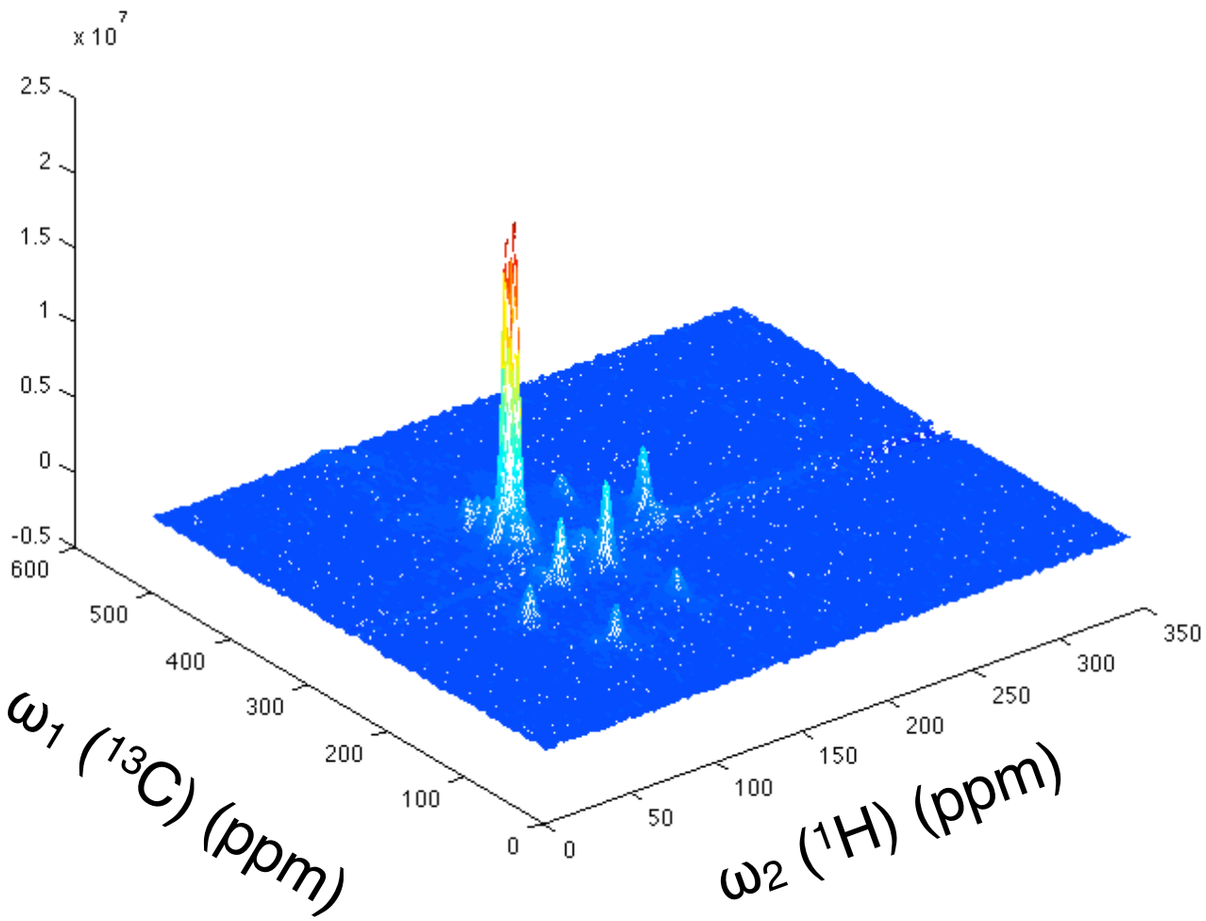


Glucose binding causes large changes in the ^{13}C-HMQC spectrum of glucokinase

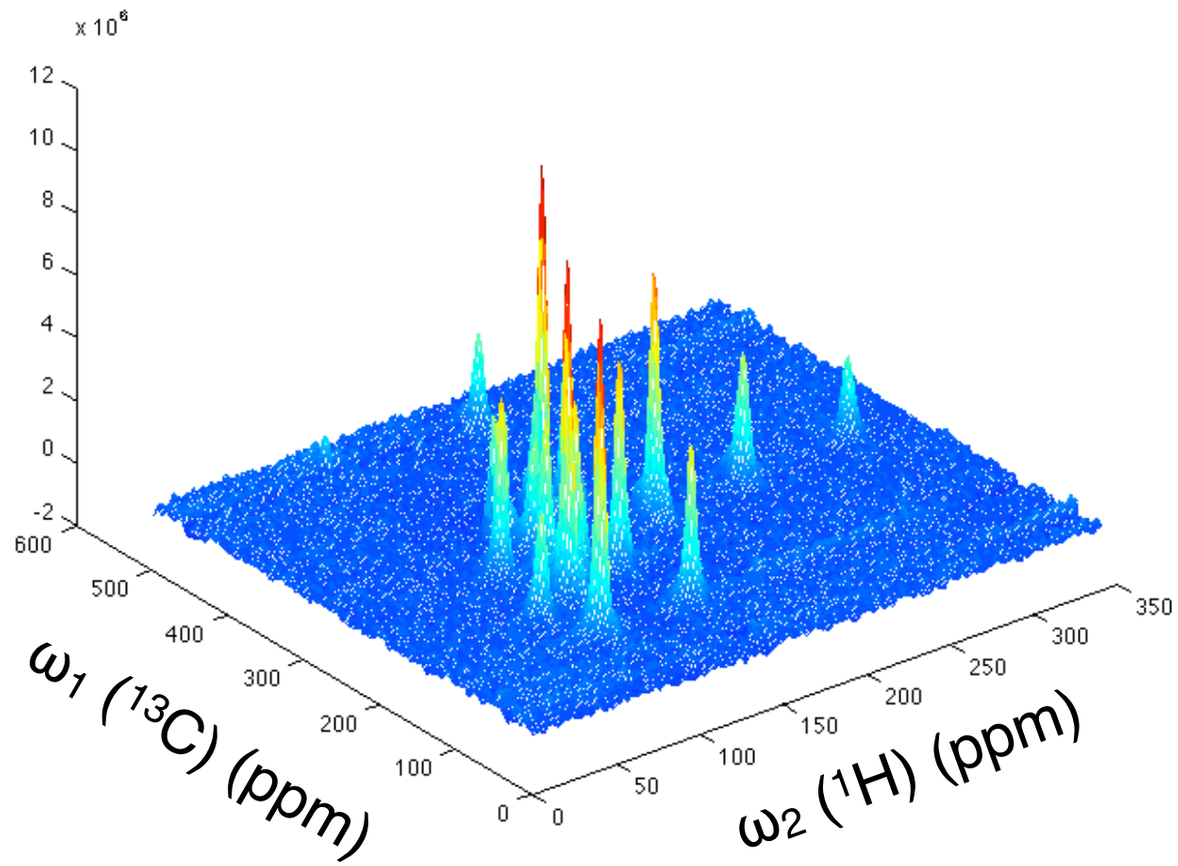


unliganded

Glucose binding causes large changes in the ^{13}C-HMQC spectrum of glucokinase

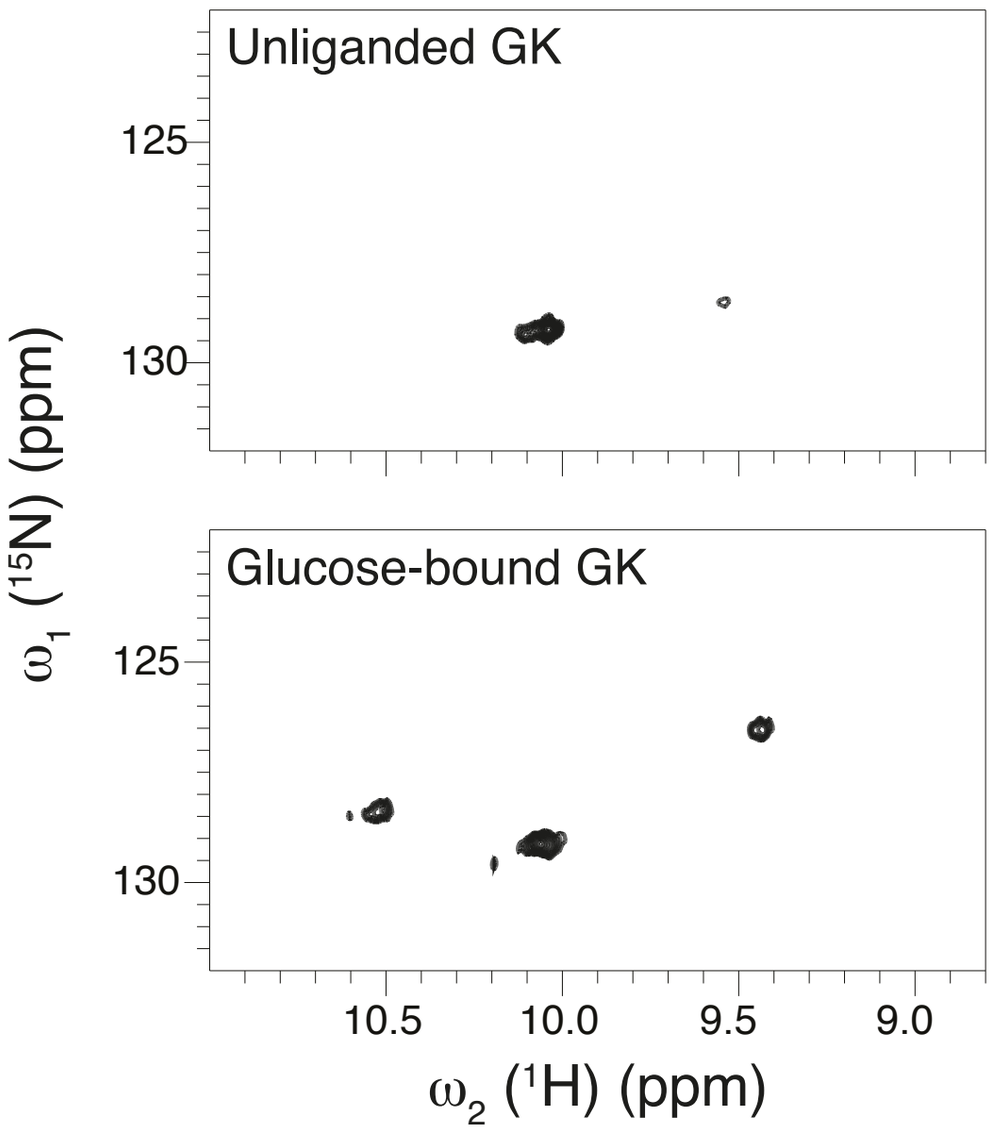


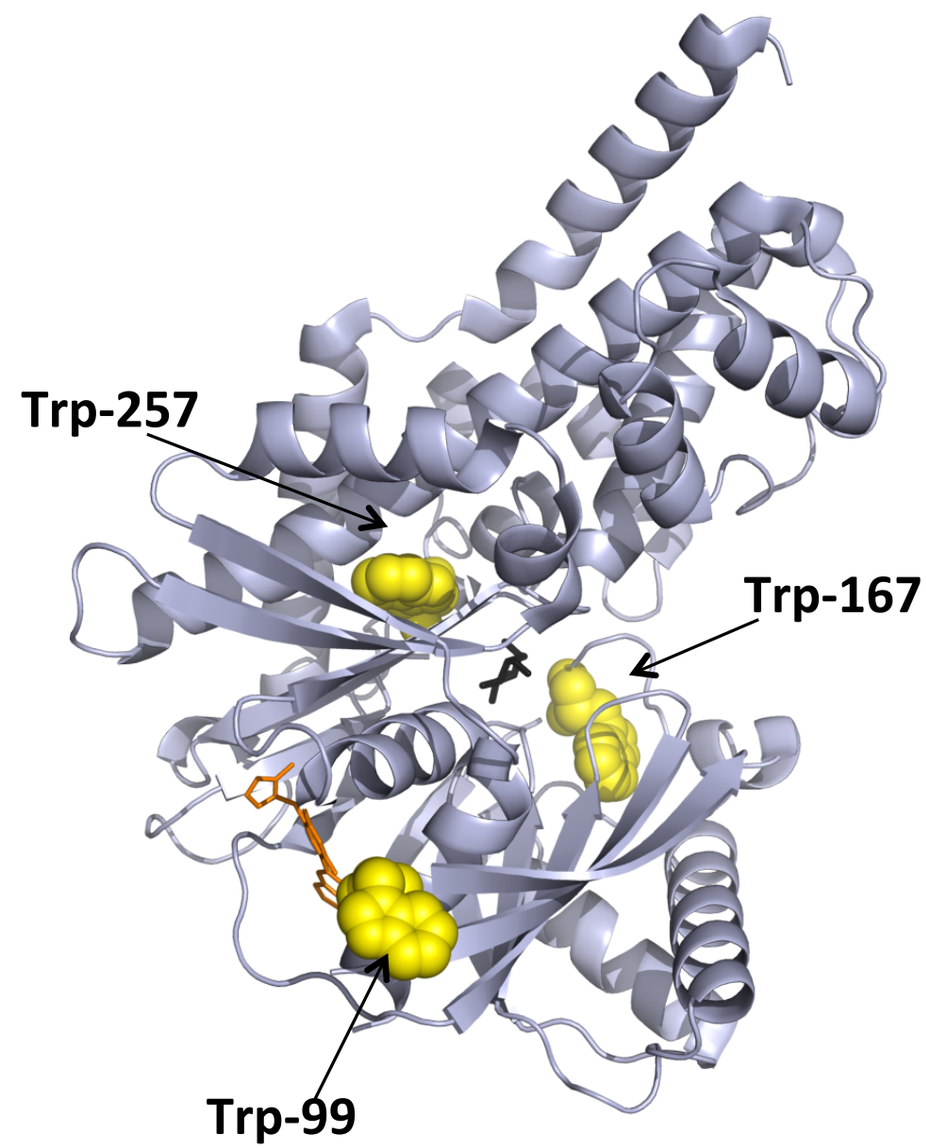
unliganded



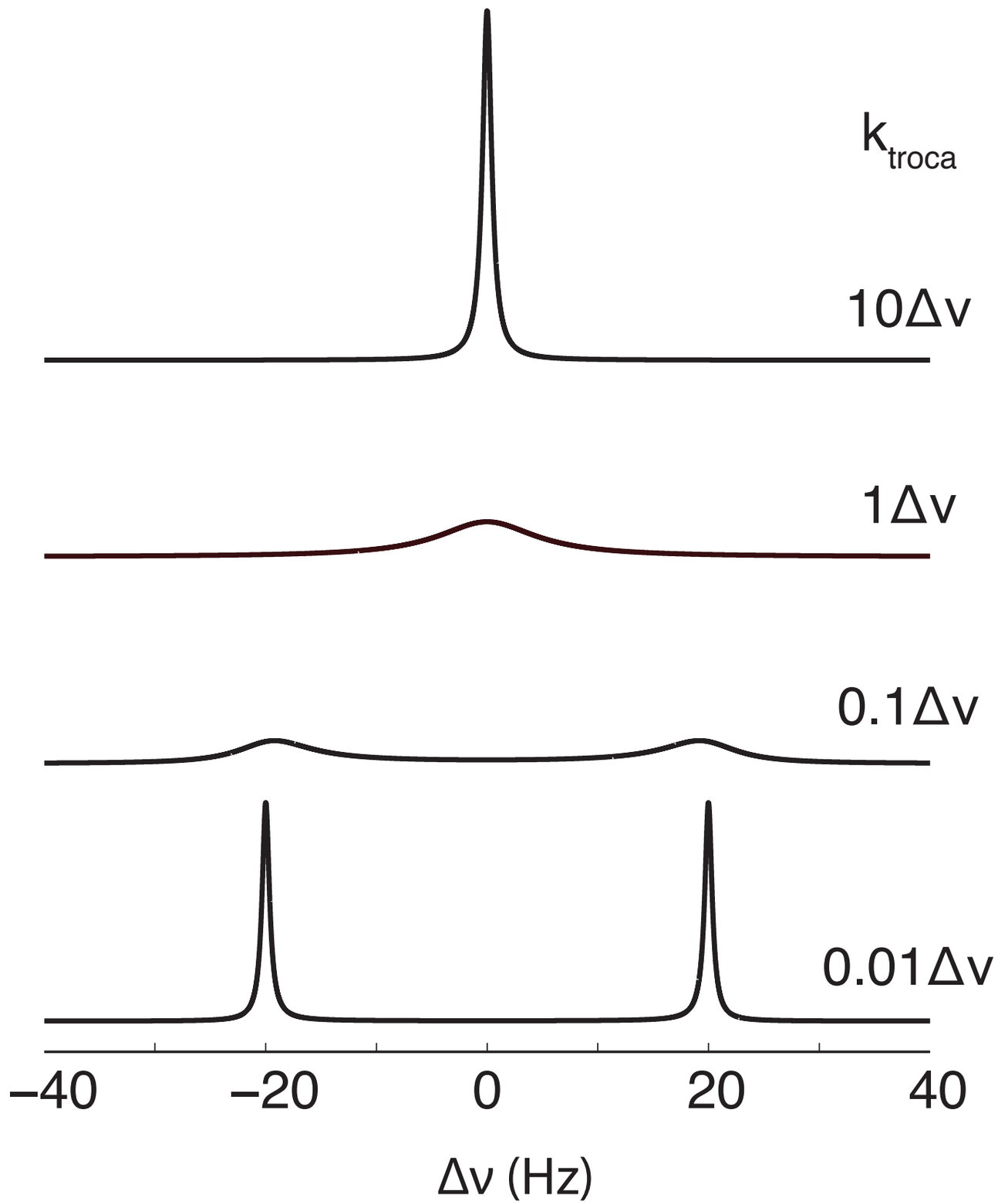
+glucose

Alterações espectrais devido à ligação de glicose também são observadas através dos sinais dos triptofanos

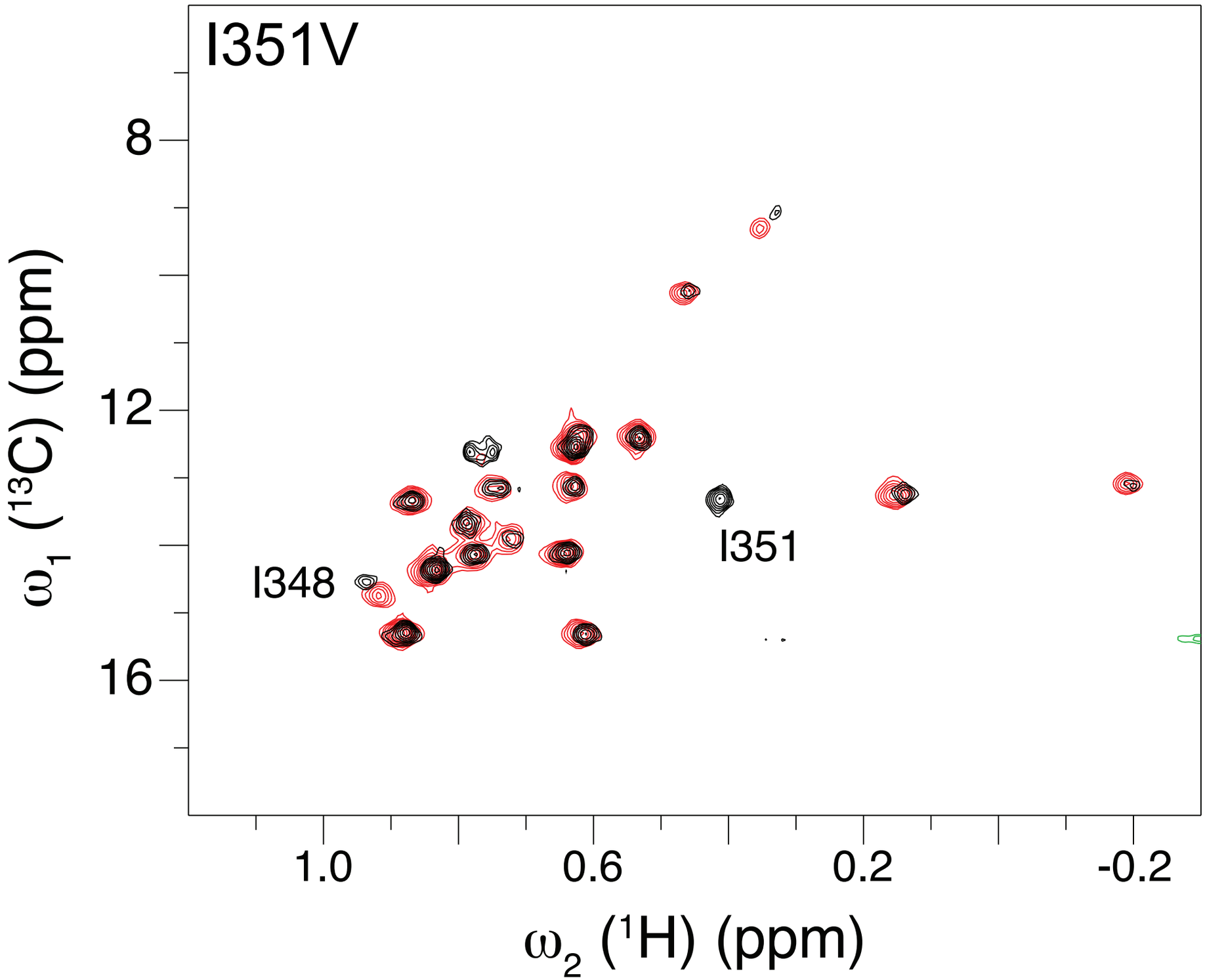




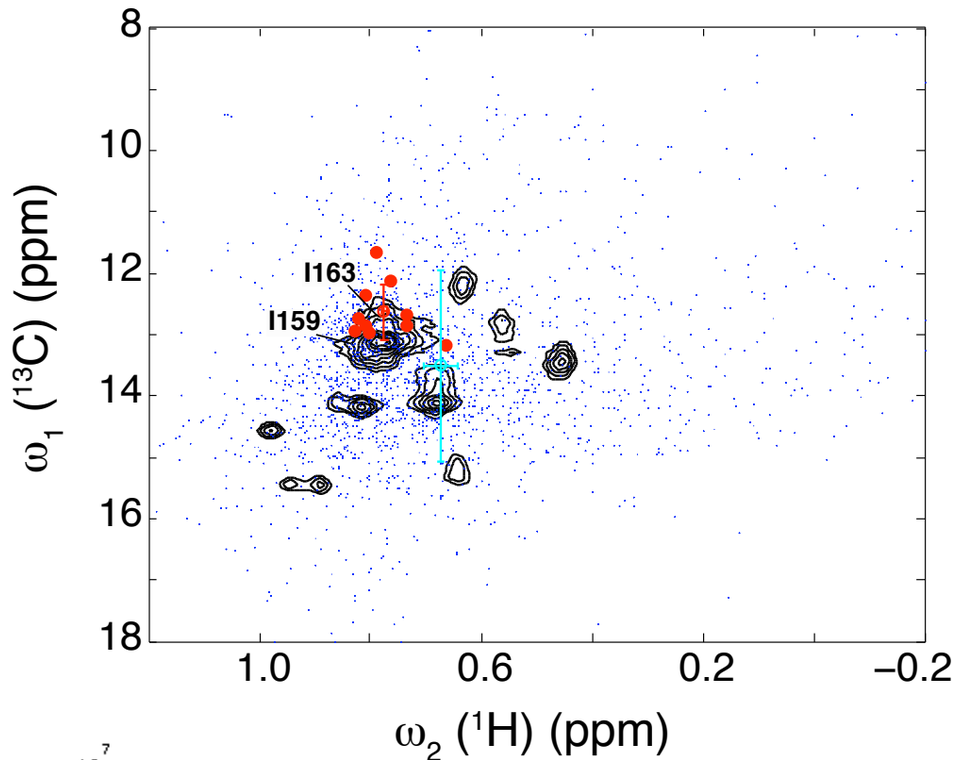
A ausência de sinais indica movimentos das cadeias laterais em escala de tempo intermediária (μs - ms)

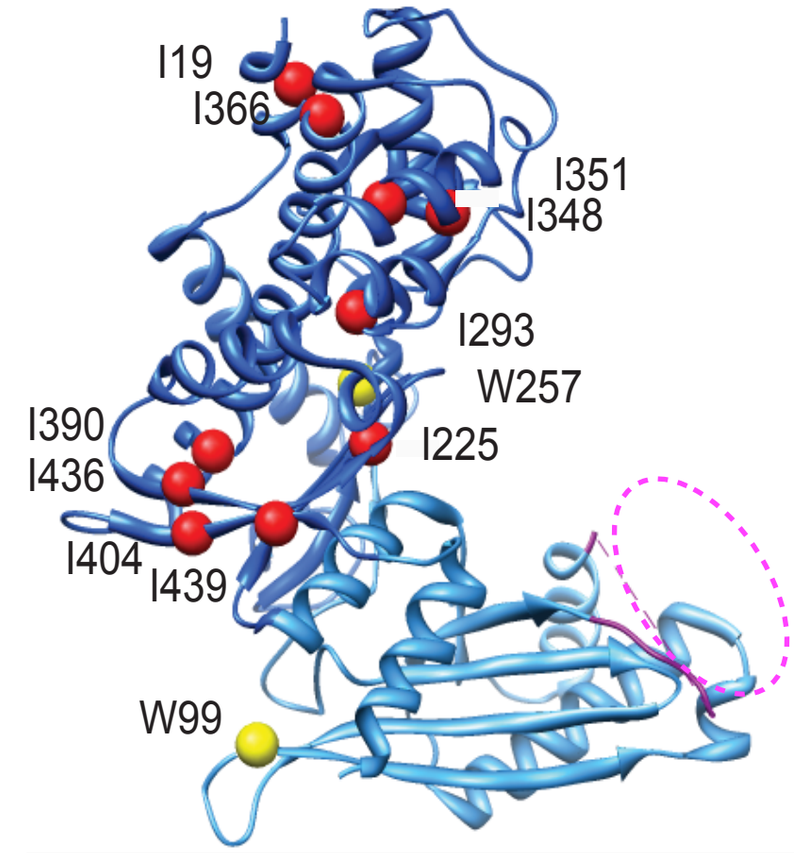


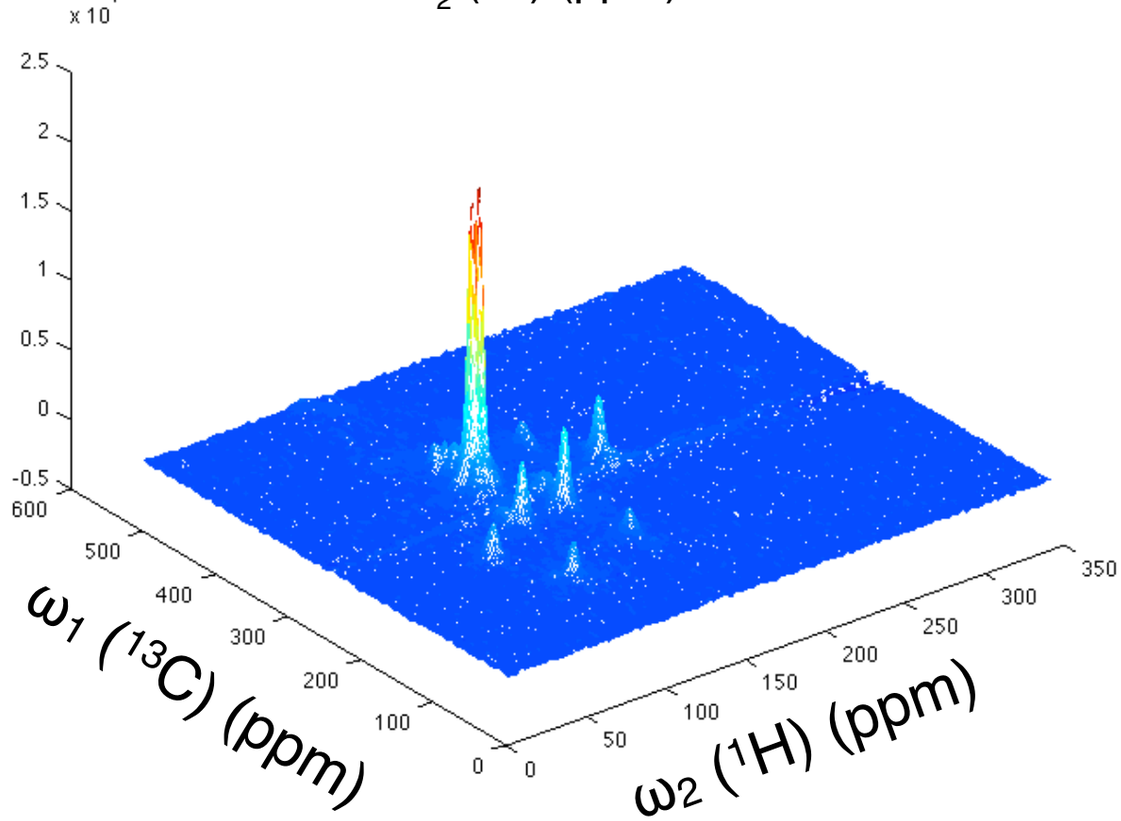
Como fazer a atribuição do espectro?

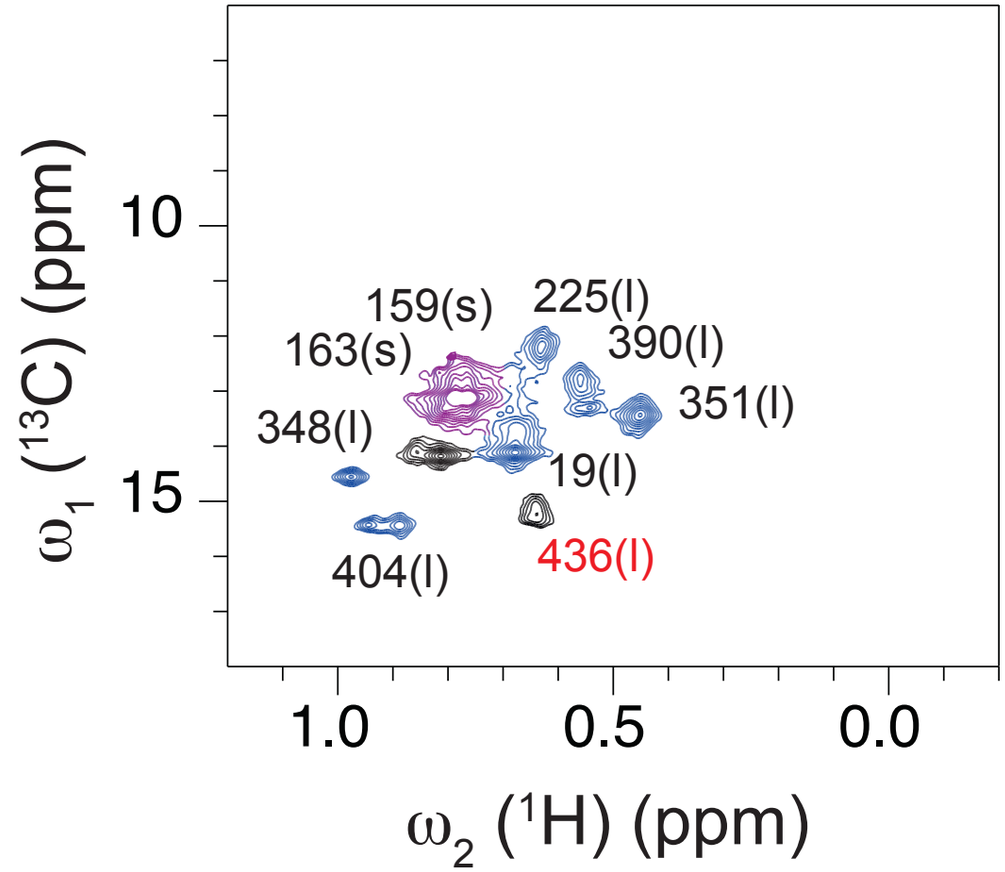


O domínio menor é intrinsecamente desordenado

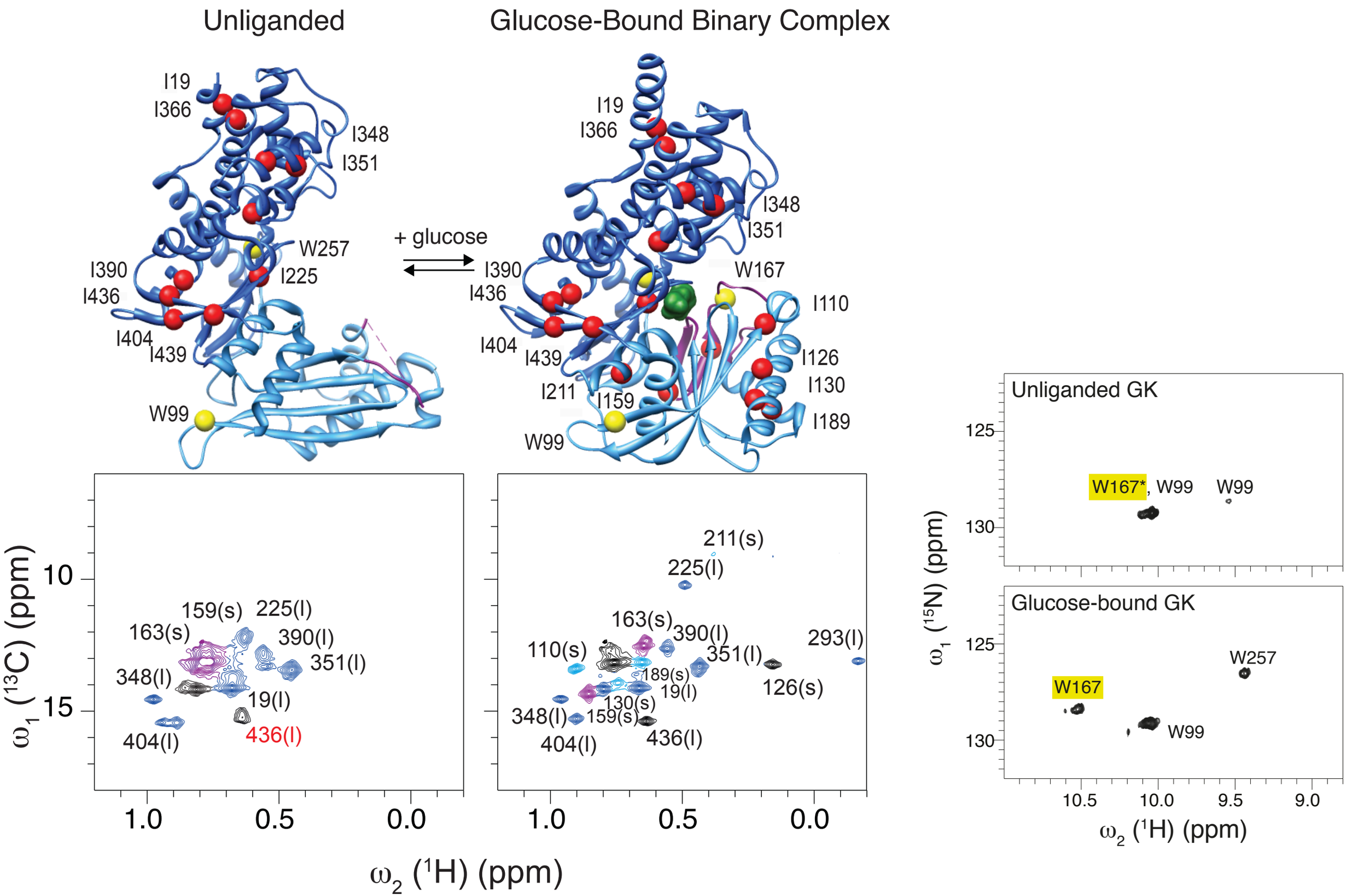




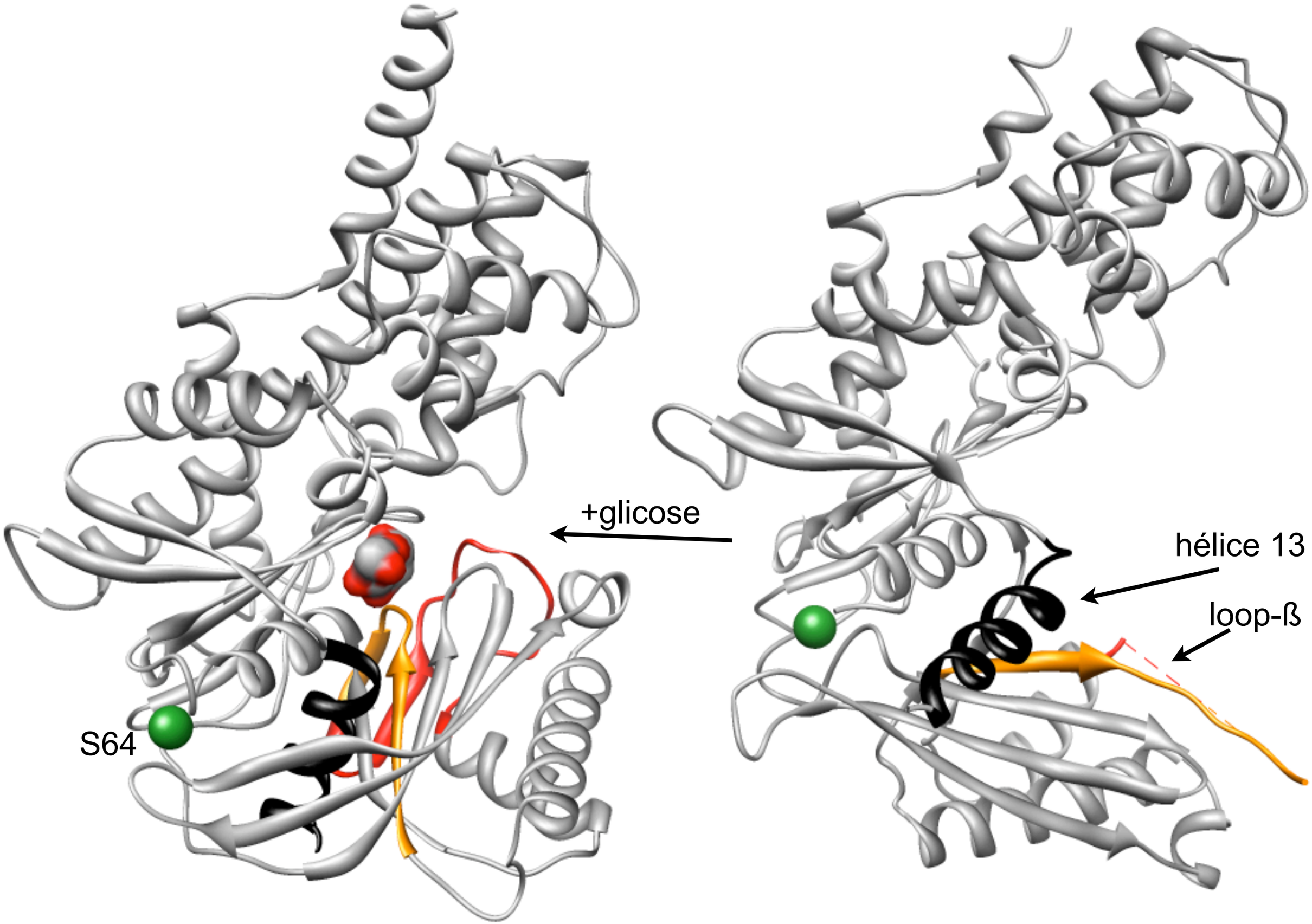




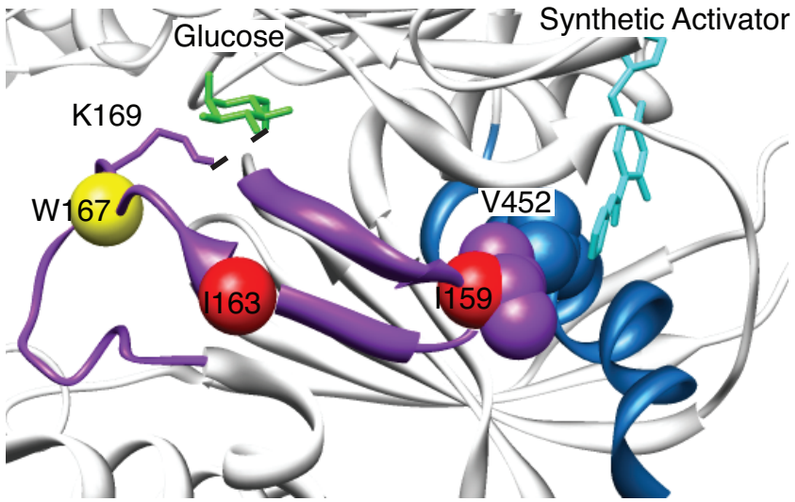
Associação com glicose induz estruturação do domínio menor

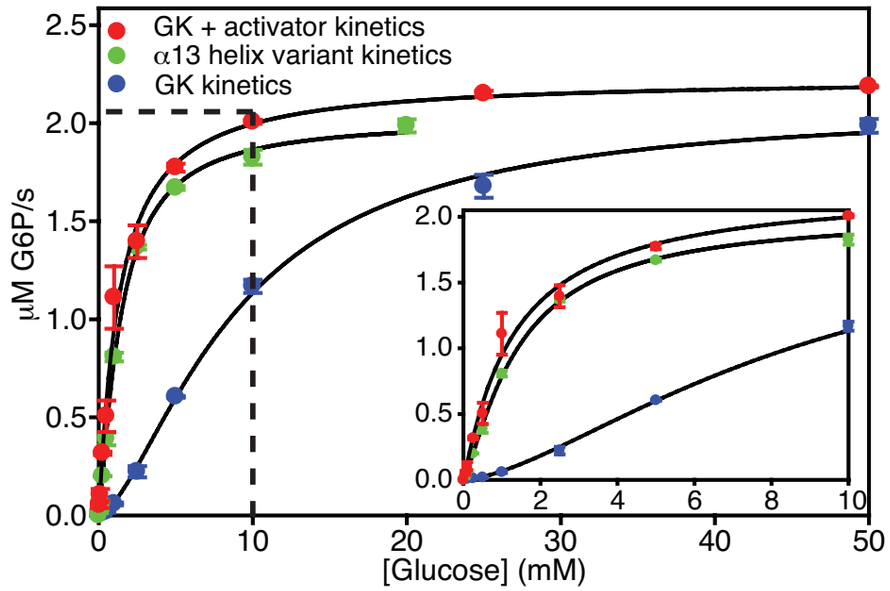


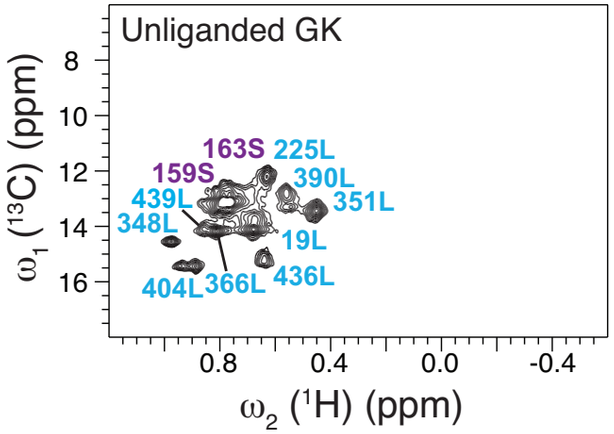
Mecanismo de ativação pelo efetor alostérico sintético e pelas mutações que causam hiperatividade

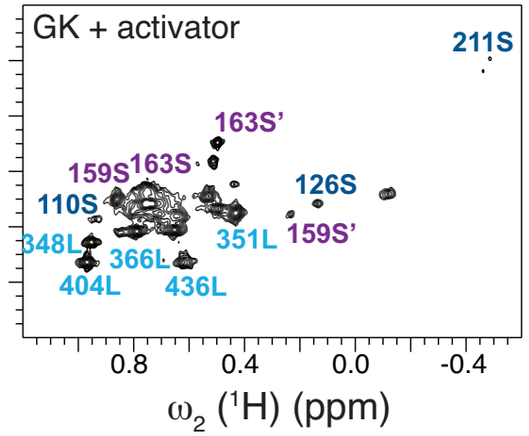


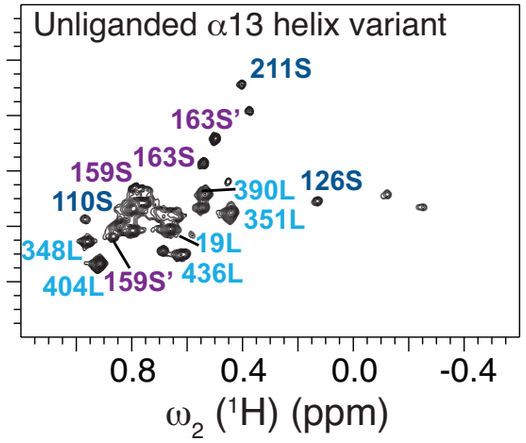
Introdução mutações que causam hiperatividade ou associação com ativador alostérico desloca o equilíbrio populacional na direção de estados mais organizados

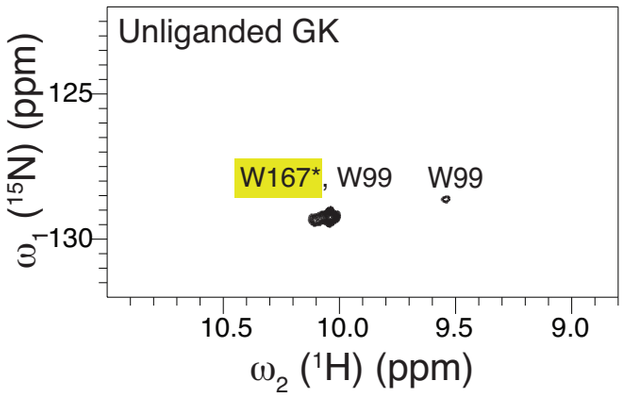


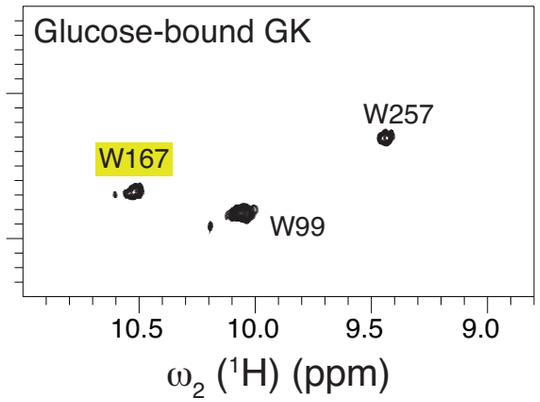


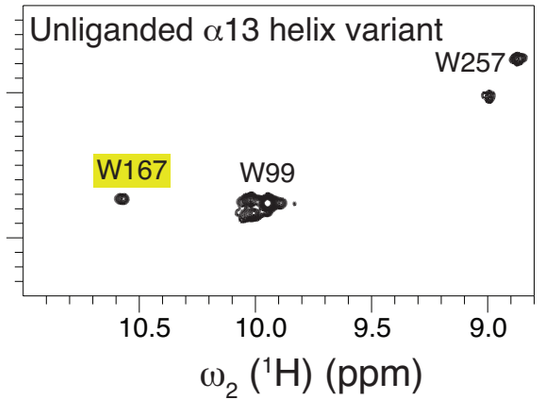












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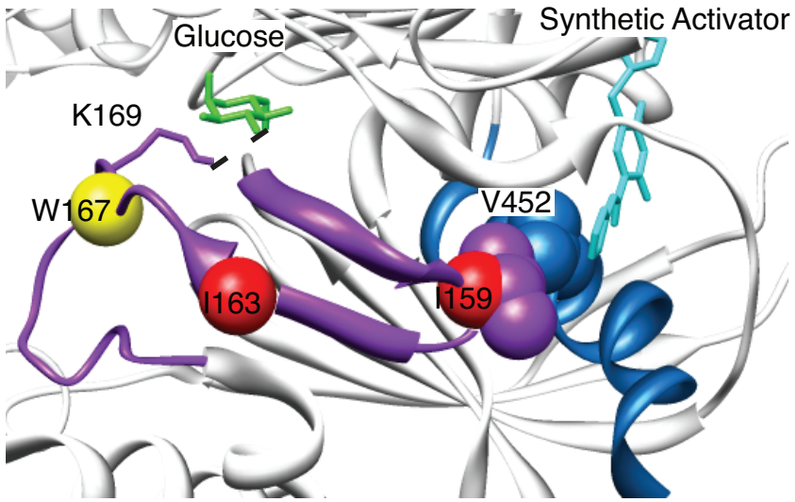
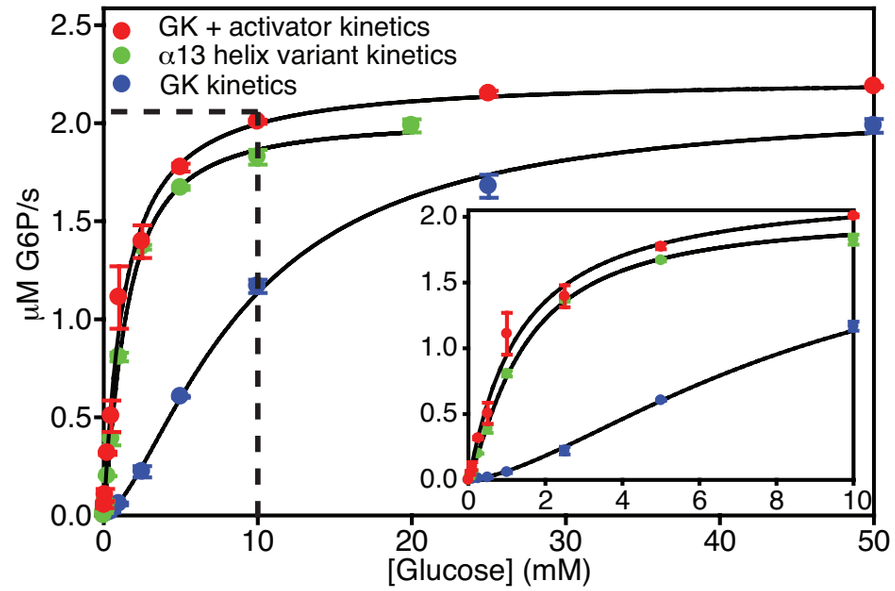
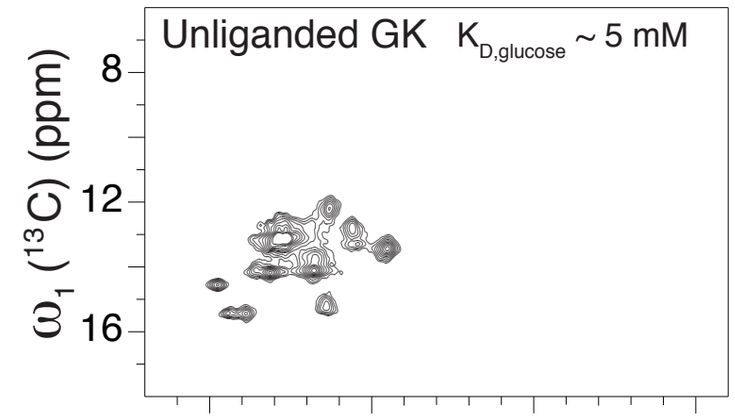
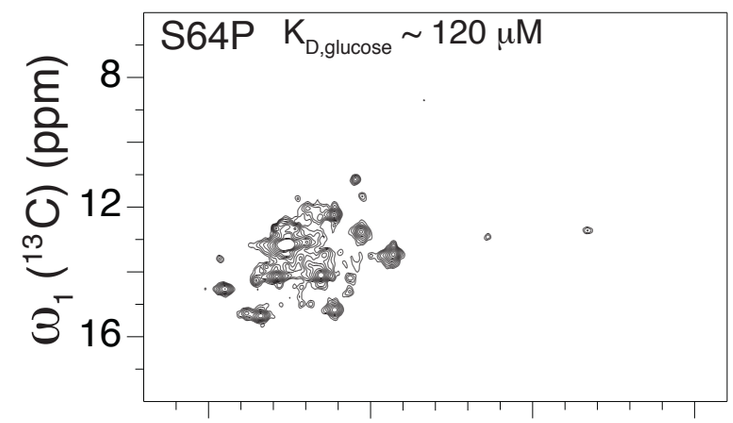
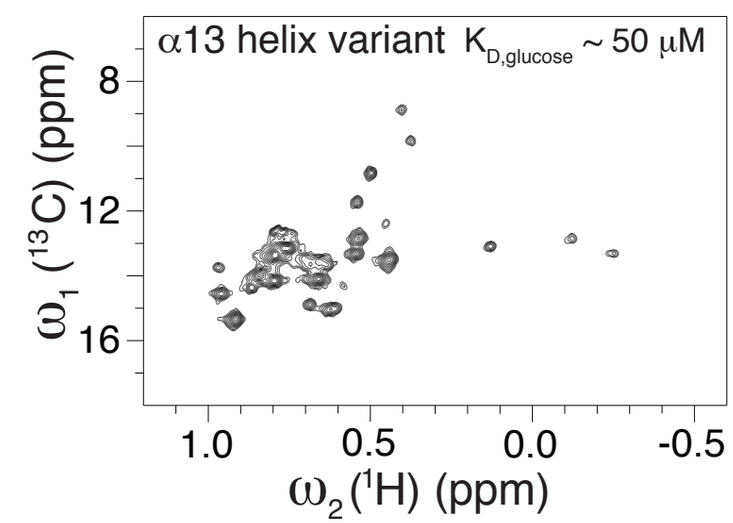
Introdução mutações que causam hiperatividade ou associação com ativador alostérico desloca o equilíbrio populacional na direção de estados mais organizados

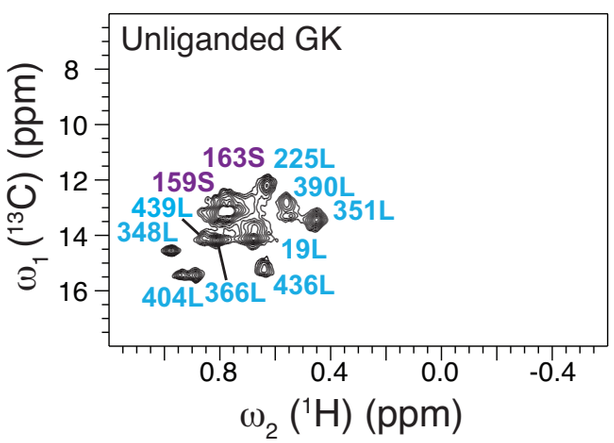


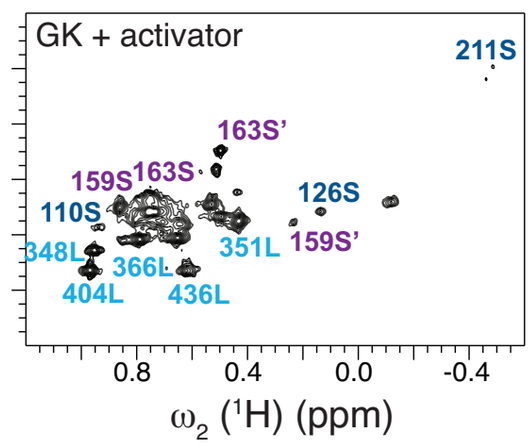


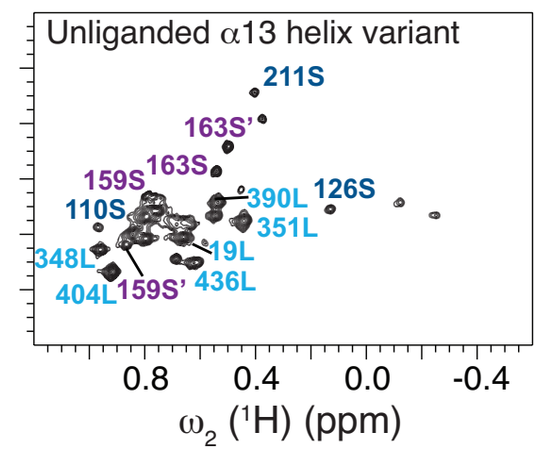


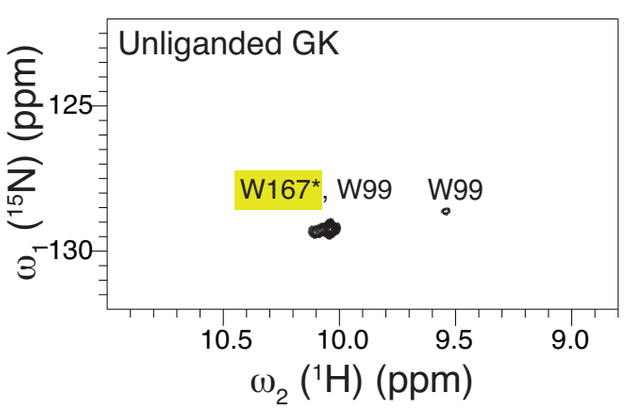


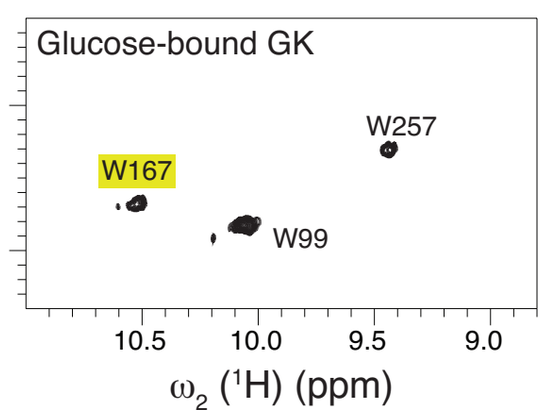


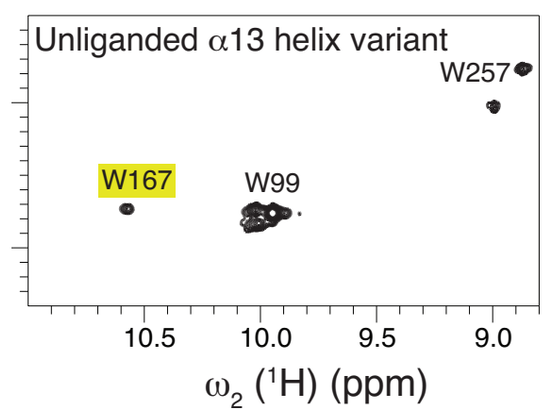












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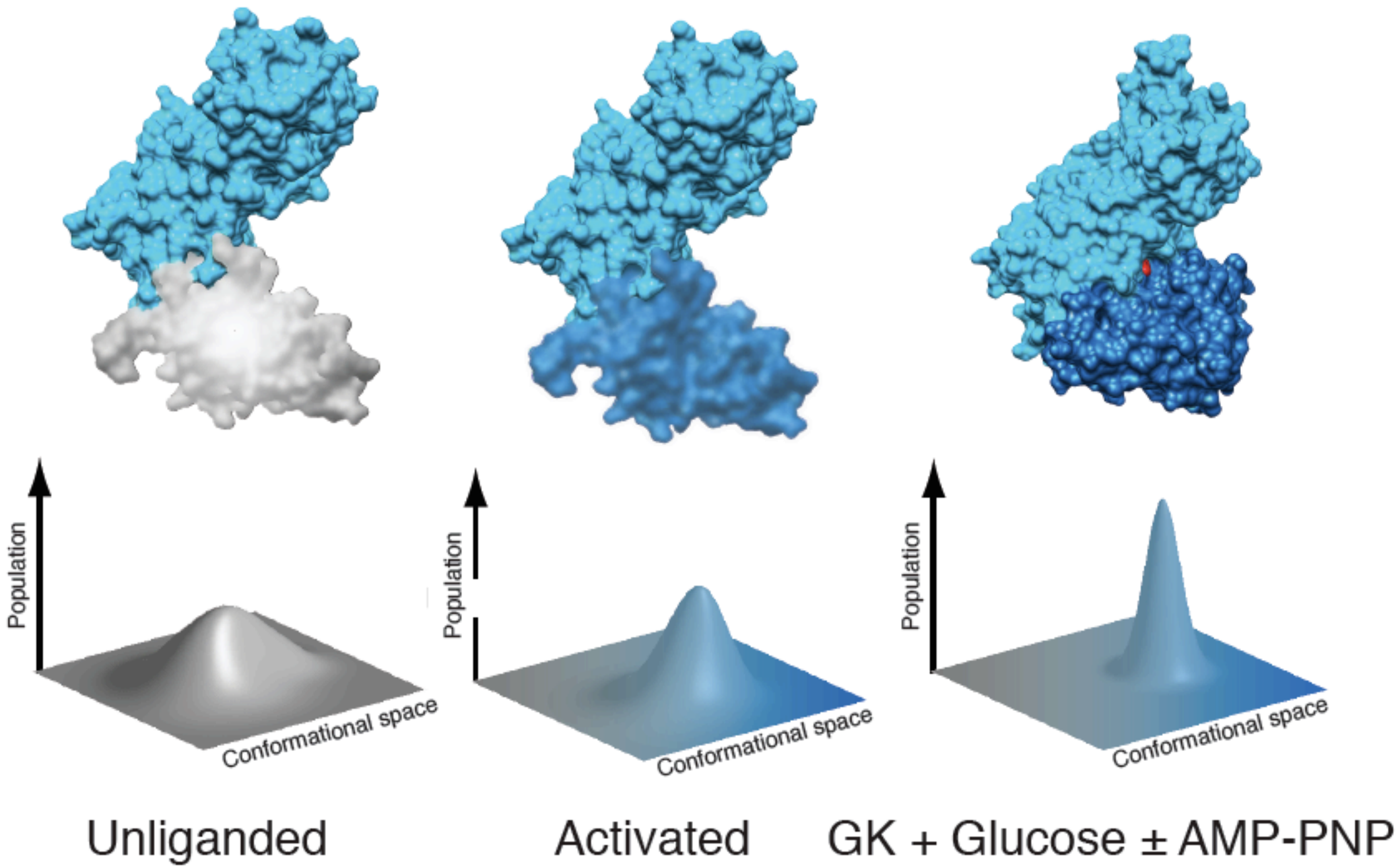
Uma proteína popula um conjunto de configurações cujas populações obedecem a uma distribuição estatística



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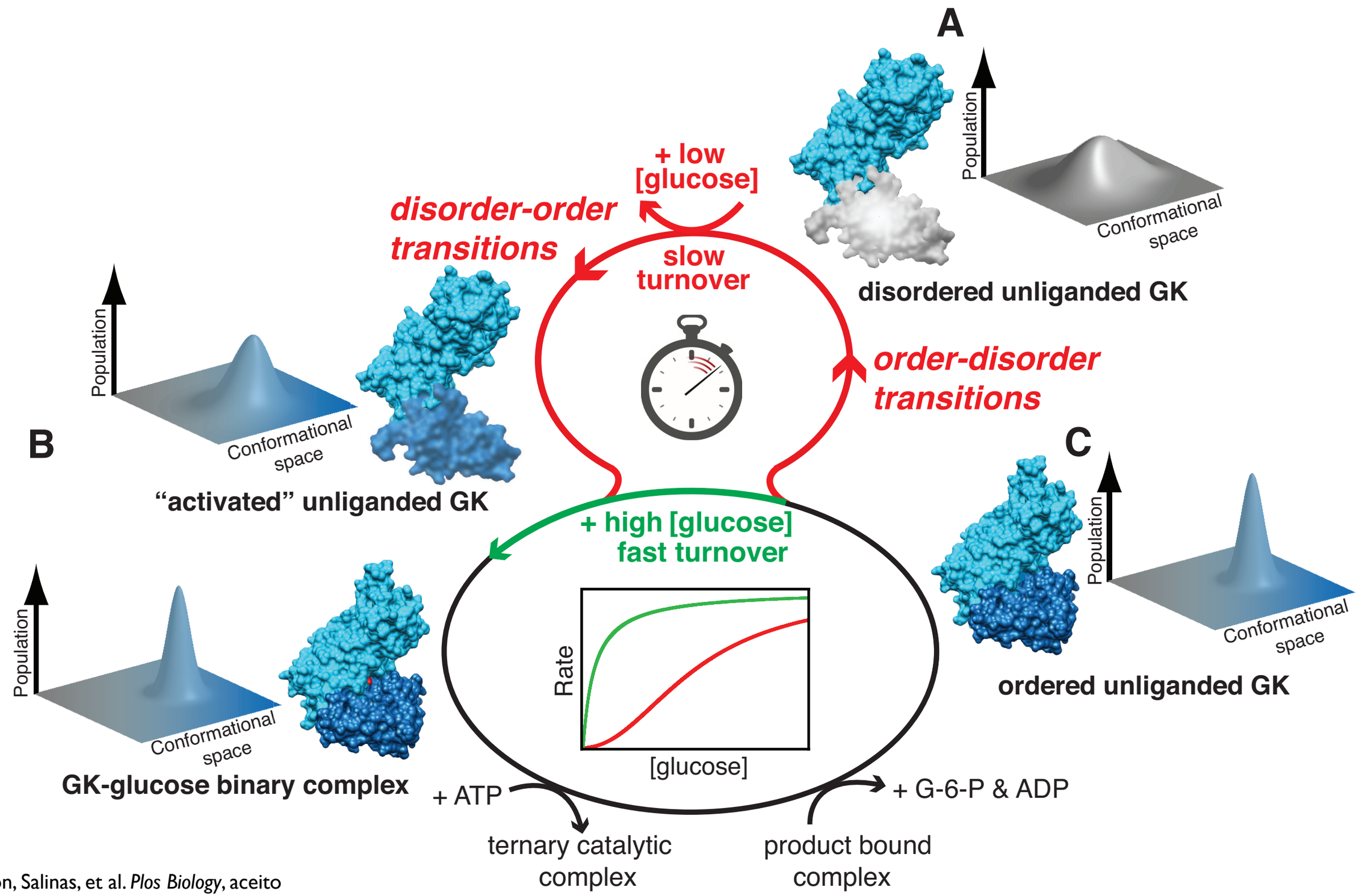
Em baixas concentrações de glicose, a dinâmica do domínio menor que ocorre e escala de tempo da mesma ordem que $1/k_{cat}$ (~12.5 ms), retarda o *turnover* da enzima



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Obrigado pela atenção