Supplementary Material

Supplementary Figure **S1**:
Sequence alignment of the full-consensus ankyrin repeat proteins NI_2C, NI_3C and the C-cap mutant NI_3C_Mut 5. The mutated residues in the C-cap are in boldface. Elements of secondary structure have been annotated according to the crystal structure of NI_3C (PDB entry 2QYJ).

Supplementary Figure **S2**:
600 MHz [^{15}N,^{1}H]-HSQC spectrum of NI_2C, 310 K, in 50 mM phosphate, 150 mM NaCl, pH 7.4.

Supplementary Figure **S3**:
600 MHz [^{15}N,^{1}H]-HSQC spectrum of NI_3C, 310 K, in 50 mM phosphate, 150 mM NaCl, pH 7.4.

Supplementary Figure **S4**:
600 MHz [^{15}N,^{1}H]-HSQC spectrum of NI_3C_Mut5, 310 K, in 50 mM phosphate, 150 mM NaCl, pH 7.4.

Supplementary Figure **S5**:
Representative strips from the 3D HNCACB (left) and HN(COCA)NH (right) spectra of NI_3C_Mut5.

Supplementary Figure **S6**:
Intramolecular attenuations of signal intensities of cross peaks in the 600 MHz [^{15}N,^{1}H]-HSQC spectra of MTSL-derivatized D28C-NI_2C (top) and D28C-NI_3C (bottom) relative to the non-spin labeled protein. Residues for which peaks could not be integrated reliably have been omitted and are marked by an asterisk directly below the horizontal axis.

Supplementary Figure **S7**:
Intermolecular attenuations of signal intensities of cross peaks in the 600 MHz [^{15}N,^{1}H]-HSQC spectra of a mixture of MTSL-derivatized unlabelled D28C-NI_3C (a), D28C-NI_3C_Mut5 (b) or D155C- NI_3C_Mut5 (c) with the non-spin labeled ^{15}N uniformly labeled corresponding proteins protected wit NEM. The ratio of signal in the mixed sample (*Mix*) over the 15N labeled sample alone (*Ref*) is plotted.

Supplementary Figure **S8**:
700 MHz [^{15}N,^{1}H]-HSQC spectra of NI_3C at 0 M (top left), 1.2 M (top right), 2.4 M (bottom left) and 3.6 M GdmCl (bottom right), 50 mM phosphate, 150 mM NaCl, pH 7.4, 310 K. Red crosses denote the positions of the original peaks in absence of GdmCl.

Supplementary Figure **S9**:
^{1}H (left) and ^{15}N (right) chemical shift changes mapped onto the structure of NI_3C (top) and NI_3C_Mut5 (bottom). The size of the spheres corresponds to the absolute change in frequency between 0 and 2.1 M or 0 and 5.0 M GdmCl for NI_3C and NI_3C_Mut5, respectively.

Supplementary Figure **S10**:
^{15}N-{^{1}H}-NOE data of NI_3C_Mut5 in presence of 2 M (a), 4 M (b) or 6 M GdmCl (c) recorded at 600 MHz. In case of the data at 6 M GdmCl no assignments were available and the values on the x-axis are chosen arbitrarily.

Supplementary Figure **S11**:
700 MHz [^{15}N,^{1}H]-HSQC spectra of NI_3C_Mut5 at 0 M (top left), 2.0 M (top right), 4.0 (bottom left) and 6.0 M GdmCl (bottom right), 50 mM phosphate, 150 mM NaCl, pH 7.4, 310 K.

Supplementary Figure **S12**:
^{15}N-{^{1}H}-NOE data of NI_2C (a), NI_3C (b) and NI_3C_Mut5 (c) recorded at 600 MHz on 0.7 mM solutions of the proteins in 50 mM phosphate, 150 mM NaCl, pH 7.4, 310 K. Gray background indicates the location of helices 1 and 2 in the repeats. Values for the second conformation of NI_2C and NI_3C are shown as red diamonds.

Supplementary Figure **S13**: Exchange times (1/rate) of selected amide protons of NI_3C in presence of 1 M (a) and 2 M (b) GdmCl. Values for residues that cannot be assigned are depicted in the separate panel on the right.

Supplementary Figure **S14:**
CD-monitored denaturation curves of NI_1C, NI_2C, NI_3C, NI_1C_Mut5 and NI_3C_Mut5 (asterisks with error-bars) and the Ising model fit (solid lines) obtained using equation 5 and the parameters reported in Table 1.

Supplementary Figure **S15**:
600 MHz [^{15}N,^{1}H]-HSQC spectrum of NI_3, 310 K, in 50 mM phosphate, 150 mM NaCl, pH 7.4.

Supplementary Figure **S16**:
Predictions of α-helix for NI_3C_Mut5 content based on the primary sequence using the program AGADIR.

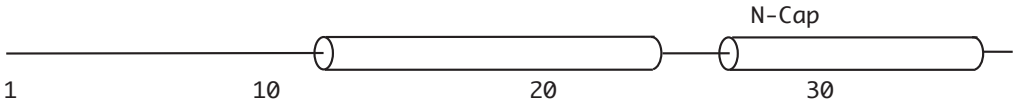
Some remarks considering the assignment:

For NI_2C, 125 (and thus 6 more than the maximally expected 119) cross-peaks were detected in the [^{15}N,^{1}H]-HSQC spectrum, for NI_3C 161 (151 expected) and for NI_3C_Mut5 152 (153 expected). In general, peaks due to residues from the His-tag and up to the first ankyrin residue (Asp13) were invisible at pH 7.4. Because of overlapping or missing peaks, we were not able to assign amide moieties of D13 in all three proteins and K101 in NI_3C. The overall completeness of the backbone assignment was 99% for NI_2C, NI_3C and NI_3C_Mut5 (see also Figures S2-S4). Amide proton, ^{15}N, Cα and Cβ chemical shifts of NI_2C, NI_3C and NI_3C_Mut5 have been deposited in the BMRB database under accession codes 16718, 16717 and 16716, respectively.

We have stated the importance that individual cross peaks are observed in the [^{15}N,^{1}H]-HSQC spectrum of the repeat protein. Resolving peaks along the amide proton frequency is in principle possible in ^{15}N-resolved NOESY experiments, but the resolution in the proton (F1) domain was often insufficient, and hence we either used the HN(CACO)NH experiment to exploit resolution in the ^{15}N domain or resorted to triple-resonance spectra with peak matching in ^{13}C. Nevertheless, the combination of knowledge of amide proton and nitrogen frequencies of sequential amide moieties greatly facilitated assignments. Since carbonyl chemical shifts usually display good signal dispersion, the 3D HNCO and HN(CA)CO experiments were recorded and additionally utilized for assignments.

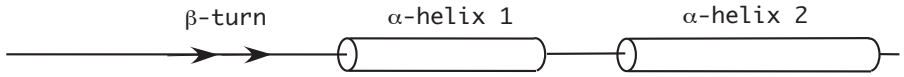
S1

Sequence Alignment




```
            1                 10                  20                  30
NI2C        M R G S H H H H H H G S D L G K K L L E A A R A G Q D D E V R I L M A N G
NI3C        M R G S H H H H H H G S D L G K K L L E A A R A G Q D D E V R I L M A N G
NI3C M5     M R G S H H H H H H G S D L G K K L L E A A R A G Q D D E V R I L M A N G
```

internal full-consensus repeat




```
                40                  50                  60                  70
NI2C        A D V N A K D K D G Y T P L H L A A R E G H L E I V E V L L K A G      I-1
NI3C        A D V N A K D K D G Y T P L H L A A R E G H L E I V E V L L K A G
NI3C M5     A D V N A K D K D G Y T P L H L A A R E G H L E I V E V L L K A G

                            80                  90                  100
NI2C        A D V N A K D K D G Y T P L H L A A R E G H L E I V E V L L K A G      I-2
NI3C        A D V N A K D K D G Y T P L H L A A R E G H L E I V E V L L K A G
NI3C M5     A D V N A K D K D G Y T P L H L A A R E G H L E I V E V L L K A G

                        110                 120                 130
NI2C        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -      I-3
NI3C        A D V N A K D K D G Y T P L H L A A R E G H L E I V E V L L K A G
NI3C M5     A D V N A K D K D G Y T P L H L A A R E G H L E I V E V L L K A G
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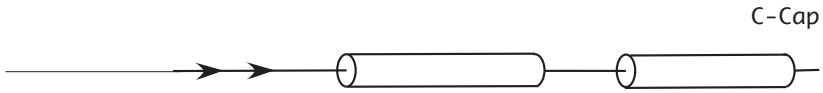


```
                140                 150                 160
NI2C        A D V N A Q D K F G K T A F D I S I D N G N E D L A E I L Q
NI3C        A D V N A Q D K F G K T A F D I S I D N G N E D L A E I L Q
NI3C M5     A D V N A Q D K F G K T P F D L A I D N G N E D I A E V L Q K A A
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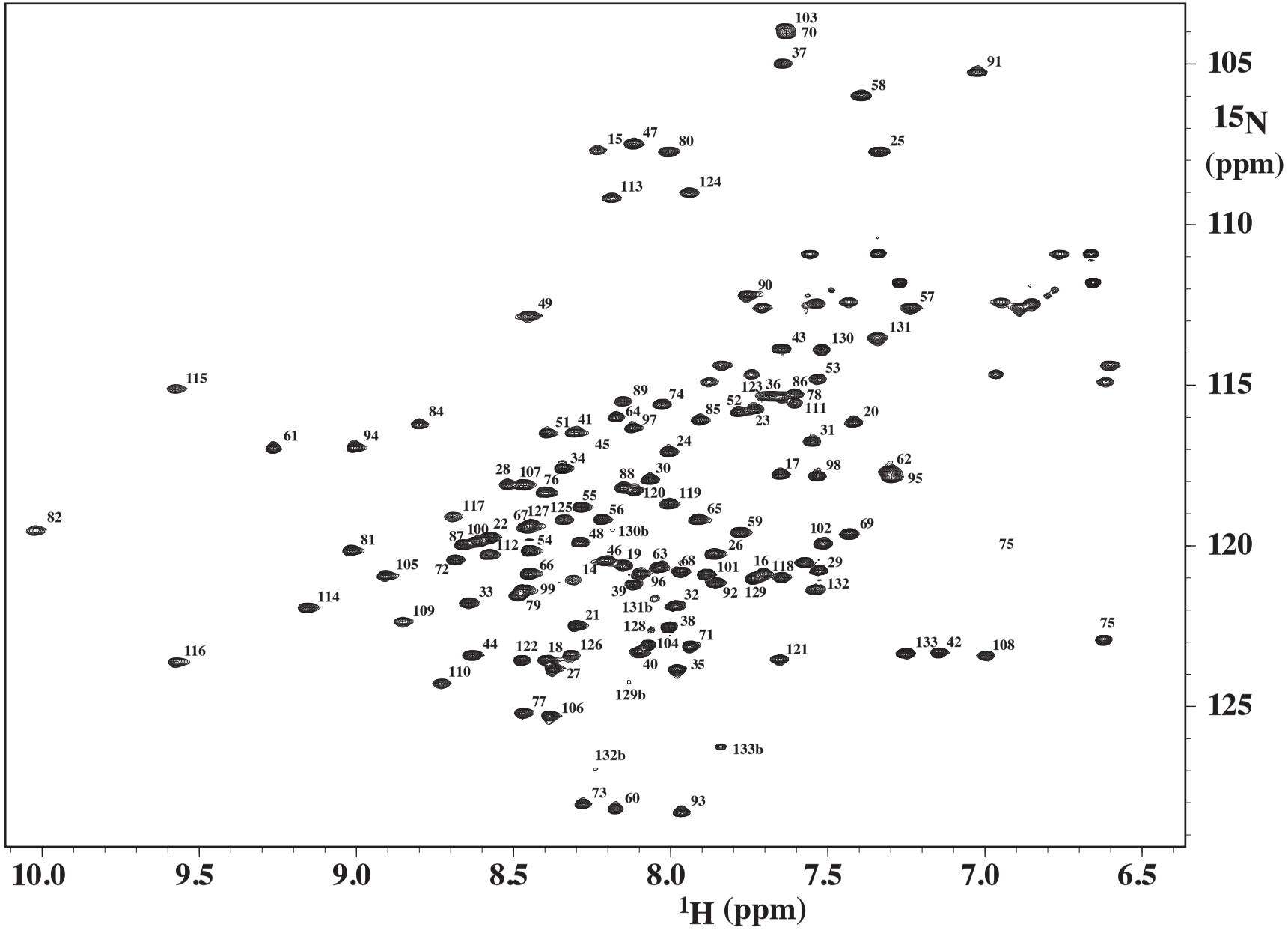
S2

[^{15}N,^{1}H]-HSQC of NI_2C



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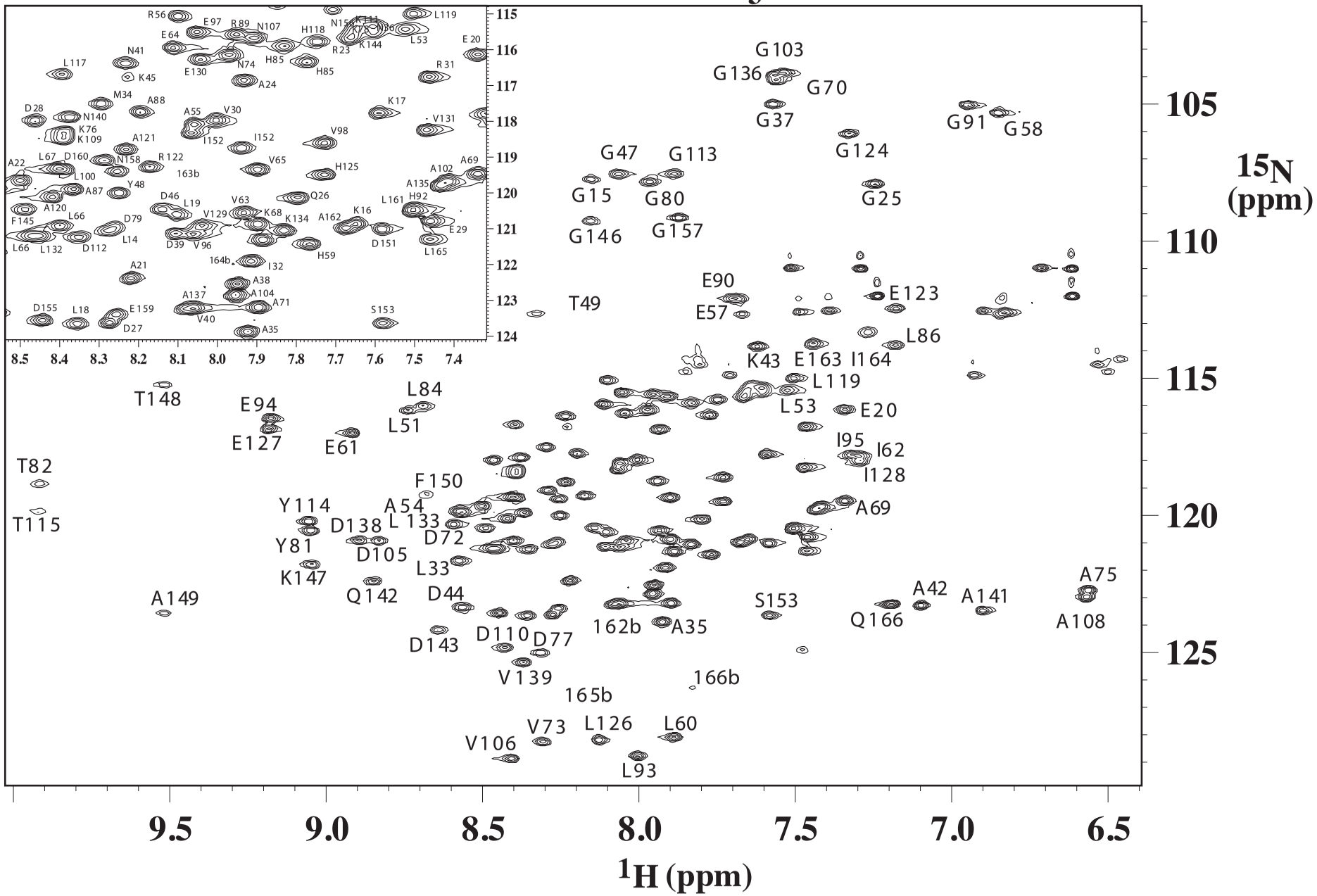
[^{15}N,^{1}H]-HSQC of NI_3C



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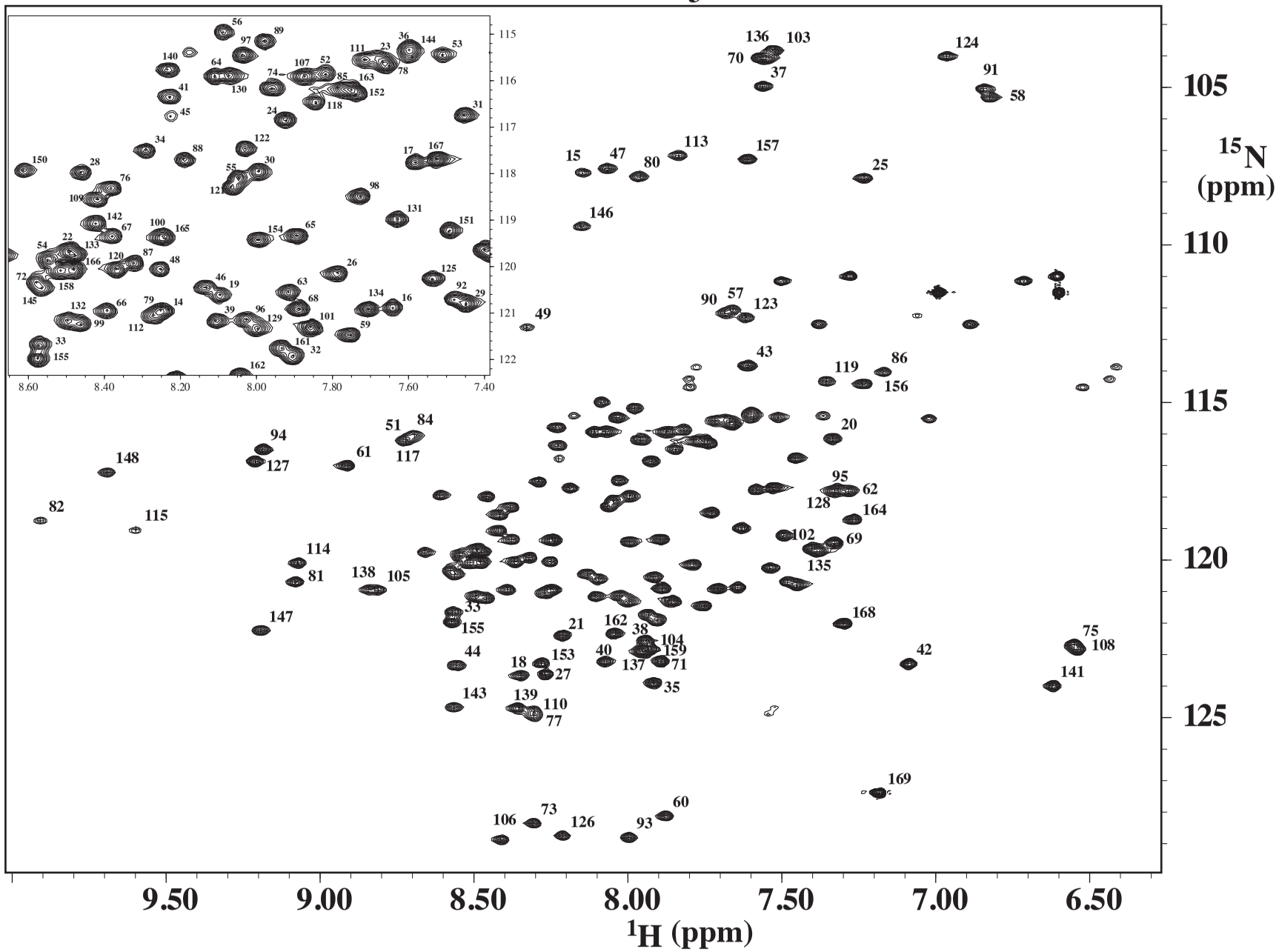
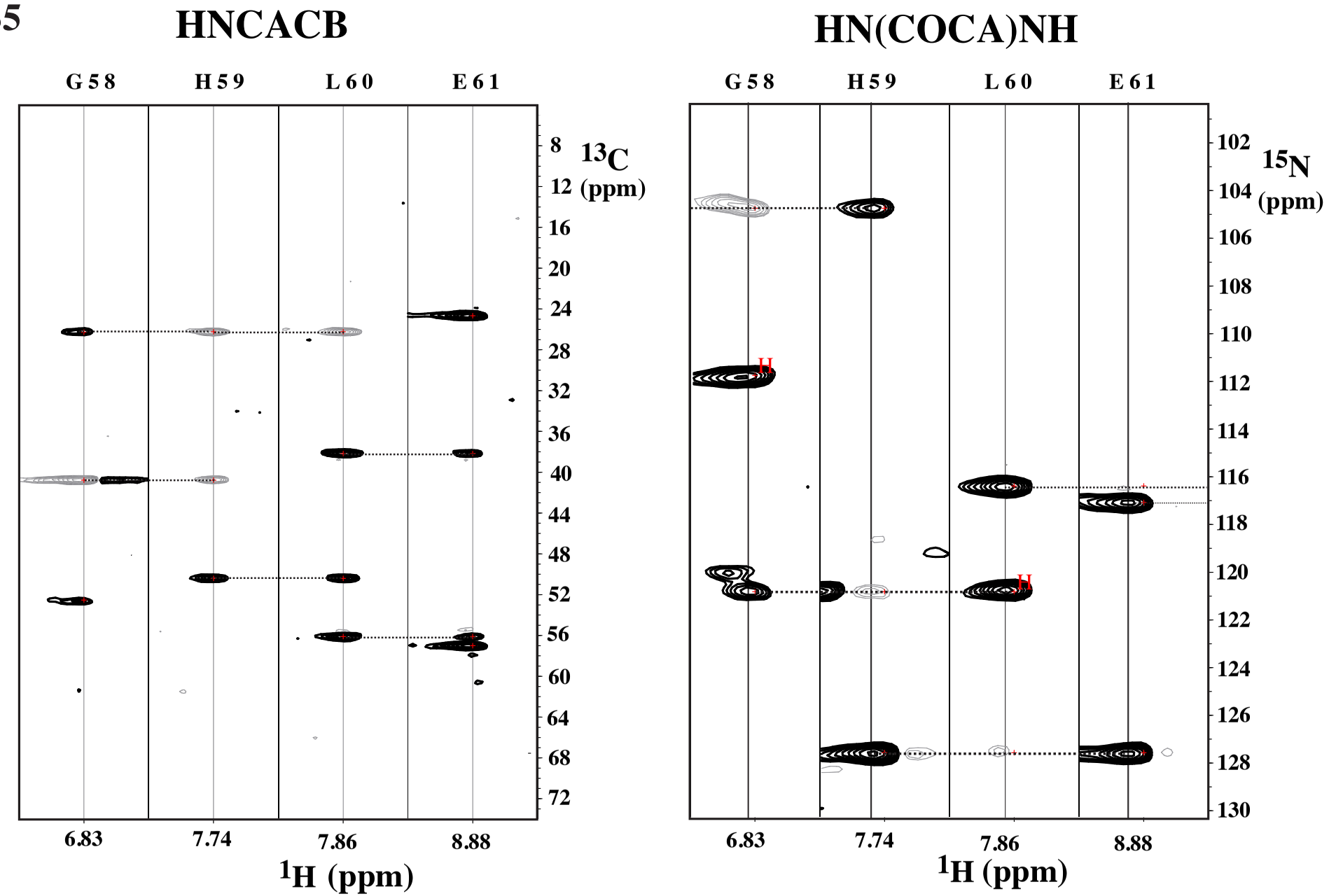
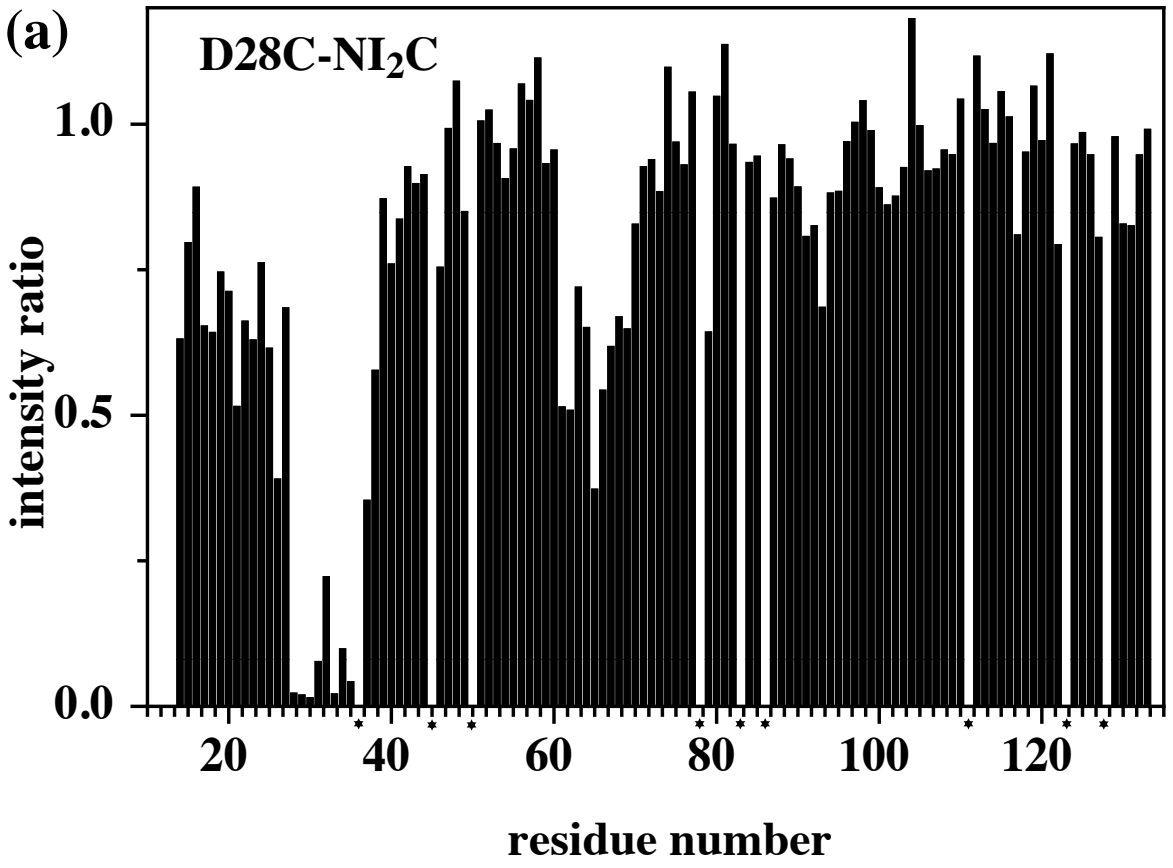
[^{15}N,^{1}H]-HSQC of NI$_3$C_Mut5



S5


HNCACB
G58
H59
L60
E61
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
13C (ppm)
6.83
7.74
7.86
8.88
1H (ppm)
HN(COCA)NH
G58
H59
L60
E61
102
104
106
108
110
112
114
116
118
120
122
124
126
128
130
15N (ppm)
H
H
6.83
7.74
7.86
8.88
1H (ppm)

S6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0.5
0.0
intensity ratio
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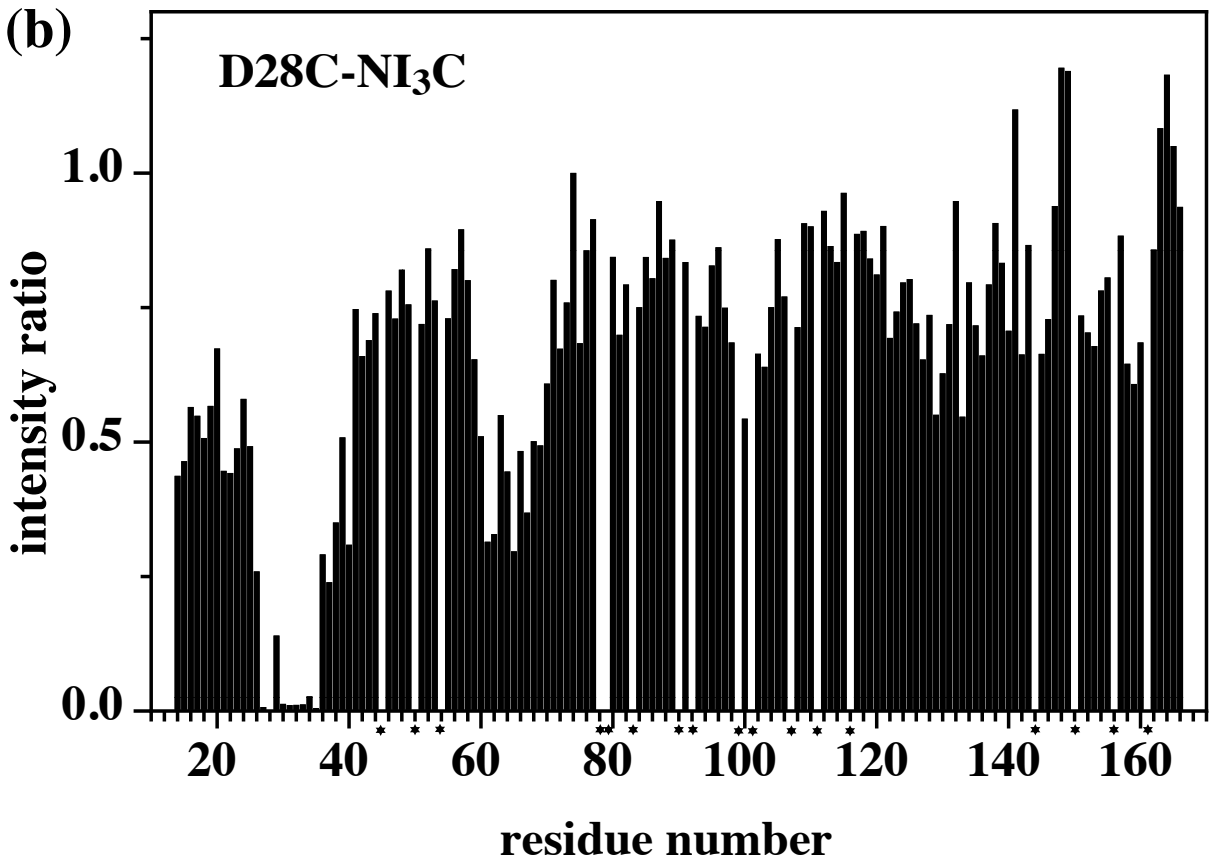
40
60
80
100
120
residue number


(b)
D28C-NI3C
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0.5
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intensity ratio
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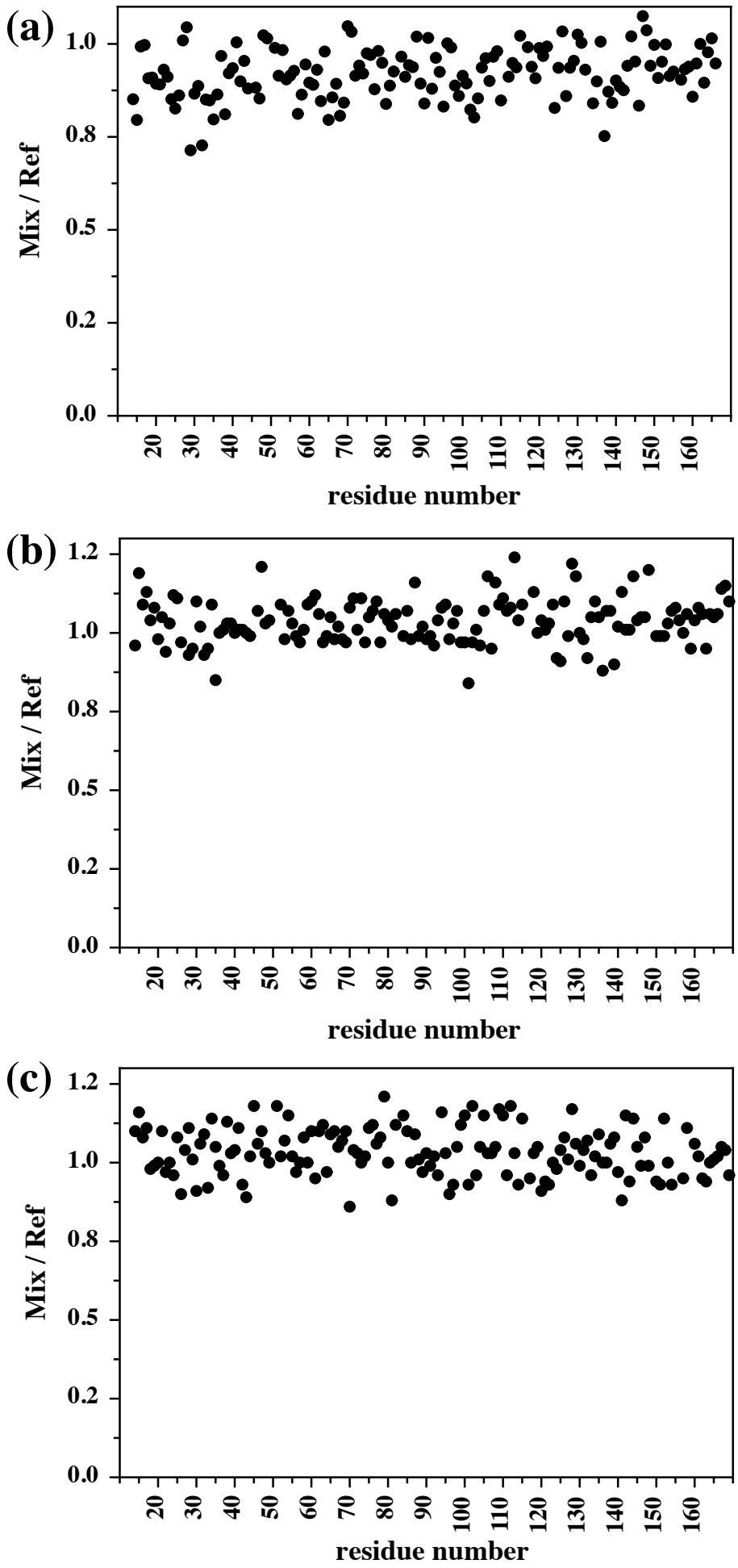
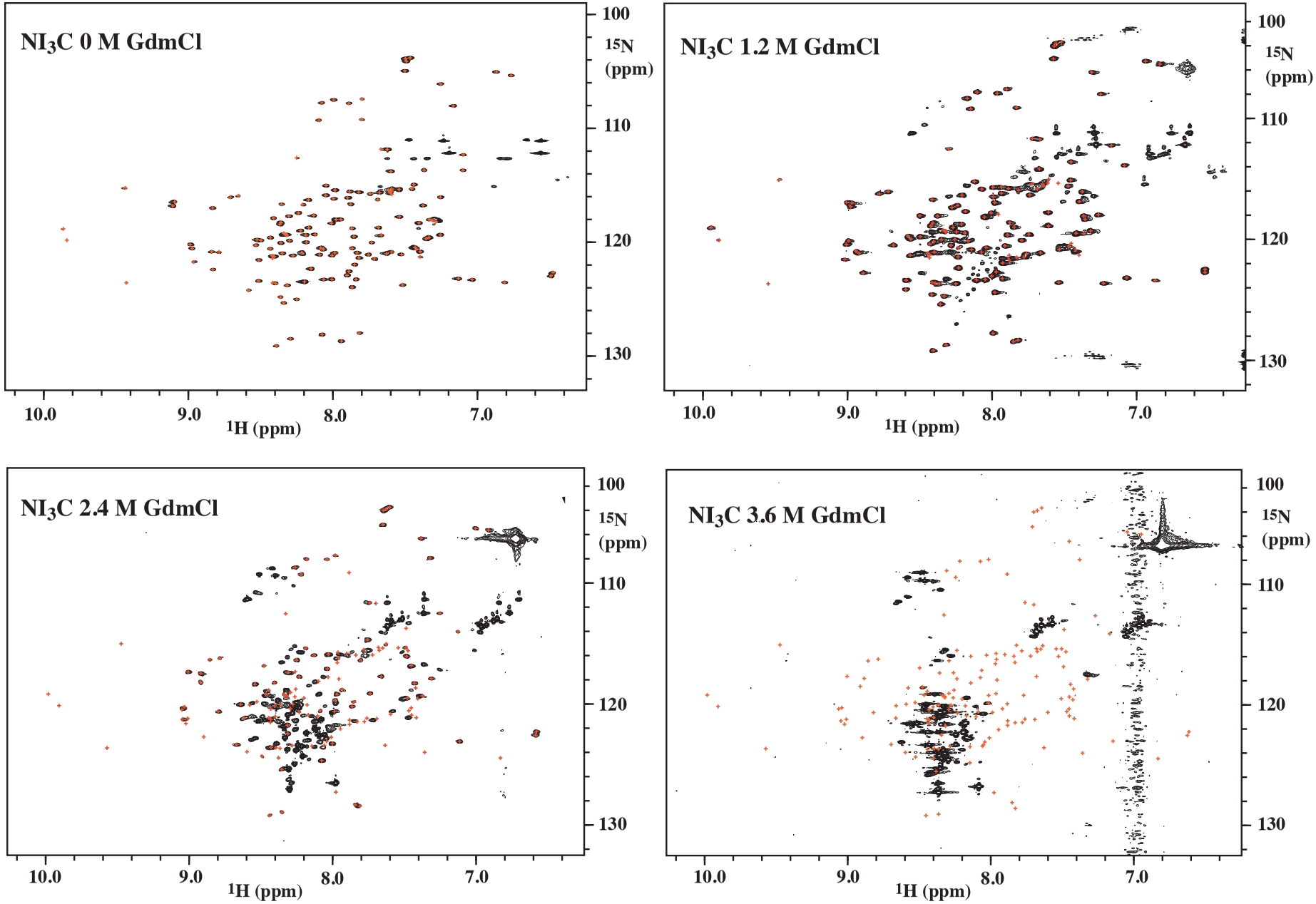
100
120
140
160
residue number

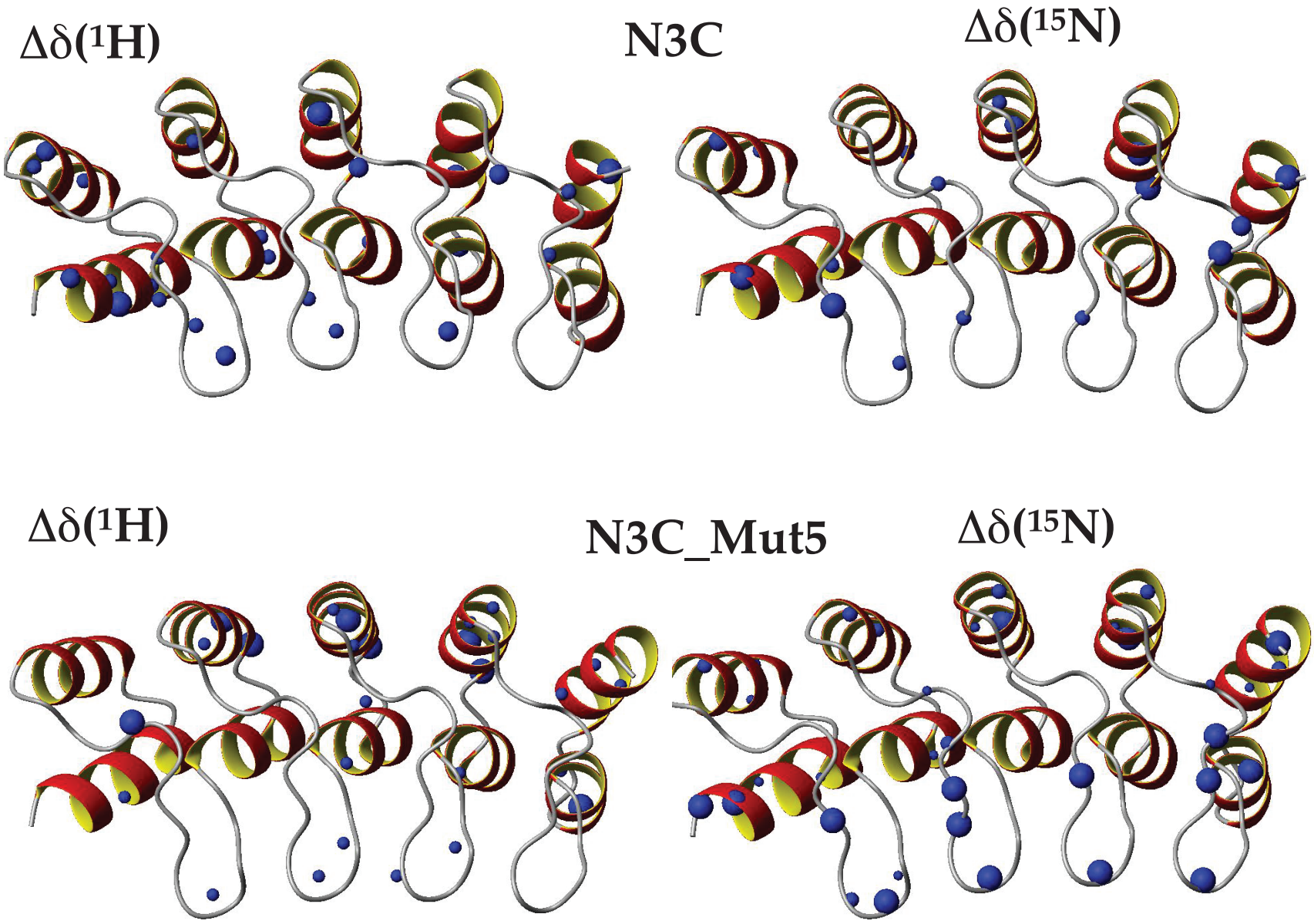
S7


(a)
Mix / Ref
1.0
0.8
0.5
0.2
0.0
20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
residue number
(b)
Mix / Ref
1.2
1.0
0.8
0.5
0.2
0.0
20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
residue number
(c)
Mix / Ref
1.2
1.0
0.8
0.5
0.2
0.0
20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
residue number

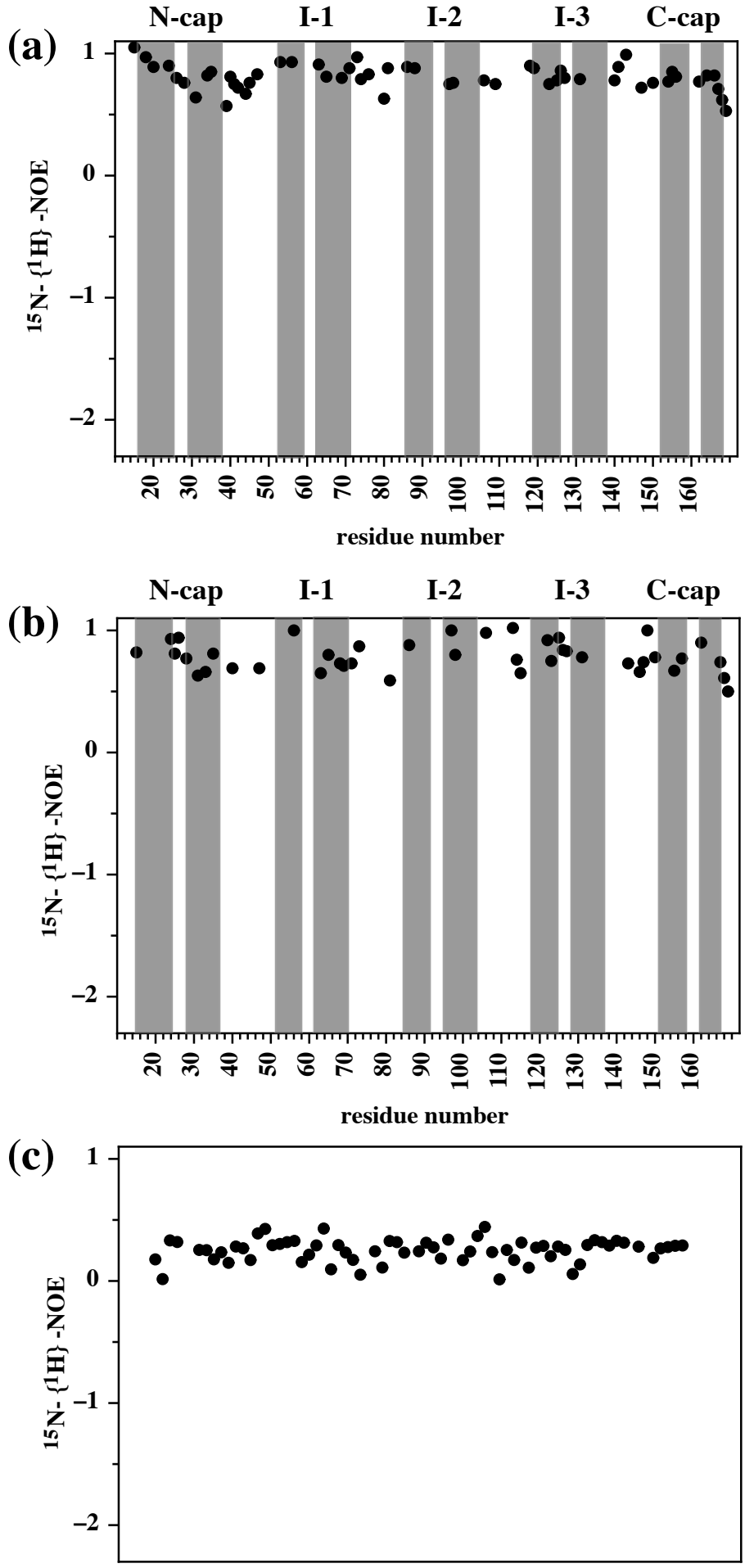
S8


NI3C 0 M GdmCl
NI3C 1.2 M GdmCl
NI3C 2.4 M GdmCl
NI3C 3.6 M GdmCl
15N (ppm)
100
110
120
130
1H (ppm)
10.0
9.0
8.0
7.0

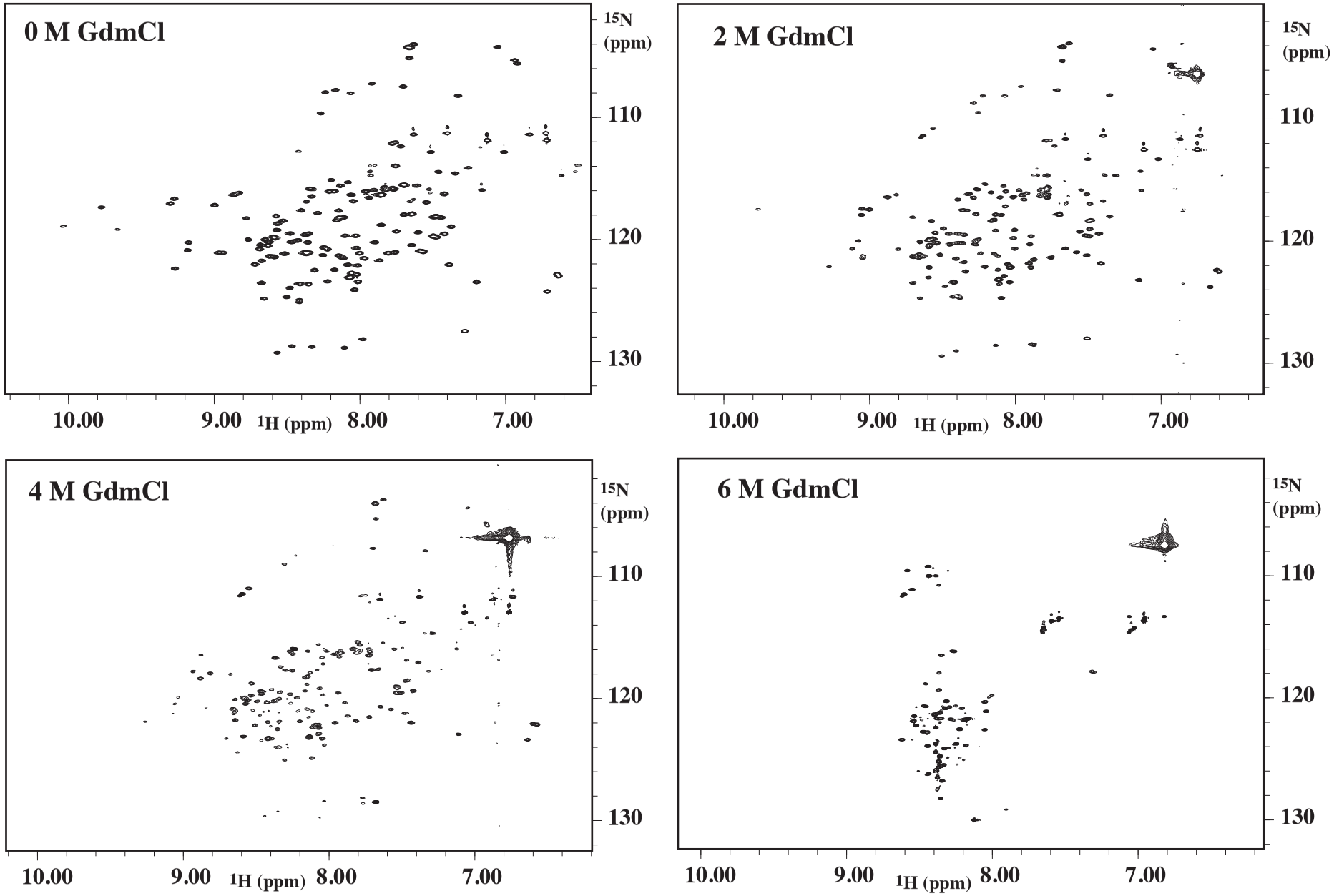
S9



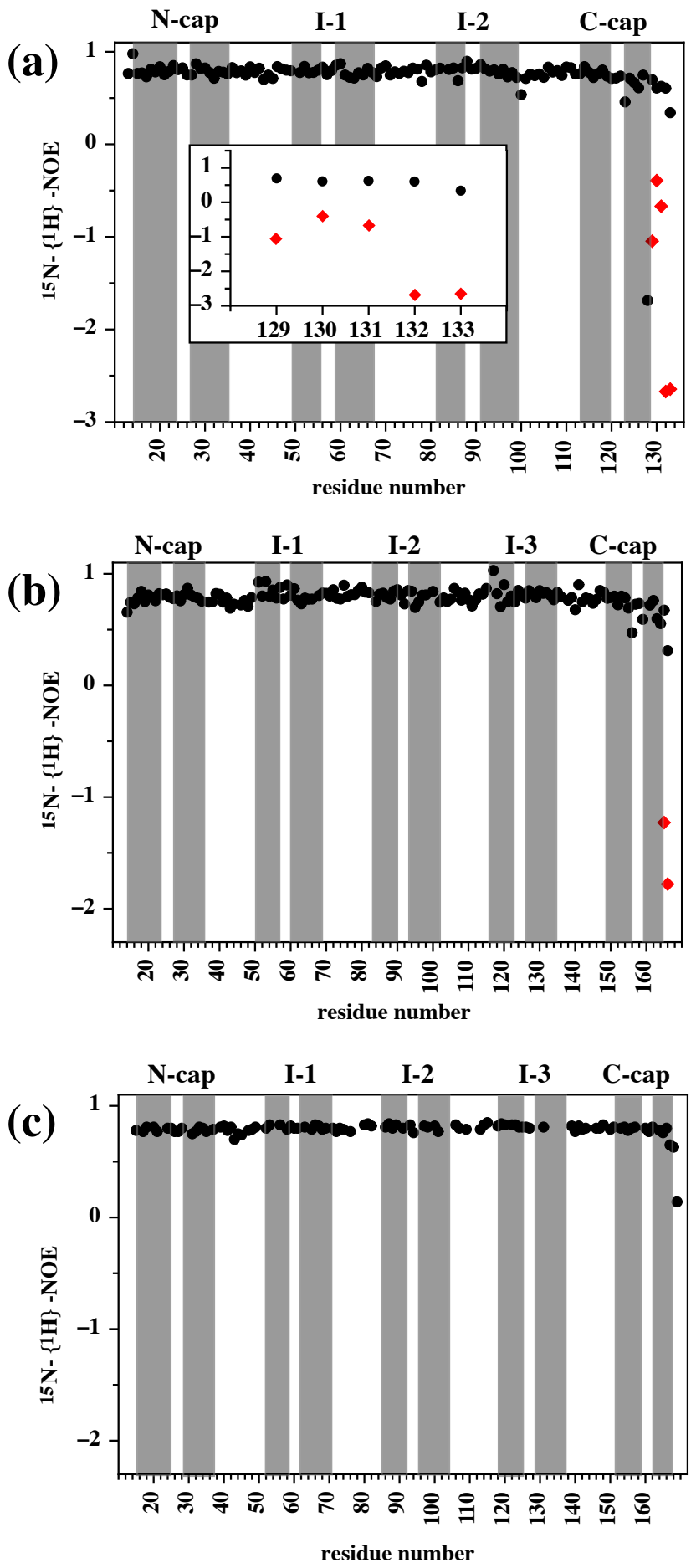
S10


(a)
N-cap
I-1
I-2
I-3
C-cap
^{15}N- {^{1}H} -NOE
residue number
(b)
N-cap
I-1
I-2
I-3
C-cap
^{15}N- {^{1}H} -NOE
residue number
(c)
^{15}N- {^{1}H} -NOE

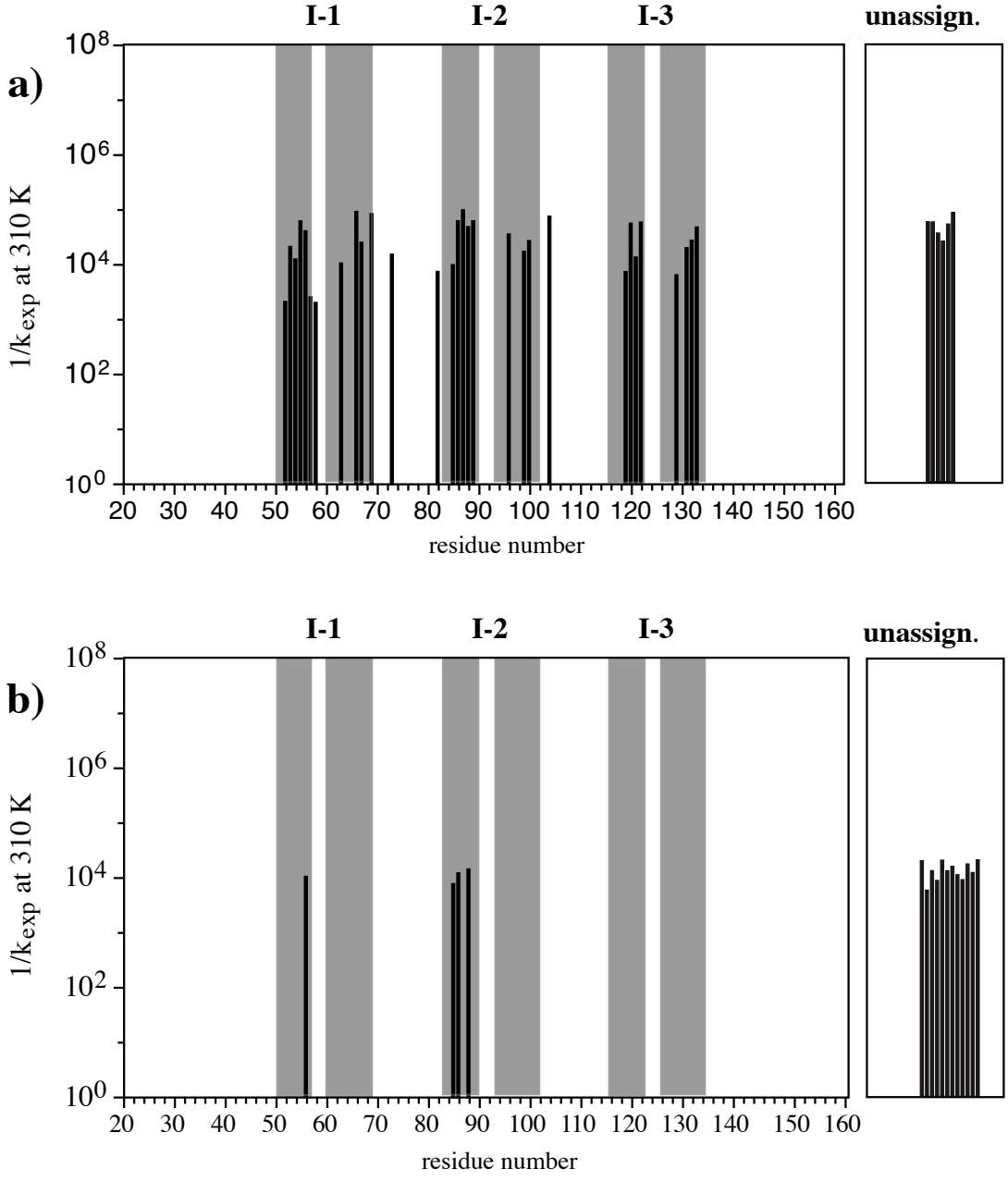
S11


0 M GdmCl
2 M GdmCl
4 M GdmCl
6 M GdmCl
15N (ppm)
110
120
130
10.00
9.00
1H (ppm)
8.00
7.00

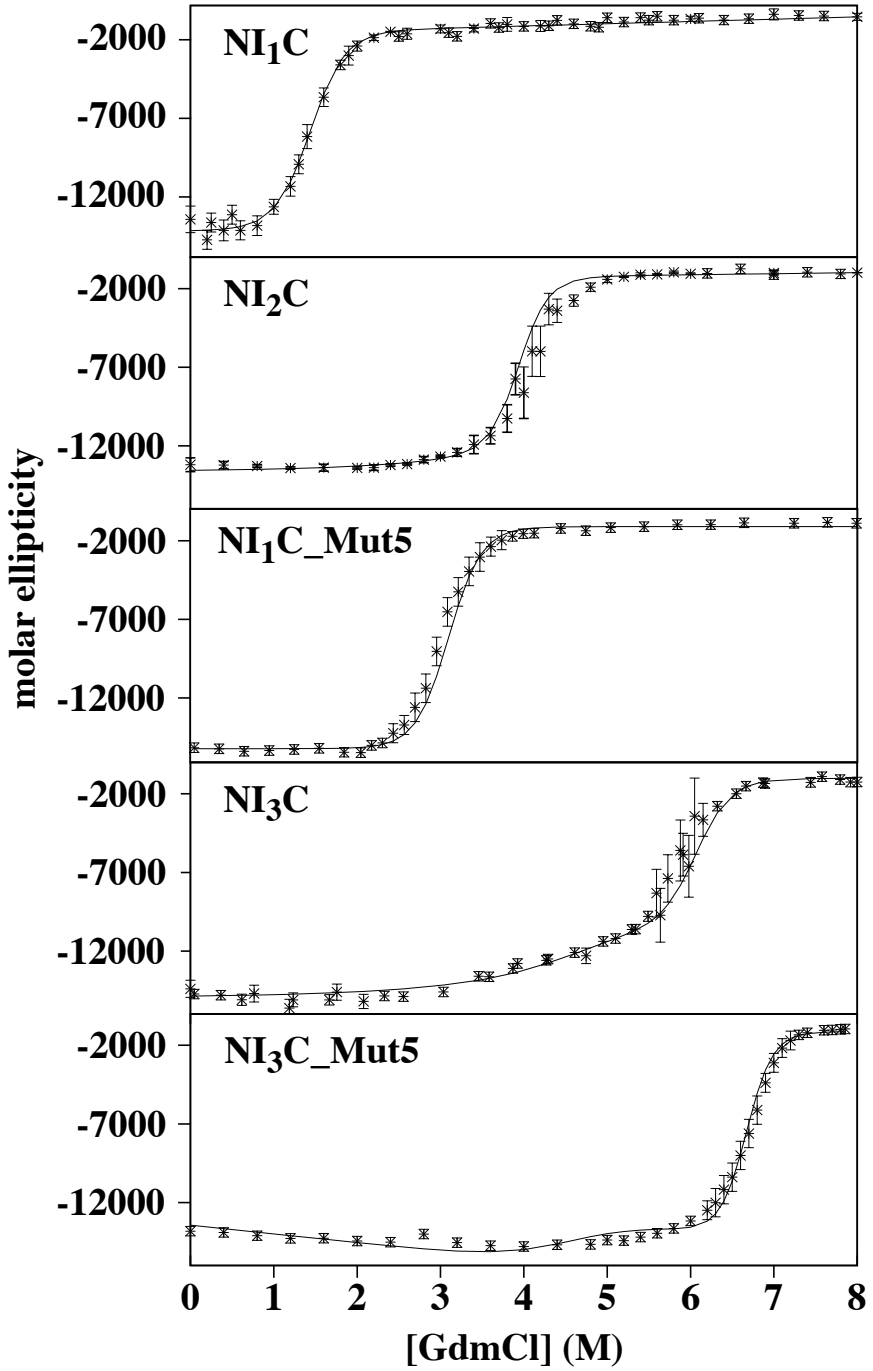
S12


(a)
N-cap
I-1
I-2
C-cap
15N- {1H} -NOE
residue number
(b)
N-cap
I-1
I-2
I-3
C-cap
15N- {1H} -NOE
residue number
(c)
N-cap
I-1
I-2
I-3
C-cap
15N- {1H} -NOE
residue number

S13

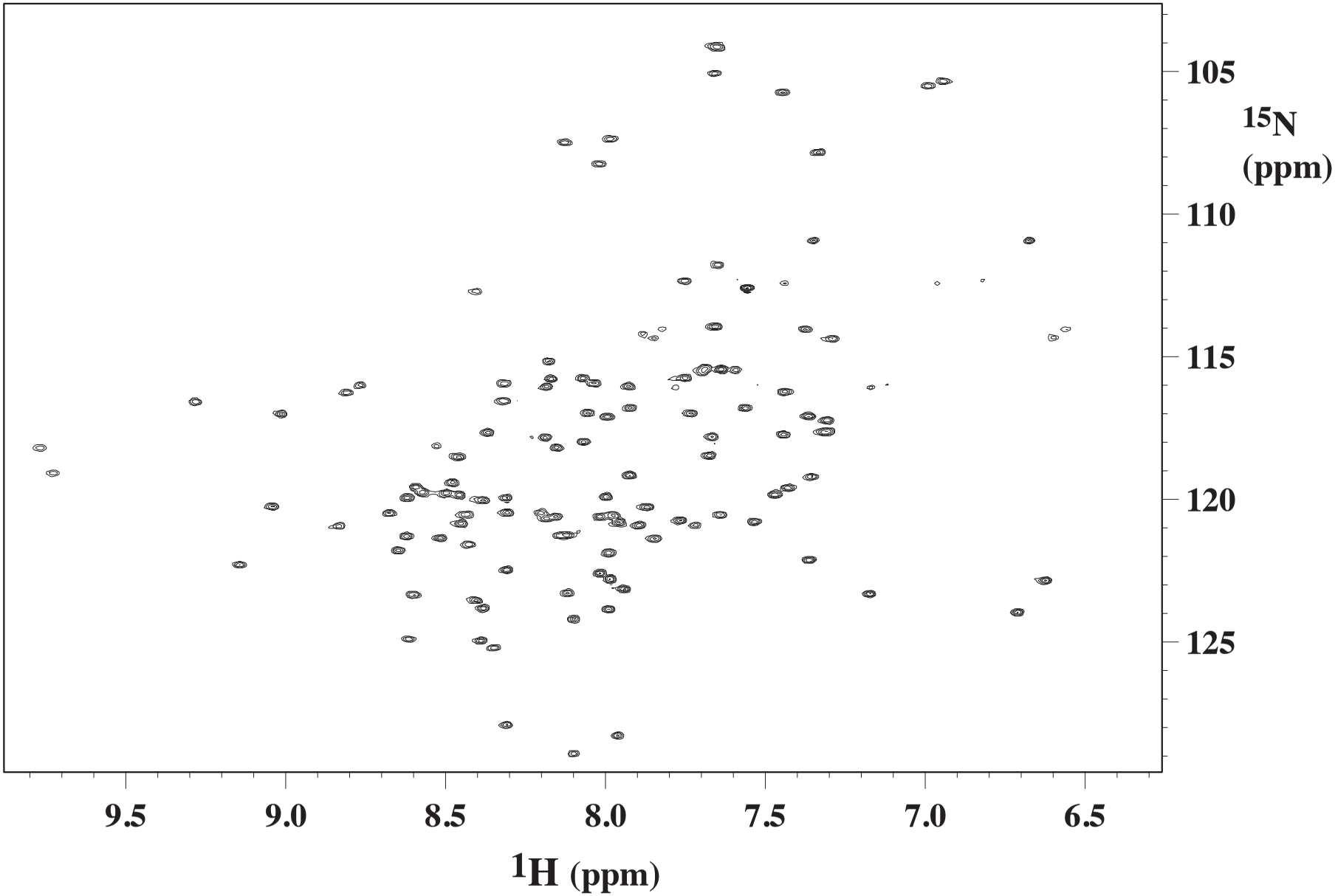

a)
I-1
I-2
I-3
unassign.
10^8
10^6
10^4
10^2
10^0
$1/k_{exp}$ at 310 K
20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
residue number
b)
I-1
I-2
I-3
unassign.
10^8
10^6
10^4
10^2
10^0
$1/k_{exp}$ at 310 K
20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
residue number

S14

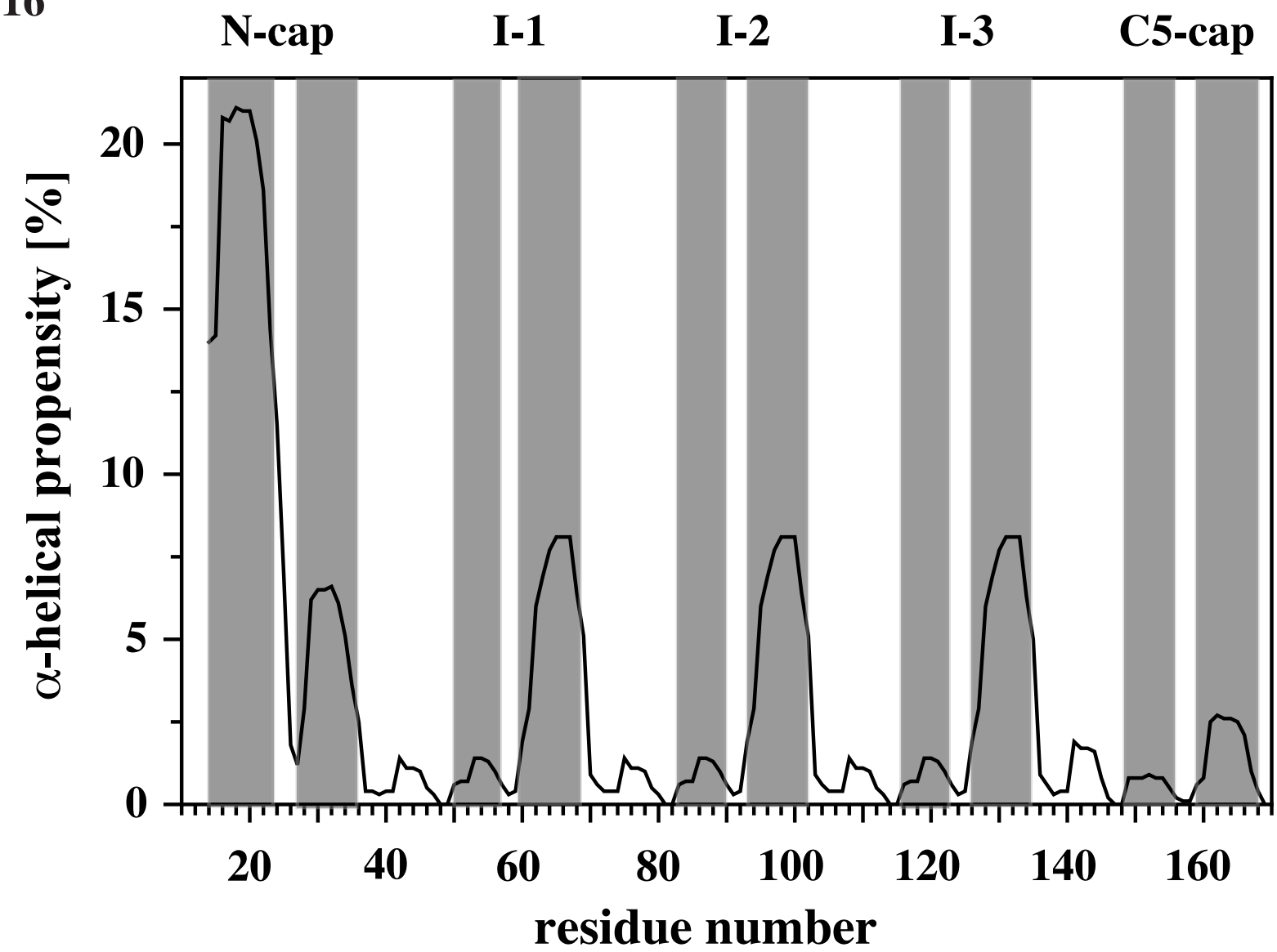

NI1C
NI2C
NI1C_Mut5
NI3C
NI3C_Mut5
-2000
-7000
-12000
molar ellipticity
0
1
2
3
4
5
6
7
8
[GdmCl] (M)

S15

[^{15}N,^{1}H]-HSQC of NI_3



S16


N-cap
I-1
I-2
I-3
C5-cap
α-helical propensity [%]
0
5
10
15
20
20
40
60
80
100
120
140
160
residue number