J-PARC Workshop 2022
Deuterium Science Entering a New Phase

Methods of uniform or site-specific deuteration of proteins and their applications to NMR analyses

蛋白質の均一重水素化あるいは部位特異的重水素化の方法とそのNMRへの応用

2023 Jan 20th（Fri）10:40-11:10

Ibaraki Quantum Beam Research Center, Tokai

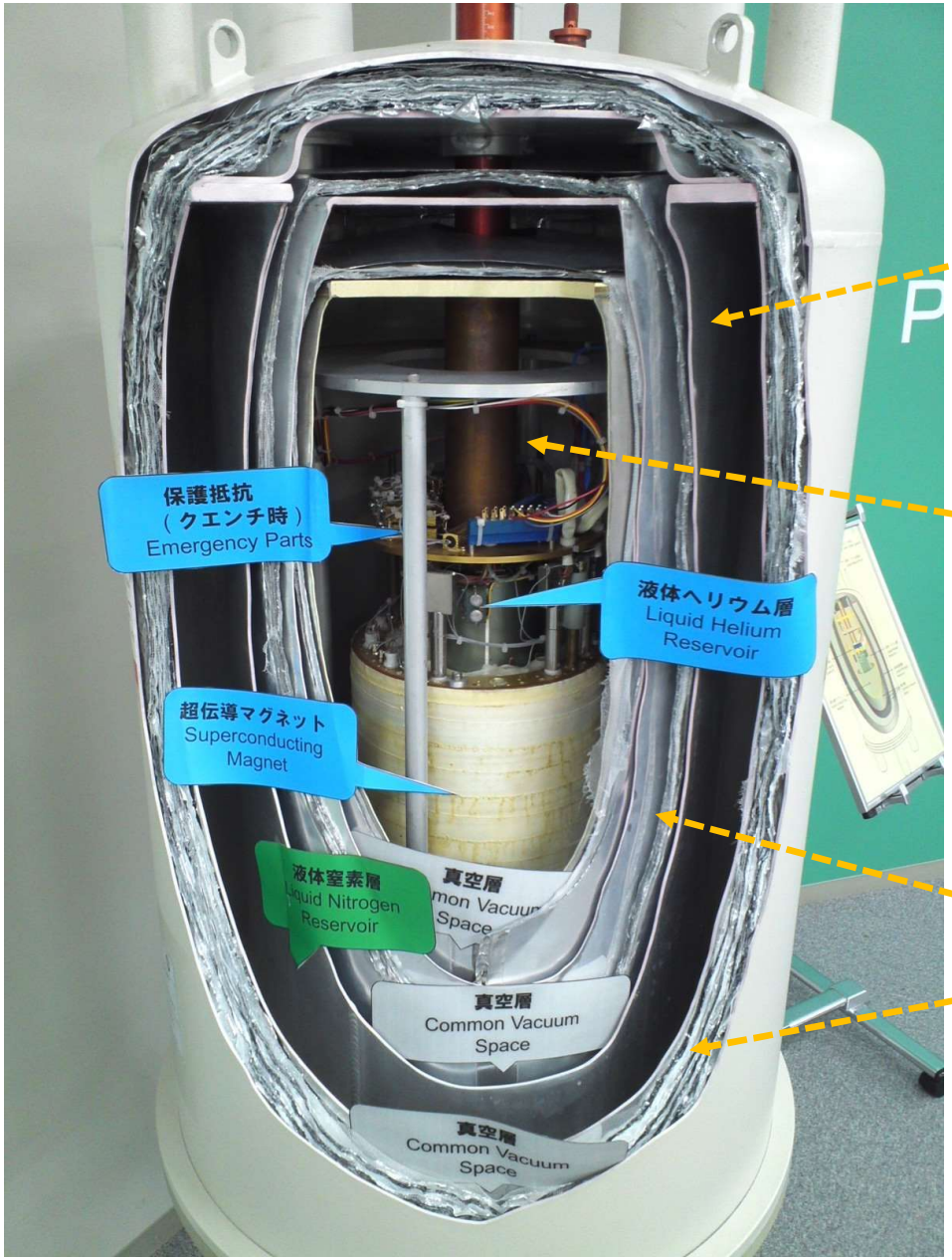
Takahisa IKEGAMI　池上貴久
Yokohama city University　横浜市立大学

NMR (nuclear magnetic resonance) 核磁気共鳴

900 MHz NMR magnet

950 MHz NMR magnet

superconducting magnet 超電導磁石



liquid N_2
-196℃

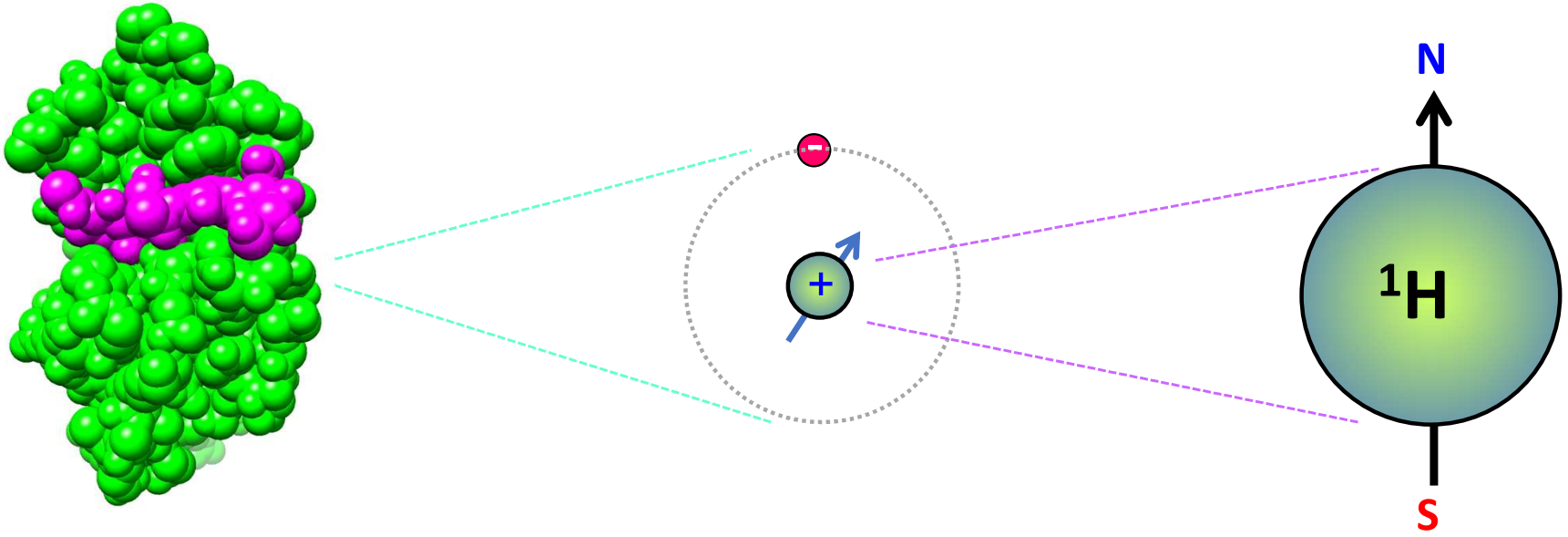
liquid He
-269℃

vacuum

a picture in RIKEN-Yokohama

1）How can we detect NMR signals?

NMR 信号の検出原理



protein molecule

^{1}H
^{12}C → ^{13}C
^{14}N → ^{15}N
^{19}F
^{31}P

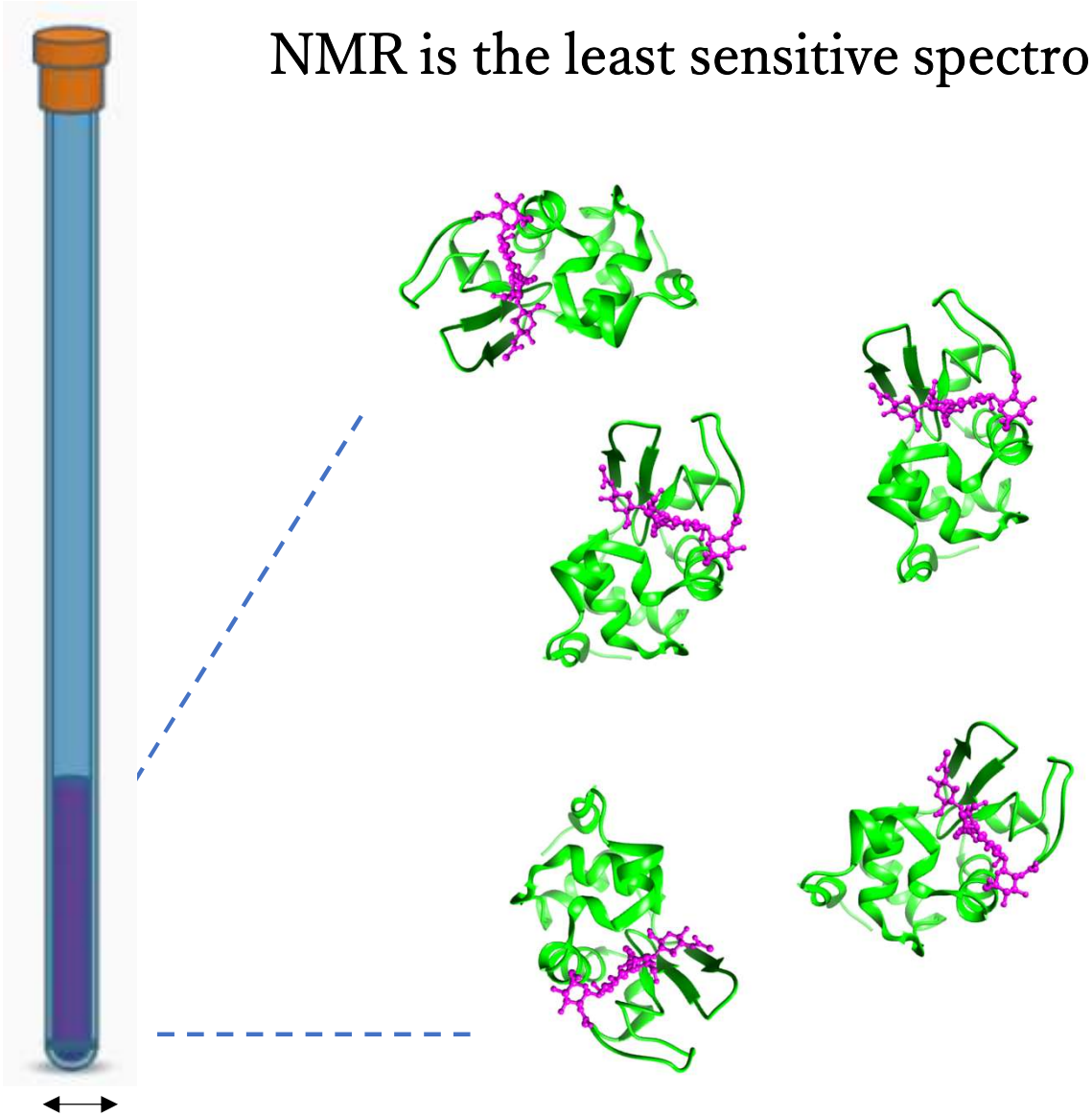
^{1}H nucleus
=
proton

Safe nuclei that emit no radiation = stable isotopes

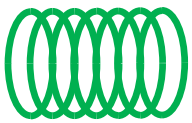
安定同位体

10^{17} protein molecules /mL solution

NMR is the least sensitive spectroscopy.



20 K to minimize the coil resistance

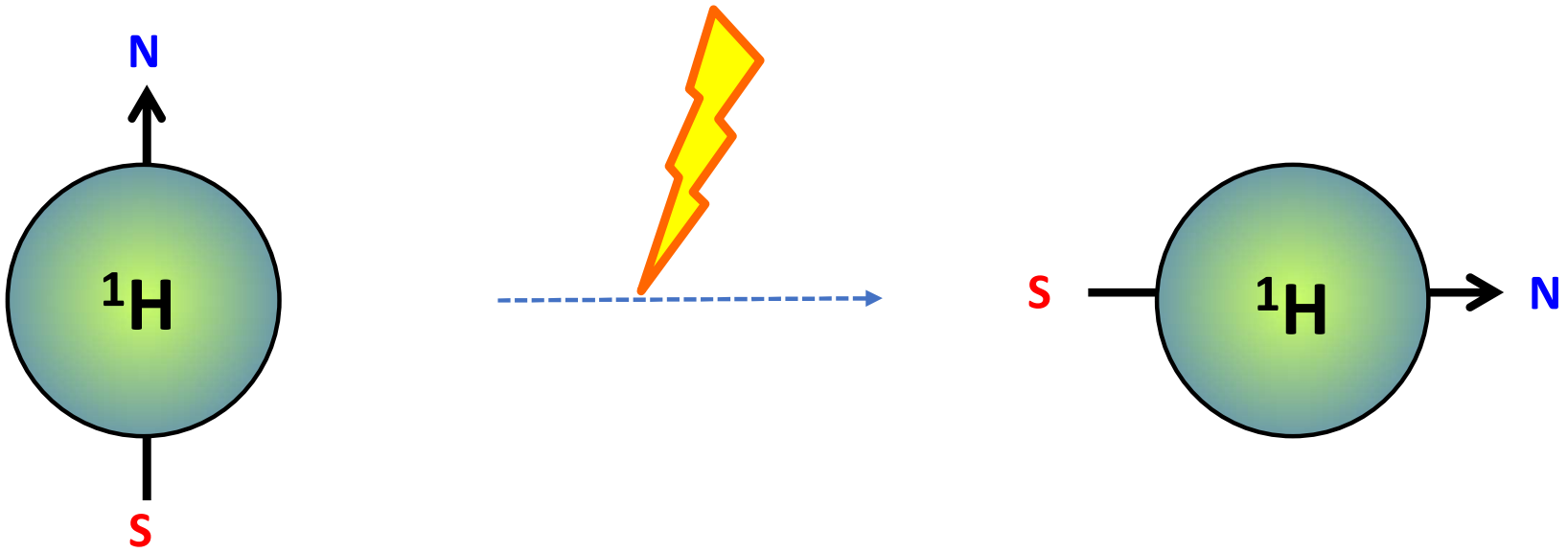


The small coil detects the electromagnetic field induced by the precession of nuclear spins.

核スピンの歳差運動による電磁誘導を検出するためのコイル

an electromagnetic field pulse with the resonance frequency

共鳴周波数をもつ電磁波パルス



in the equilibrium state

熱平衡状態

$$|0\rangle$$

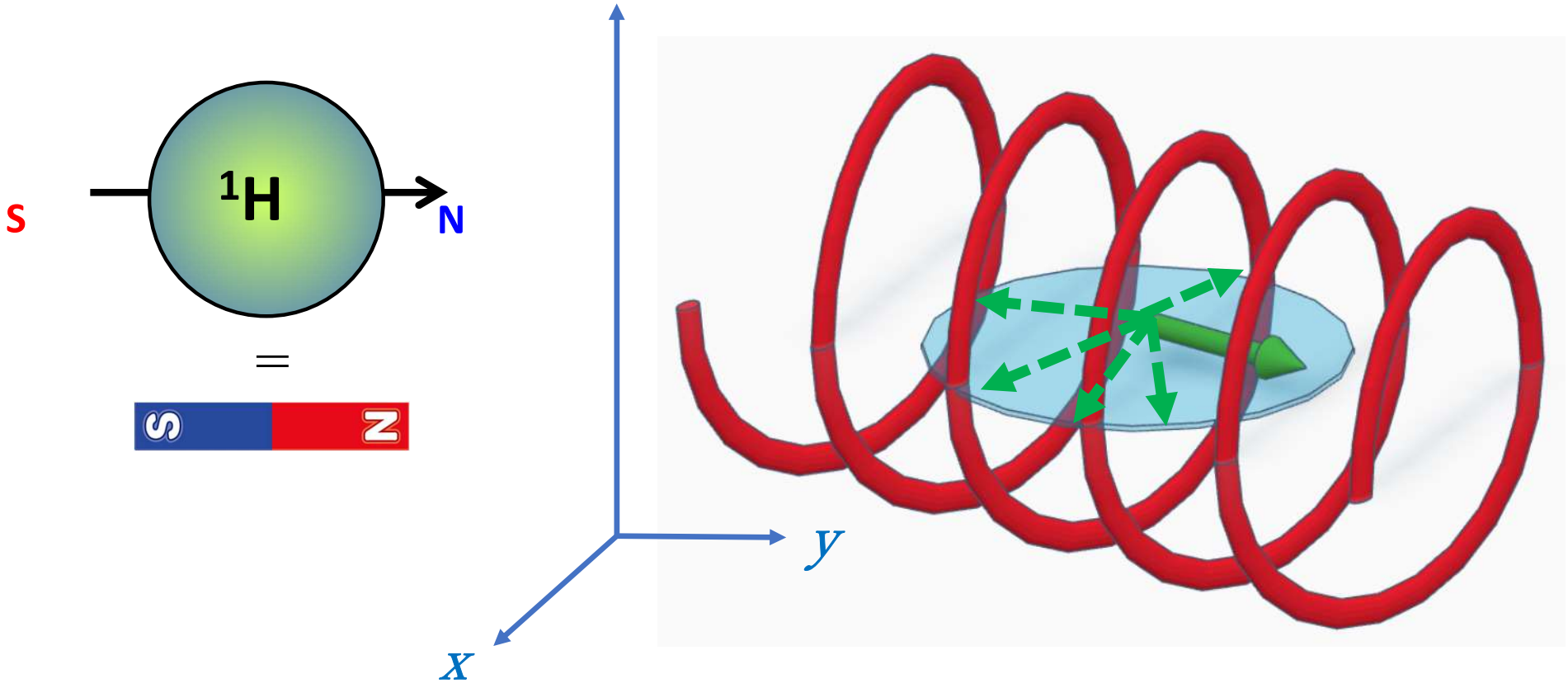
in the superposition state

重ね合わせの状態

$$\frac{1}{\sqrt{2}}(|0\rangle + |1\rangle)$$

Faraday's electromagnetic induction, 1831
ファラデーの電磁誘導

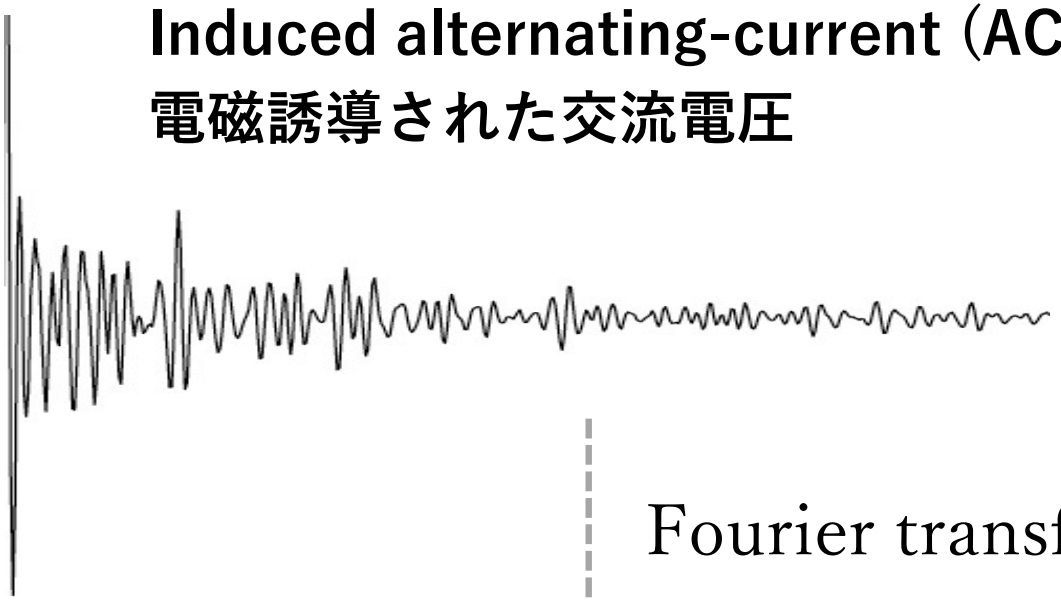
NMR's large static magnetic field (z axis)



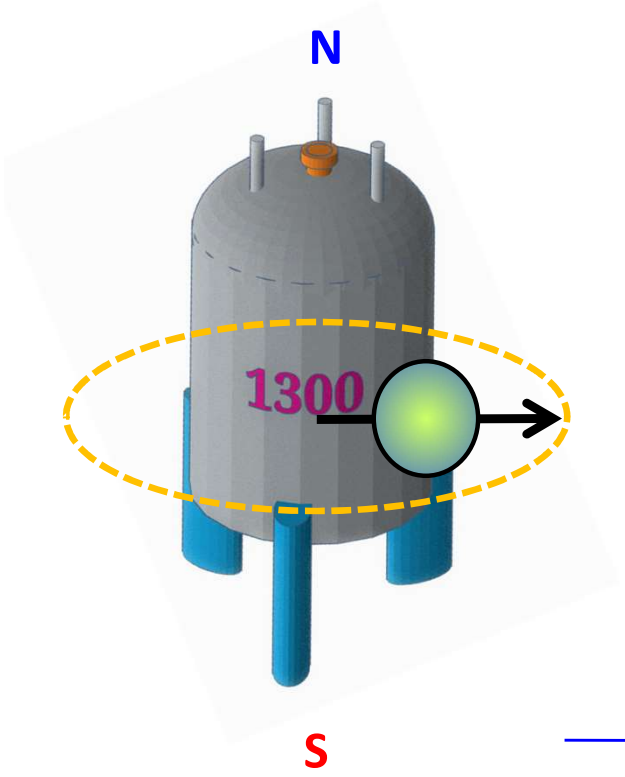
Nuclear spins precess on the x/y horizontal plane about the z axis.

Induced alternating-current (AC) voltage

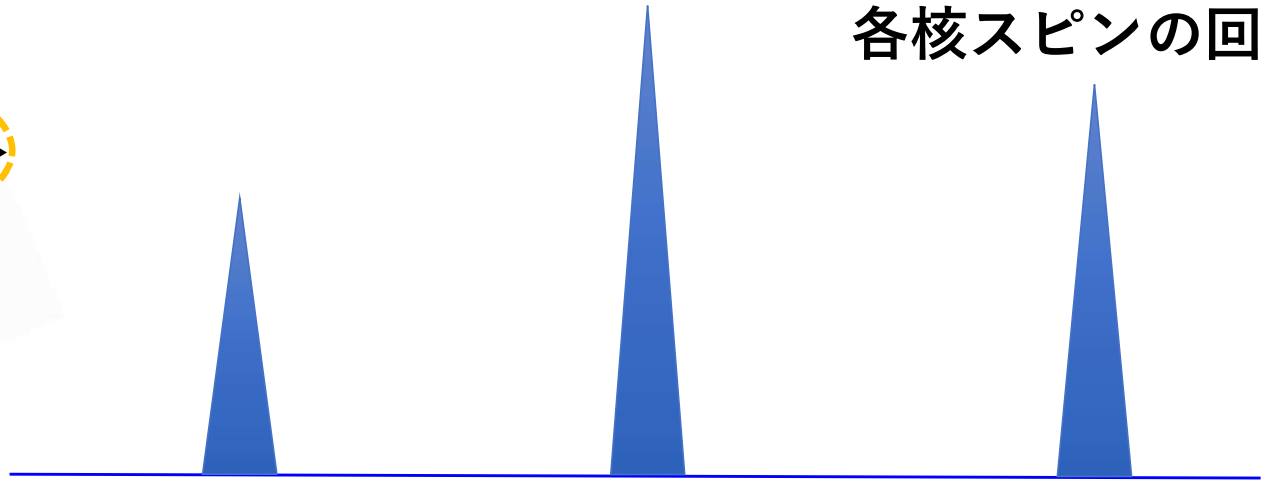
電磁誘導された交流電圧



Fourier transform

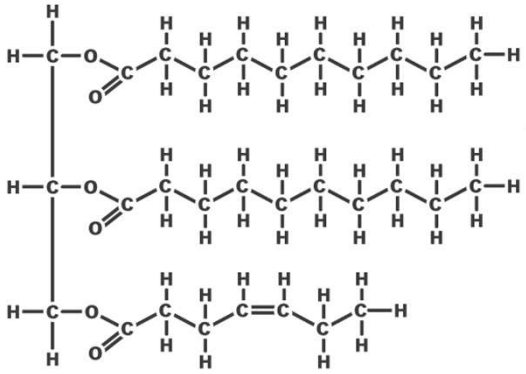


各核スピンの回転速度



The rotational speed of each spin
= chemical shift (*ppm*)

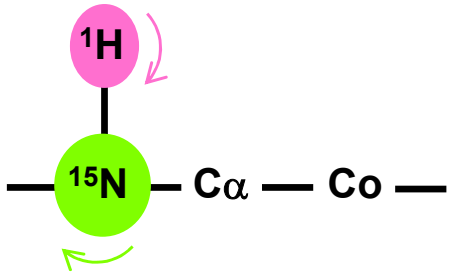
^{1}H-^{13}C NMR spectra

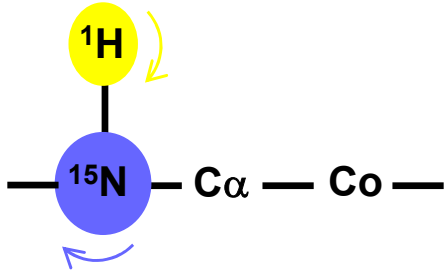


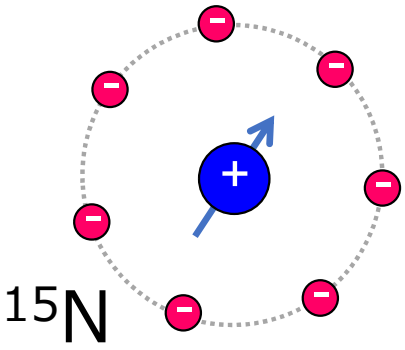
coffee fresh (UCC)

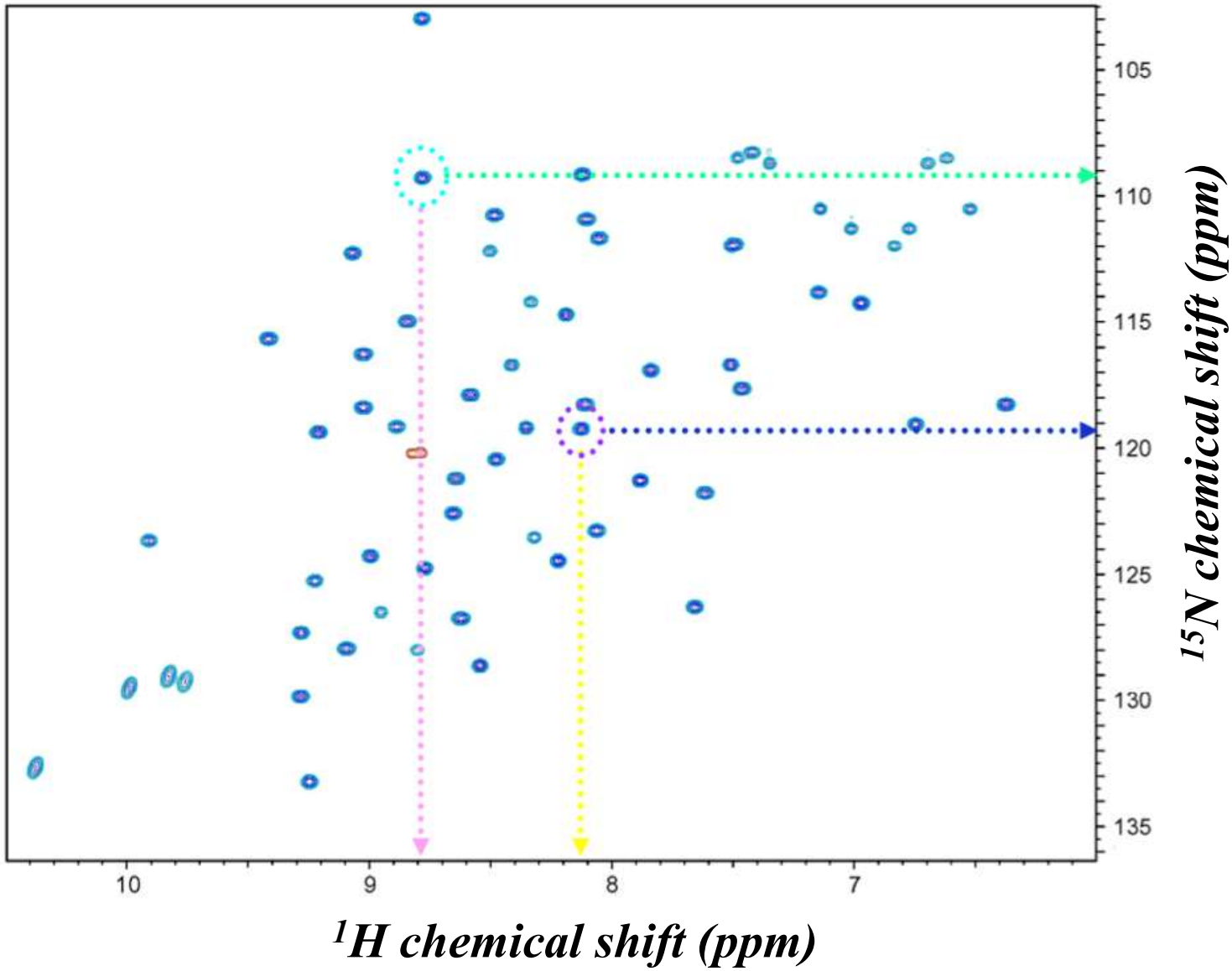
milk

2D ^{1}H-^{15}N correlation HSQC spectrum
二次元 ^{1}H-^{15}N 相関 HSQC スペクトル



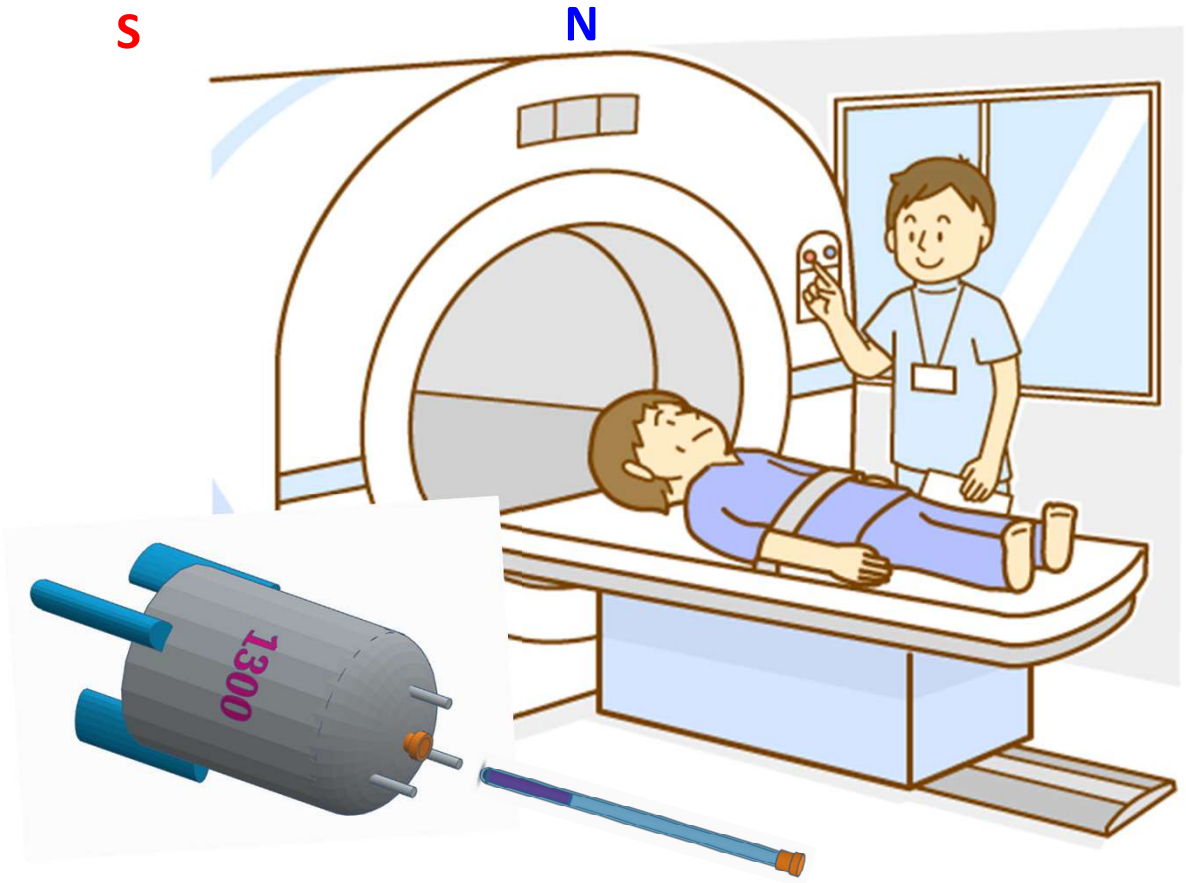




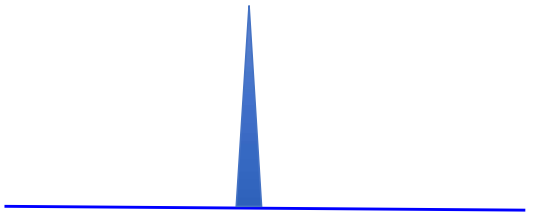


# of peaks ≈ # of amino acids

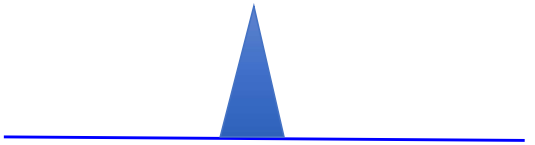
MRI detects ^1H spins of H_2O in each part of your body.

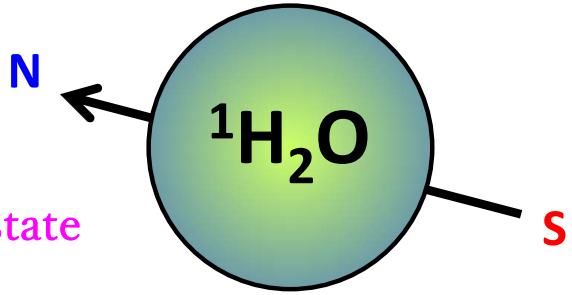


blood＝low viscosity



cells＝high viscosity





in the equilibrium state

熱平衡状態

2）What can we get from protein deuteration?

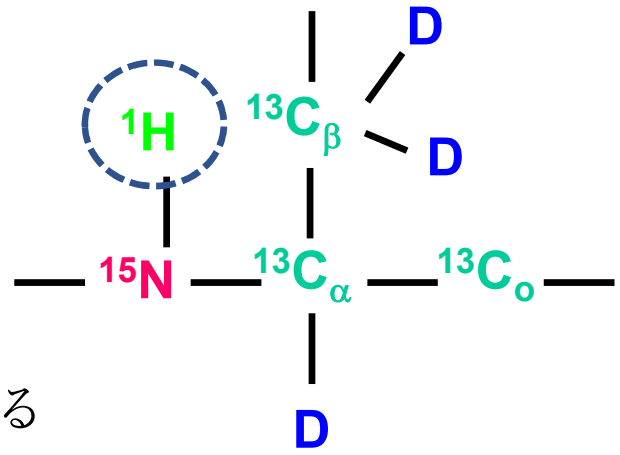
蛋白質の重水素化で何が得られるのか？

NMR-detectable isotopes in proteins

$^{2}H^{N}$ is exchanged with water ^{1}H during purification.

↓

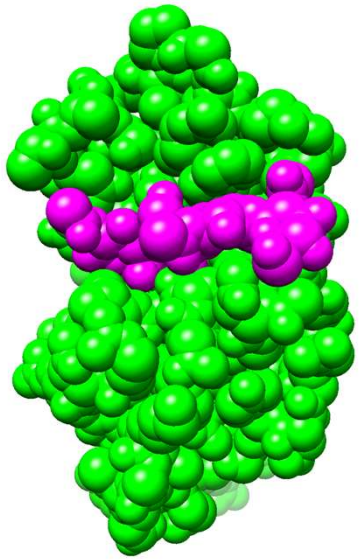
2D ^{1}H-^{15}N spectra

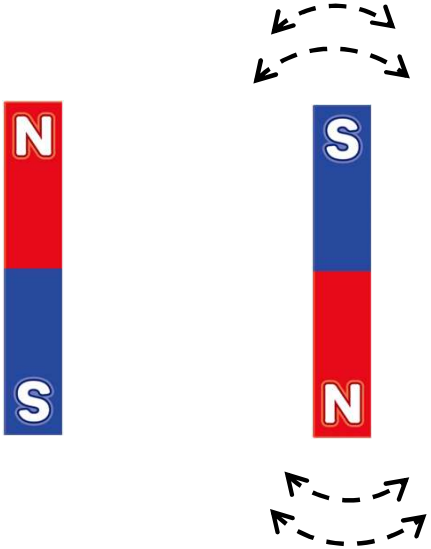


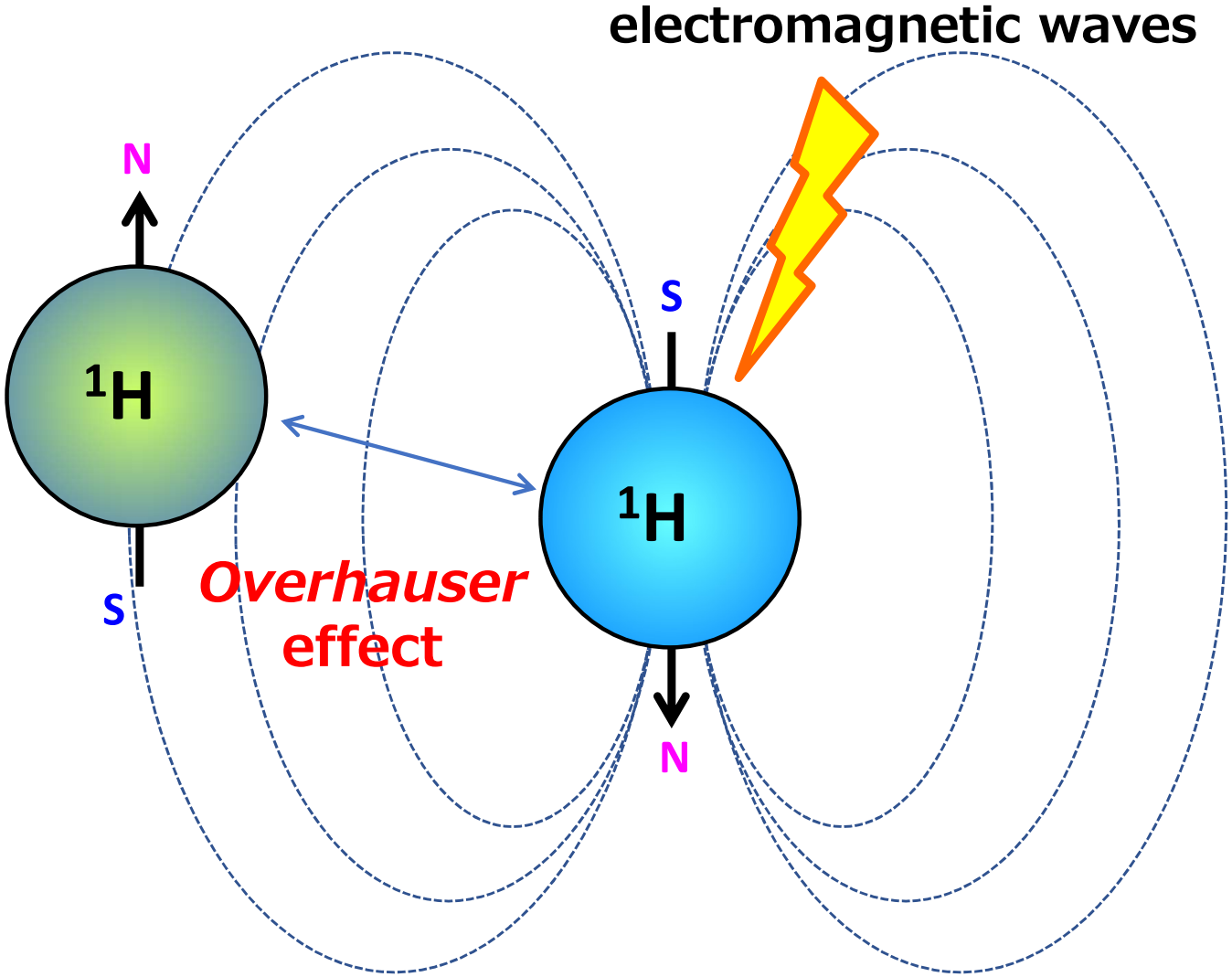
アミド基 $^{2}H^{N}$ は精製中に溶媒水 ^{1}H に交換される

	N. A. (%)	
H-1	99.98	highest sensitivity and strong *dd* interactions
H-2	0.015	lower sensitivity and weak *dd* interactions (compared to ^{1}H)
H-3	0	high sensitivity but radio-active ! not allowed to use
C-13	1.108	[^{13}C]-glucose supplied to bacteria, $^{13}CO_2$ to chlorella
N-15	0.37	$^{15}NH_4Cl$ supplied to bacteria
F-19	100	often introduced to artificial medicine (synthesized drugs)
P-31	100	DNA, RNA, ATP, NAD ..., phospholipid

Magnetic dipole-dipole interactions tell us the distances between ^{1}H spins → structures, interactions

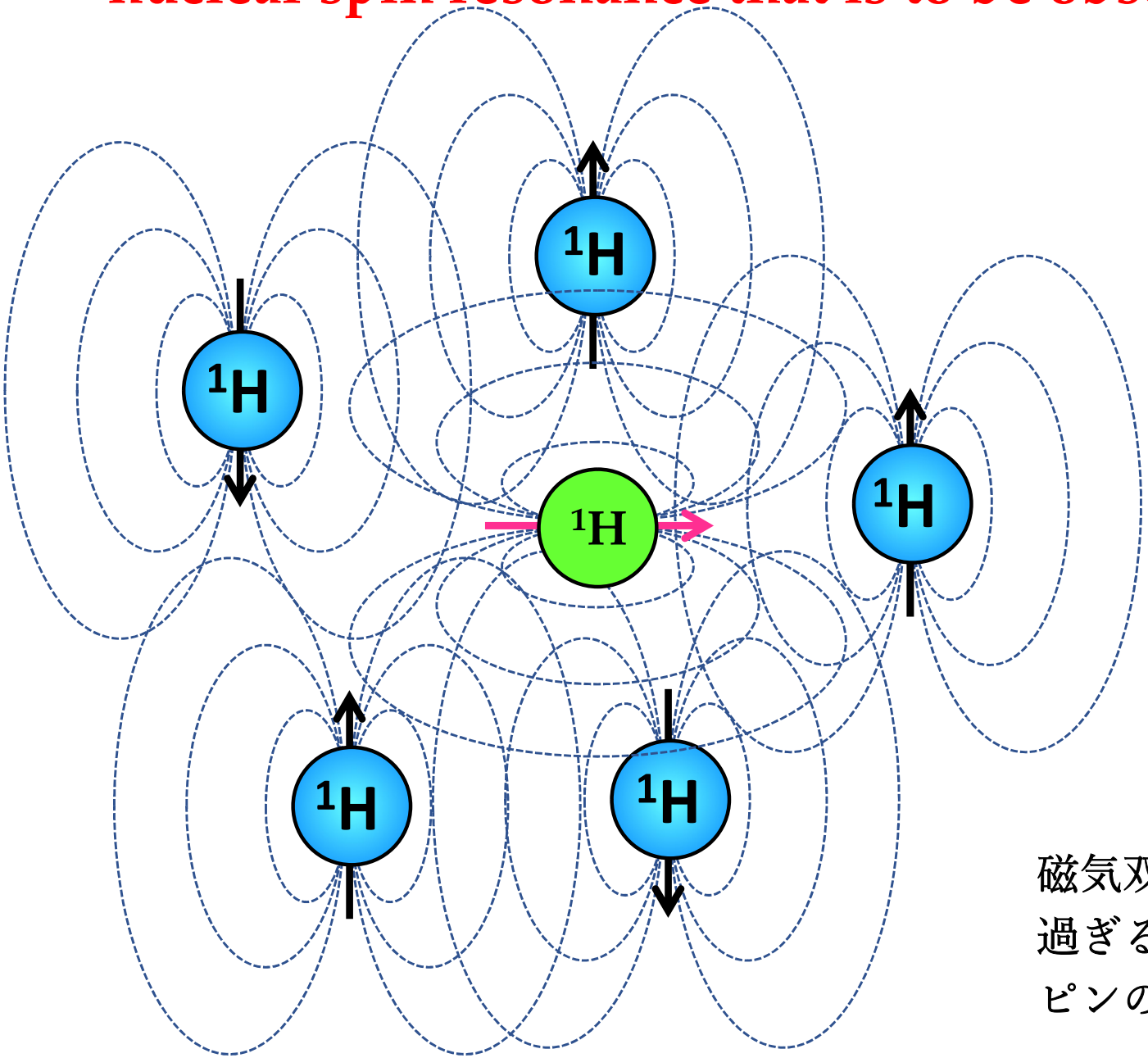






磁気双極子間相互作用により ^{1}H スピン間の距離が分かる → 構造、分子間相互作用

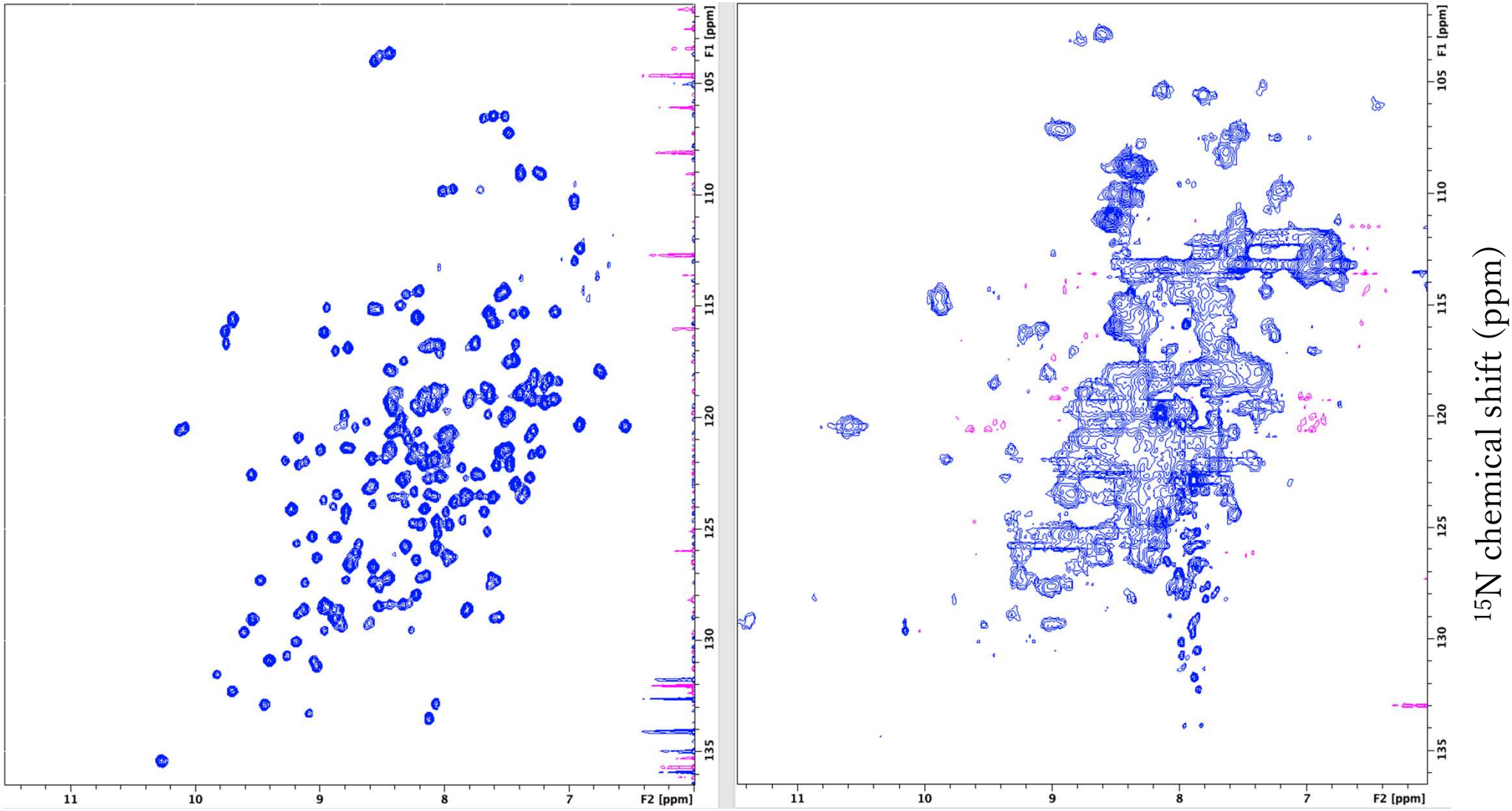
Too many magnetic *dd* interactions disturb the nuclear spin resonance that is to be observed.



磁気双極子間相互作用が多過ぎると、観測したい核スピンの共鳴値が乱れる

The perturbation by *dd* interactions becomes worse as the molecular weight increases.

高分子量になるほど、双極子間相互作用による攪乱が酷くなる



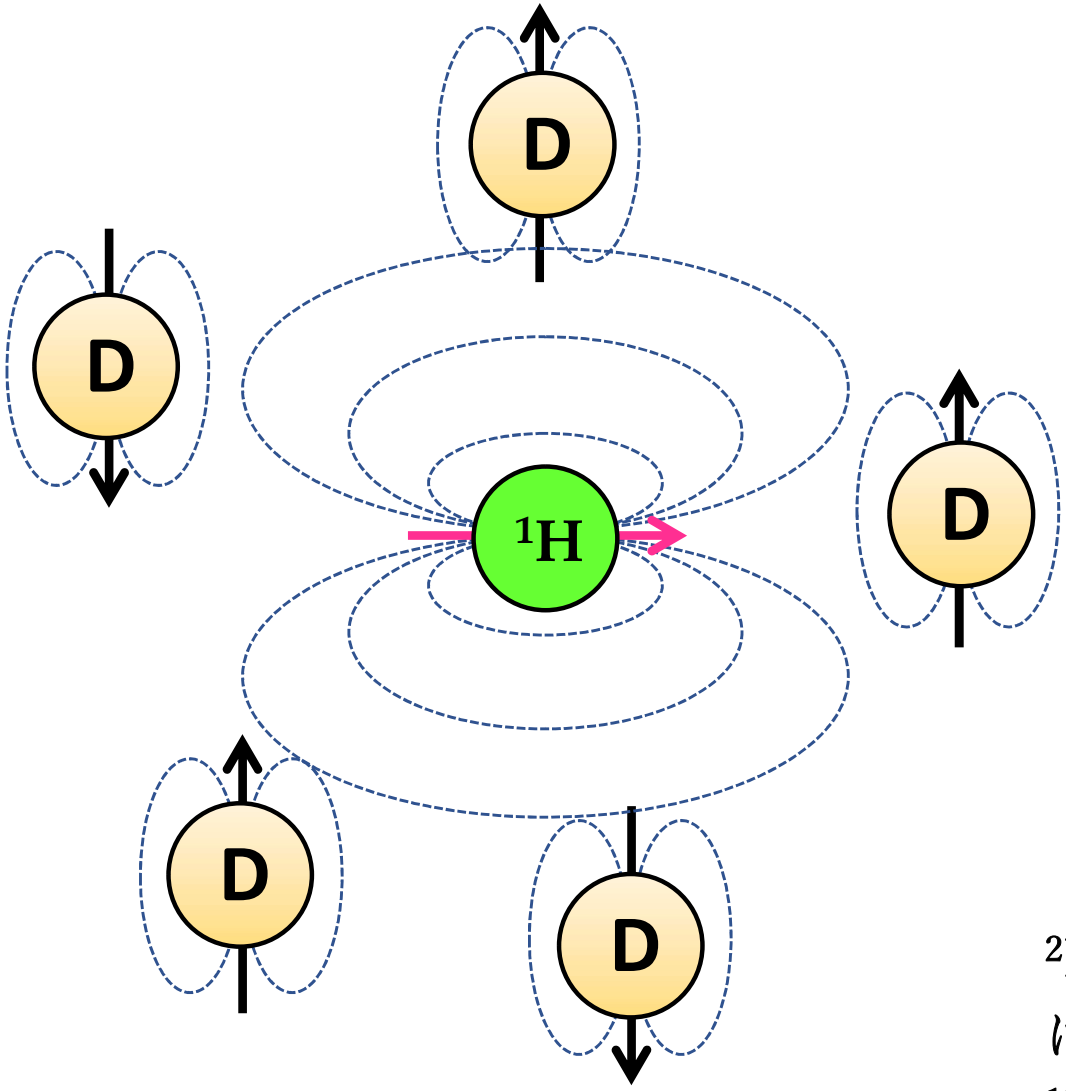
^{15}N chemical shift (ppm)

36 kDa

^{1}H chemical shift (ppm)

36 *4 = 144 kDa

^{2}H spins have smaller *dd* interactions than ^{1}H spins.



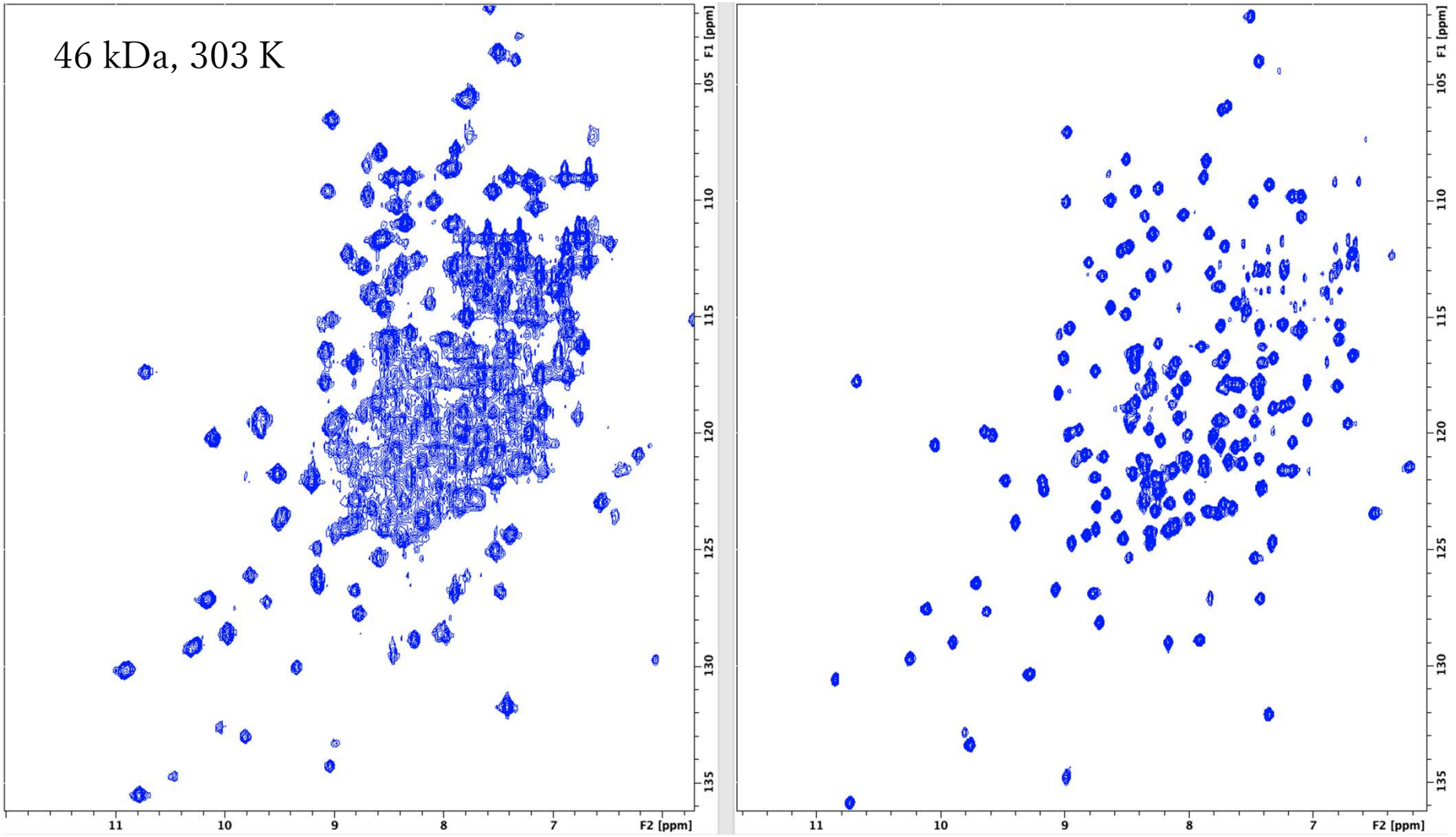
$\gamma_{1H} = 267 \times 10^6$ rad/s/T
$\gamma_{2H} = 41 \times 10^6$ rad/s/T

$\gamma_{1H} = 6.5\ \gamma_{2H}$

^{2}H との磁気双極子間相互作用は小さいので、観測対象の ^{1}H/^{13}C ピークはシャープ

Deuteration can provide sharp peaks even for high molecular-weight proteins.

高分子量であっても重水素化すれば、シャープなピークが得られる。



protonated protein　　^{1}H chemical shift (ppm)　　deuterated protein

3）How can we introduce ^{2}H to proteins?

どのようにして重水素を蛋白質に導入するか？

目的タンパク質をコードする遺伝子をプラスミド DNA に挿入する

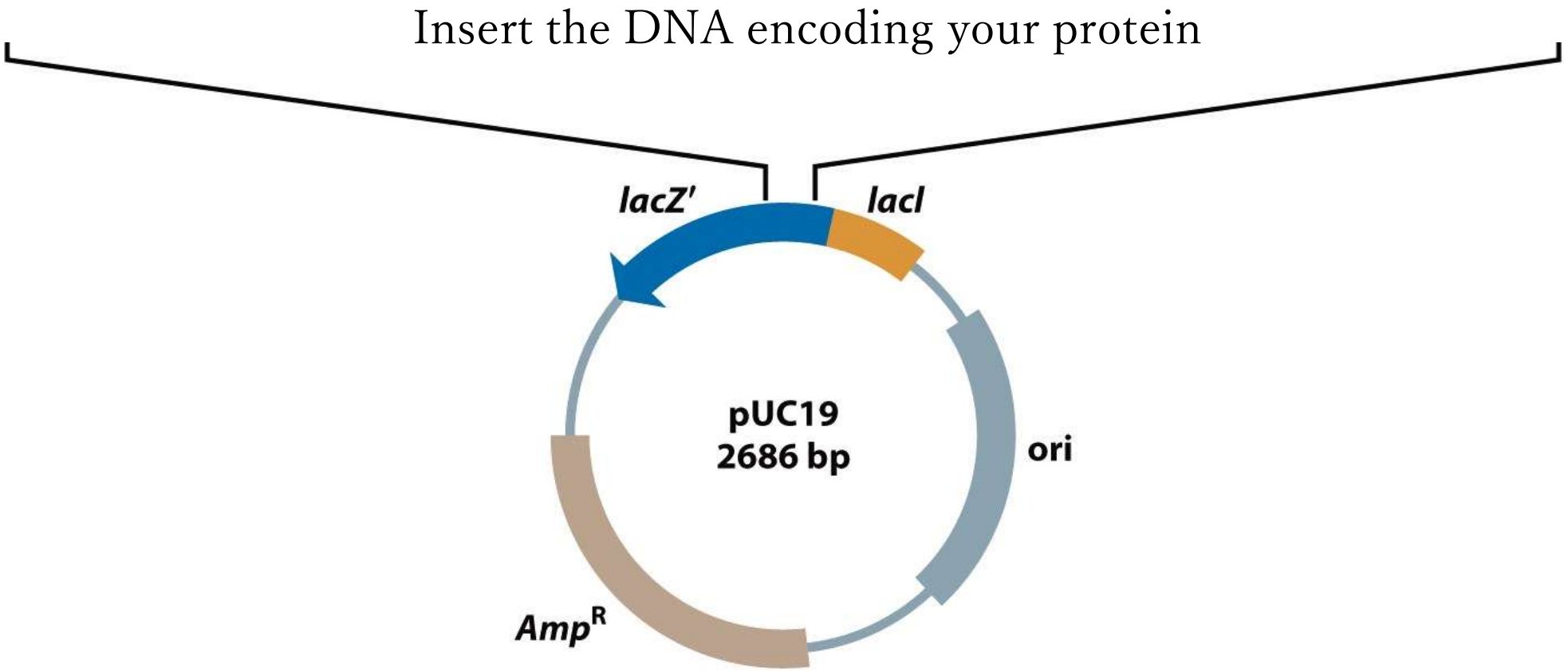
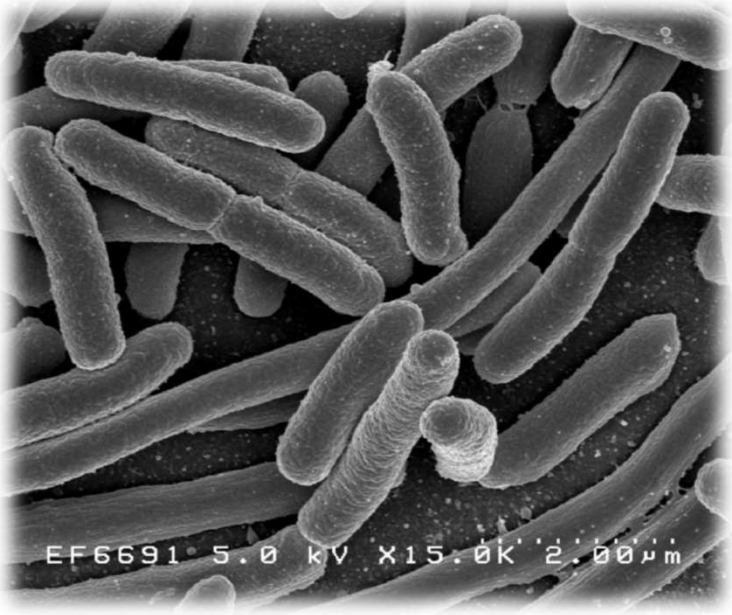


Figure 6.4 Human Molecular Genetics, 4ed. (© Garland Science)

Plasmids replicate to > 100 in bacteria



プラスミドを大腸菌に組み込む

Bacterial cells are transformed with the plasmids.

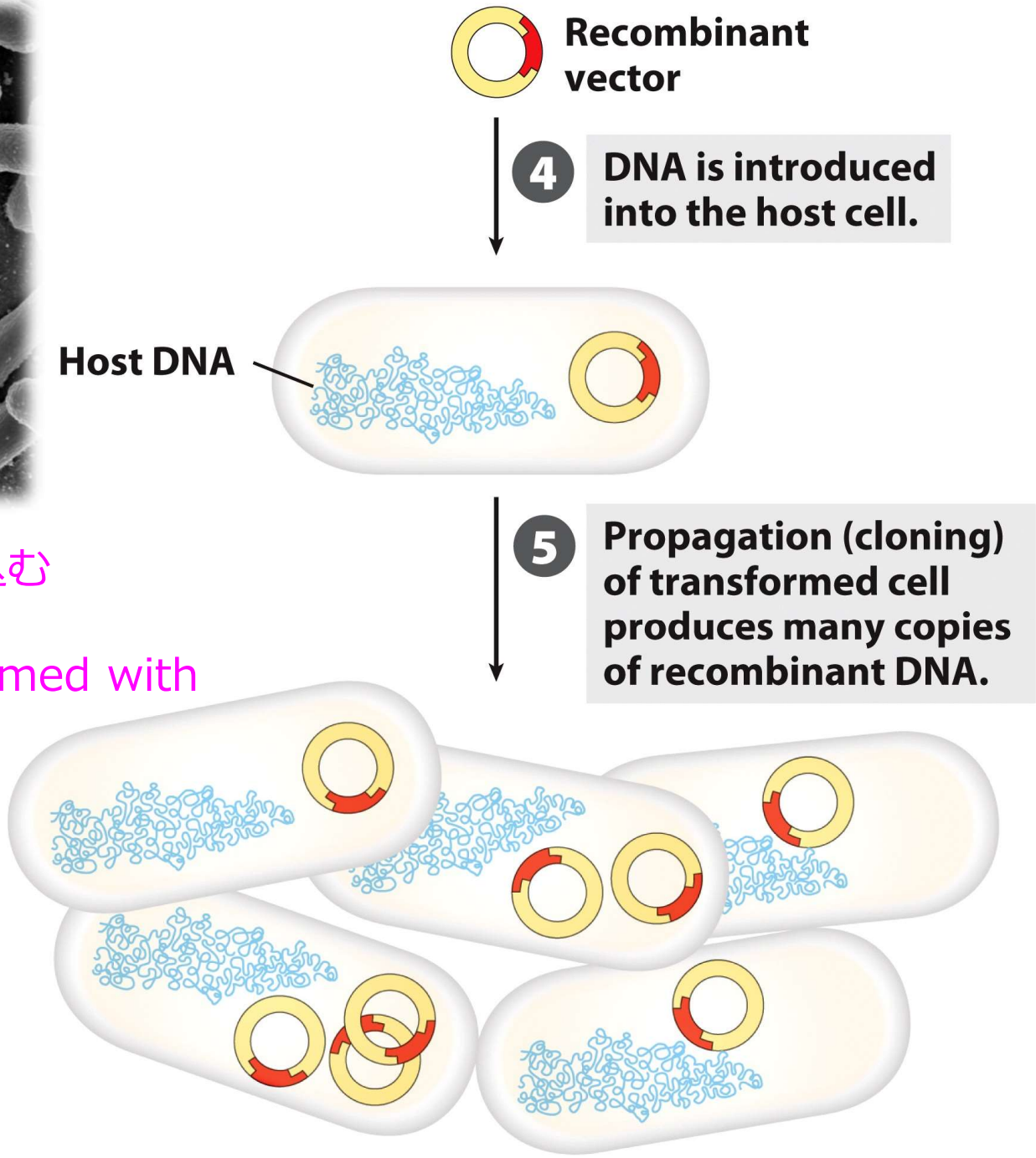
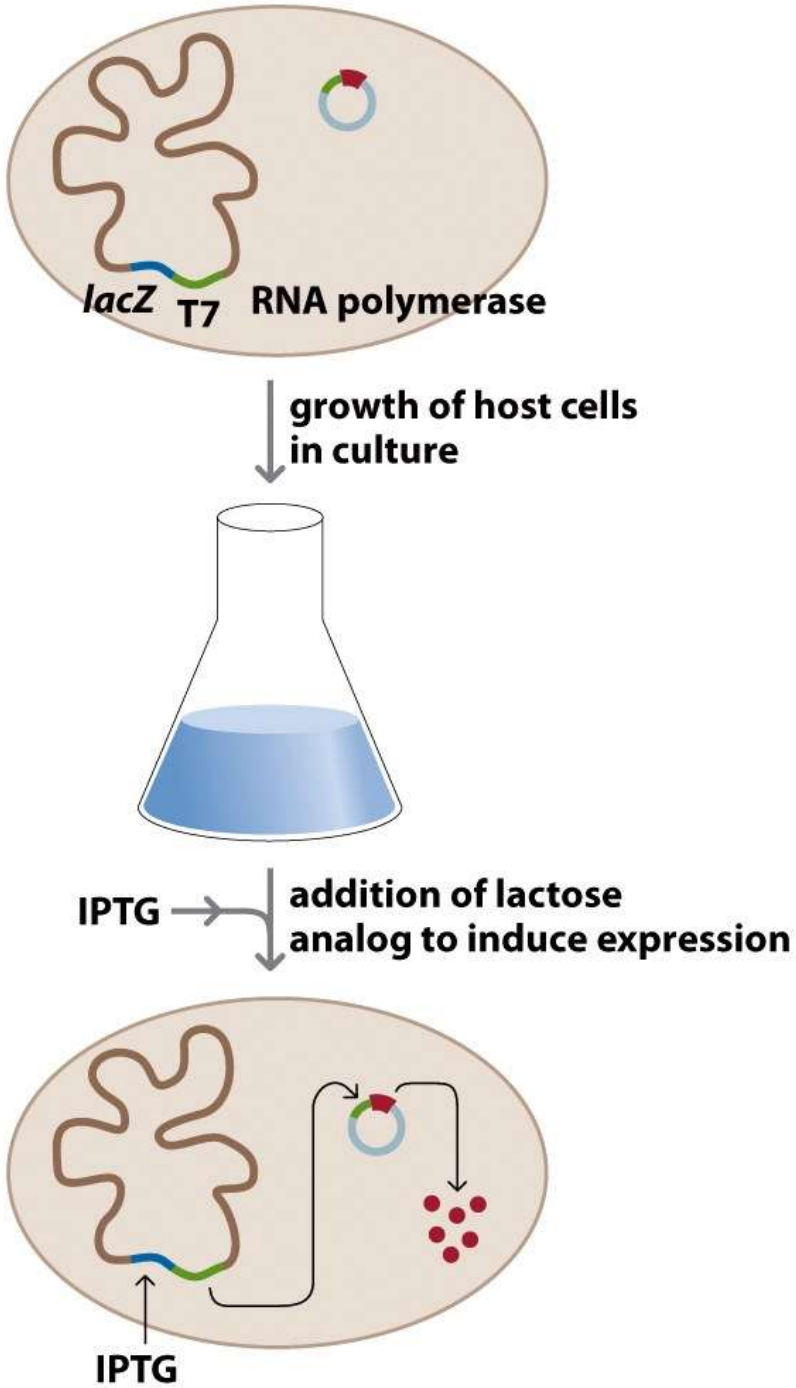


Figure 9-1 part 2
Lehninger Principles of Biochemistry, Sixth Edition

grinded powder of yeast (nutritious)　栄養豊富な酵母の粉末

10^{12} *E. coli* bacterial cells /L

8 mL of the culture medium = the population of the earth



形質転換されたバクテリアは、挿入されたプラスミド DNA を設計図として、大量の目的タンパク質を合成する。

Transformed bacteria synthesize large amounts of the desired proteins by translating the inserted plasmid DNAs.

Figure 6.11 part 2 of 2 Human Molecular Genetics, 4ed. (© Garland Science)

1L M9 minimum medium for ^{2}H, ^{15}N, ^{13}C culture

(1) 10 x salt	**Do not autoclave!**	
Na_2HPO_4	7.0 g	Do not use hydrated ones
KH_2PO_4	3.0 g	pH becomes 7.15 automatically
NaCl	0.5 g	the total concentration becomes 130 mM
(2) vitamin & nucleic-acids	**Do not autoclave!**	
thymidine (T)	20 mg	nucleosides (need not be nucleotides)
adenosine (A)	20 mg	
guanosine (G)	20 mg	
cytidine (C)	20 mg	
thiamine	20 mg	vitamin B_1
biotin	20 mg	vitamin H (difficult to be dissolved in water)
10 mM $FeCl_3$	1.0 mL	
1M $MgSO_4$	2.0 mL	not $MgCl_2$!
50 mM $MnCl_2$	1.0 mL	
(3) stable-isotope	filter	
$^{15}NH_4Cl$	2.0 g	
^{2}H, ^{13}C-glucose	2.0	
(4) 50 mM $CaCl_2$	2.0 mL	
(5) [^{2}H]-glycerol	1/1000 (=1mL)	only for ^{2}H, ^{15}N-single-labelling
(6) ampicillin	50-100 ug/mL	
(7) $ZnCl_2$	20 uM	only for zinc-finger proteins

Dissolve (1)-(7) into 1L D_2O

Very low calories with only salts, minerals and vitamins

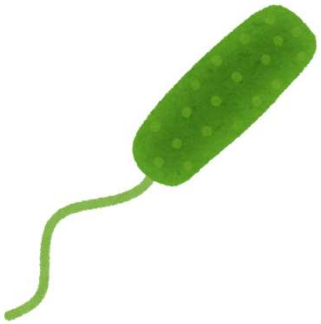
塩、ミネラル、ビタミンのみの超低カロリー食餌

Supplements

(1) folic acid (folate)	(vitamin M)	1mg
(2) choline chloride	(vitamin B)	1mg
(3) nicotine-amide	(vitamin B)	1mg
(4) D-pantothenic acid	(vitamin B)	1mg
(5) pyridoxal	(vitamin B_6)	1mg
(6) riboflavin	(vitamin B_2,G)	0.1mg
(7) inositol		2mg

drinks of only vitamin and mineral

^{2}H, ^{15}N, ^{13}C



beer yeast powder

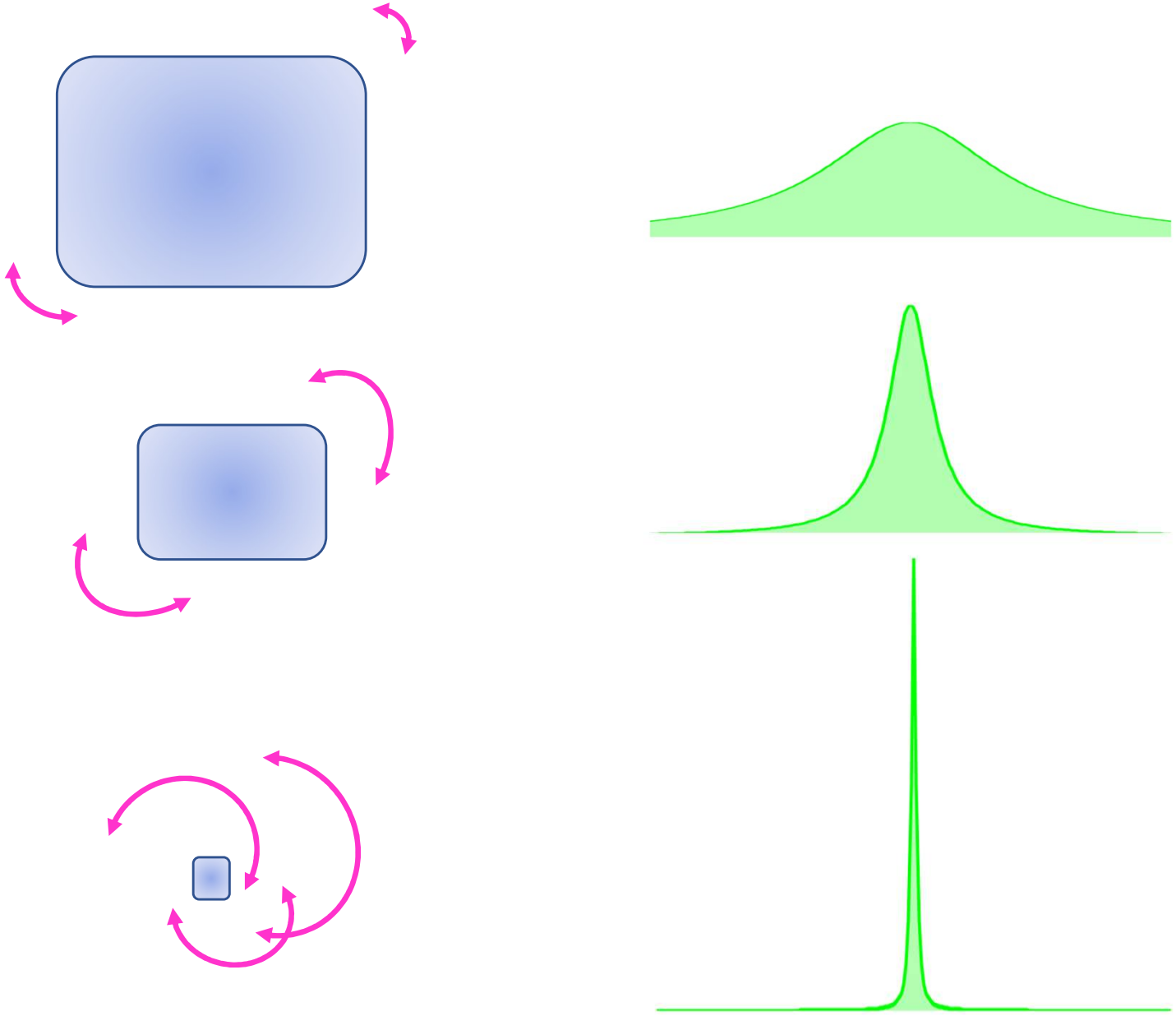
^{1}H, ^{14}N, ^{12}C

4）How can we observe even larger molecules?

さらに大きな生体分子を観測するには？

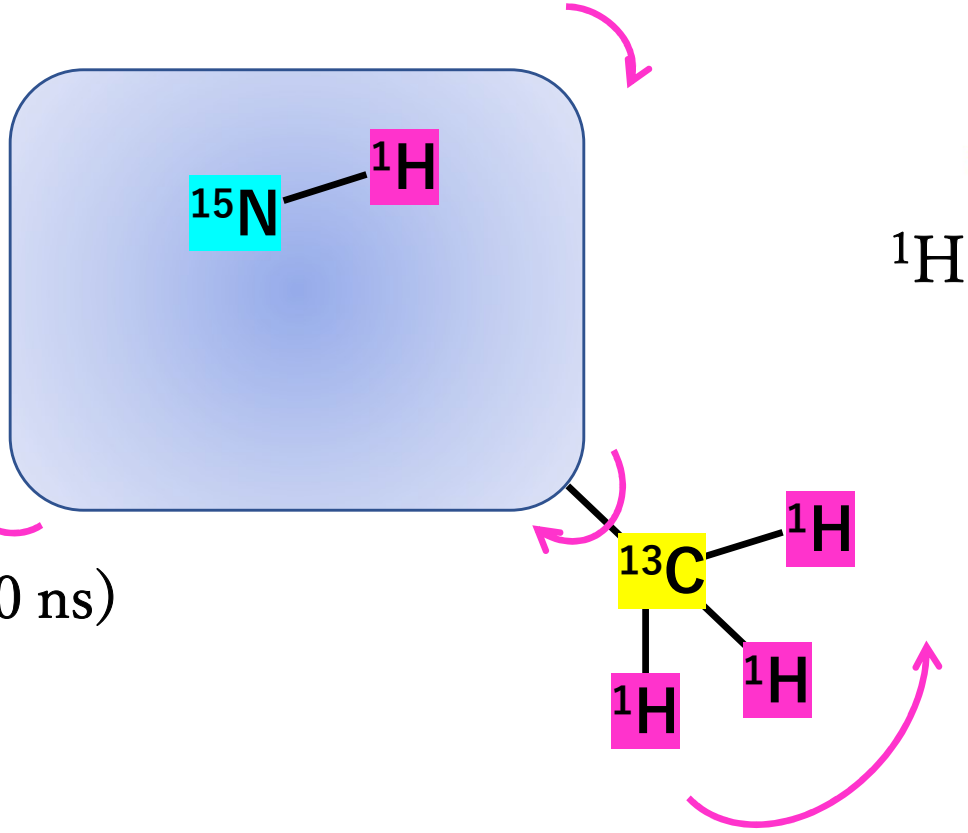
The faster the Brownian rotation, the sharper the NMR peaks.

速くブラウン回転するほど、NMR ピークがシャープになる。

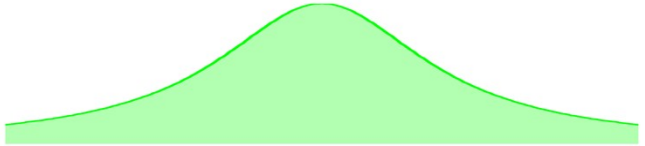


Methyl groups provide sharper and stronger peaks than amide groups.

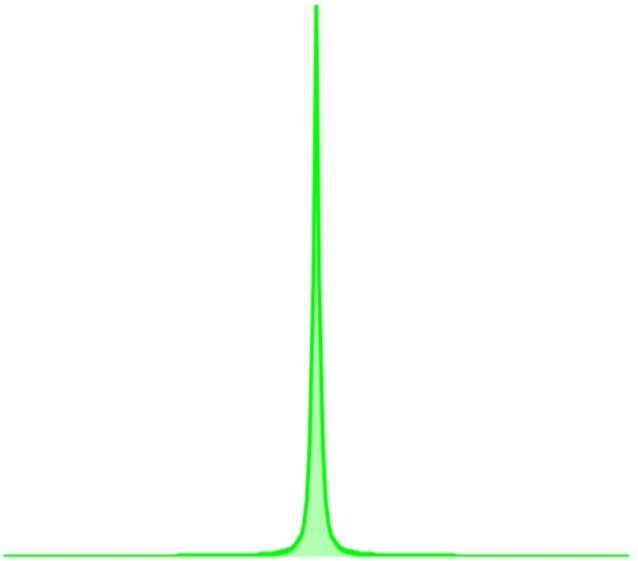
メチル基のピークは、アミド基のピークよりもシャープで強い。



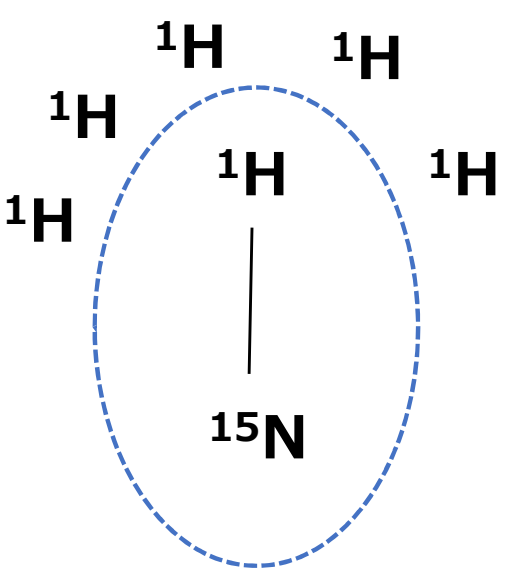
(~10 ns)



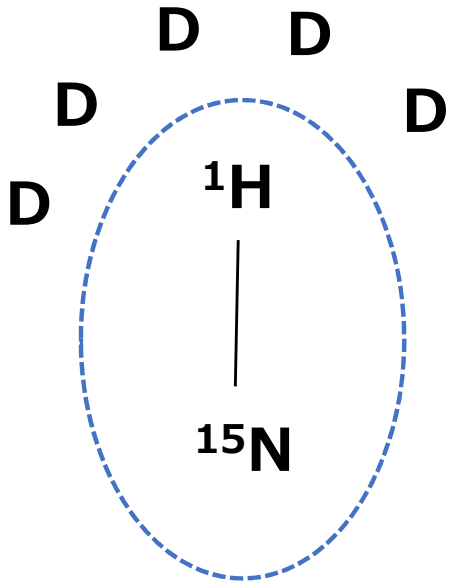
^{1}H-^{15}N: fixed to the main chain



^{13}C-$^{1}H_3$: rotates around the chemical bond at a high speed (~10 ps)



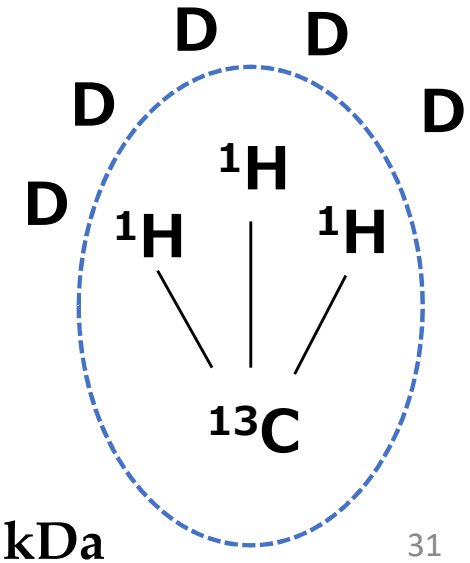
20 kDa



60 kDa

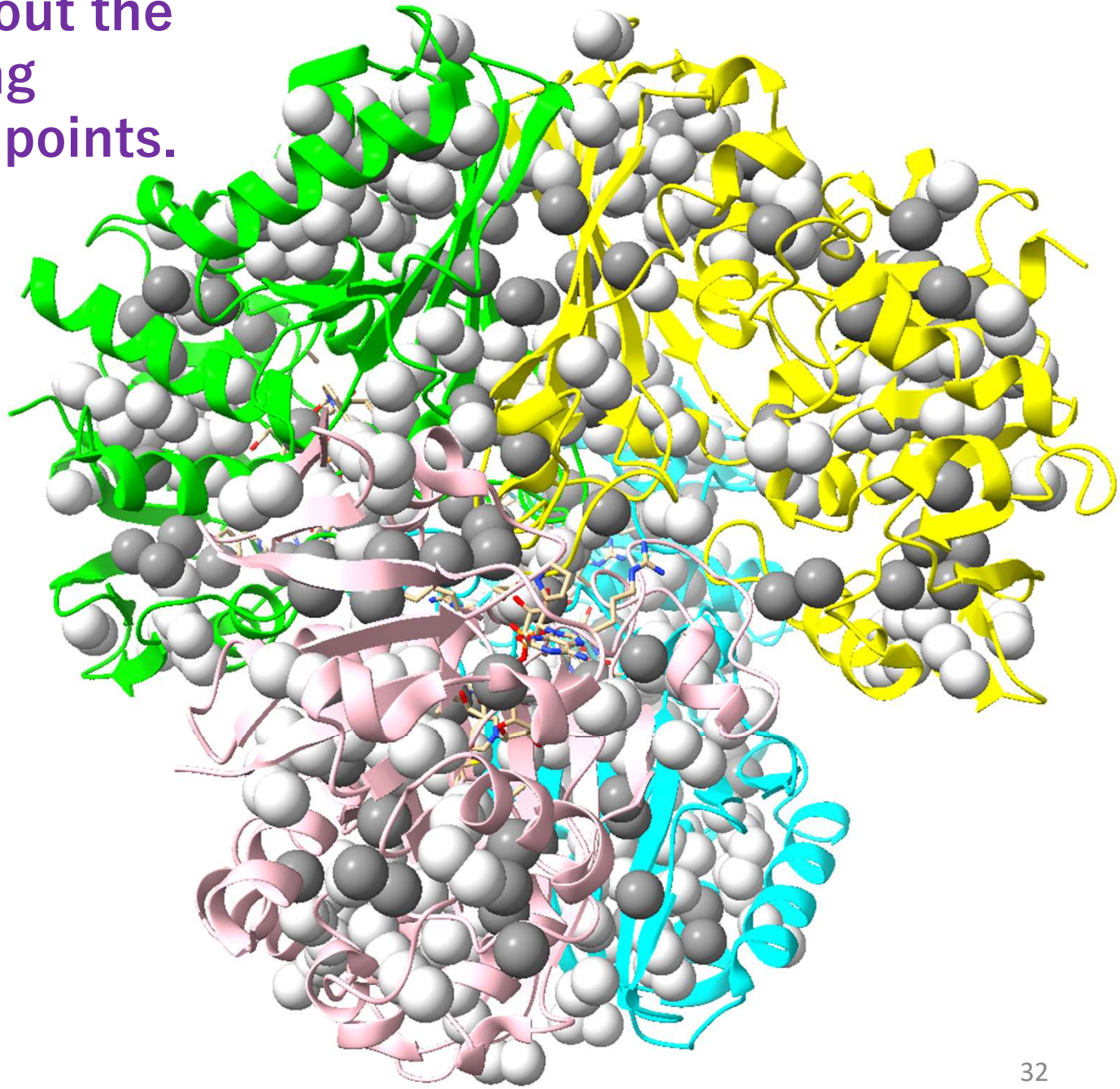
Deuteration of all but methyl groups allows for observation of even larger proteins.

メチル基以外を重水素化すると、より大きなタンパク質を観察できる。



> 300 kDa

Methyl groups are scattered throughout the structure, providing many observation points.



メチル基は構造全体に散らばり、多くの観測ポイントとしての役割を果たす。

White: Leu, Val
Grey: Met, Ile

Proteins are deuterated except for the methyl groups of Ile, Leu, and Val

[^{2}H]-glucose, 100% D_2O

$^{13}CH_3$-CD_2-CO-COO^-

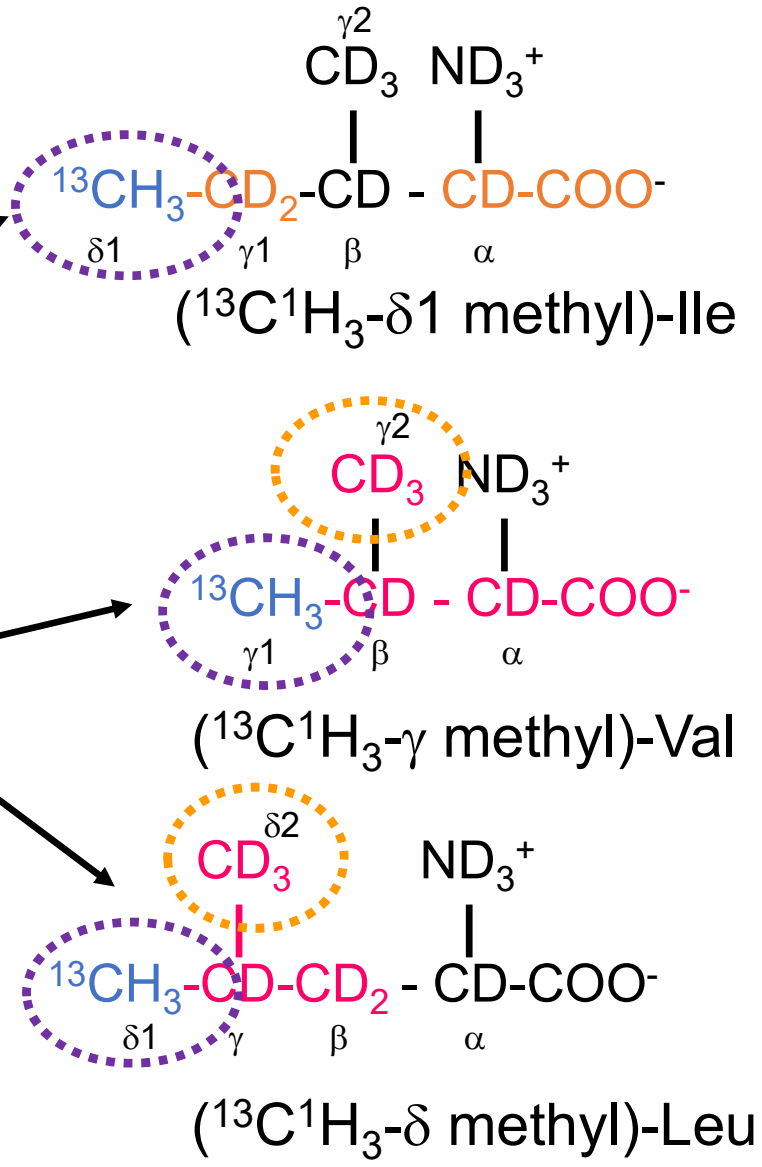
2-keto-3,3-d_2-4-^{13}C-butyrate
α-ketobutyric acid methyl-^{13}C 3,3-d_2

2 -ketobutyrate

CD_3
|
$^{13}CH_3$-CD-CO-COO^-

2-keto-3-methyl-d_3-3-d_1-4-^{13}C-butyrate
α-ketoisovaleric acid 3-methyl-^{13}C 3,4,4,4-d_4

2 -ketoisovalerate



Tugarinov, V. *et al.* (2004) *J. Biomol. NMR* 28, 165.

The bacterial metabolic pathways for biosynthesis of Val, Leu, and Ile

Almost no scrambling to other amino acids

pyruvate

2-ketobutyrate

2-ketoisovalerate

Val

Leu

Ile

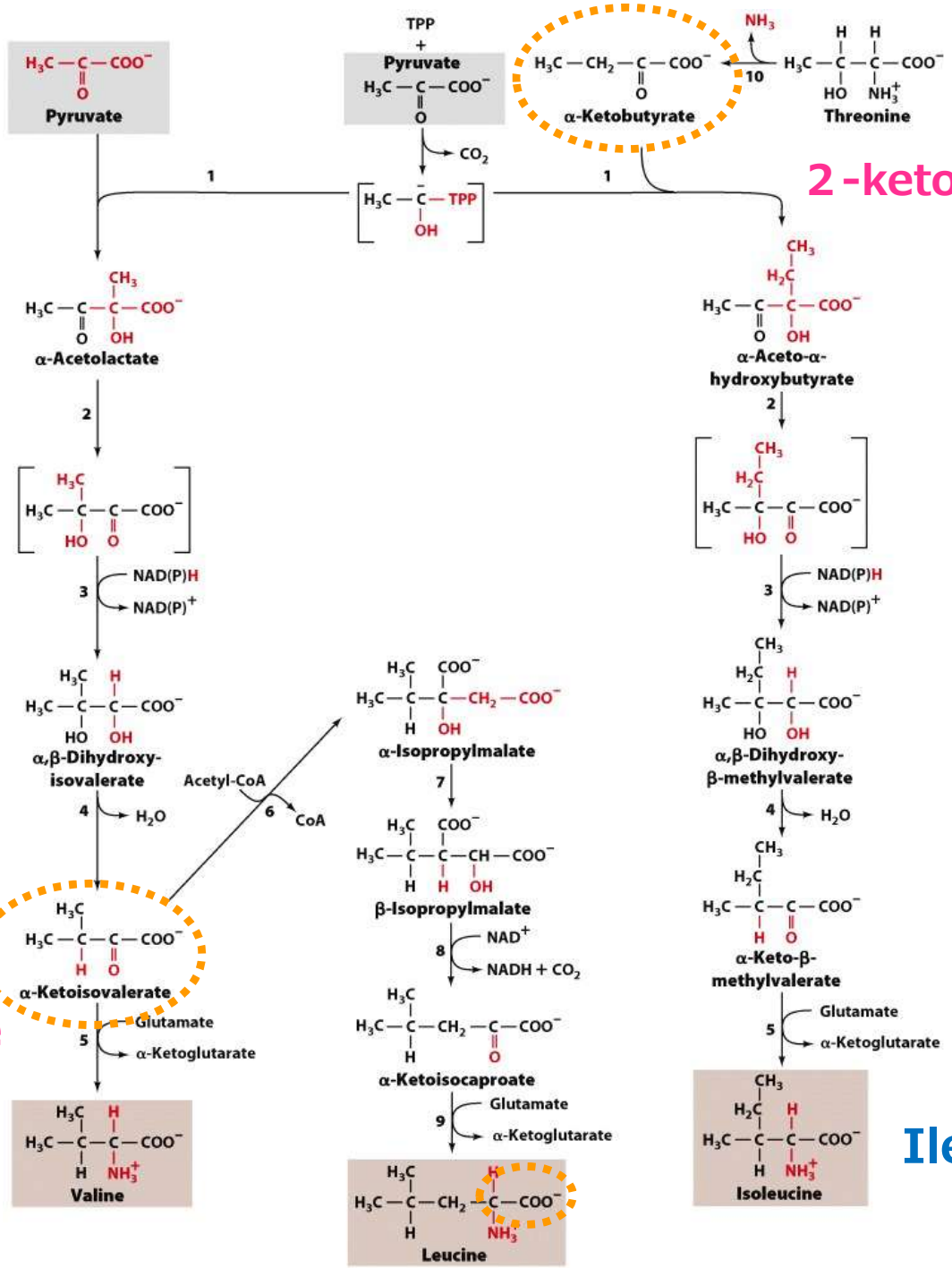
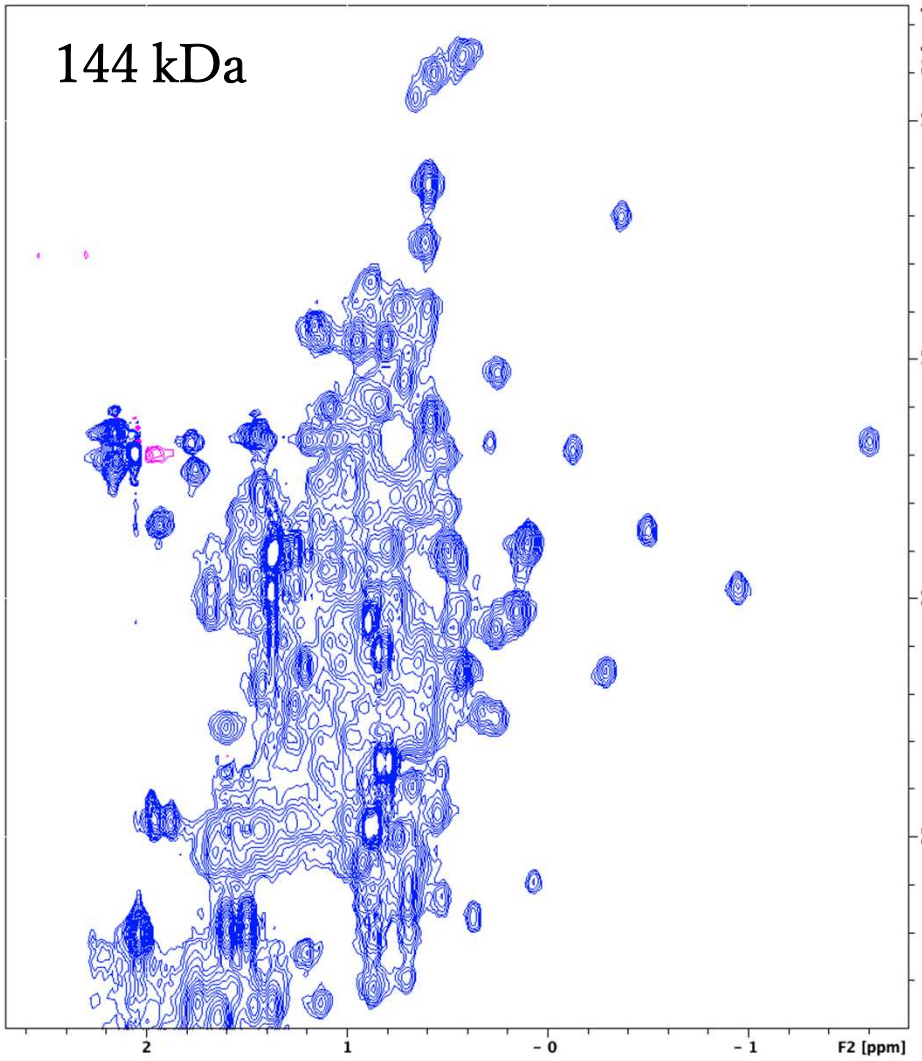
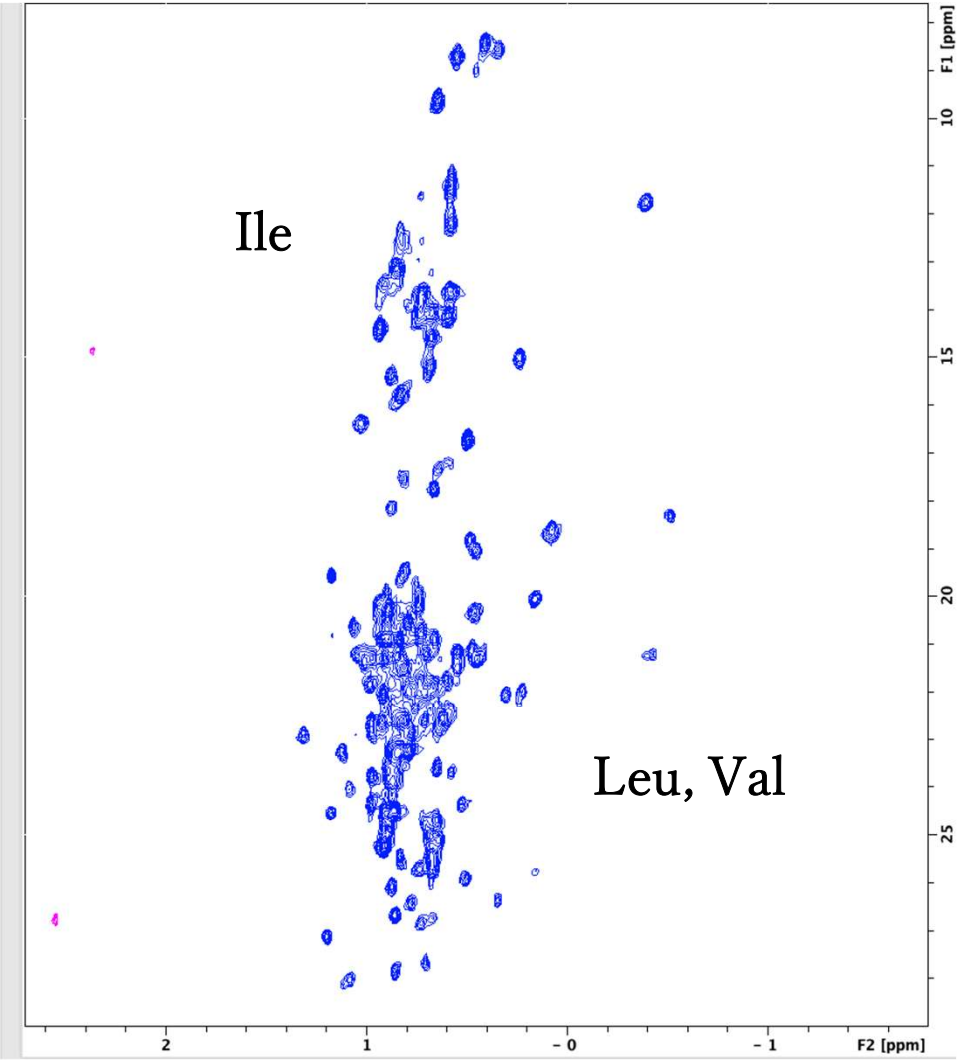


Figure 26-61

Methyl-specific labeling will be the cornerstone of future protein NMR.

メチル基以外を重水素化することで、より大きなタンパク質を観測できる

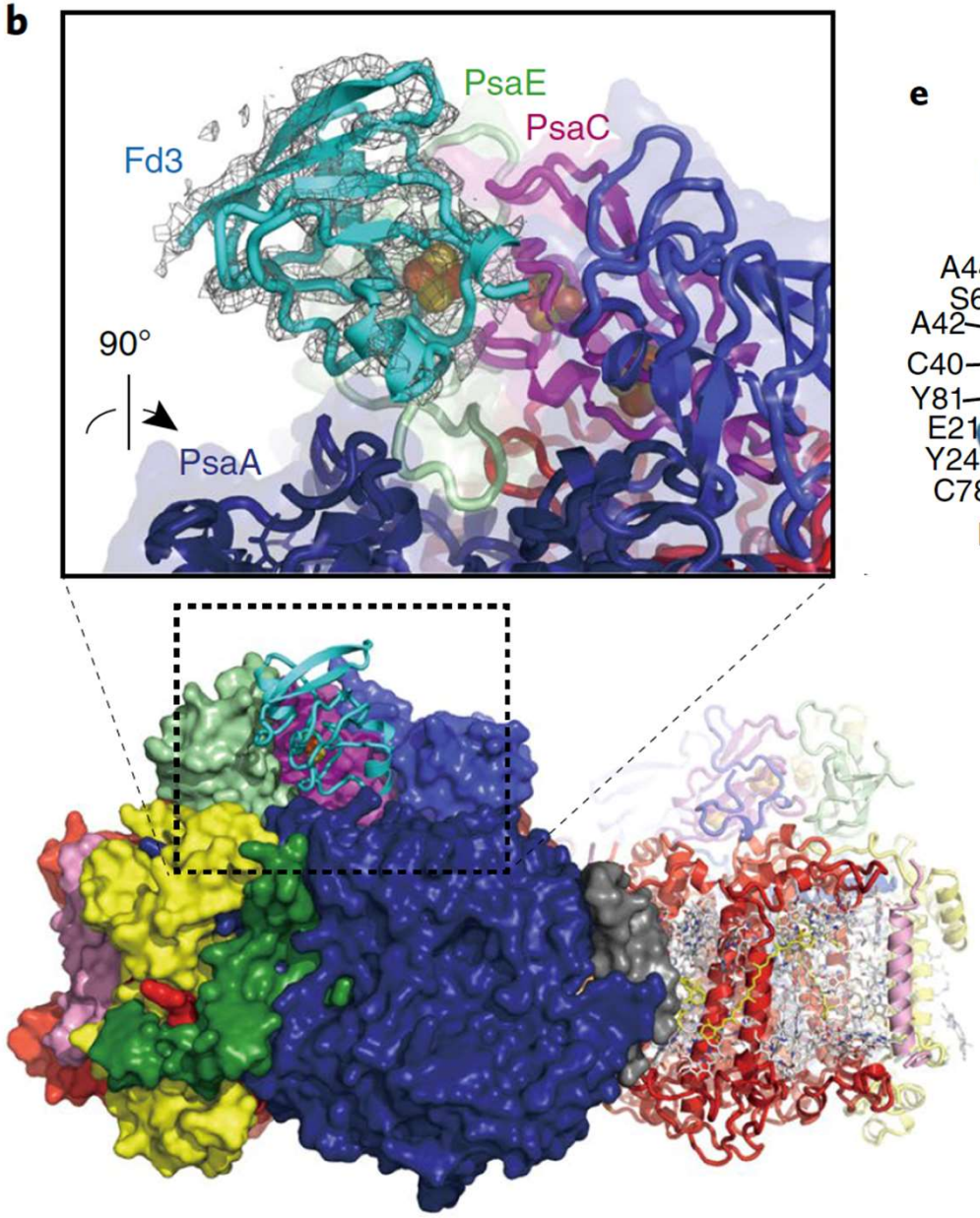


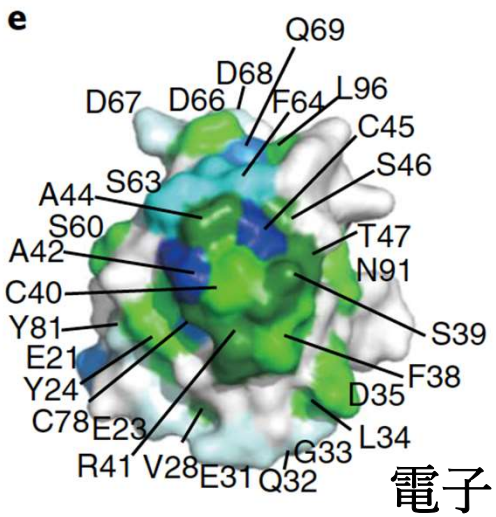


Uniformly ^{13}C, ^{1}H labeled

specifically methyl ^{13}C-$^{1}H_3$, otherwise ^{2}H

Interaction analyses





[^{2}H, ^{15}N]-Fd
receiving e-

10 kDa

電子を受け取る
フェレドキシン（^{2}H, ^{15}N）

[non-label]-Photosystem I
of cyanobacteria

2,200 kDa

シアノバクテリアの光合成に
関する膜タンパク質（非標識）

Kubota-Kawai, *et al*. (2018) X-ray structure of an asymmetrical trimeric ferredoxin–photosystem I complex. *Nat. Plants* 4, 218.

Expression of deuterated proteins using insect and mammalian cells

- Cells do not grow in D_2O.
- Deuterated amino acids are added to the medium.
- However, they are expensive.
- ^1H contaminates from the solvent water.

- Yeast (*Pichia pastoris*) would be a better choice.
- Cell-free expression system (*E. coli*) is also preferred.
  D_2O: 250,000 yen/L (90,000 yen/L in 2021)