Protein Folding and Dynamics based on Chemical Exchange/ Hydrogen Exchange

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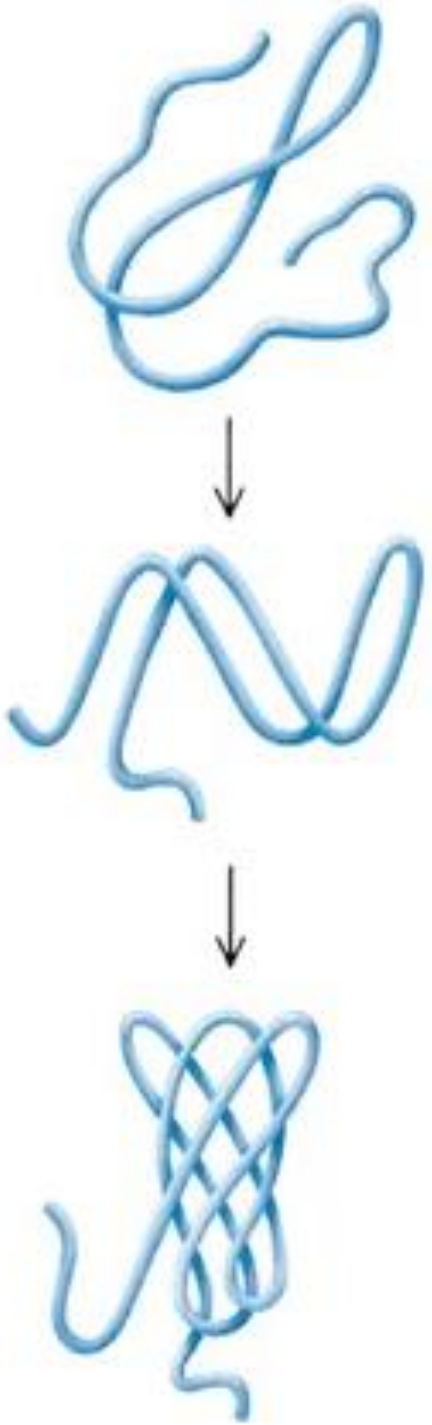
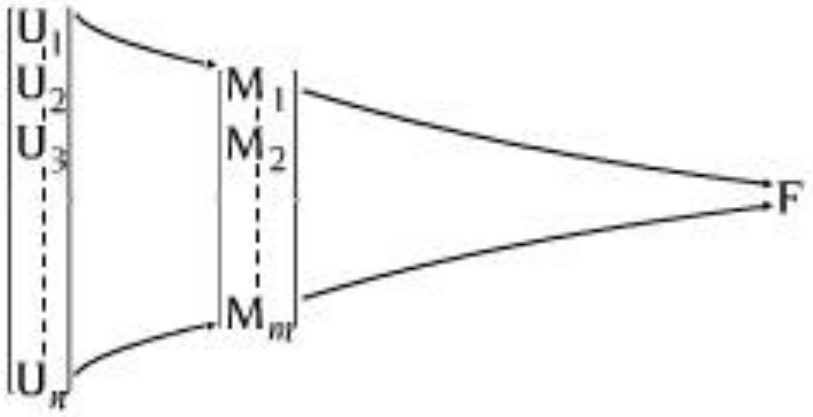
- Protein folding and misfolding
- Amide exchange and applications
- Monitoring folding and dynamics by detecting intermediates and excited states
  - EXSY, CPMG, RDC/PRE

Protein Folding

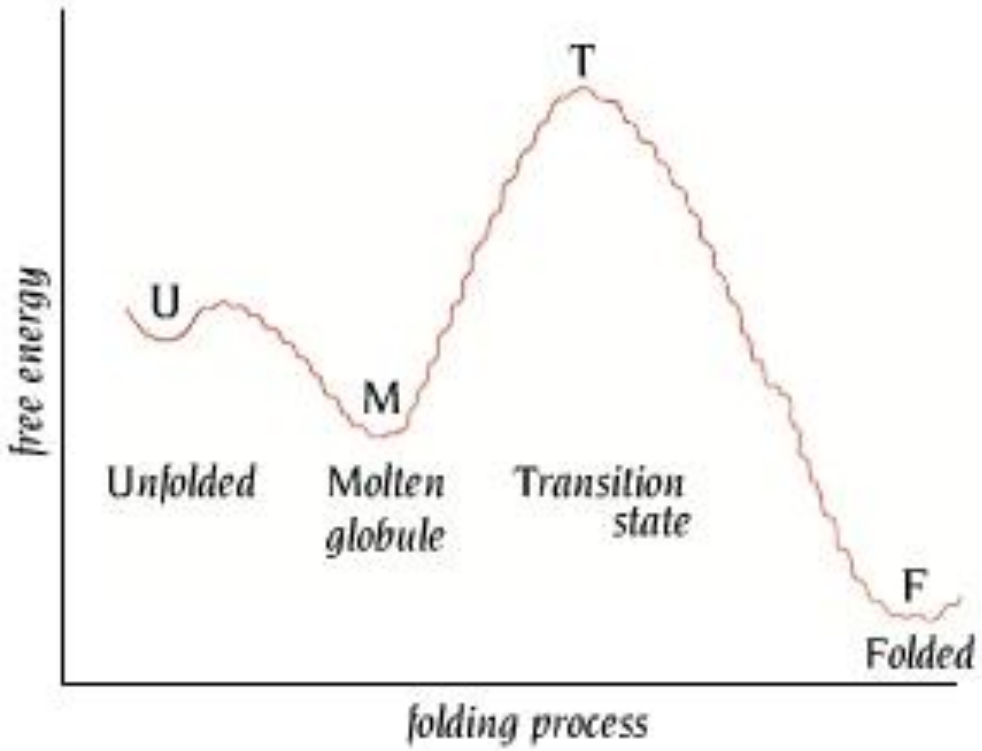
- Protein folding considers the question of how the process of protein folding occurs, i. e. how the unfolded protein adopts the native state.
- This has proved to be a very challenging problem. It has aptly been described as the second half of the genetic code, and as the three-dimensional code, as opposed to the one-dimensional code involved in nucleotide/amino acid sequence.
  - Predict 3D structure from primary sequence
  - Avoid misfolding related to human diseases
  - Design proteins with novel functions



Unfolded State



- The unfolded state is an ensemble of a large number of molecules with different conformations.



The Folding Funnel/ Energy Landscape

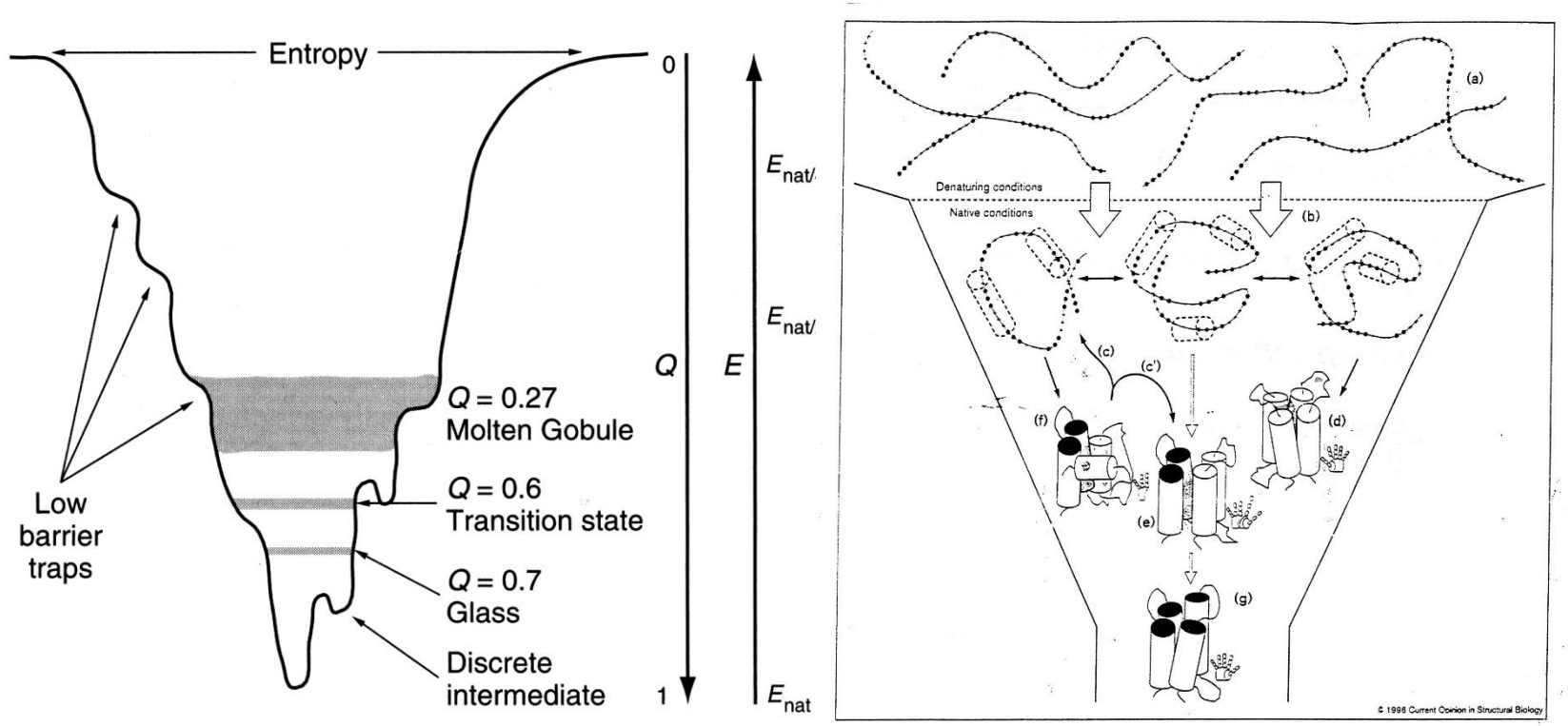
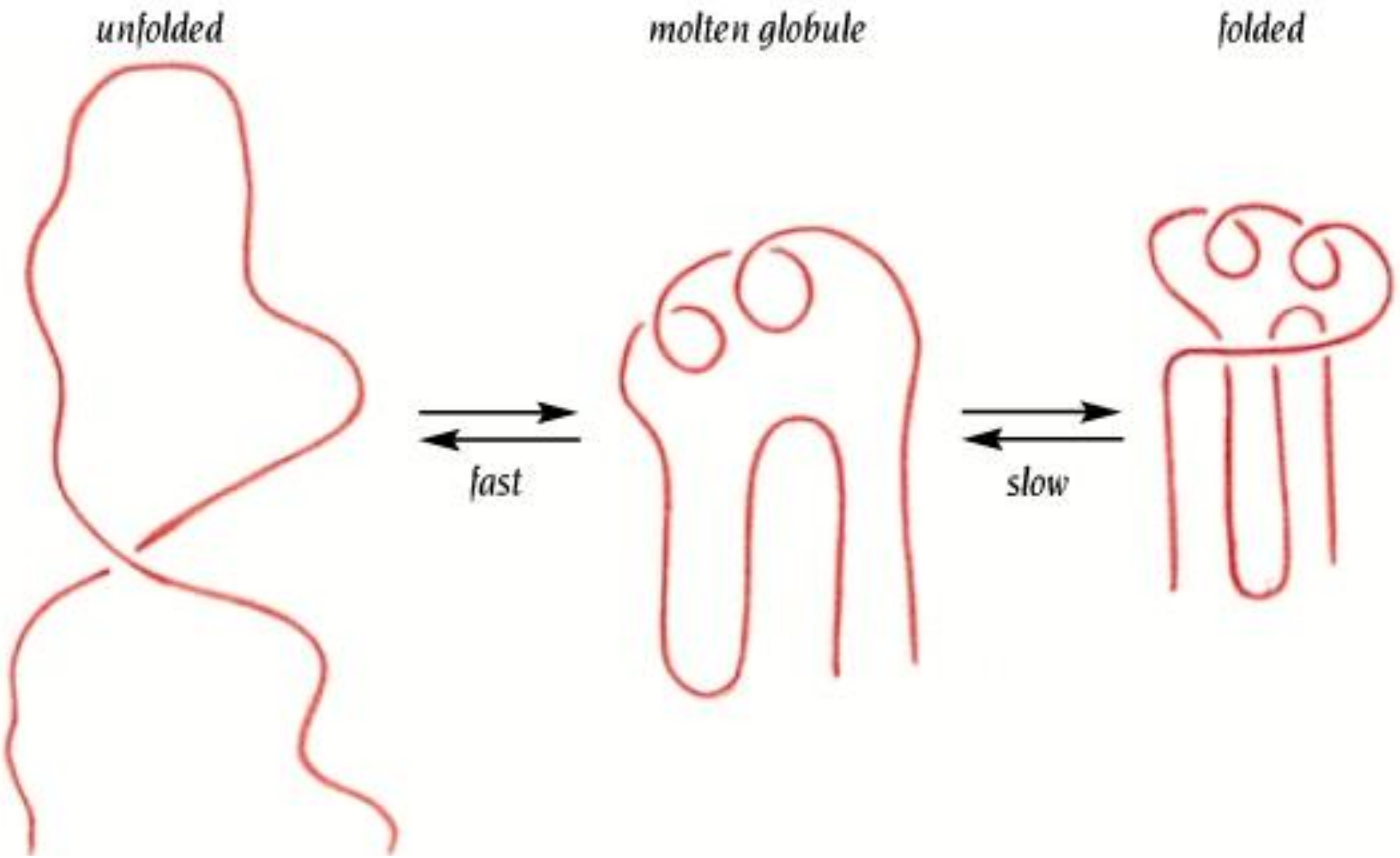


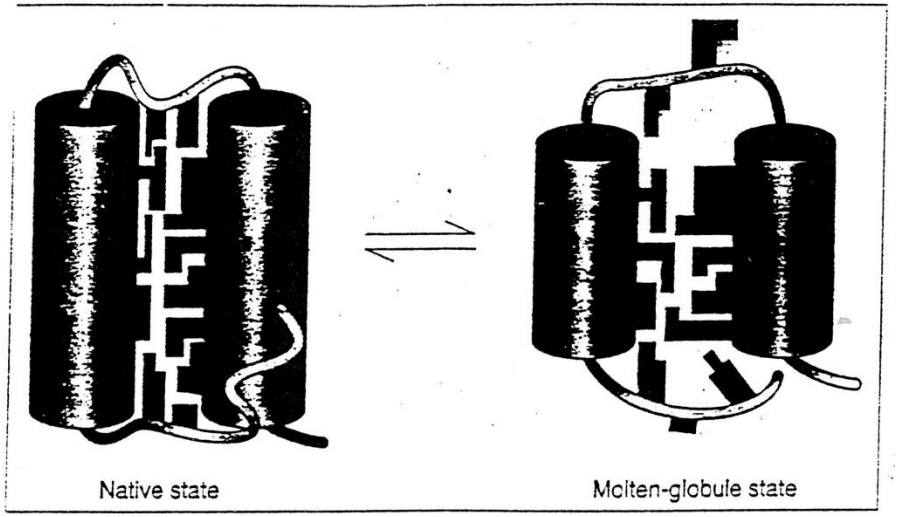
Figure 19.16 Cross section through a folding funnel. *E* corresponds to free energy. [Courtesy of P. G. Wolynes]

- A new view of protein folding suggested that there is no single route, but a large ensemble of structures follow a many dimensional funnel to its native structure.
- Progress from the top to the bottom of the funnel is accompanied by an increase in the native-like structure as folding proceeds.

MG is a Key Kinetic Intermediate



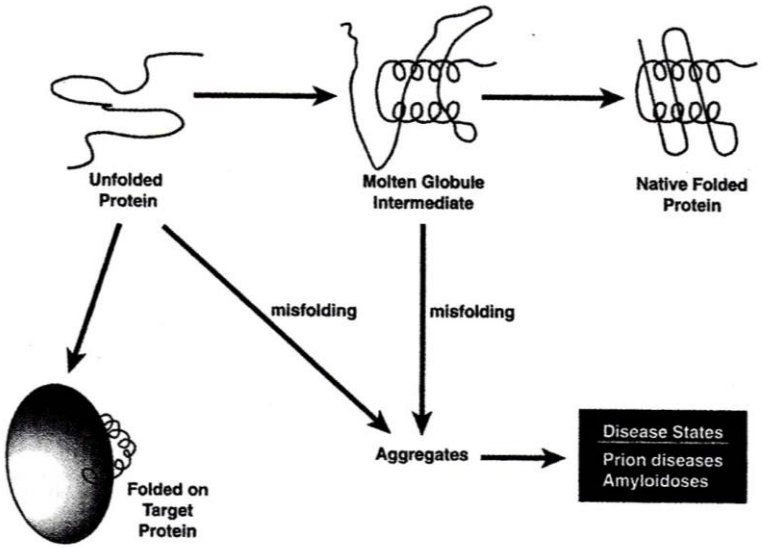
Molten Globule State (MG)



- It is an intermediate of the folding transition U→MG→F
- It is a compact globule, yet expanded over a native radius
- Native-like secondary structure, can be measured by CD and NMR proton exchange rate
- It has a slowly fluctuating tertiary structure which gives no detectable near UV CD signal and gives quenched fluorescence signal with broadened NMR chemical peaks
- Non-specific assembly of secondary structure and hydrophobic interactions, which allows ANS to bind and gives an enhanced ANS fluorescence
- MG is about a 10 % increase in size than the native state

MG for folding and misfolding

Unfolding/Folding and Misfolding



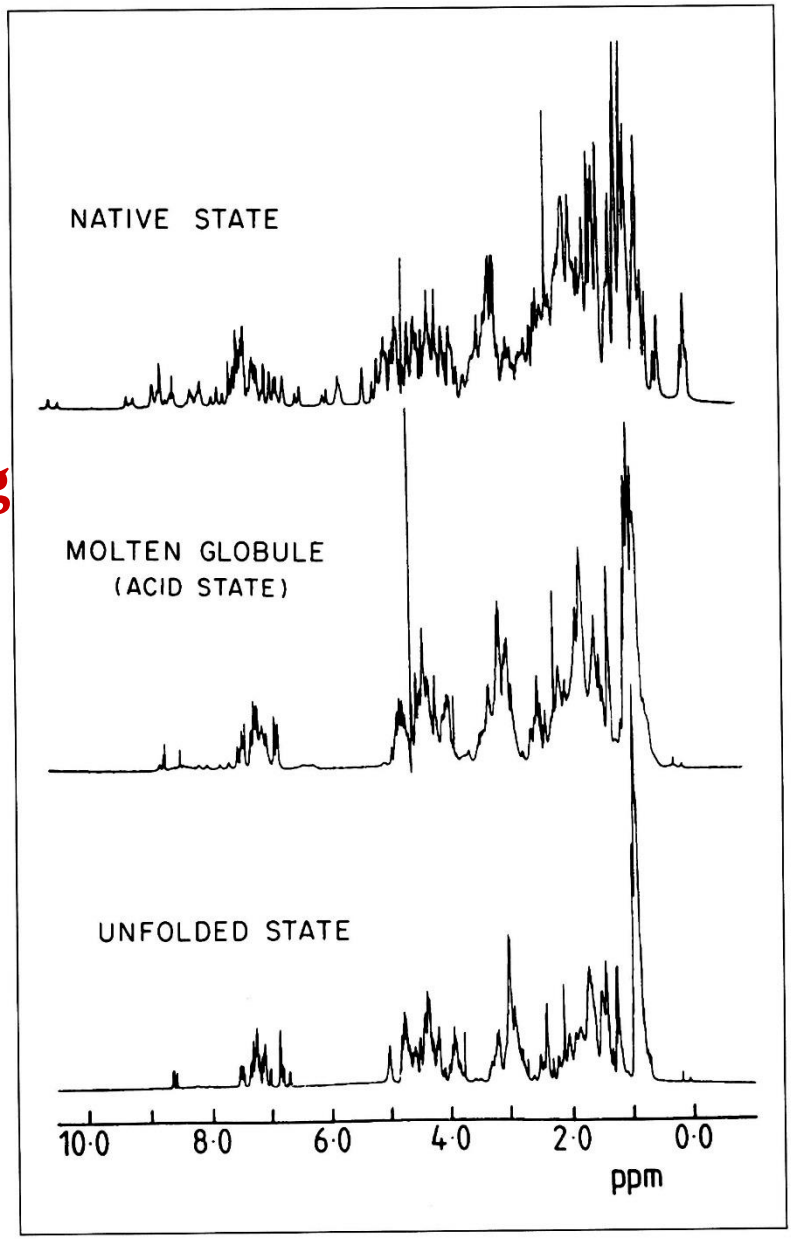
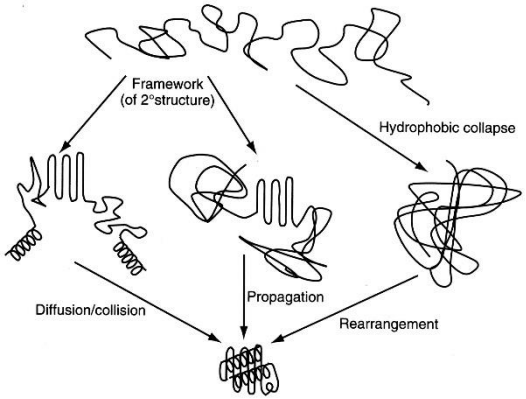


FIGURE 6–4. *Five hundred–MHz ^{1}H-NMR spectra of guinea pig α-lactalbumin in the native (pH 5.4), acid (pH 2.0), and unfolded (in 9-M urea) states recorded at 52°C. (Adapted from Baum et al., 1989)*

Classic Models of Protein Folding



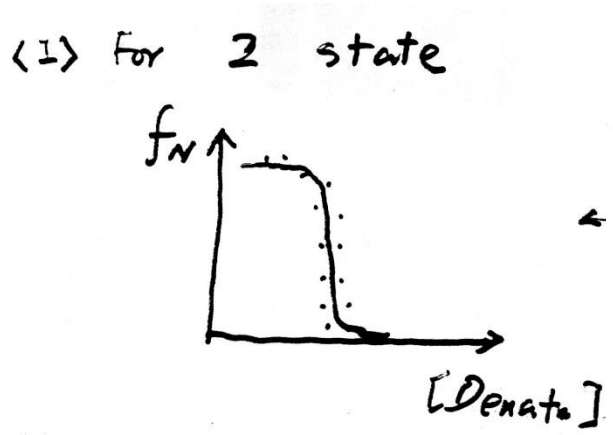
- The Framework model -Local elements of native local secondary structure could form independently of tertiary structure (Kim and Baldwin).
- Diffusion-collision model-These preformed 2nd elements would diffuse until they collided, successfully adhering and coalescing to give the tertiary structure (Karplus & Weaver).
- The classic nucleation model -some neighboring residues in the sequence would form native secondary structure that would act as a nucleus from which the native structure would propagate, in a stepwise manner. Thus, the tertiary structure would form as a necessary consequence of the secondary structure (Wetlaufer).
- The hydrophobic-collapse model -a protein would collapse rapidly around its hydrophobic sidechains and then rearrange from restricted conformational space occupied by the intermediate. 2nd structure would be directed by native-like tertiary structure (Ptitsyn & Kuwajima).

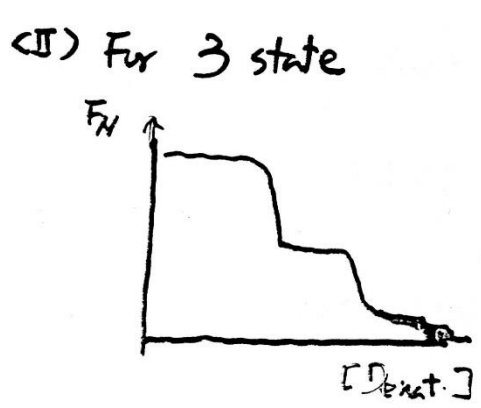
Kinetic Folding Pathways

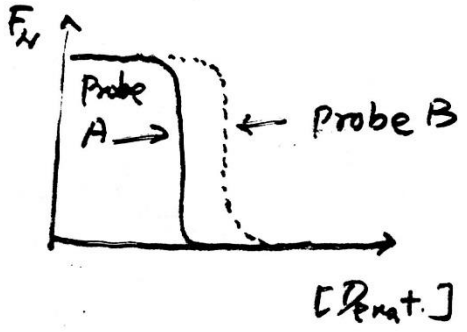
- U→ I →II → N
- Not all steps have the same rate constants.
- Intermediates accumulate to relatively low concentrations, and always present as a mixture
- Identify kinetic intermediates
- Measuring the rate constants
- Figure out the pathways
- Slow folding
  - Formation of disulfile bond
  - Pro isomerization

Equilibrium Unfolding

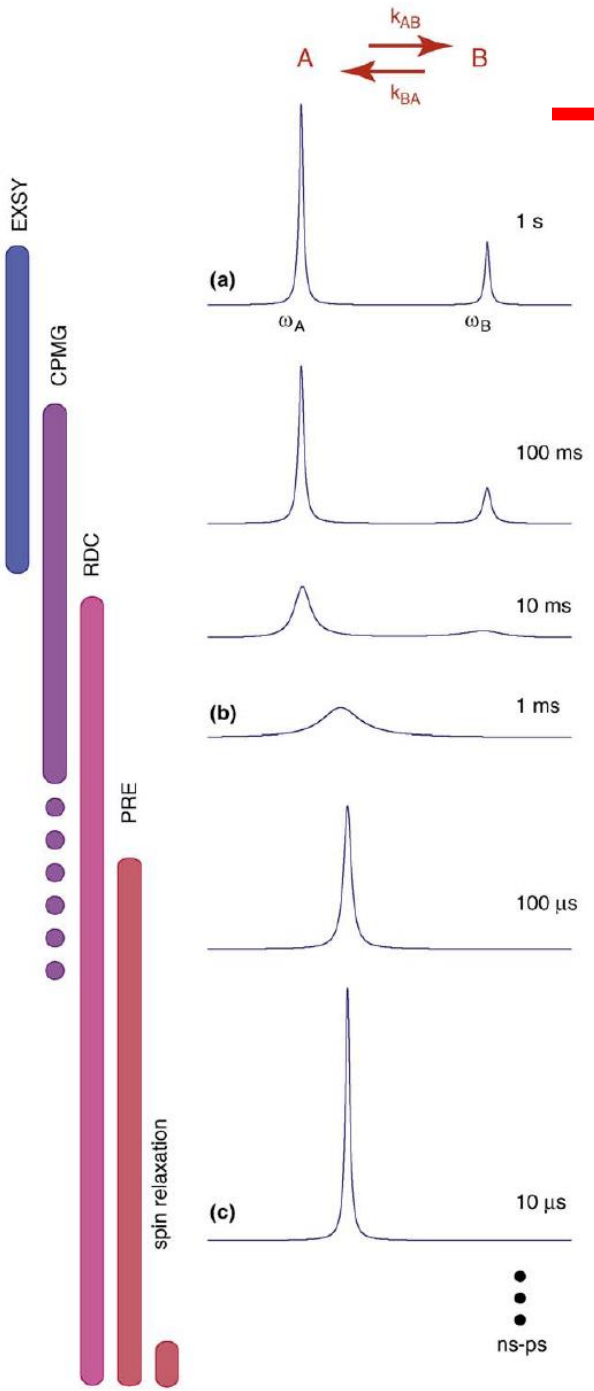
- Using many probes to investigate the number of transitions during unfolding and folding
- For 2-state unfolding, all probes give the same transition curves. Single domains or small proteins usually have two-state folding behavior.
- For 3-state unfolding, there are more than one transitions or different probes have different transition curves







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 s^{-1} to over 50 s^{-1}

CPMG relaxation dispersion: chemical shifts100~2000s^{-1},

R1rho can extend to more rapid exchange (dot)

Residual dipolar coupling (RDC) and PRE

Spin relaxation for ns-ps

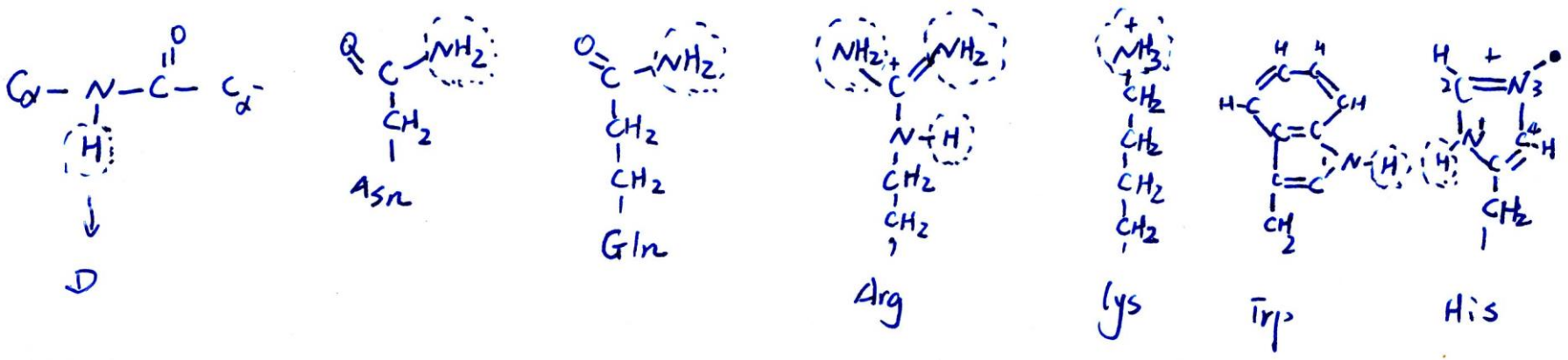
CPMG and PRE are sensitive to low-lying excited states with populations > 0.5%

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

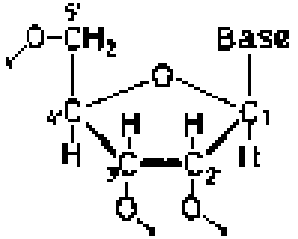
A.Mittermaier, L. Kay (2009) *Trends Biochem. Sci.* **34**, 601.
A. Mittermaier, L. Kay (2006) *Science.* **312**, 224
K. Wuthrich, G Wagner,(1978) *Trends Biochem. Sci.* **3, 227**

Hydrogen Exchange Method

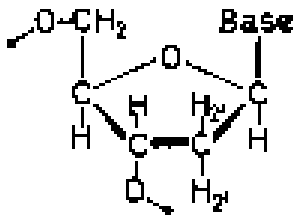
Hydrogen exchange (HX) techniques is described for measuring the approximate exchange rates of the more labile amide protons in a macromolecule. The exchangeable amides in proteins are:



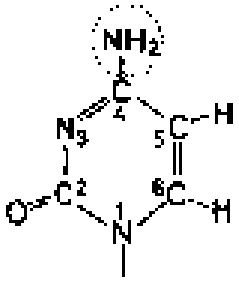
Exchangeable Nucleotides



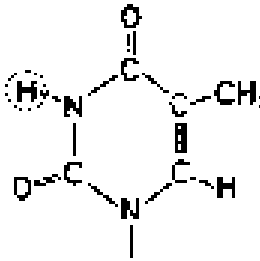
β-D-Ribose
XWTPMA



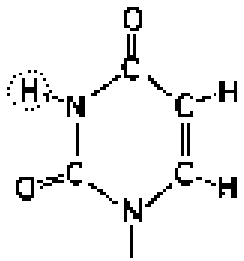
2"-Deoxy-β-D-Ribose
XAMWTNP



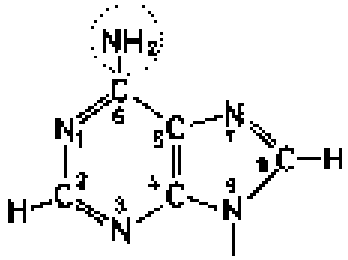
Cytosine, C
AX



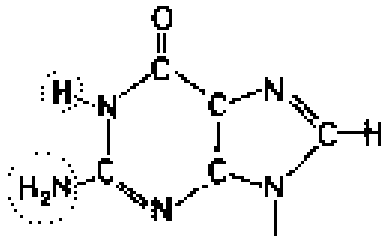
Thymine, T
A_3X



Uracil, U
AX

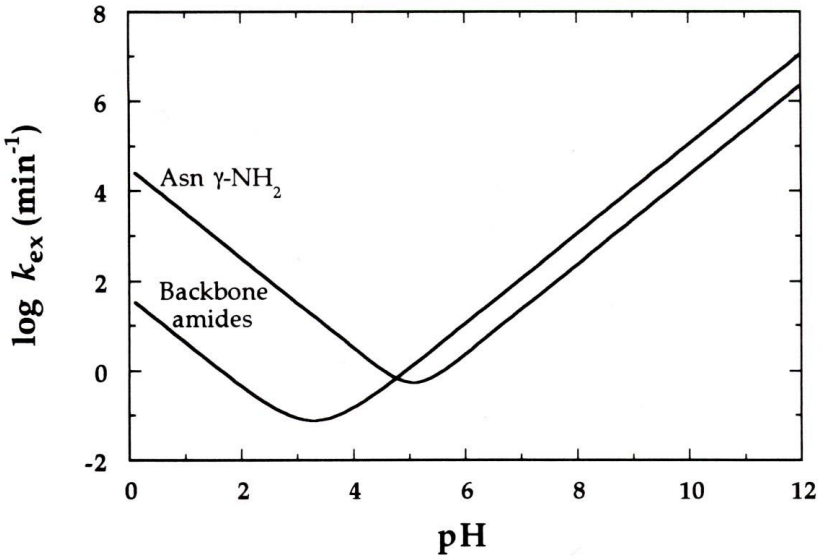


Adenine, A
A+A



Guanine, G
A

Hydrogen-Exchange Chemistry



A minimum ~ pH 3.5

> 1hr at pH 3

< 1ms at pH 10

- Hx rate is catalyzed by OH^- and H^+

$k_{intrinsic}$

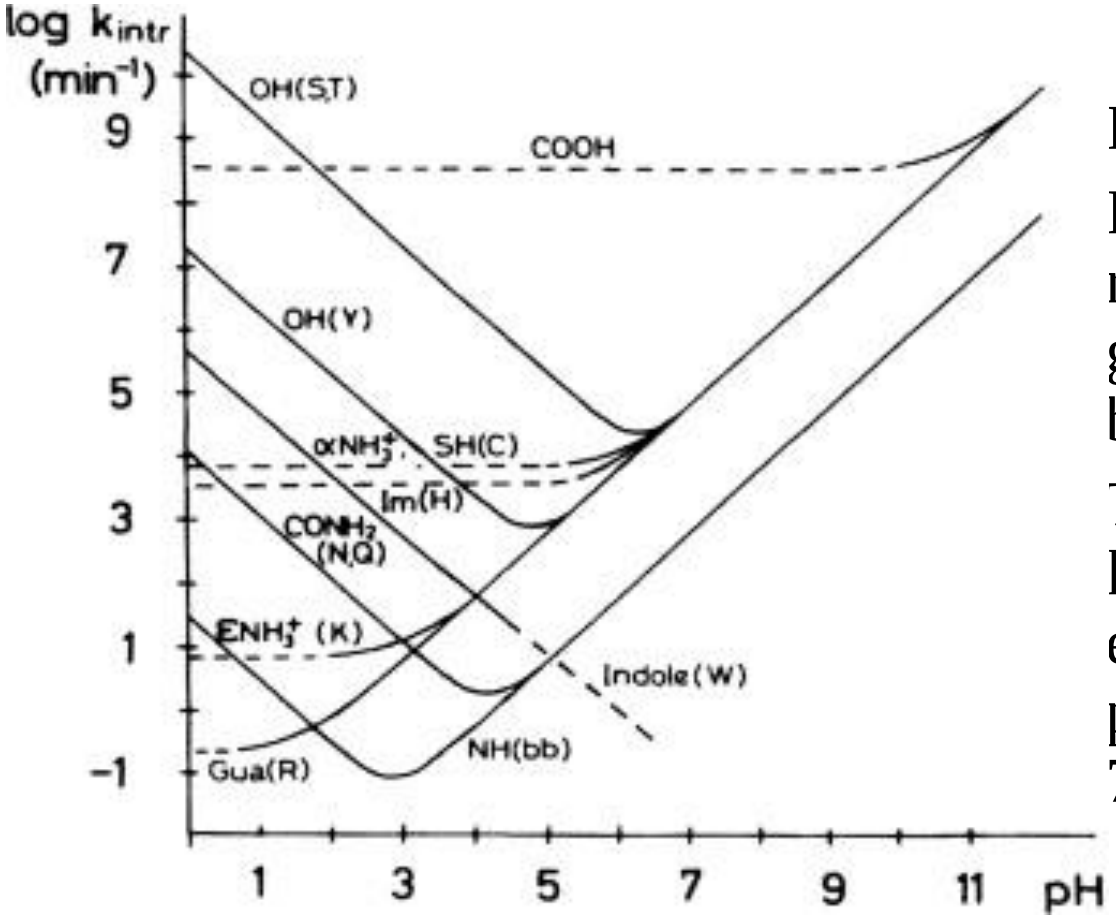
$k_{ex} = k_{oH}[OH^-] + k_H[H^+] + k_w$

- All exchange rates are referenced to random coil polyAla at 0 C.
- HX rates are sensitive to pH,local chemical environment, solvent, sidechain type, neighboring amino acids and temperature
- $k_{intrinsic}$ for each amino acid is different

  pD = pH* + 0.4

Bai. And Englander. (1993) Proteins, 17, 75;
Koide S.. and Wright PE J Biomol NMR. 1995 Nov;6(3):306-12.

Simulated Exchange Rates for Labile Protons of Polypeptides

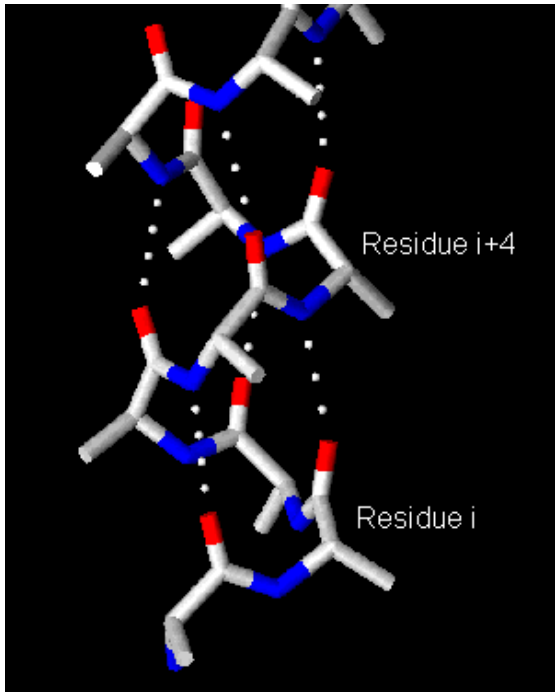


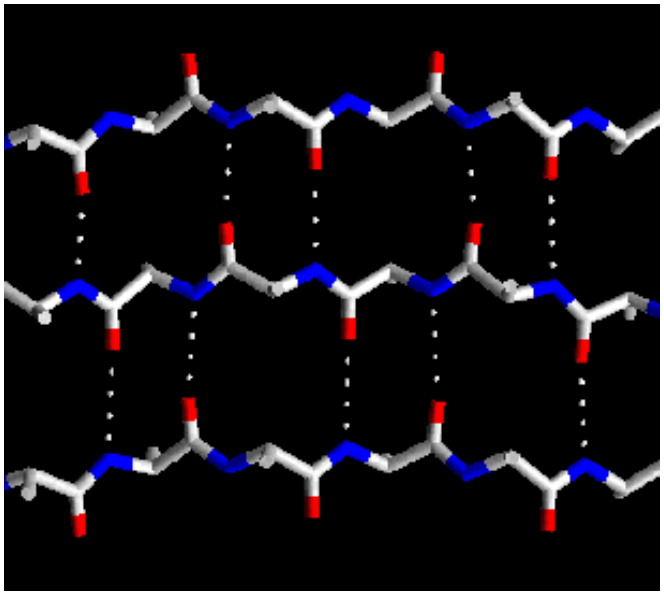
In H_2O solution at 25 °C.

Im stands for imidazole ring NH, Gua for guanidinium NH, bb for backbone.

The amide protons have a large range of possible exchange rates under physiological pH (pH 6.5–7.5).

Wuthrich &Wagner JMB 1979





HX vs. Protein Structure

In proteins, HX rates can be altered:

- H-bonding
- Shielding in the center of protein
- Shielding by binding another molecules
- pH and temperature

Extremely slow exchange can be months,yrs

Protection factor $\theta p = k_{intrinsic}/k_{obs}$

$\theta p > 10^6$-10^7 for slow exchange

Amide exchange rate contains information about secondary structural elements

Hx Mechanism (Ex1/EX2)

$$\text{Close} \underset{k_{cl}}{\overset{k_o}{\rightleftharpoons}} \text{Open} \xrightarrow{k_{intrinc}} \text{Exchanged}$$

-Hvidt & Nielsen, 1966

- Solvent penetrates protein secondary structure
- A protected amide hydrogen is 'closed' to exchange and becomes accessible to exchange through an 'open' state at the exchange rate for an unstructured peptide.

Ex1: $k_{intrinc} >> k_{cl}$ $k_{obs} = k_{op}$ independent of pH

Ex2: $k_{intrinc} << k_{cl}$ $k_{obs} = k_{op}k_{intrinc}$ pH dependent

Ex2 is typically encounted in proteins under conditions where folded state is stable and intrinsic exchange is relative slow

HX is an excellent way to look at the stability of proteins

- The rates of amide proton exchange for individual protons can be related to equilibrium constants for opening of individual hydrogen bonds. Knowing the equilibrium constants, one can calculate the free energy for the conformational transition which allows exchange to occur.
- When certain protons are only exposed in the completely unfolded form then the equilibrium constants and ΔGs correspond to the global unfolding reaction. These protons are usually the slowest exchanging protons in the molecule.

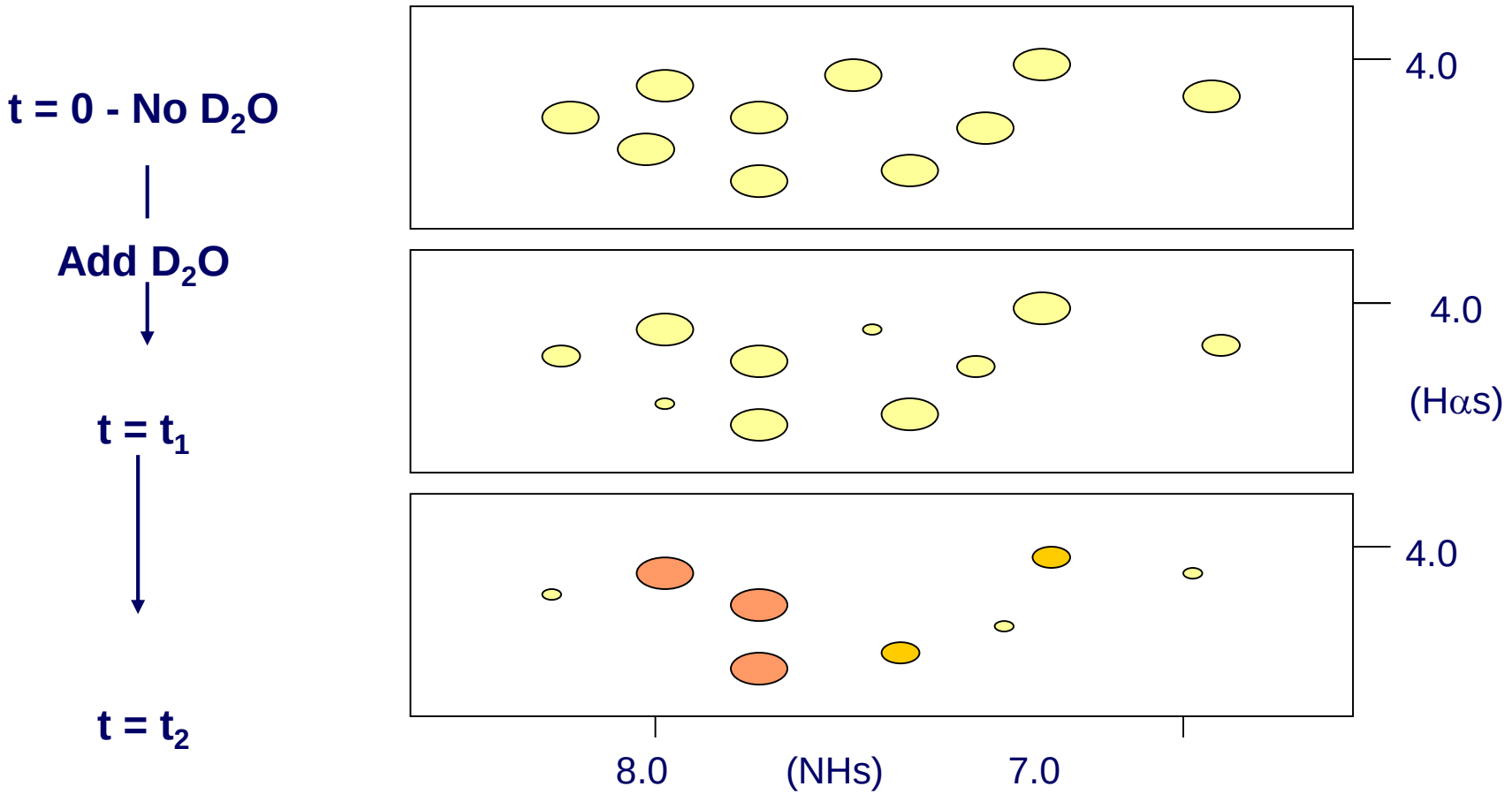
$$\Delta G_{HX} = -RT \ln(k_{obs} / k_{intrinc})$$

- For mutation, the change of stability:

$$\Delta\Delta G_{HX} = (\Delta G_{HX})_{wt} - (\Delta G_{HX})_{mut} = -RT \ln (kex_{wt} / kex_{mut})$$

Amide Exchange Rates

•Adding D_2O to our H_2O solution and take spectra at different times, signals from different amide protons will decrease in size at different rates. We look at the NH to Hα fingerprint at different times in DQF-COSY or HSQC.



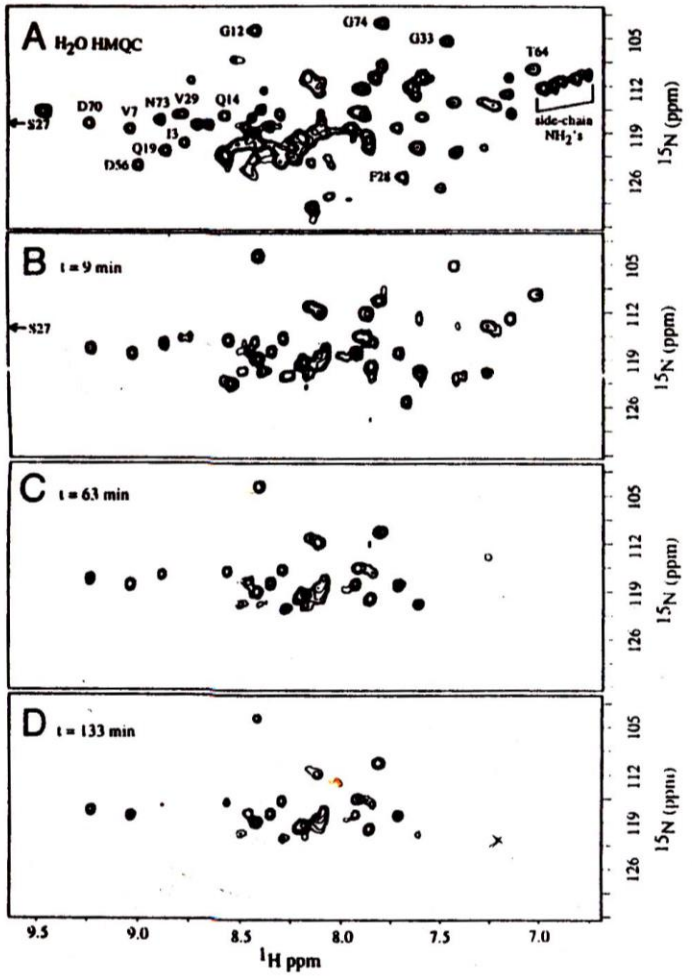


Fig. 1. Selected spectra from the amide H_2O/D_2O exchange time-course. **A:** HMQC spectrum obtained in H_2O. **B, C, D:** Spectra obtained 9, 63, and 133 min after addition of D_2O (see the Materials and methods). Peak corresponding to Ser 27 (10.07, 116.2 ppm) is visible in the spectra corresponding to A and B but is not shown in the plot limits of this figure. This signal does not appear in the spectra of C and D.

Protein Science (1995), *4*:983–993. Cambridge University Press. Printed in the USA.

Amide exchange rates in *Escherichia coli* acyl carrier protein: Correlation with protein structure and dynamics

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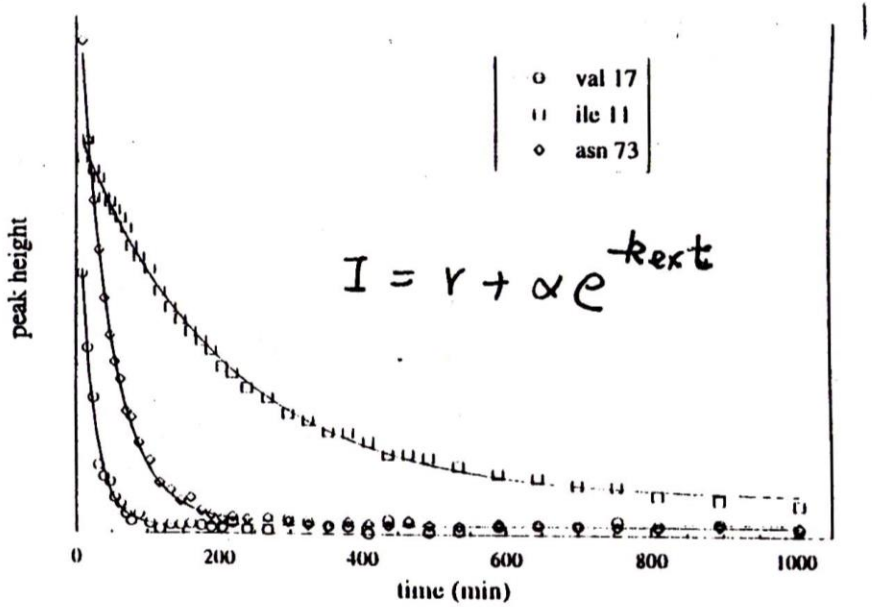
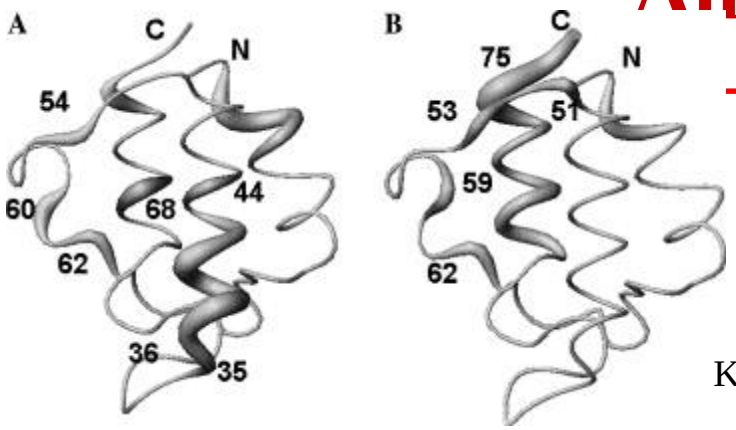
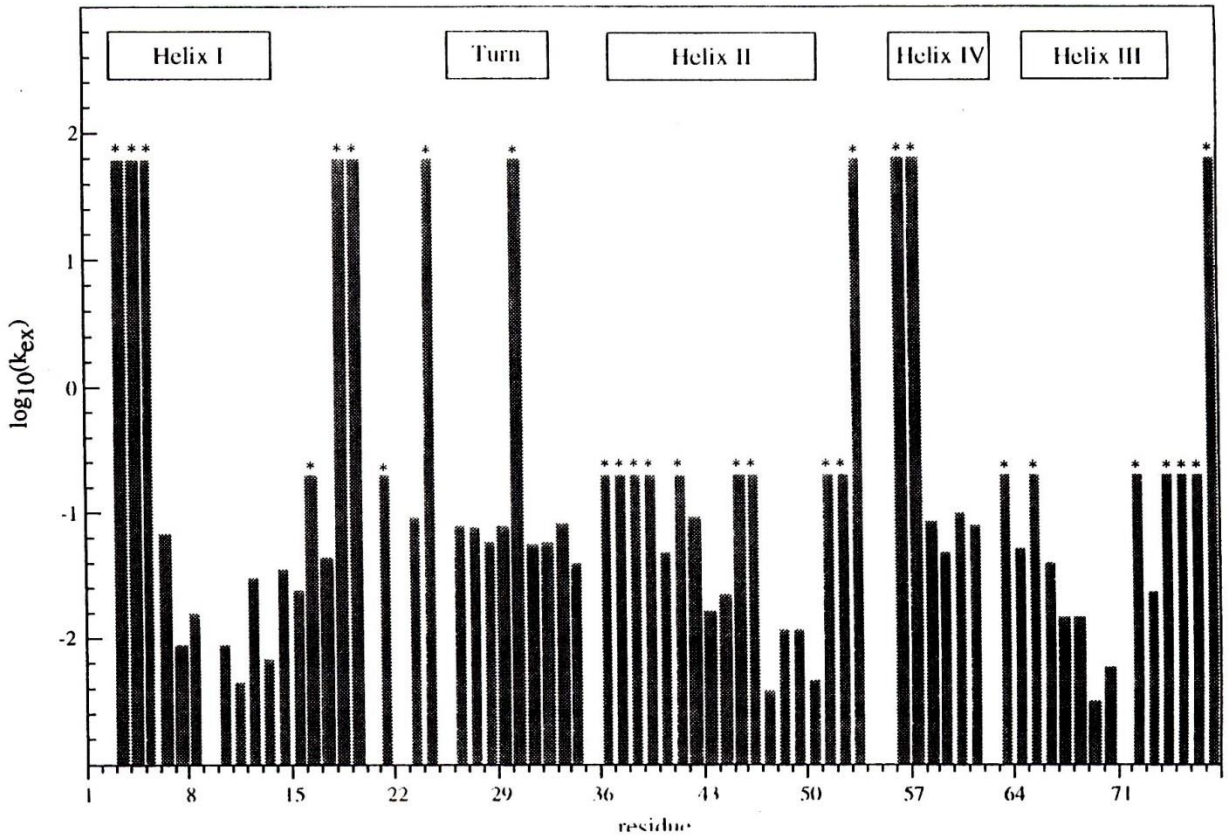


Fig. 3. Examples of signal loss due to D_2O exchange as a function of time after addition of D_2O for three selected residues. Peak height is in arbitrary units.

Amide Exchange Rates in ACP



Kima Y, et al, **BBRC,** 2006

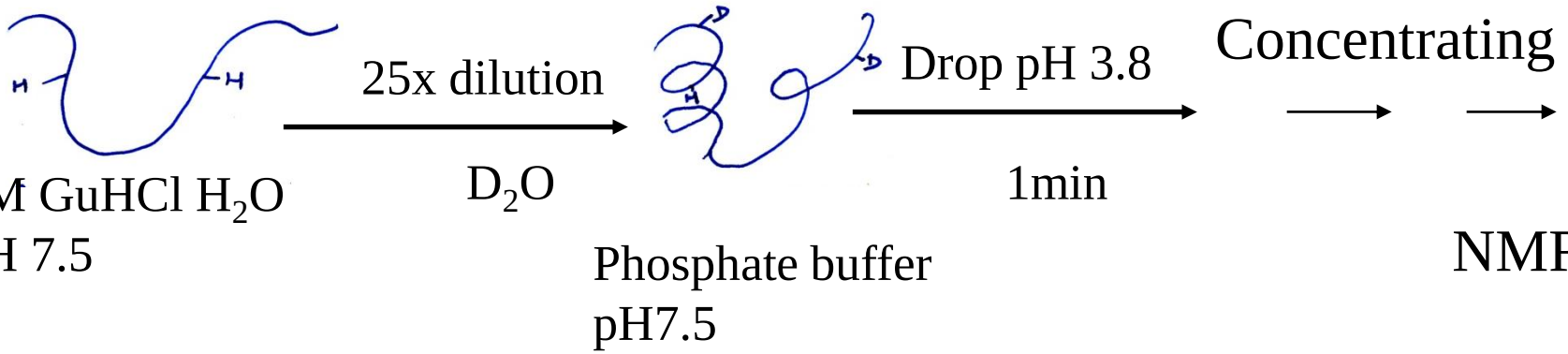


- Residues at the center of helices and hydrophobic core have slow exchange rates

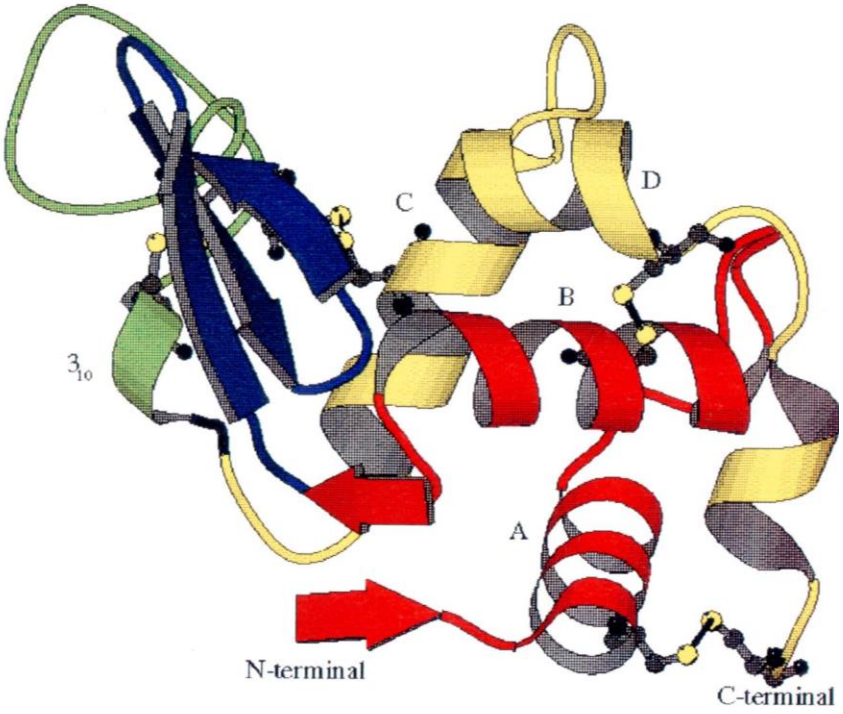
  The overall protection factors ($< 10^{4.5}$) are smaller than other proteins suggesting that ACP has high mobility

  Helix II exchanges faster than helix I and helix III suggesting that Helix II is highly dynamic.

Competition Hydrogen Exchange



- The refolding experiment involved dilution of droplets of protein denatured in 6 M GuHCl in H_2O solution into a denaturant free solution of D_2O to initiate refolding and hydrogen exchange simultaneously.
- After folding completed, HX is quenched by lowing the pH.
- Comparing 2D NMR spectrum of the refolded protein with that was not denatured. Residues protected early in refolding can be detected using NMR.

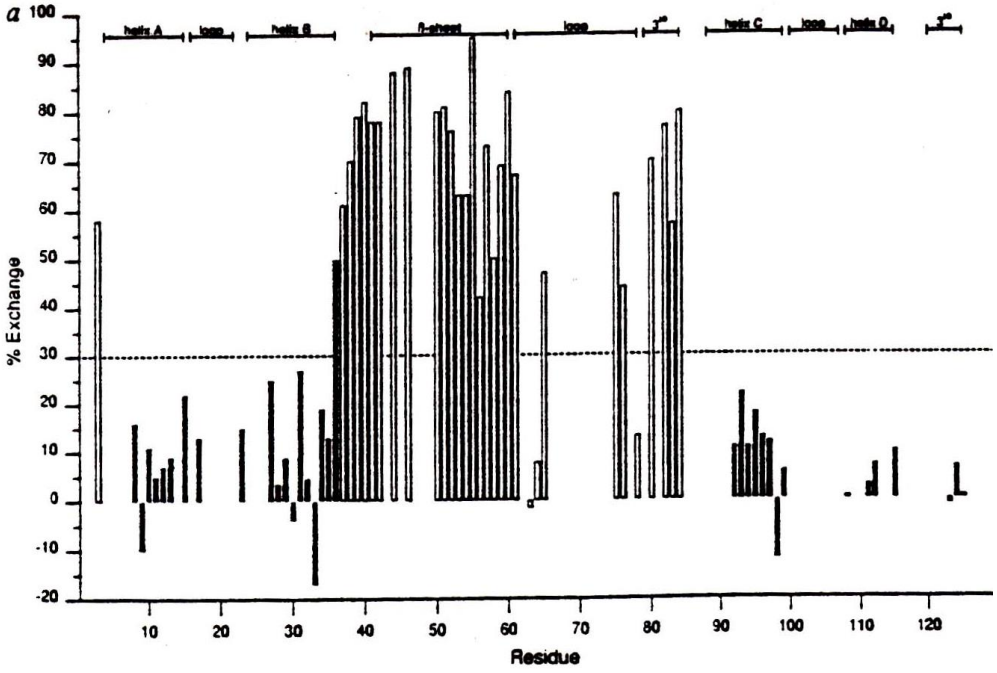


Competition HX of lysozyme

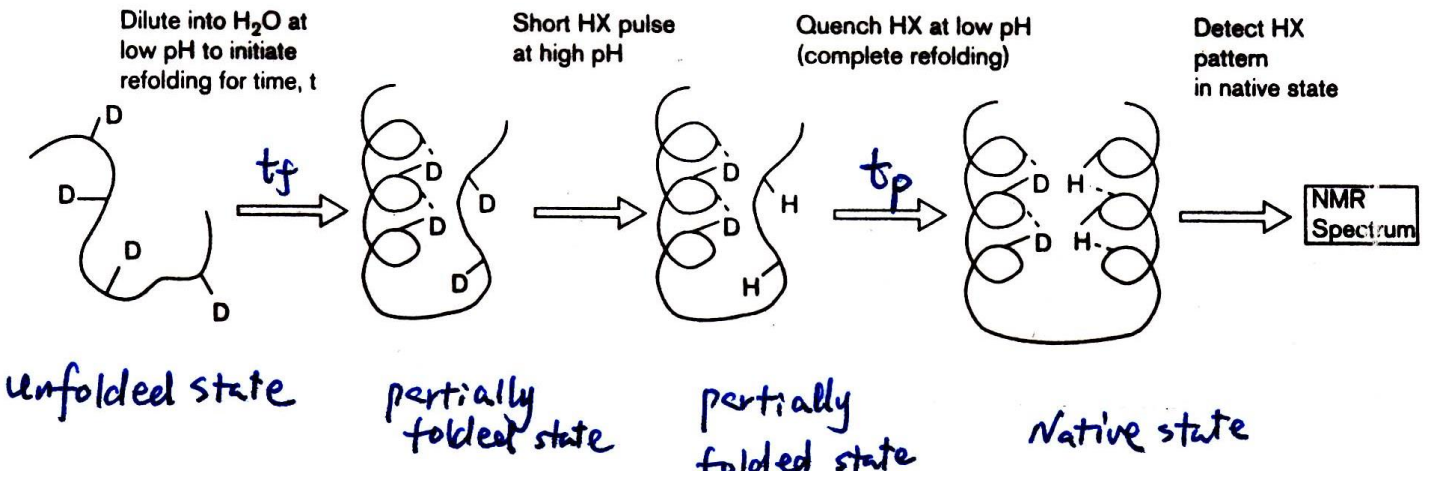
- Using 65 slowly exchanging amide hydrogen as probes.

The majority of residues in β-domain have exchange >30%. The majority of residues in α-domain have exchange <30% suggesting that two structural domains of lysozyme are folding domains that differ significantly in the extent to which protected structure accumulates early in the folding process.

Miranker et al., Nature, 1991



Pulsed-Label Hydrogen Exchange



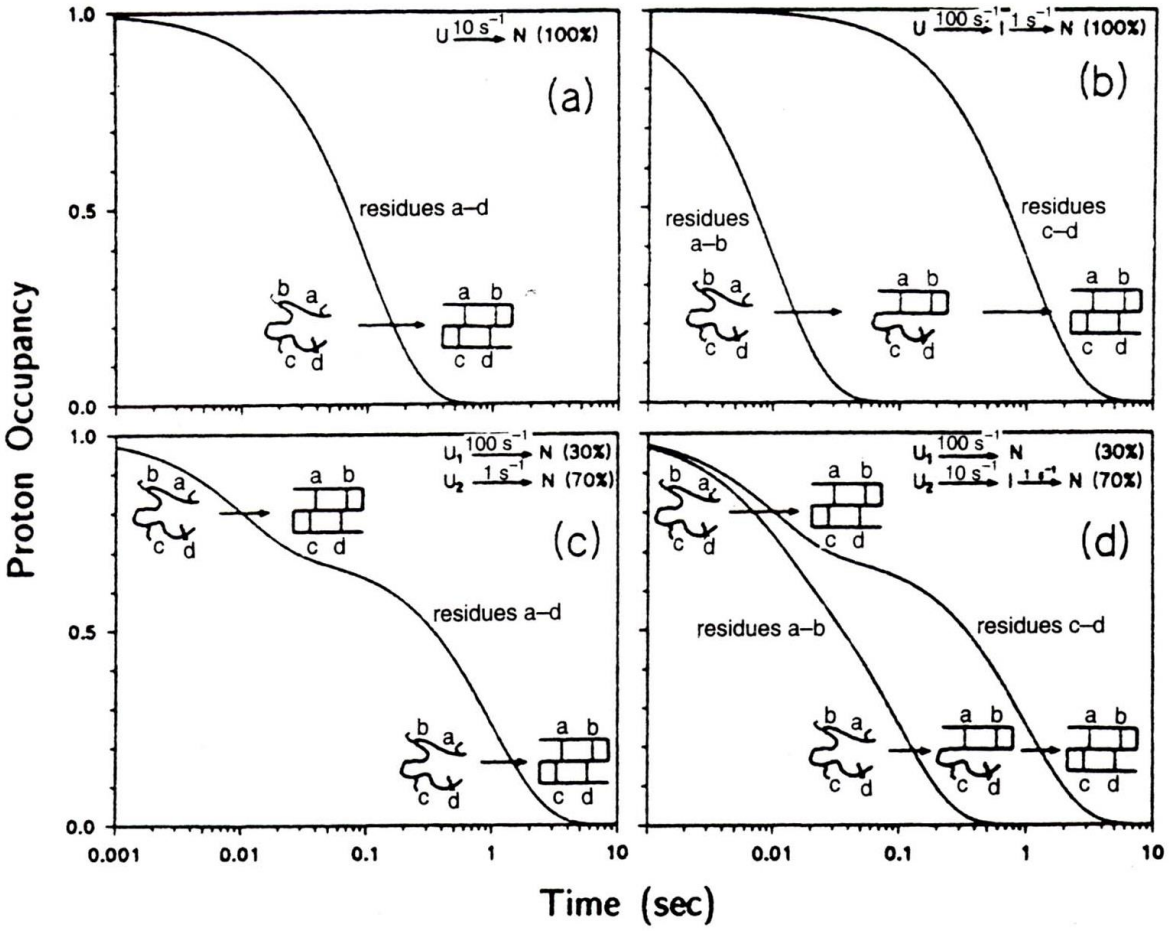
•After an adjustable refolding time, t_f, the protein is subjected to a short high pH pulse, where exchange of the unprotected NHs is very fast. NHs protected by structure within the folding time does not exchange during the short pulse

•After a pulse time t_p. The D to H exchange is quenched by rapidly lowering the pH.

•After folding completed, the pattern of NH and ND labels in the refolded protein is analyzed by 2D NMR.

•Increasing t_f time, proton occupancies measured in the NMR spectrum decreases. Plotting proton occupancy vs. folding time t_f.

Identifying Folding Pathway by HX Pulse-Labeling



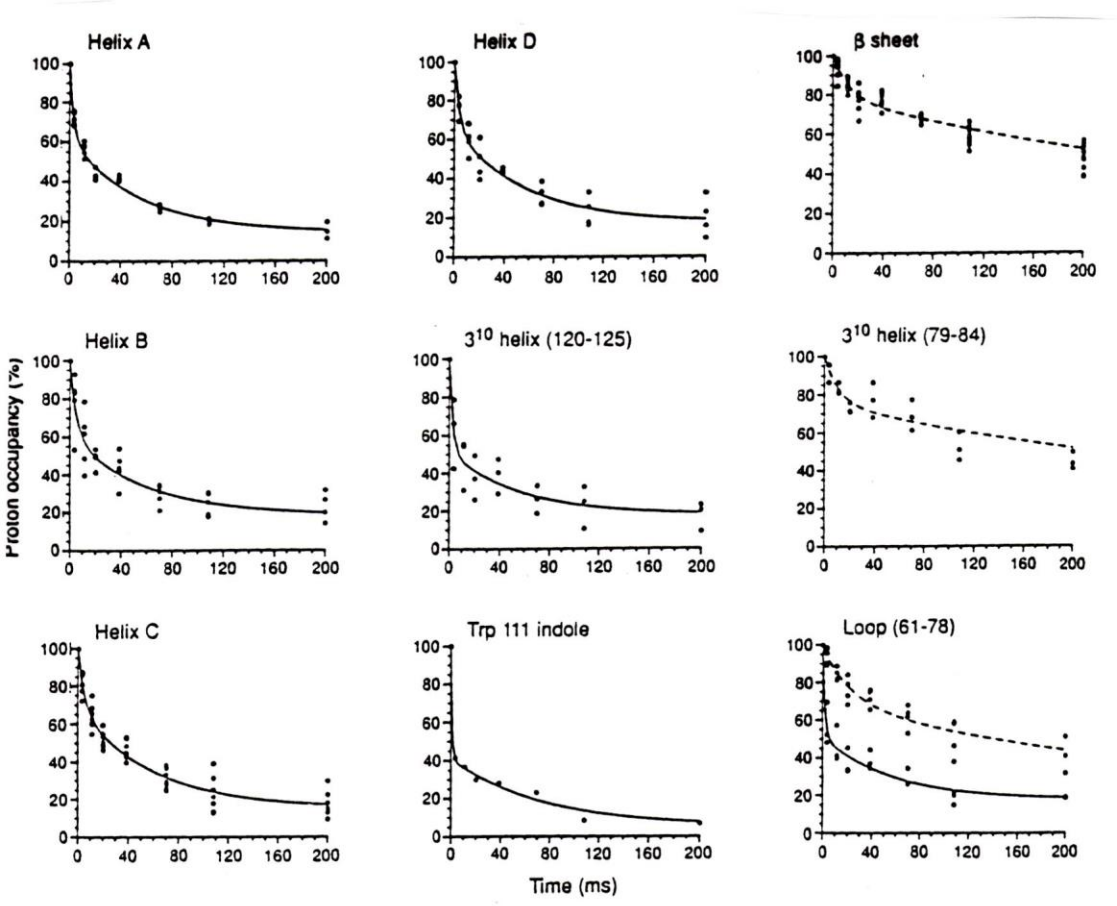
(a) pure 2-state
All probes achieve 100% protection at the same rate in a single kinetic step.

(b) U -> I -> N sequential,
I has A&B H-bonds with the same HX constant

(c) U1 -> N(30%)
U2 -> N(70%) two heterogeneous parallel paths

(d) U1-> N
U2->I->N contribution of intermediate and heterogeneous folding

Parallel Folding Pathway of Lysozyme



- All probes (50% of 126 amides) have one fast phase and one slow phase
- Fast phase rates for both α- and β domains are 10 ms
- Slow phase rates for α is 65 ms and β is 350 ms, respectively.

- α domains folds before β domain and different populations of molecules folded by kinetically distinct pathways

Additional Methods for Amide-Water Exchange

- Hwang TL, Mori S. Shaka, AJ, and van Zijl PC, Application of Phase-Modulated CLEAN Chemical EX-change Spectroscopy (CLEANEX-PM) to detect water-protein proton exchange and intermolecular NOEs. JACS, 1997, 119,6203-6204.
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  J Am Chem Soc. 2005 Dec 21;127(50):17626-7.

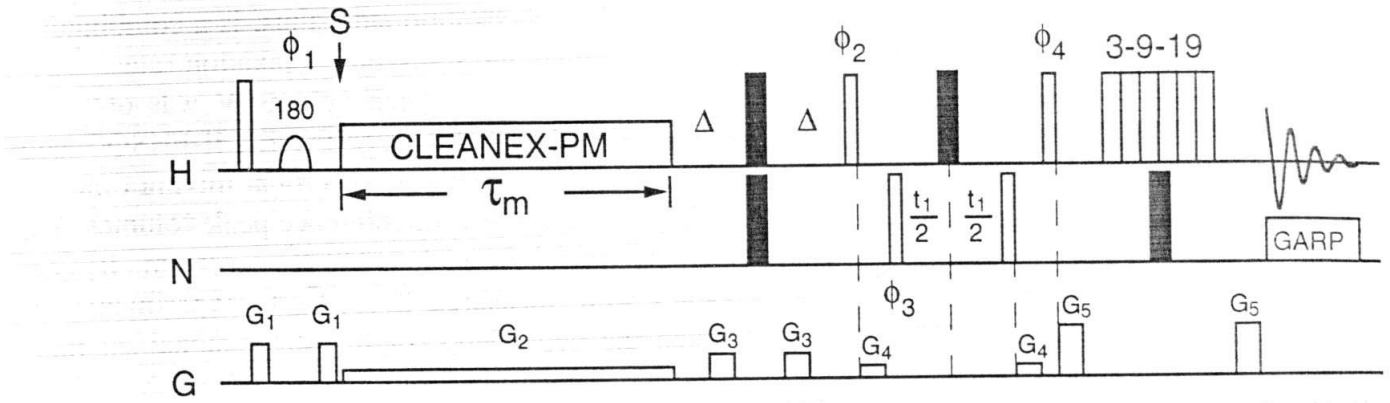


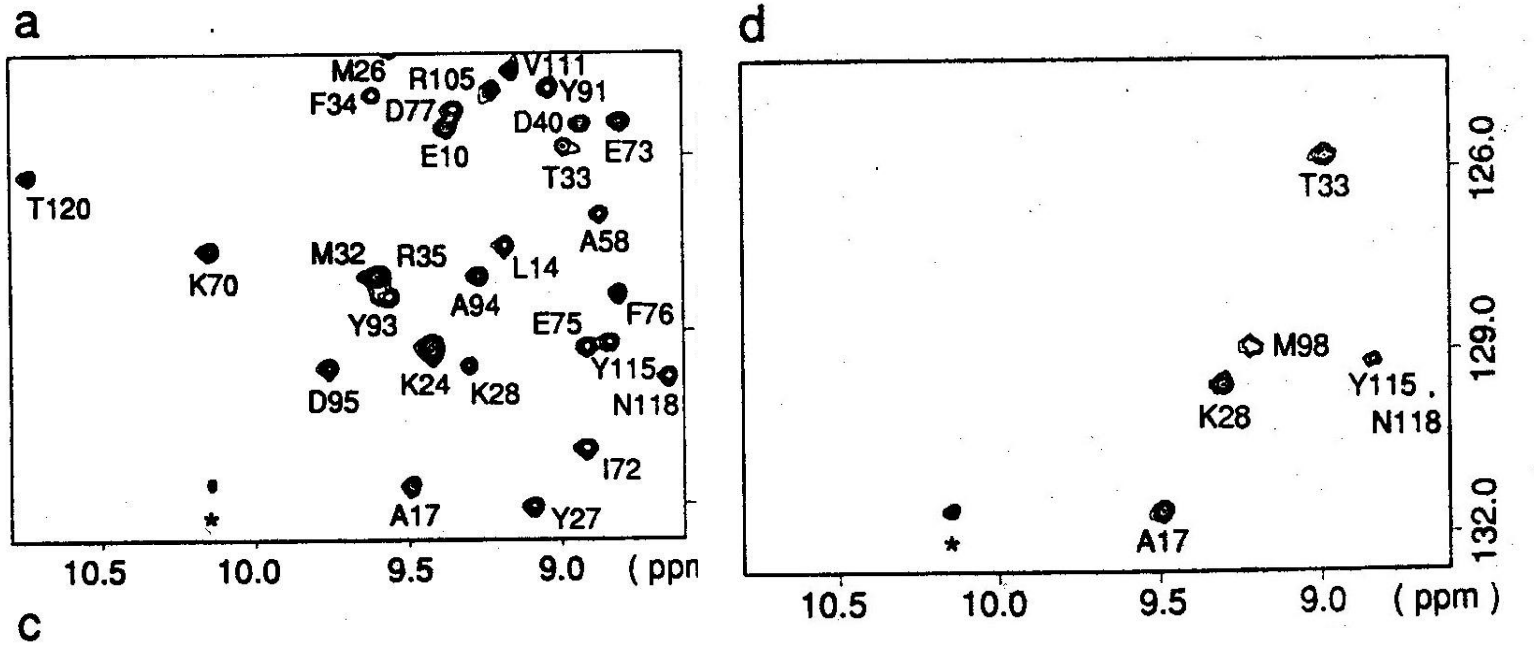
Figure 1. Timing diagram for (CLEANEX-PM)-FHSQC. Open and solid squares represent 90° and 180° pulses, respectively. At 500 MHz, the selective 180° pulse is a 7.5 ms Gaussian. The gradient strengths are $G_1 = 7.0$, $G_2 = 0.1$, $G_3 = 4.4$, $G_4 = 0.2$, and $G_5 = 28.0$ G/cm; the gradient lengths of G_1, G_3, and G_5 are 1 ms, G_4 0.5 ms, and G_2 is applied throughout the mixing period. Phase cycle: ϕ_1 {x,x,y,y}, ϕ_2 {y,y,y,y,−y,−y,−y,−y}, ϕ_3 {x,−x,x,−x}, ϕ_4 {x,x,−x,−x}, and rec.: {x,−x,x,−x,−x,x,−x,x}; the pulse trains in CLEANEX-PM and the 3-9-19 module start from the x direction. Other unspecified pulses are applied in the x direction. The ^{15}N decoupling is accomplished by GARP (Shaka et al., 1985). The interpulse delay in the 3-9-19 pulse module (Sklenář et al., 1993) is 220 μs. The saturation level of water is measured at point S (see text).

CLEANEX-PM spin-locking sequence:
135°(x) 120° (-x) 110° (x) 110°(-x) 120°(x) 135° (-x)

CLEANX-PM has the ability to specifically monitor water-proton exchange without 1) exchange relayed NOE/ROE from rapidly exchanging protons (hydroxyl or amide groups) in the macromolecules, 2) intra-molecular NOE/ROE peaks from protein CαH protons which has chemical shifts coincident with water, or TOCSY-type interactions.

FHSQC

CLEANEX-PM



The FHSQC indicates proton signal that remain at the amide resonance through out the pulse sequence.

The CLEANEX-PM indicates ^{1}H signals that initiate in the $^{1}H_2O$ resonance and then transfer to ^{1}H amide resonance during the mixing period of the pulse sequence.

Staphylococcal nuclease –Hwang et al., 1998

Journal of Biomolecular NMR **28:** 385–390, 2004.
KLUWER/ESCOM

Quantitation of rapid proton-deuteron amide exchange using hadamard spectroscopy

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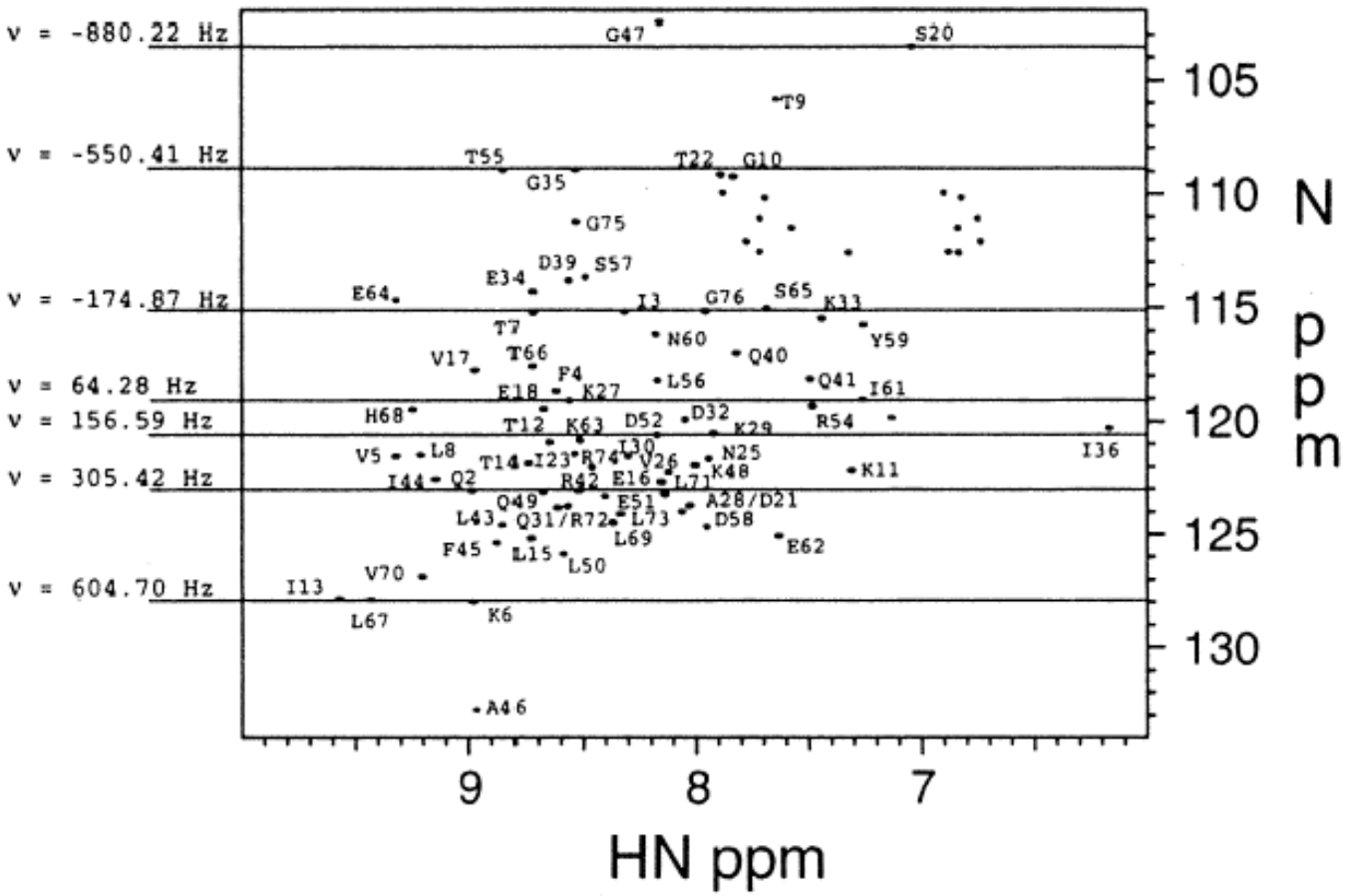
Key words: HSQC, ^{15}N, NMR, protein structure, ubiquitin

Abstract

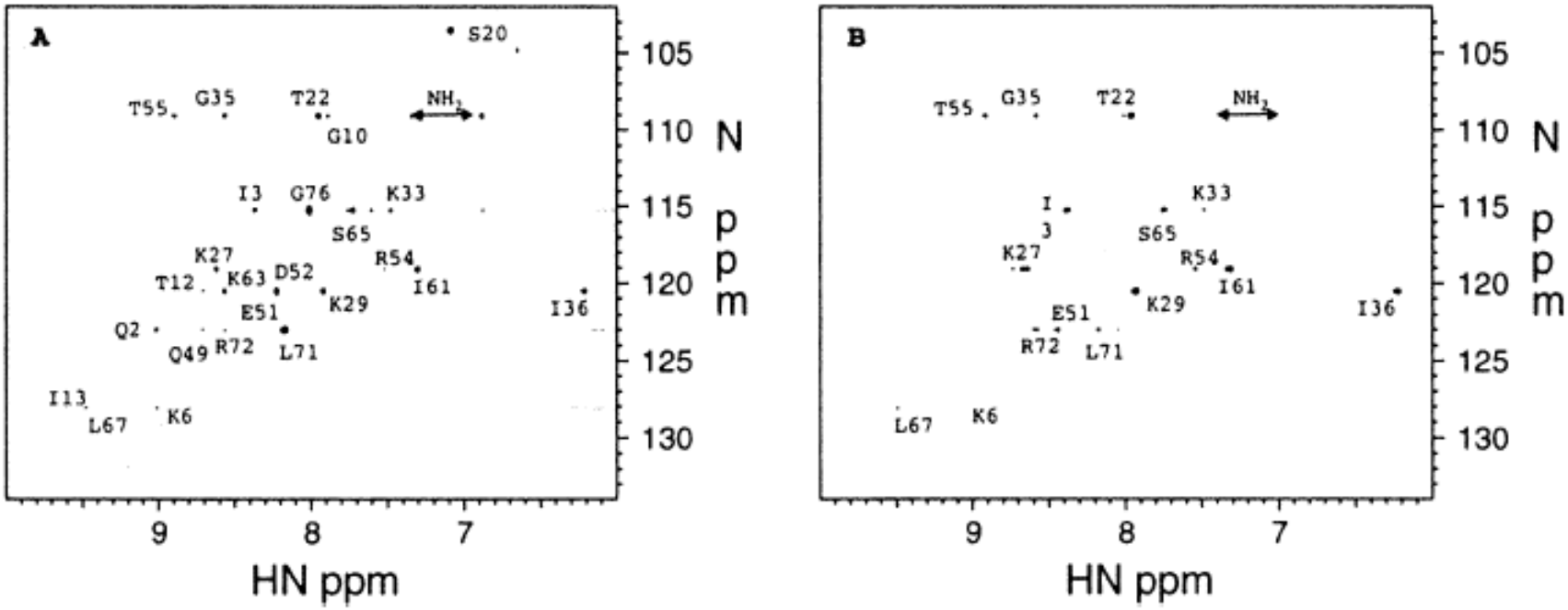
The rates of amide proton exchange in protein backbones are very useful reporters of accessibility and structural stability of specific residues and secondary structure elements. Measurement by monitoring changes in intensity of cross-peaks in standard ^{15}N-^{1}H HSQC spectra as protons are replaced by solvent deuterons has become widely accepted. However, these methods are limited to relatively slow rates due to time limitations of the conventional 2D HSQC experiment. Here we show that a Hadamard encoded version of the HSQC, which relies on a multiplexed, frequency selective, excitation in the ^{15}N dimension, extends application to rates that are as much as an order of magnitude faster than those previously accessible.

Conventional [1H,15N]-HSQC



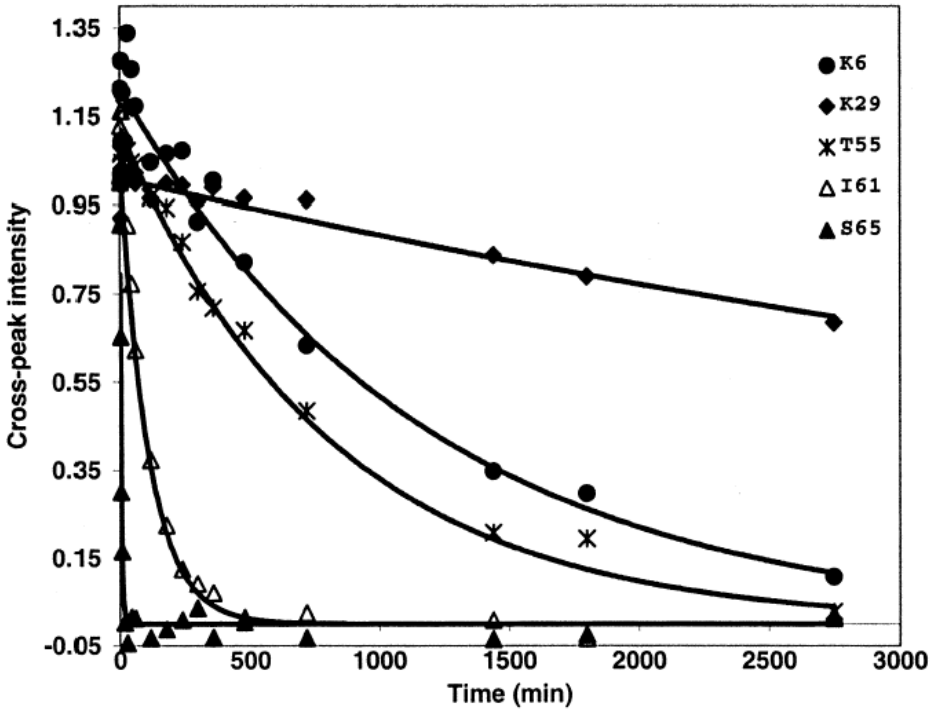
A 0.5 mM15N-labeled sample was prepared in 50 mMpotassium phosphate buffer in H2O/D2O 5/95, pH=6.2 600 MHz with cryoprobe. it required approximately 21 min using 128 t1 time increments and 4 scans per increment.

Reconstructed Hadamard [1H,15N]-HSQC Spectra for Ubiquitin

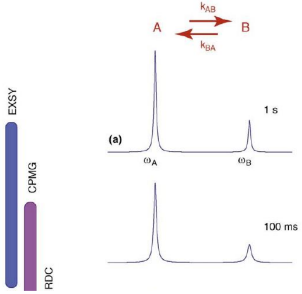


(A) Data in $^{1}H_2O$ collected with 128 t1 increments in 20 min. The sample was then lyophilized overnight and brought back to its initial volume with 99.9% $^{2}H_2O$ and immediately returned to the spectrometer for rapid collection of a series of Hadamard spectra.
(B) First point after 1 min in $^{2}H_2O$ collected with 4 scans in 42 s.

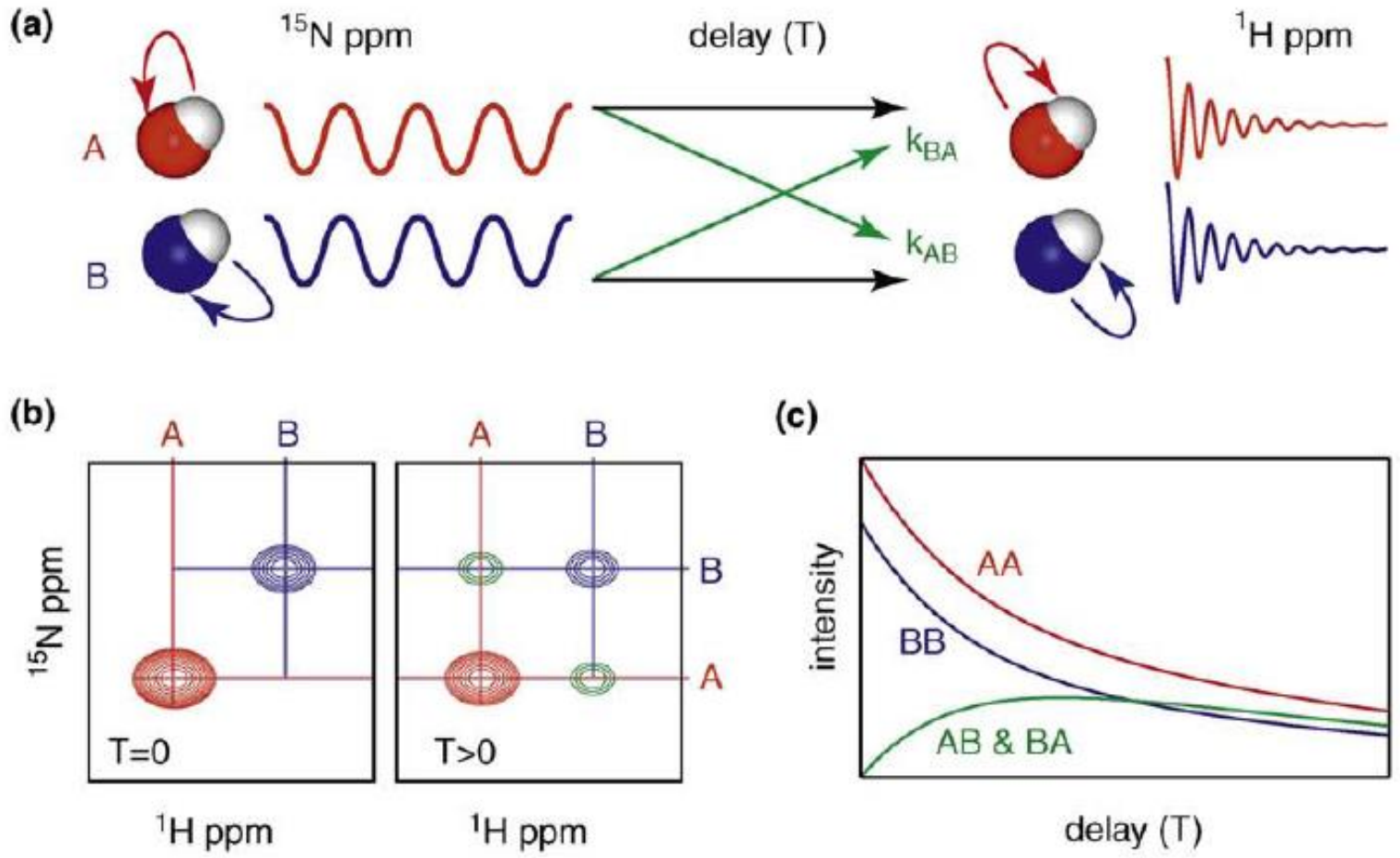
Cross-peak intensities as a function of time



Lines are best fits to $I(t) = I_o(\exp(-kt)+\text{const})$.
The precision of the data is quite high with the estimated errors for rates in the range of 1×10^{-3} min-1 being on the order of 5%. Rates derived also show reasonable agreement with previously published rates.

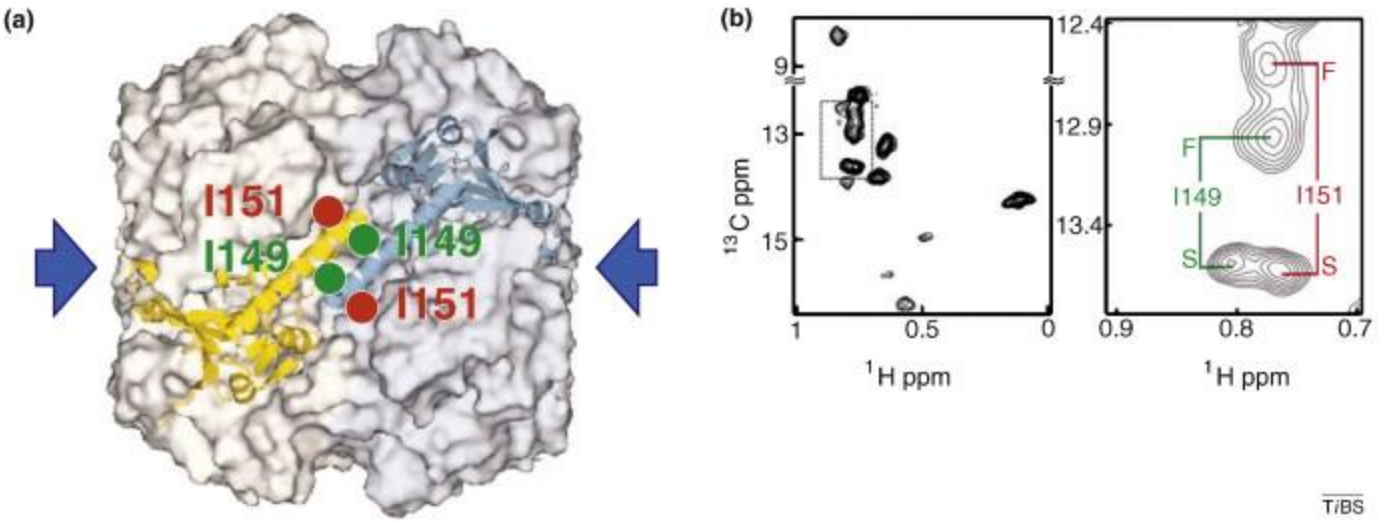


Exchange Spectroscopy (EXSY)



Mittermaier et al (2009) *Trends Biochem. Sci.* **34**, 601.

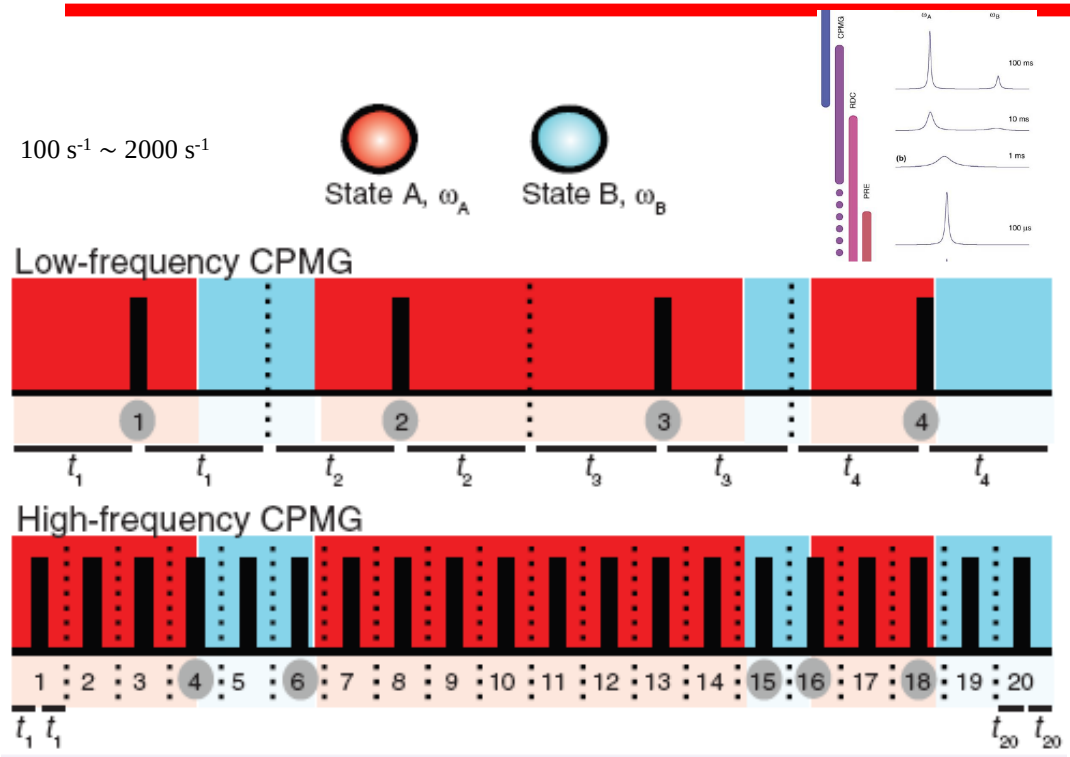
Slow conformational exchange in the protease ClpP

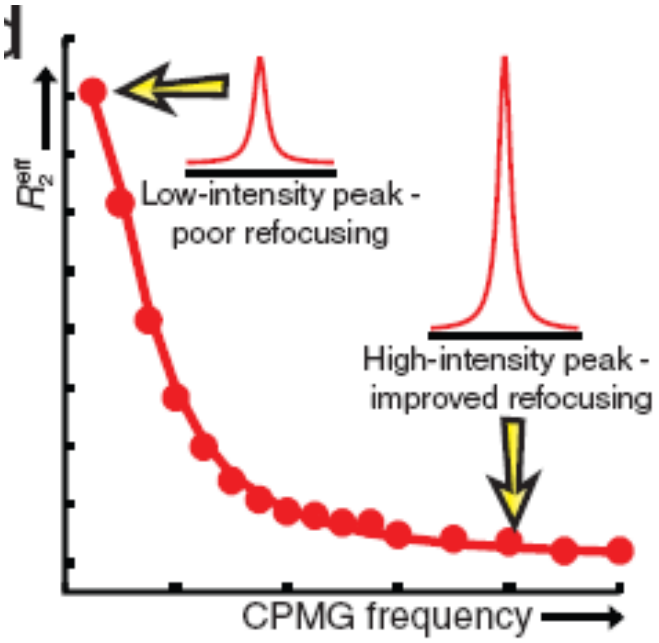


ClpP, an oligomeric protease comprising 14 subunits with a total molecular mass of 300 kDa. (a) Surface representation of ClpP with two monomers shown as yellow and blue ribbons. Locations of dynamic isoleucine residues are identified by green and red circles. Substrate entry pores are indicated with blue arrows. (b) The $^{1}H/^{13}C$ methyl TROSY correlation spectrum collected for a uniformly [^{15}N, ^{2}H], Ile δ1 [^{13}C,^{1}H] labeled ClpP sample. I149 and I151 are each associated with two δ1 methyl peaks, designated F and S, reflecting slow exchange between two distinct, functionally important, conformations. ($5^{0}C$, rotational correlational time >0.4 us)

R.Sprangers, PNAS, et al.2005 Wang JM, Cell, 1997

Carr–Purcell–Meiboom–Gill (CPMG) Relaxation Dispersion





Exchange between ground state and excited state is in the millisecond time scale

$$A \underset{k_{BA}}{\overset{k_{AB}}{\rightleftharpoons}} B$$

$k_{ex} = k_{AB} + k_{BA}$

$k_{BA} > k_{AB}$

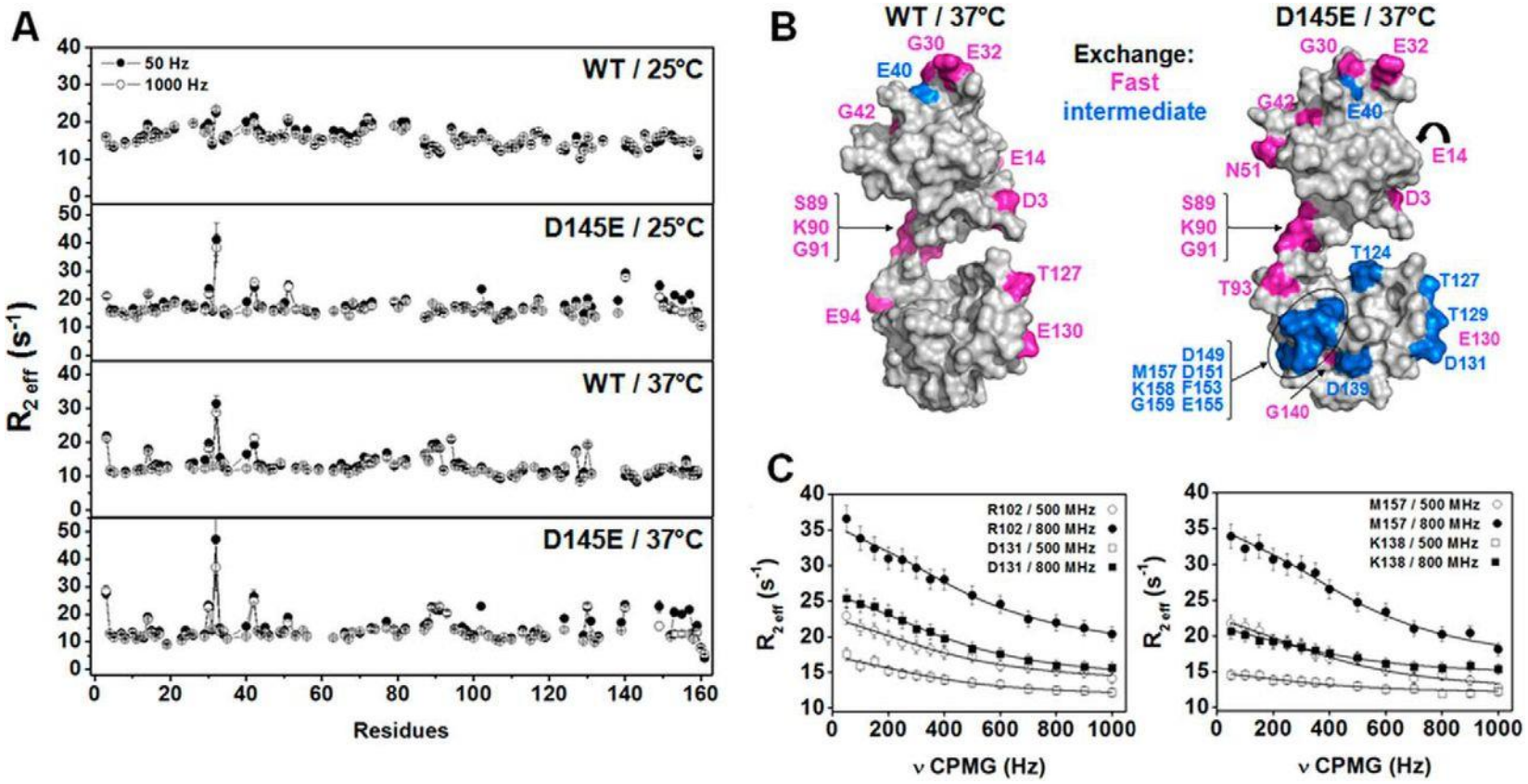
Shape of the dispersion depend on:

- populations of the two states
- chemical shift difference
- the rate of exchange

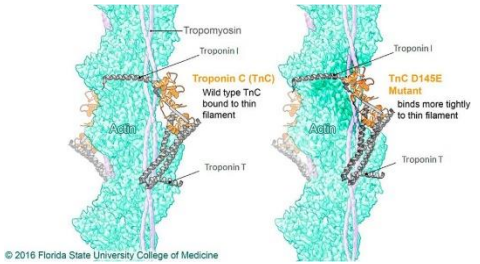
In a typical series of experiments, variable numbers of refocusing pulses are applied to magnetization as it evolves under the influence of a chemical shift that varies stochastically due to the exchange process

Baldwin et al (2009) *Nature Chem Biol* **5**, 808.

Allosteric Transmission along a Loosely Structured Backbone Allows a Cardiac Troponin C Mutant to Function with Only One Ca2+ Ion.

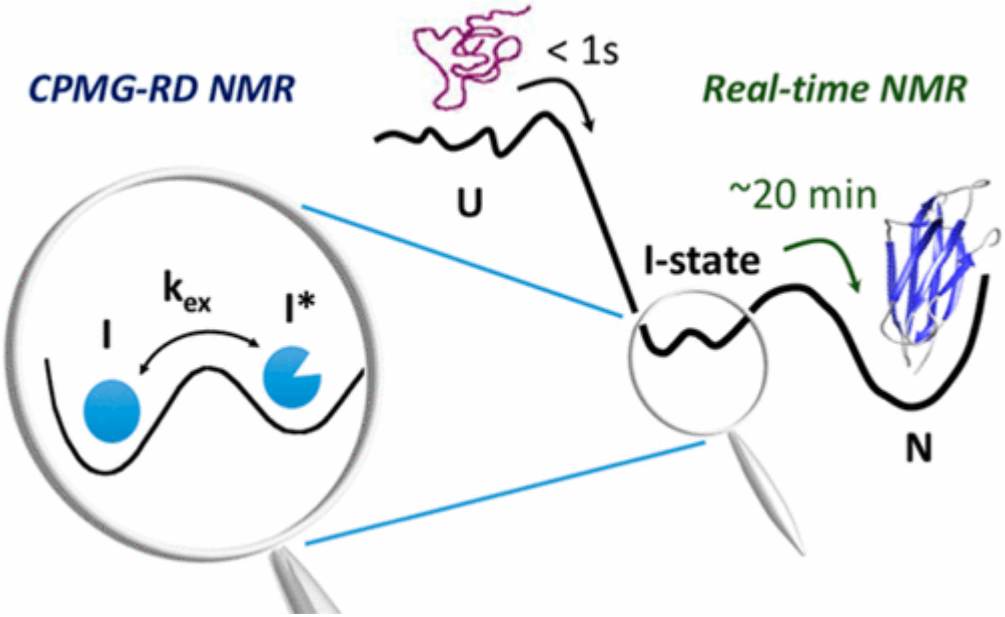


Quantitative analysis of exchange dynamics in cTnC D145E using CPMG



Mayra de A. Marques et al. J. Biol. Chem. 2017;292:2379-2394

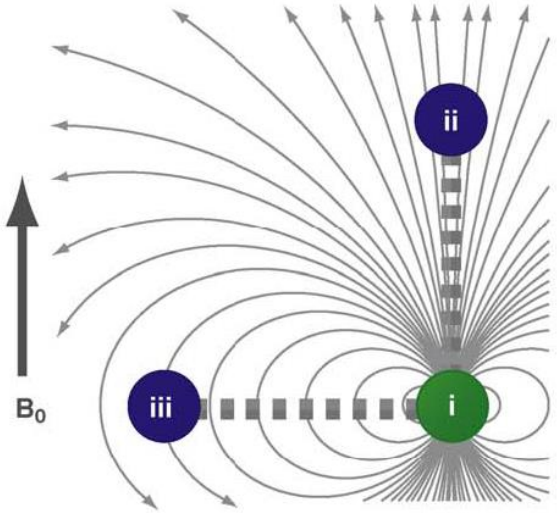
Probing Conformational Exchange Dynamics in a Short-Lived Protein Folding Intermediate by Real-Time Relaxation−Dispersion NMR

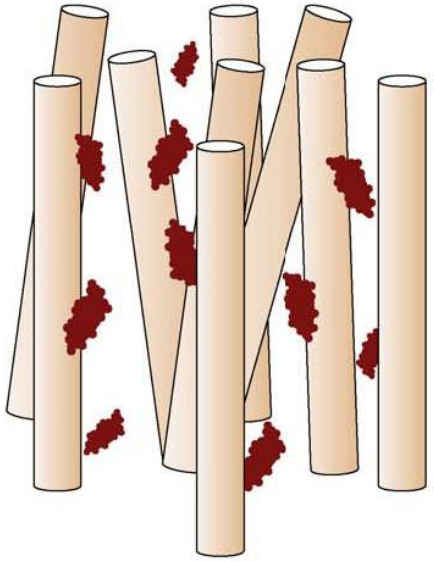


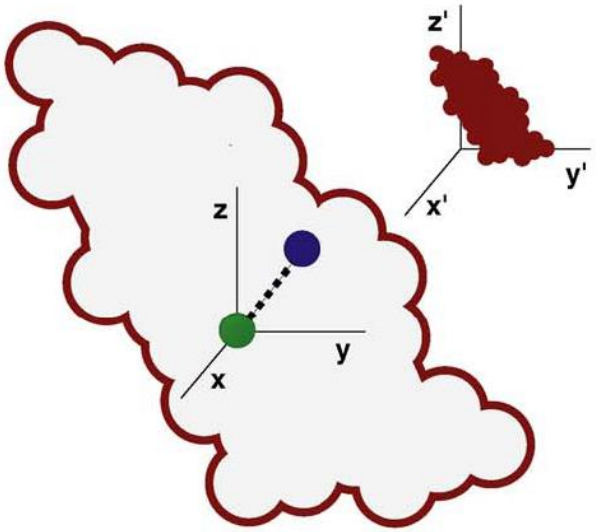
- Using relaxation–dispersion NMR and real-time NMR to reveal the conformational exchange dynamics present in short-lived excited protein states, such as those transiently accumulated during protein folding. Amyloidogenic protein β2-microglobulin folds via an intermediate state which is believed to be responsible for the onset of the aggregation process leading to amyloid formation.

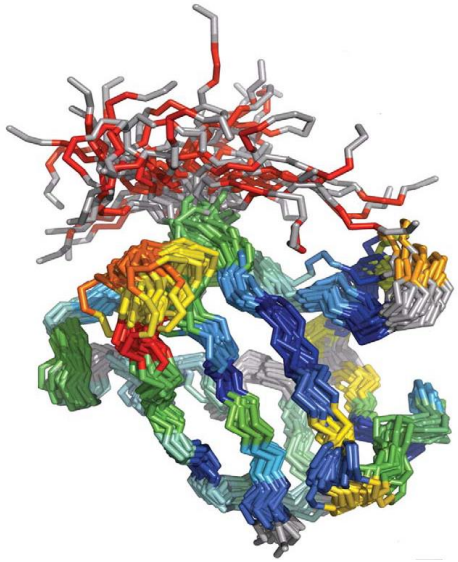
- Rémi Franco, et al JACS, 2017

Residual Dipolar Coupling (RDC)



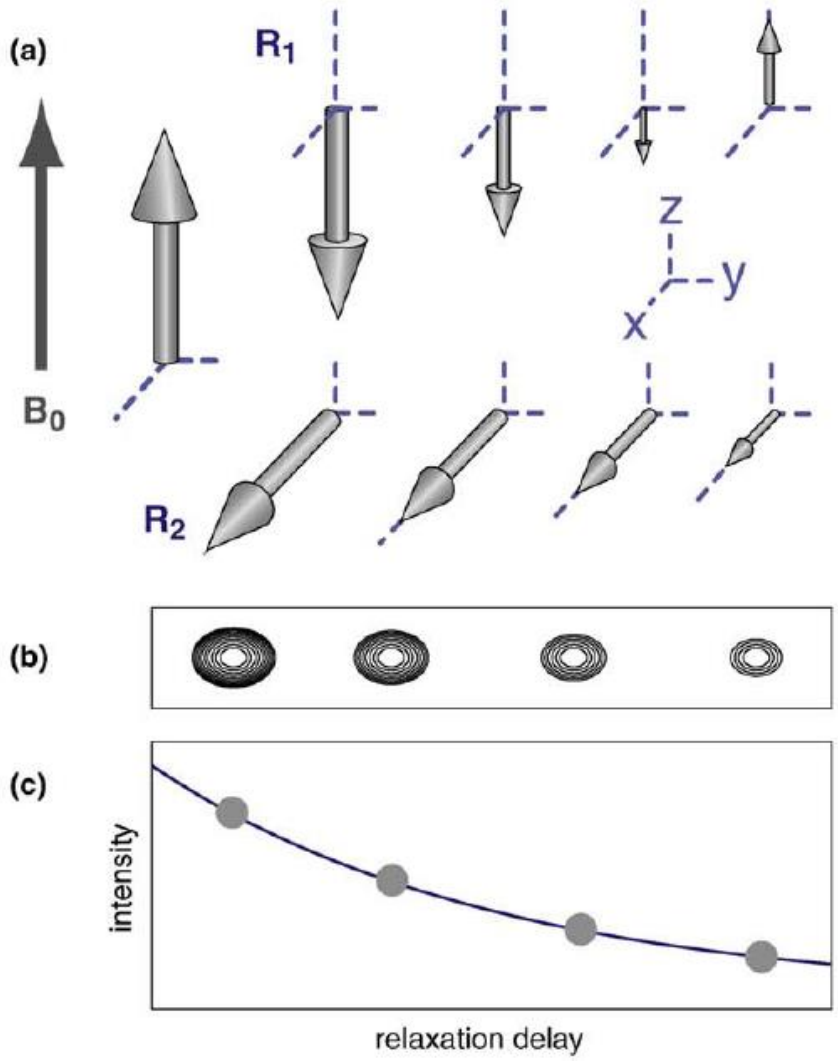


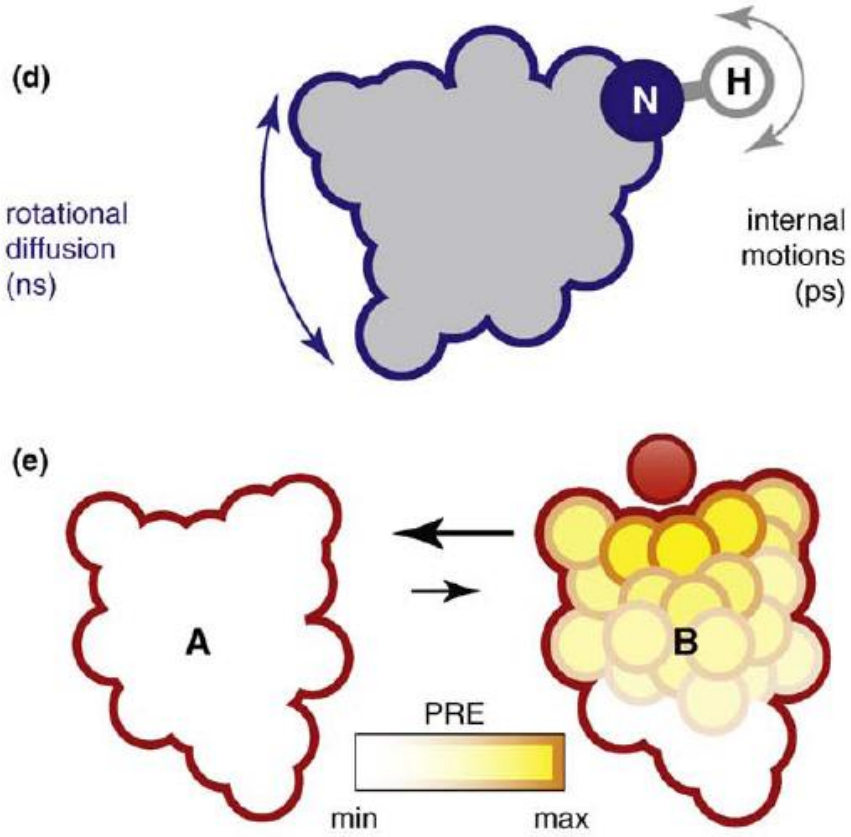




Mittermaier et al (2009) *Trends Biochem. Sci.* **34**, 601.

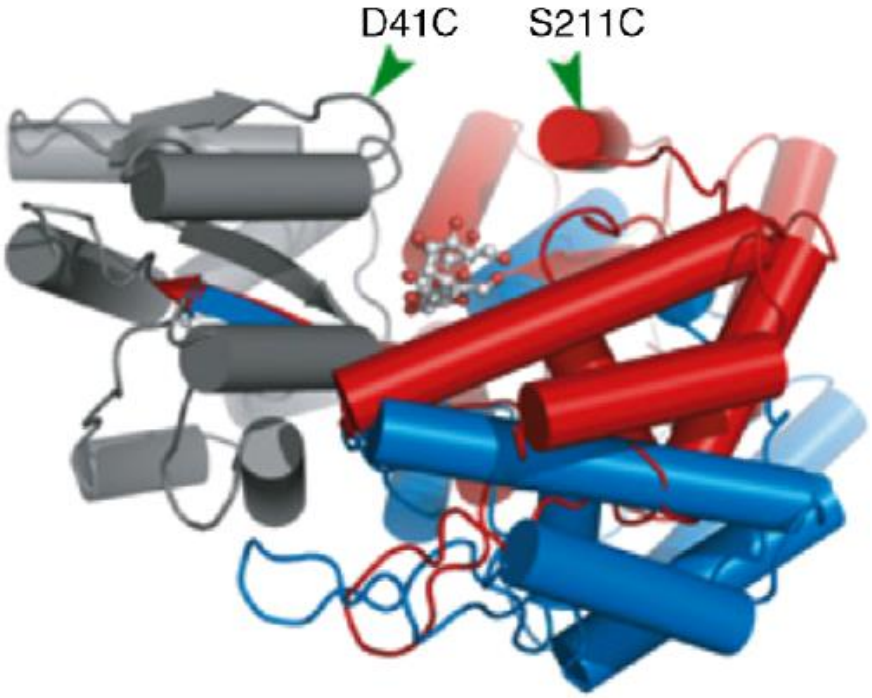
Spin Relaxation and Paramagnetic Relaxation Enhancement





Mittermaier et al (2009) *Trends Biochem. Sci.* **34**, 601.

PRE Study of Maltose Binding Protein



Grey: N-terminal domain

Blue: C-terminal domain, apo

Red: C-terminal domain, holo

- PRE of holo form agree with X-ray structure
- PRE of apo form is larger than X-ray structure
- The apo form is in a transient close form (5%)

Mittermaier et al (2009) *Trends Biochem. Sci.* **34**, 601.