NMR spectroscopy beyond 20 kDa

general problems with increasing molecular weight:

Increasing number of resonances result in severe overlap

slower molecular tumbling increases transverse relaxation

larger linewidth

ineffective magnetization transfer

low signal / noise in spectra

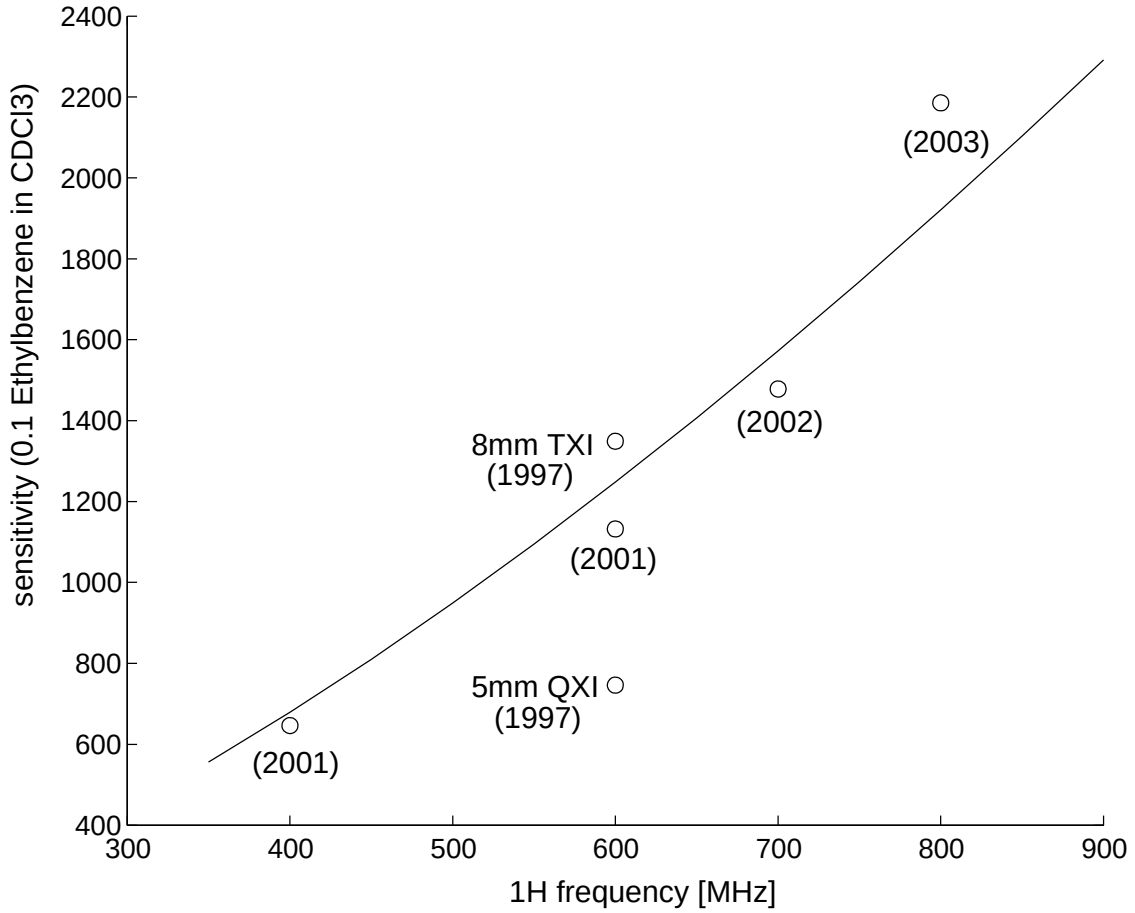
high solubility required !

1 mM sample (500 µl) 5 mg of a 10 kDa protein

20 mg of a 40 kDa protein

Higher sensitivity - Increasing of B_0 field strength

Signal / Noise ratio is proportional to $B_0^{3/2}$



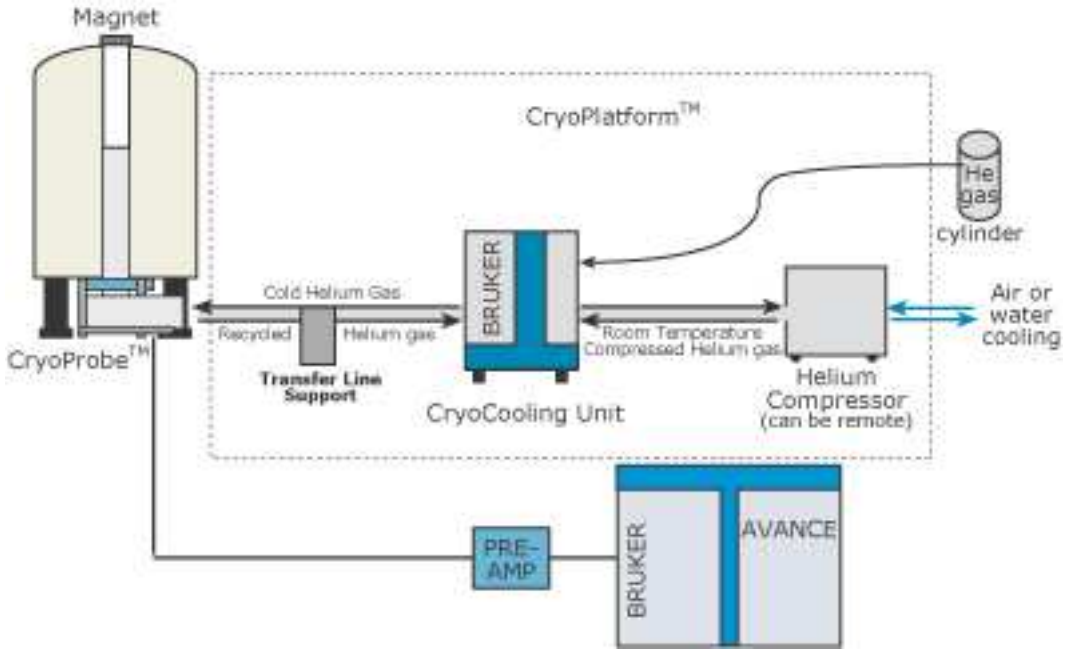
Cyroprobes

cooling down the receiver coil (to 15-30 K) reduced thermal noise

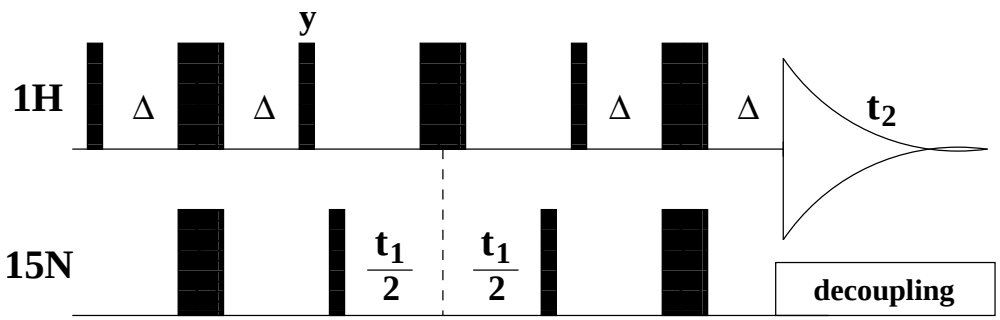
S/N using the standard Ethylbenzene test

6800:1 700 MHz system in Bayreuth

8000:1 800 MHz system in Bayreuth

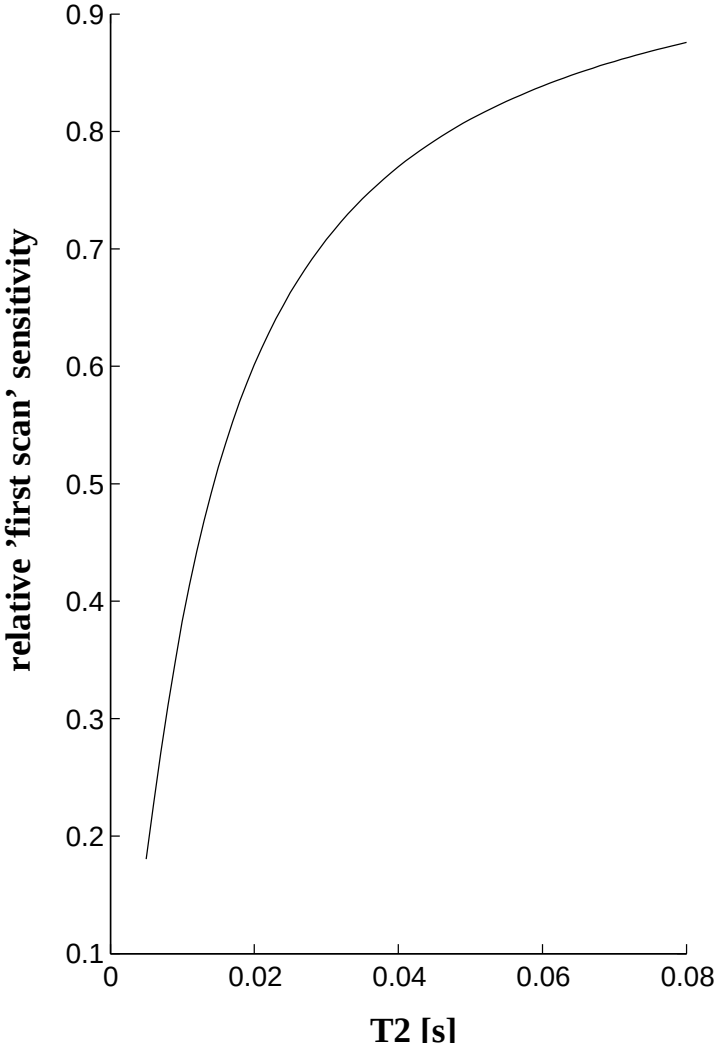


Transfer Effiency in a 1H, 15N HSQC

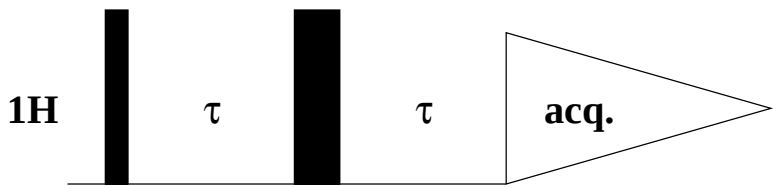


$$\Delta = \frac{1}{4J(H,N)} \qquad \Delta_{opt} = \frac{1}{2\pi J}\,\mathrm{atan}(\pi J T_2)$$

$$\text{'first scan' sensitivity} = \exp\{-4\Delta/T_2\} * \sin^2(2\pi\Delta J)$$



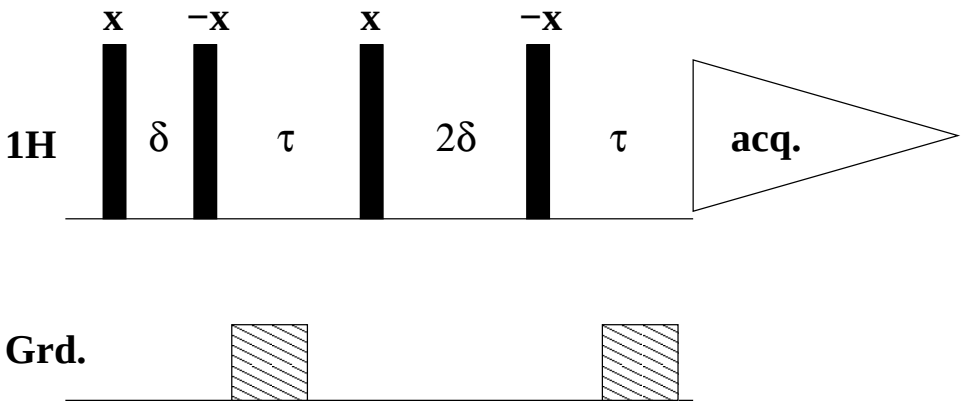
Simple Estimation of Transverse 1H Relaxation Rates



$S(\tau) = S(\tau=0)*\exp(-2\tau/T2)$

T2 can be estimated from the two independent experiments

practical protein version (watersuppresion) (Sklenar, V. & Bax, A., 1987)



zero excitation at carrier frequency

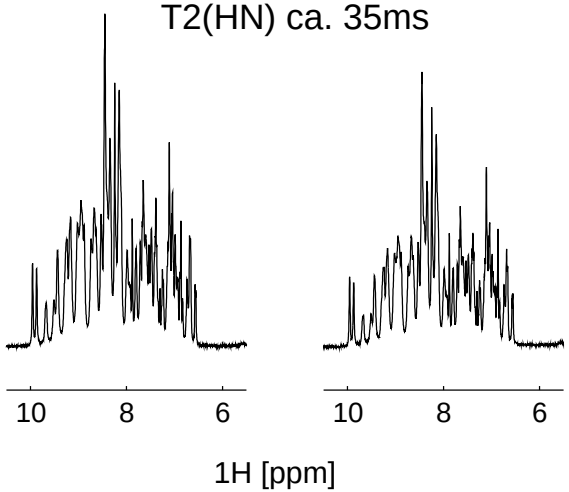
excitation maximum at $1/(4\delta)$

Transverse Relaxation of Amide Protons

(Two spin−echo experiments with 9 ms difference)

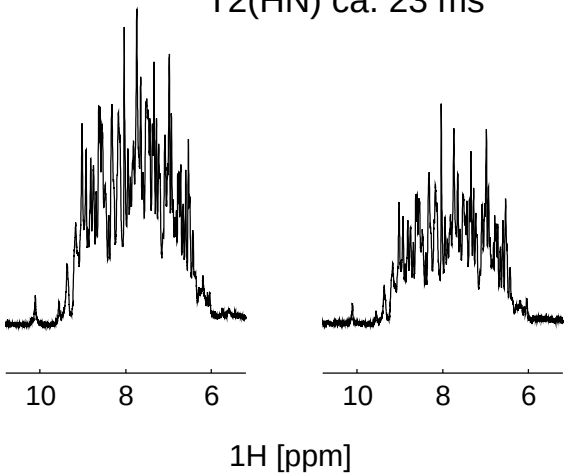
LynSH3 (7 kDa, 400 MHz, 298 K)

T2(HN) ca. 35ms



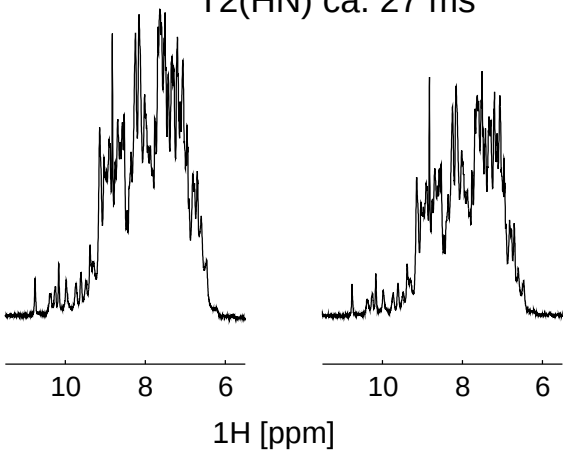
H. salinarum Fdx (14 kDa, 600 MHz, 288 K)

T2(HN) ca. 23 ms



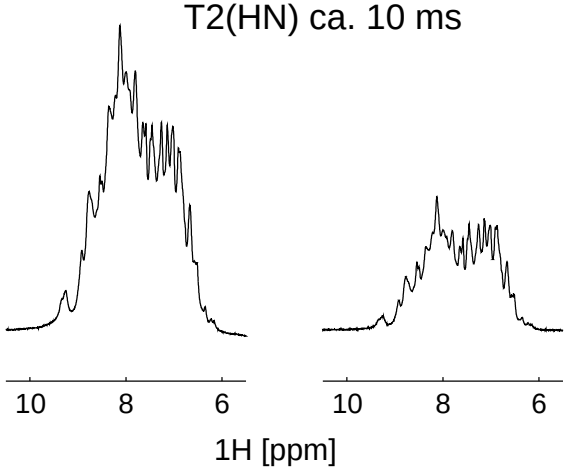
Lysozyme (14 kDa, 400 MHz, 298 K)

T2(HN) ca. 27 ms

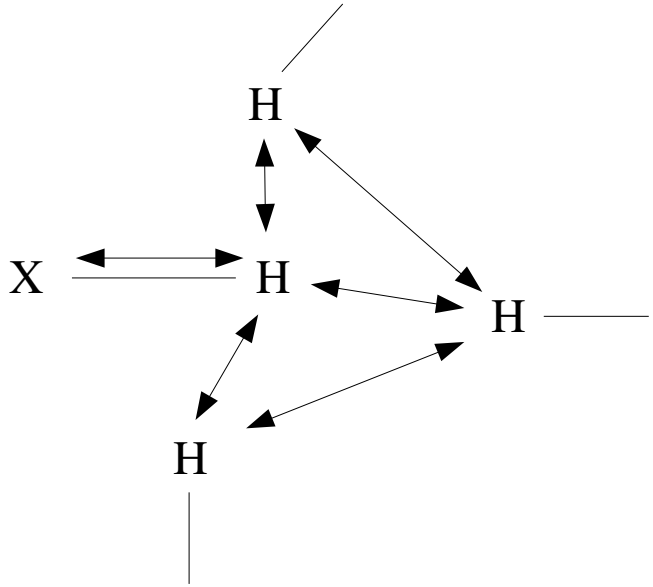


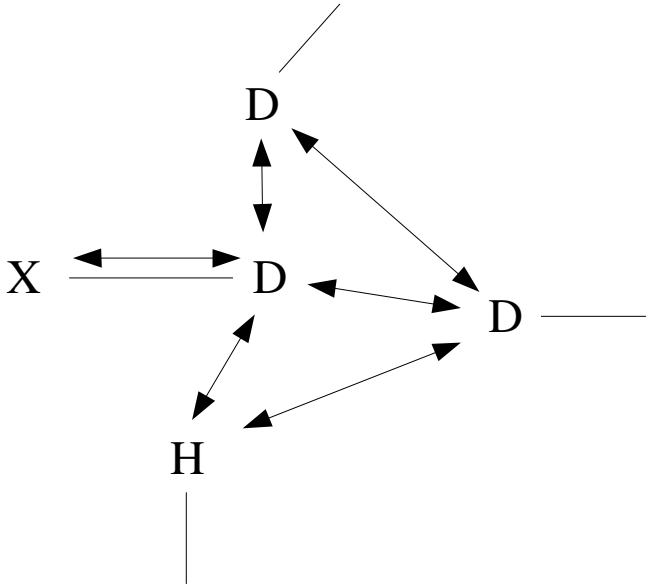
Tom70−16K (16 kDa (momomer), 400 MHz, 298 K)

T2(HN) ca. 10 ms



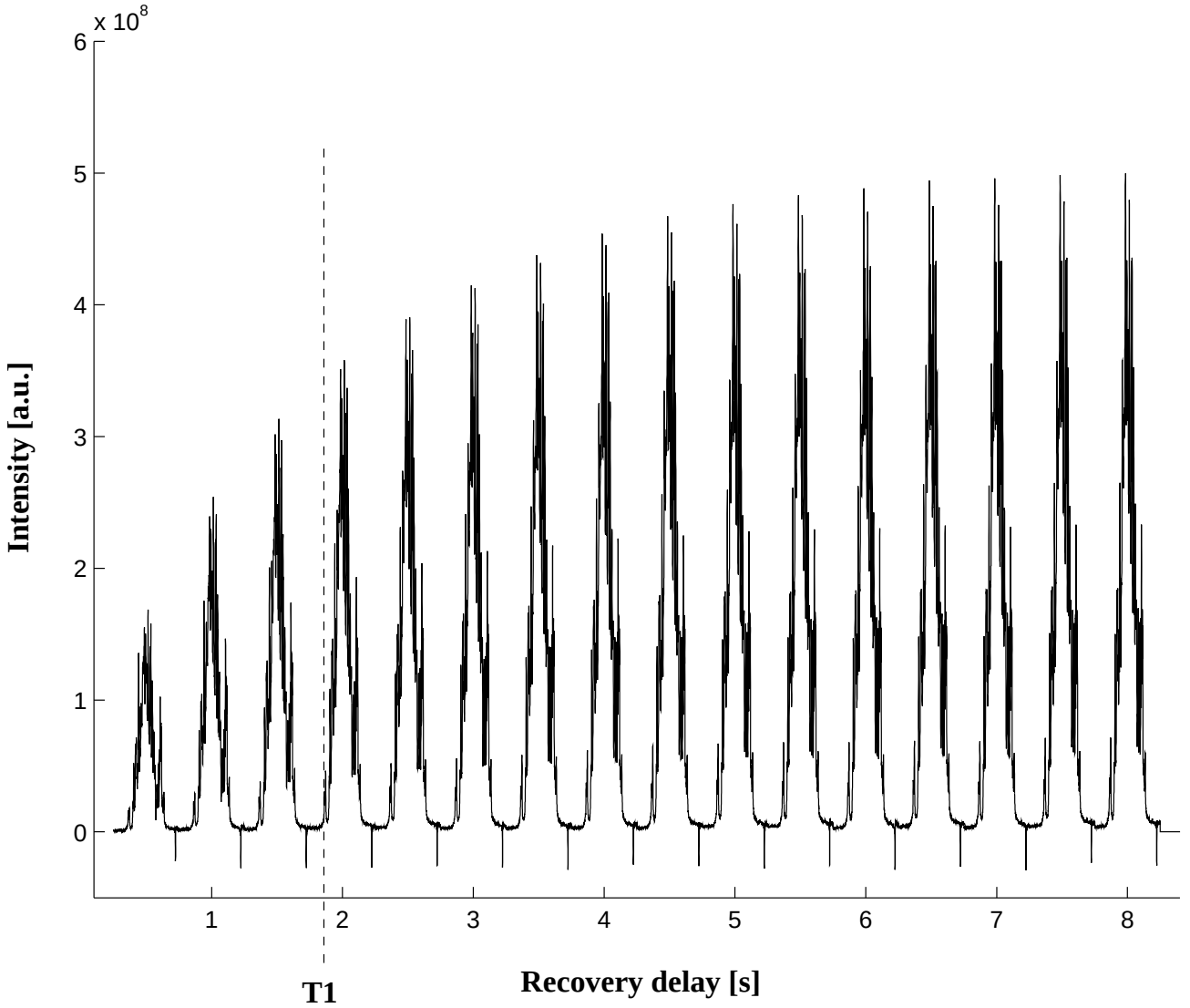
Dipole-Dipole interaction is main mechanism for relaxation in proteins



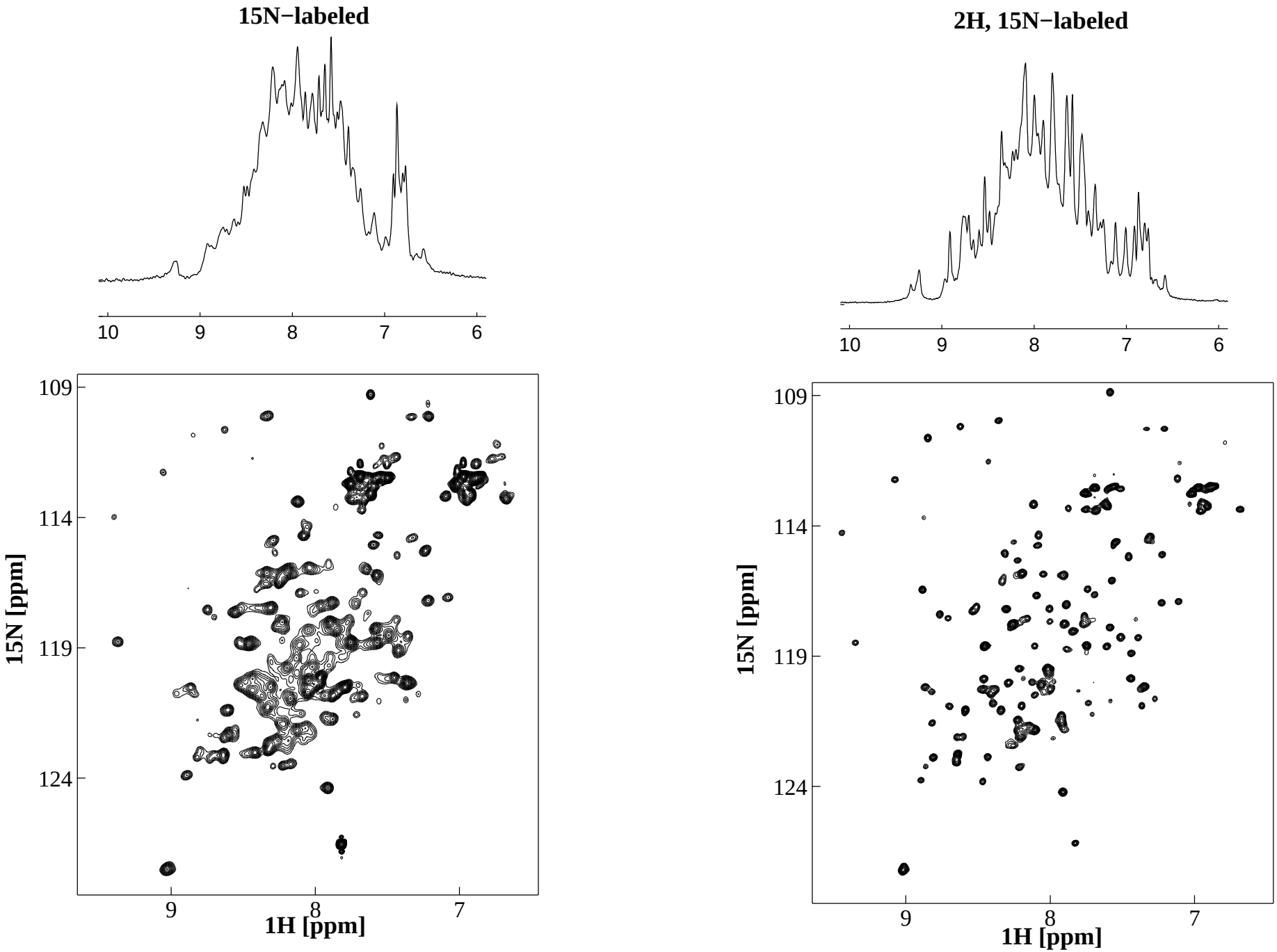


Substitution ^{1}H -> ^{2}D reduces transverse relaxation for the direct bound X-nucleus (mainly ^{13}C) and for the ^{1}H spins due to reduced proton density ! $\frac{\gamma(^{1}H)}{\gamma(^{2}D)} = \frac{6.5}{1}$

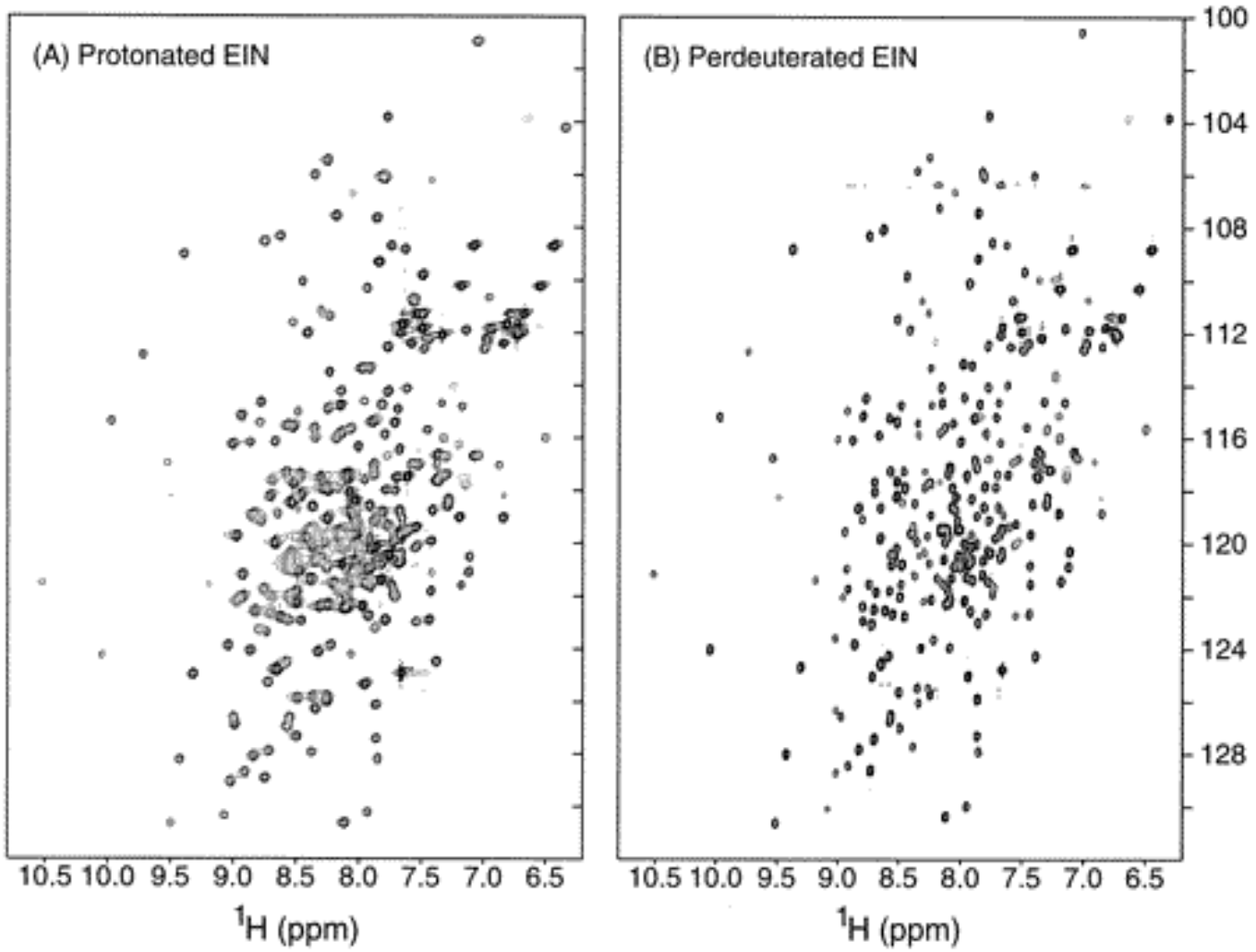
Amide proton signal recovery of [2H,15N] Tom70−16K during interscan delay



1H, 15N HSQC of Tom70−16K (800 MHz)



^{1}H, ^{15}N HSQC spectra of a 30 kDa protein



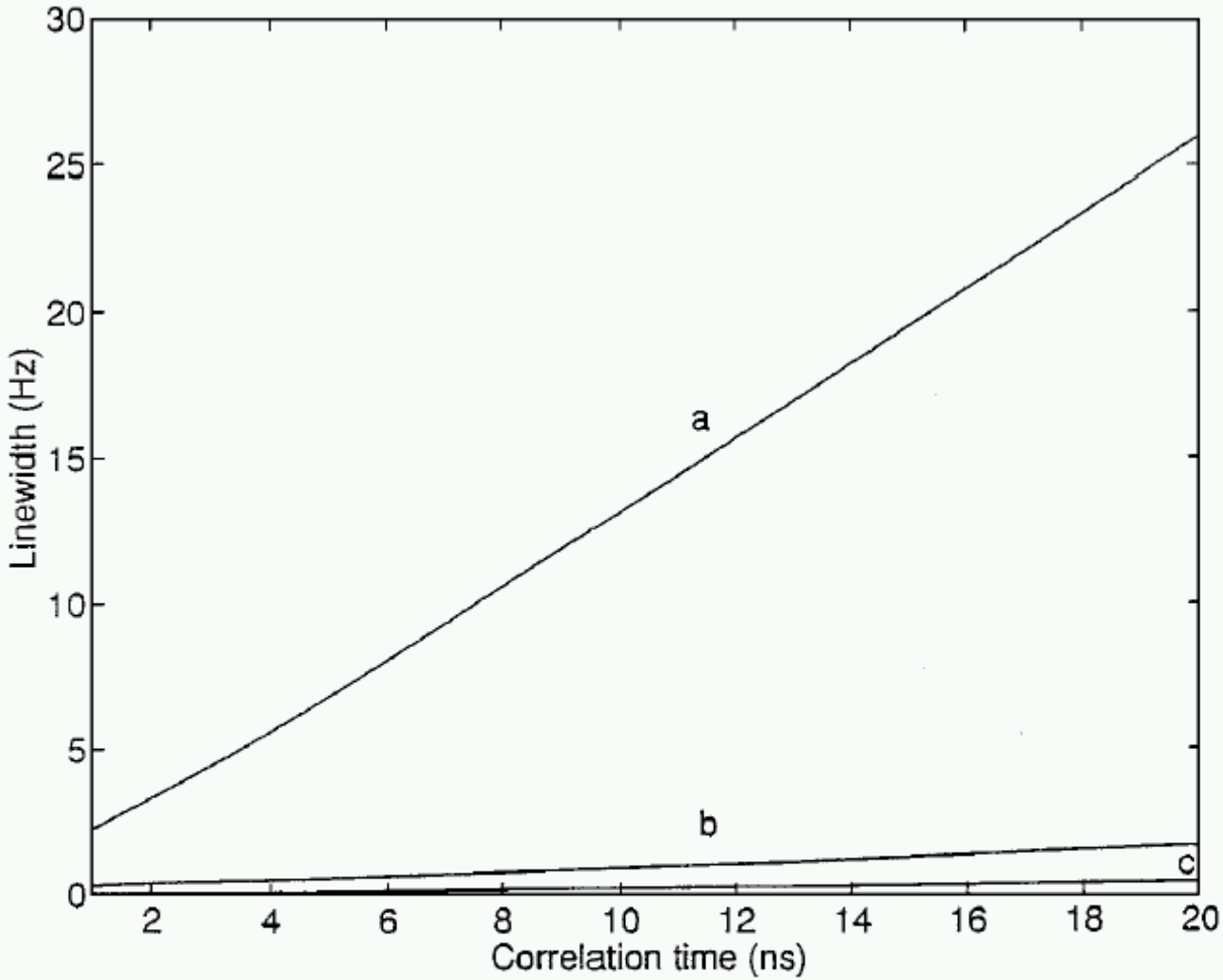


Figure 7 Expected contributions to the $^{13}C\alpha$ line-width from dipolar and CSA relaxation mechanisms assuming an isolated $^{13}C\alpha$-$^{1}H\alpha$ or $^{13}C\alpha$-$^{2}H\alpha$ spin pair as part of an isotropically tumbling molecule in solution. (*a*) Contribution to the $^{13}C\alpha$ line-width from $^{13}C\alpha$-$^{1}H\alpha$ dipolar interactions (assuming a 1.10 Å carbon-proton separation) (*b*) Contribution from $^{13}C\alpha$-$^{2}H\alpha$ dipolar interactions. (*c*) Contribution from $^{13}C\alpha$ CSA (assuming an axially symmetric $^{13}C\alpha$ CSA tensor, $\Delta\sigma = 34$ ppm). (170, by permission.)

HNCA spectrum of a protonated and a deuterated protein

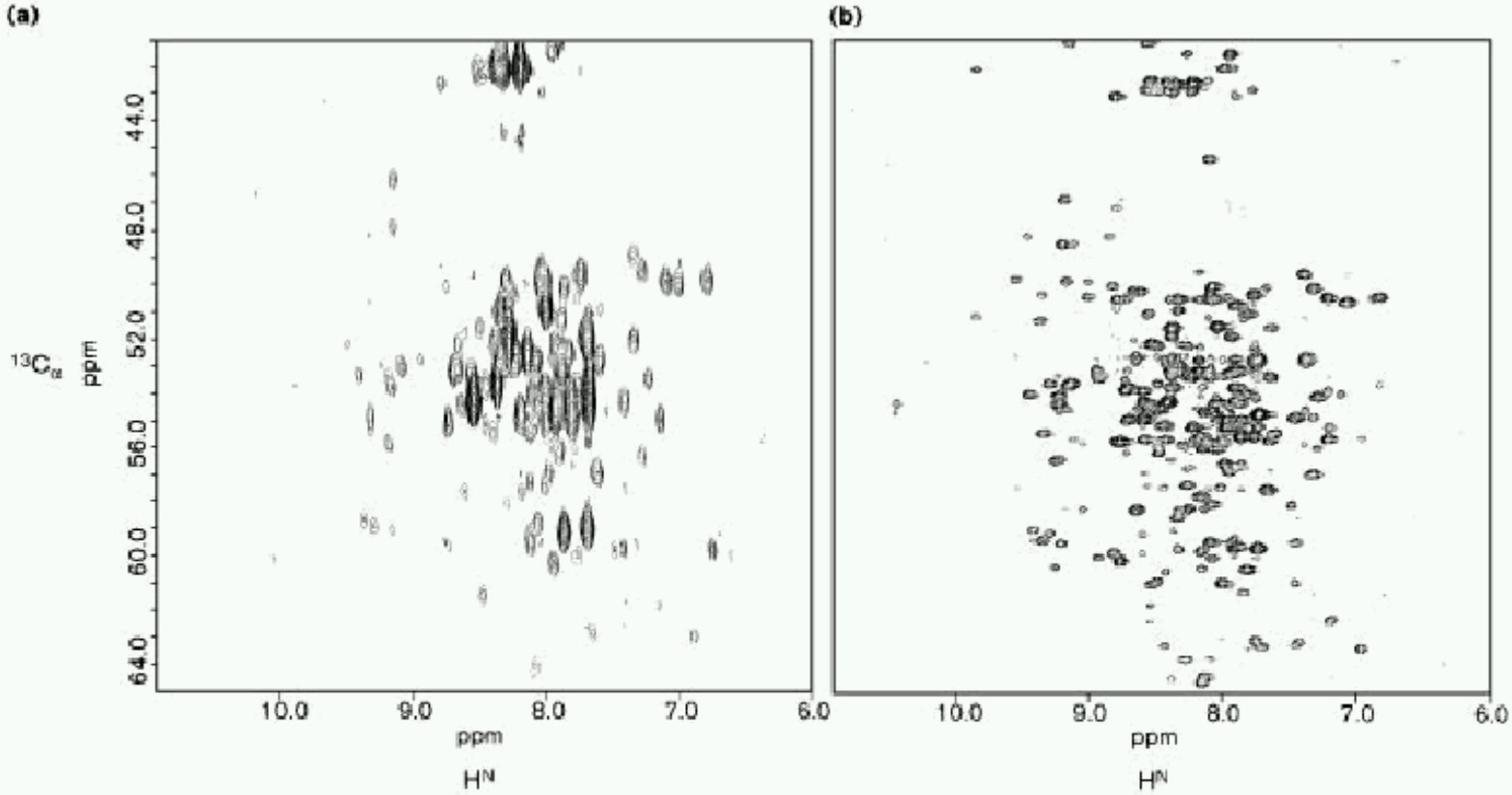
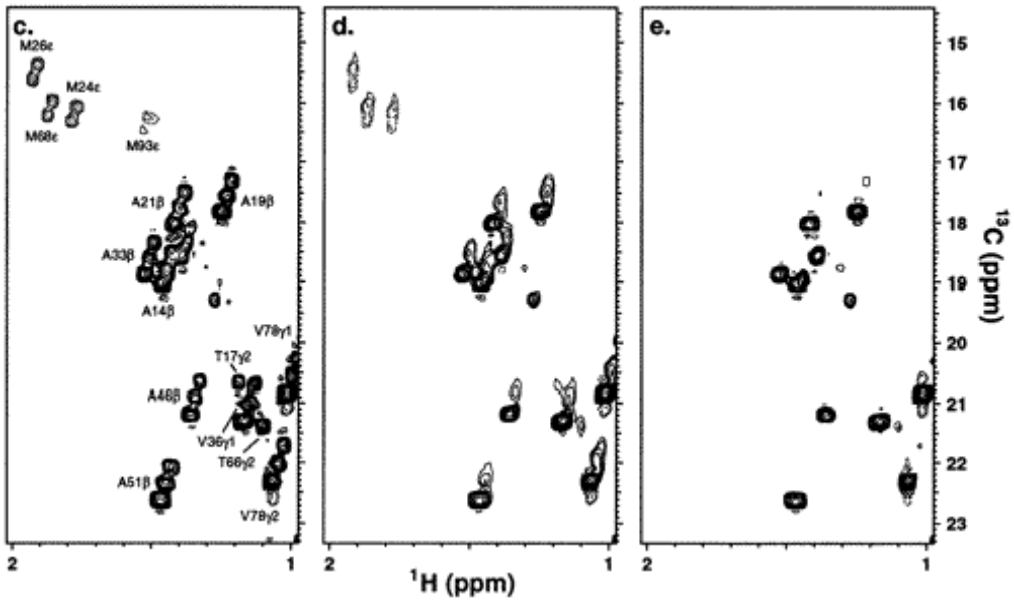


Figure 2 Comparison of 2D ($^{13}C\alpha$, NH) projections from 3D HNCA spectra recorded of a 23 kDa Shc PTB domain/phosphotyrosine peptide complex. (*a*) ^{15}N, ^{13}C, ^{1}H uniformly labeled Shc PTB domain. (*b*) ^{15}N, ^{13}C, 75% ^{2}H uniformly labeled Shc PTB domain. (136, by permission.)

Isotope effects for methyl carbons in the case of deuteration

^{13}C ct-HSQC
with ^{2}H-decoupling

suppression of
CH_2D and CHD_2 isotopomers



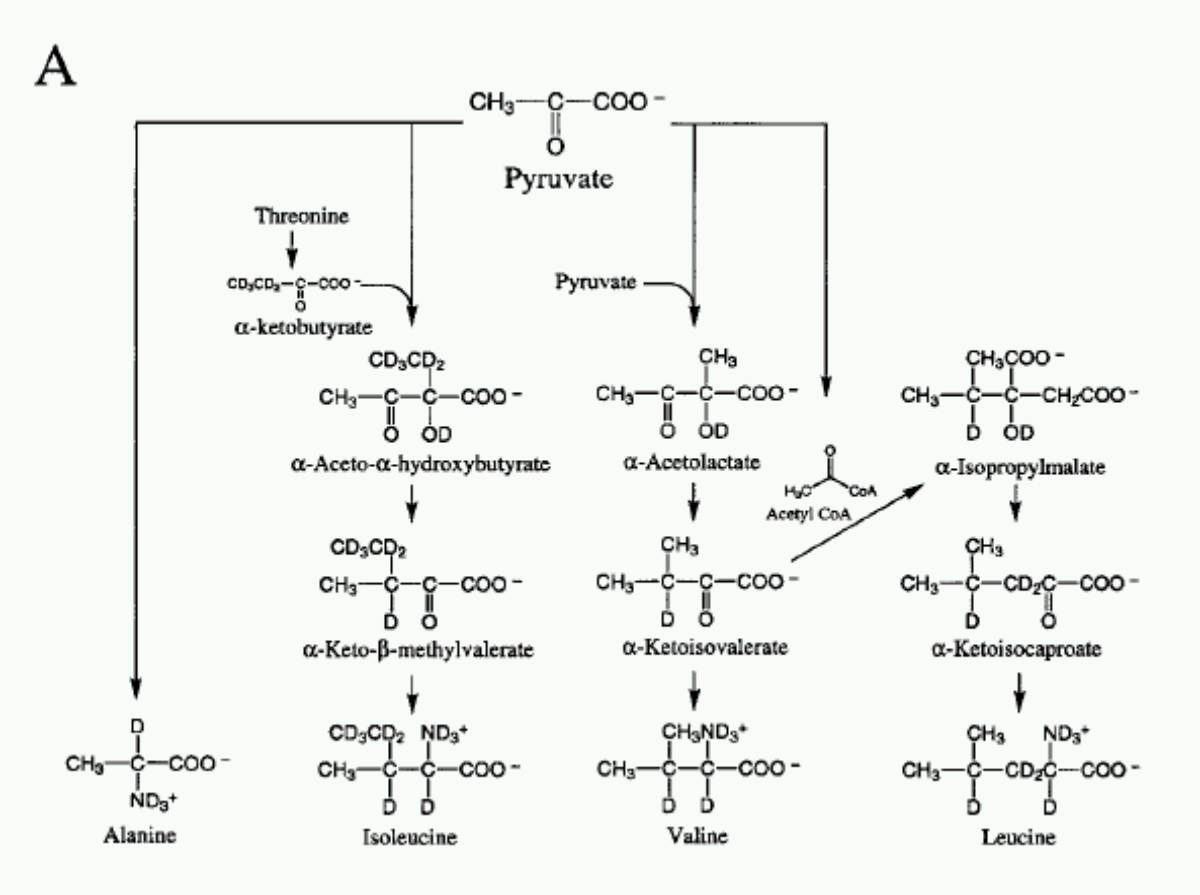
^{13}C ct-HSQC
without ^{2}H decoupling

selective protonation

Instead of a partial, random deuteration it is possible to deuterate proteins with a high degree and introduce protons selectively !

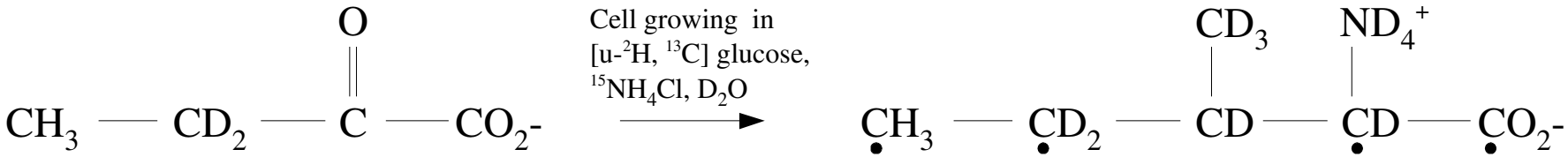
Important amino acids for structure characterization:	aromatic sidechains
	methyl groups
	main components of hydrophobic cores
Introduction of protonated amino acids:	adding protonated (^{13}C,^{15}N) labeled amino acids (F, Y, W, H) to a deuterated medium for cell growing
Introduction of protonated methyl groups	addding of defined labeled precursors of the corresponding amino acids

Pyruvate as methly precursor



Addition of [u-^{13}C] labeled pyruvate to the M9 minimal medium (D_2O (99%), [u-^{13}C] glucose, $^{15}NH_4Cl$)

α-keto-acids as precursor

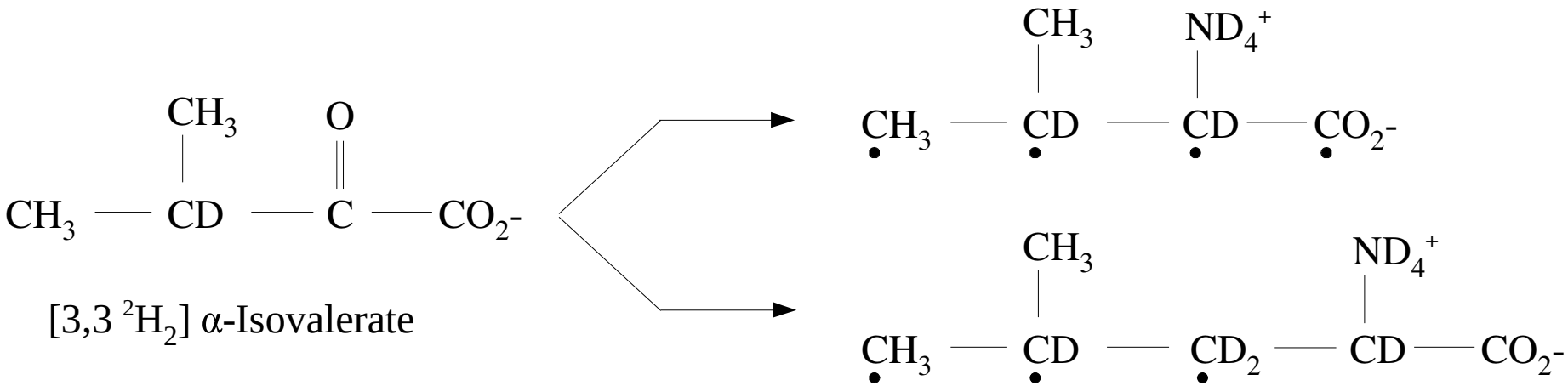


[3,3 $^{2}H_2$] α-Ketobutyrate

• C-atom from precursor

250 mg [u-^{13}C, 3,3 $^{2}H_2$] ca. 1000 Euro

500 mg [u-^{13}C] ca. 1600 Euro, 3,3 deuteration: 2-3h, 45°C, pH 12.5 in D_2O)



[3,3 $^{2}H_2$] α-Isovalerate

250 mg ca. 825 Euro

approximate costs (per litre growing medium)

4 g	[u-^{2}H, u-^{13}C] glucose	4 * 605 Euro
1 g	$^{15}NH_4Cl$	40 Euro
50 mg	[u-^{13}C, 3,3 $^{2}H_2$] α-Ketobutyrat	200 Euro
100 mg	[u-^{13}C, 3-^{2}H] α-Ketoisovalerat	330 Euro
1 l	D_2O (99%)	300 Euro
	total	3290 Euro

c.f. standard ^{13}C, ^{15}N M9 (glucose, ammoniumchloride) ca. 560 Euro / l medium

Strategies for assignment of deuterated proteins

1. protein backbonet — out&back-HNX-tripleresonance experiments

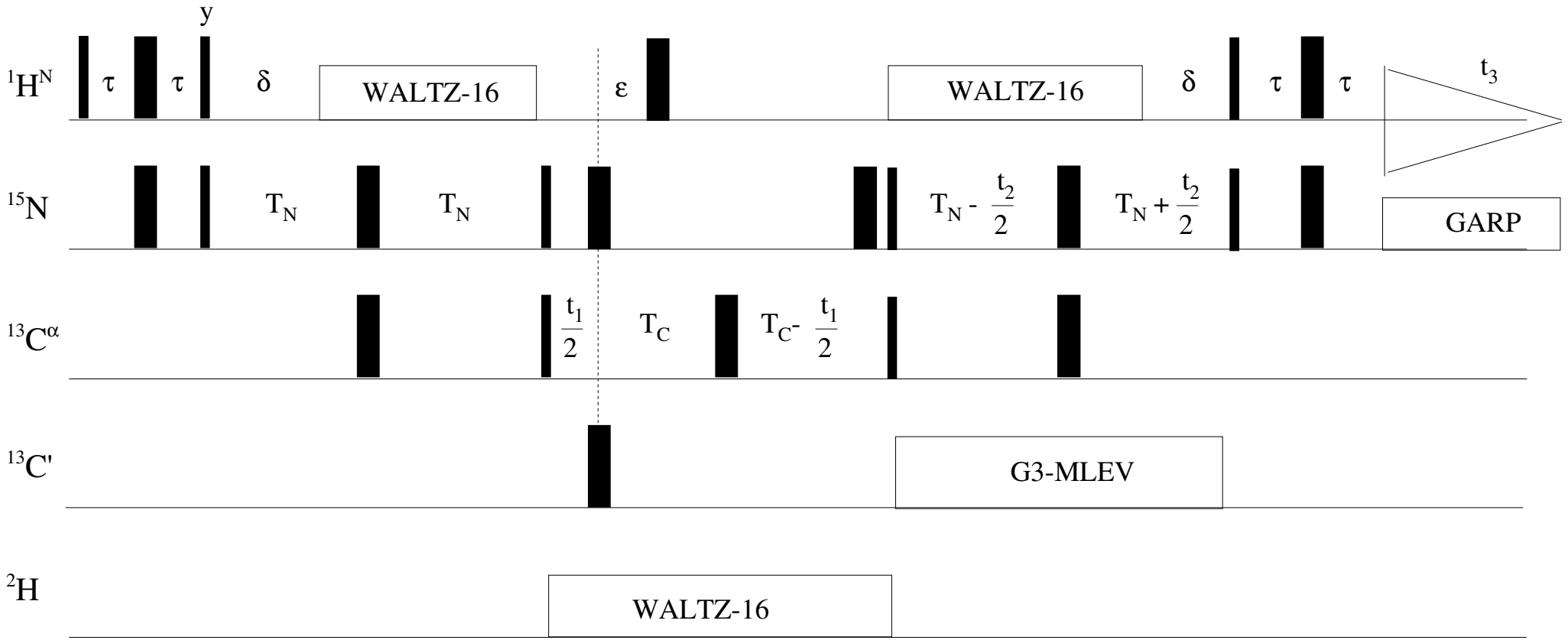
HN(CA)CO	/	HNCO
HNCA	/	HN(CO)CA
HNCACB	/	HN(CO)CACB
		(CBCA(CO)NH not possible !)

slow $^{13}C^{\alpha}$ -^{2}H relaxation allows sampling of $^{13}C^{\alpha}$-evolution in a constant-time manner -> high resolution

For helical proteins: $^{1}H^{N}$-$^{1}H^{N}$ NOEs

general: high degree of deuteration preferred
$^{13}C^{\alpha}$-$^{1}H^{\alpha}$ isotopomers need to be suppressed (for high resolution)

Basic pulse sequence for a ^{13}C constant time HNCA with ^{2}H decoupling



τ = 2.5 ms δ = 5.5 ms T_N = 12 ms T_C = 13 ms ε = 1.7 ms

Assignment of sidechain resonances

For partial random deuterated proteins the classical side chain experiments can be performed

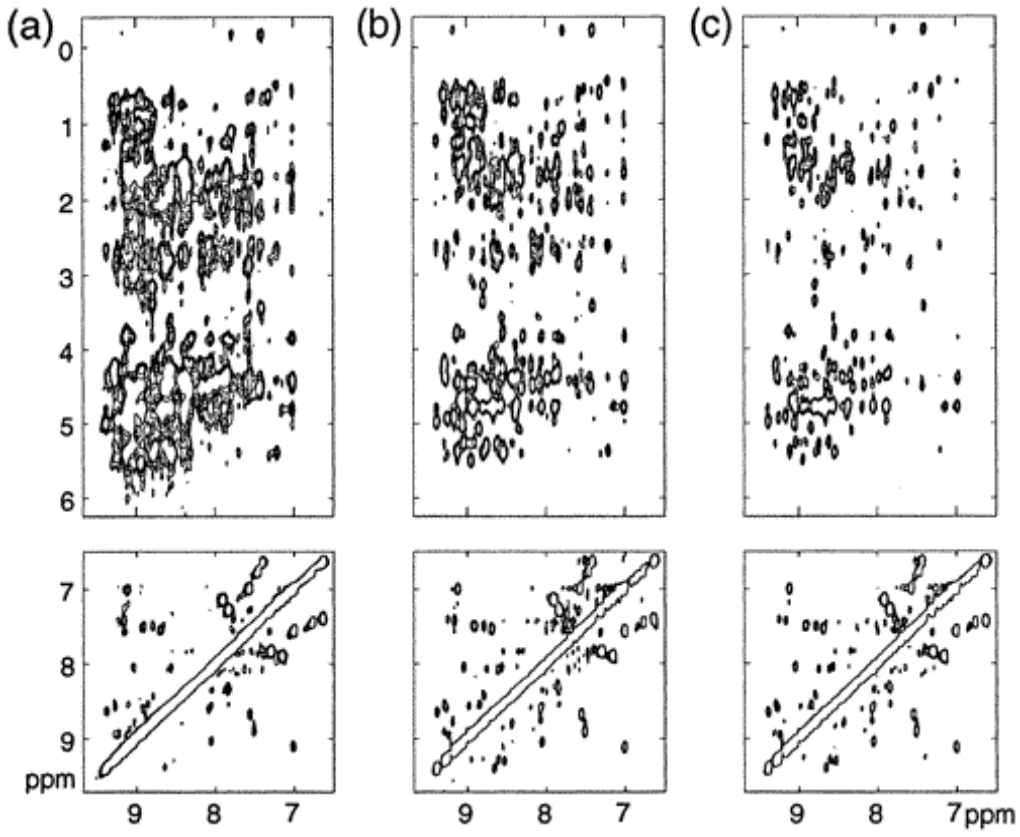
HBHA(CO)NH
C(CO)NH
H(CCO)NH
HCCH-COSY/TOCSY

the reduced transverse relaxation often compensates the lower protons concentration

In the case of selectively labeled mehyl groups experiments for correlating these methyl groups with the amide resonances are developed (e.g. C(CO)NH and H(CCO)NH).

NOESY experiments with deuterated proteins

1. partial (random) deuterated proteins

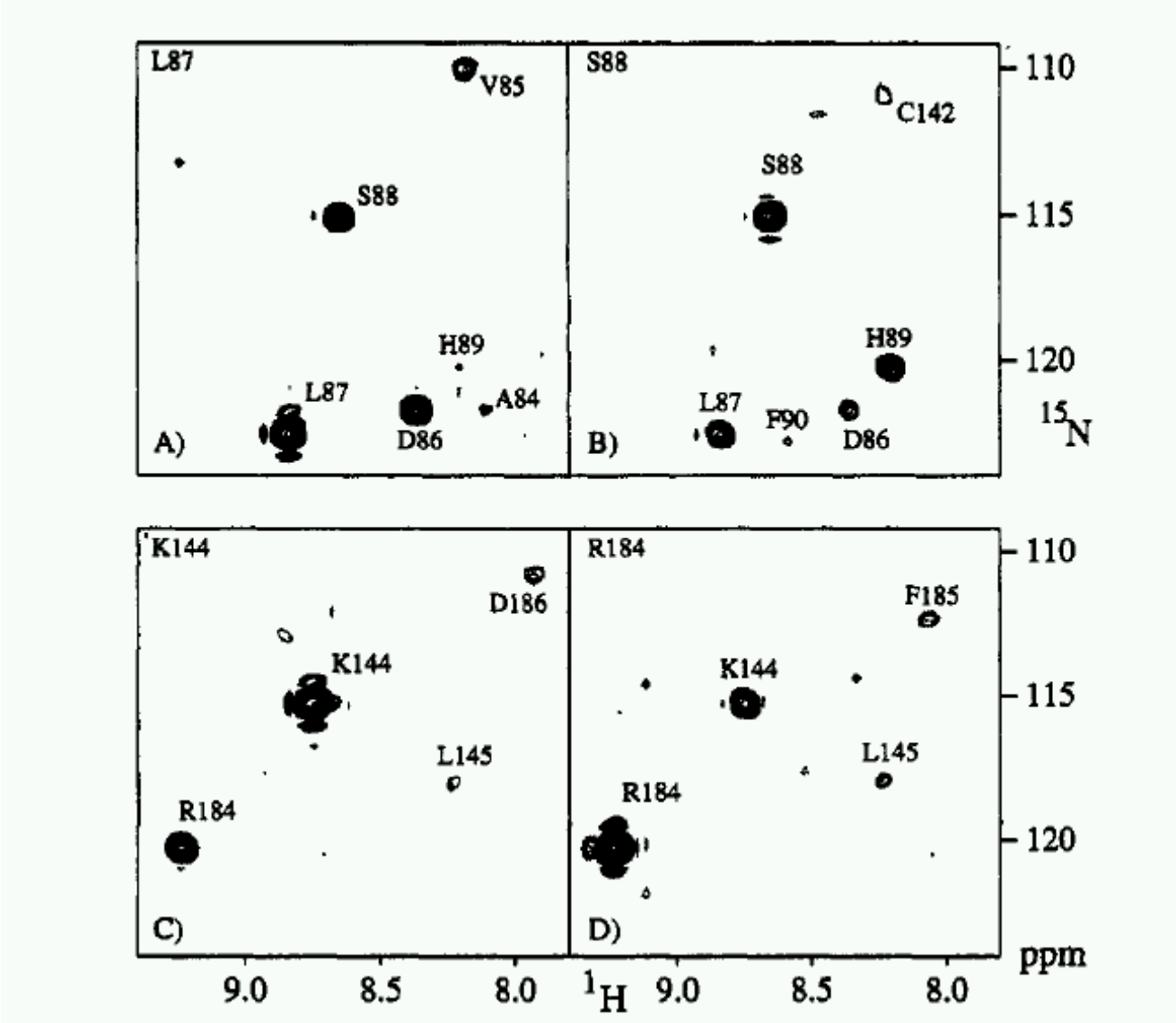


0 % ^{2}H 50 % ^{2}H 75 % ^{2}H

4D H^N,H^N NOESY experiments with a perdeuterated protein

Nef (39-206), 0.6 mM
perdeuterated
200 ms mixing time

long mixing time possible,
because low proton density
reduces spin diffusion



3D ct-methyl- CCH-NOESY

Maltose-binding-protein (370 aa)
selective methyl protonation
100 ms mixing time