SUPPLEMENTAL MATERIALS

Ultrafast hydrogen exchange reveals specific structural events during the initial stages of folding of cytochrome *c*

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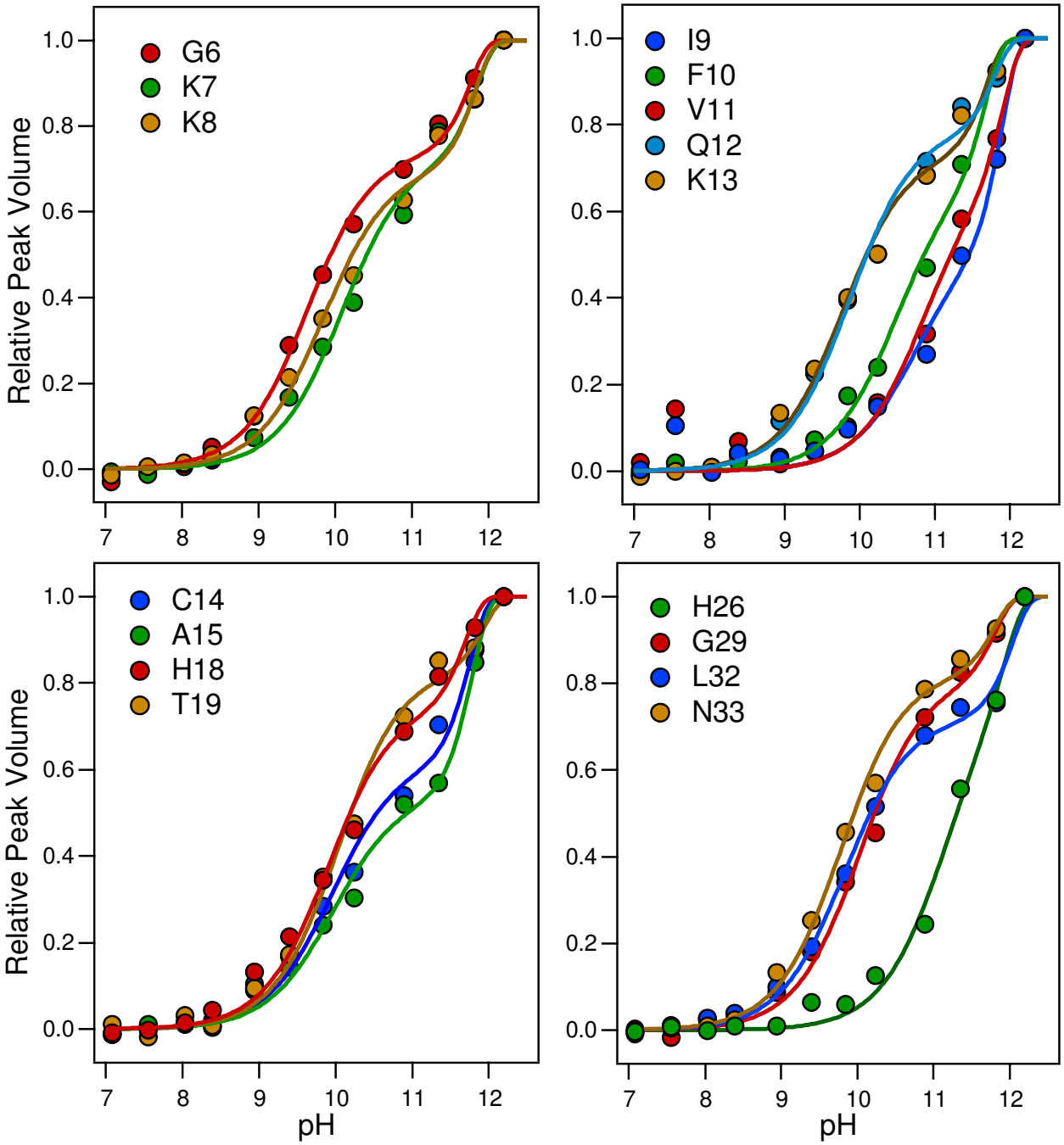
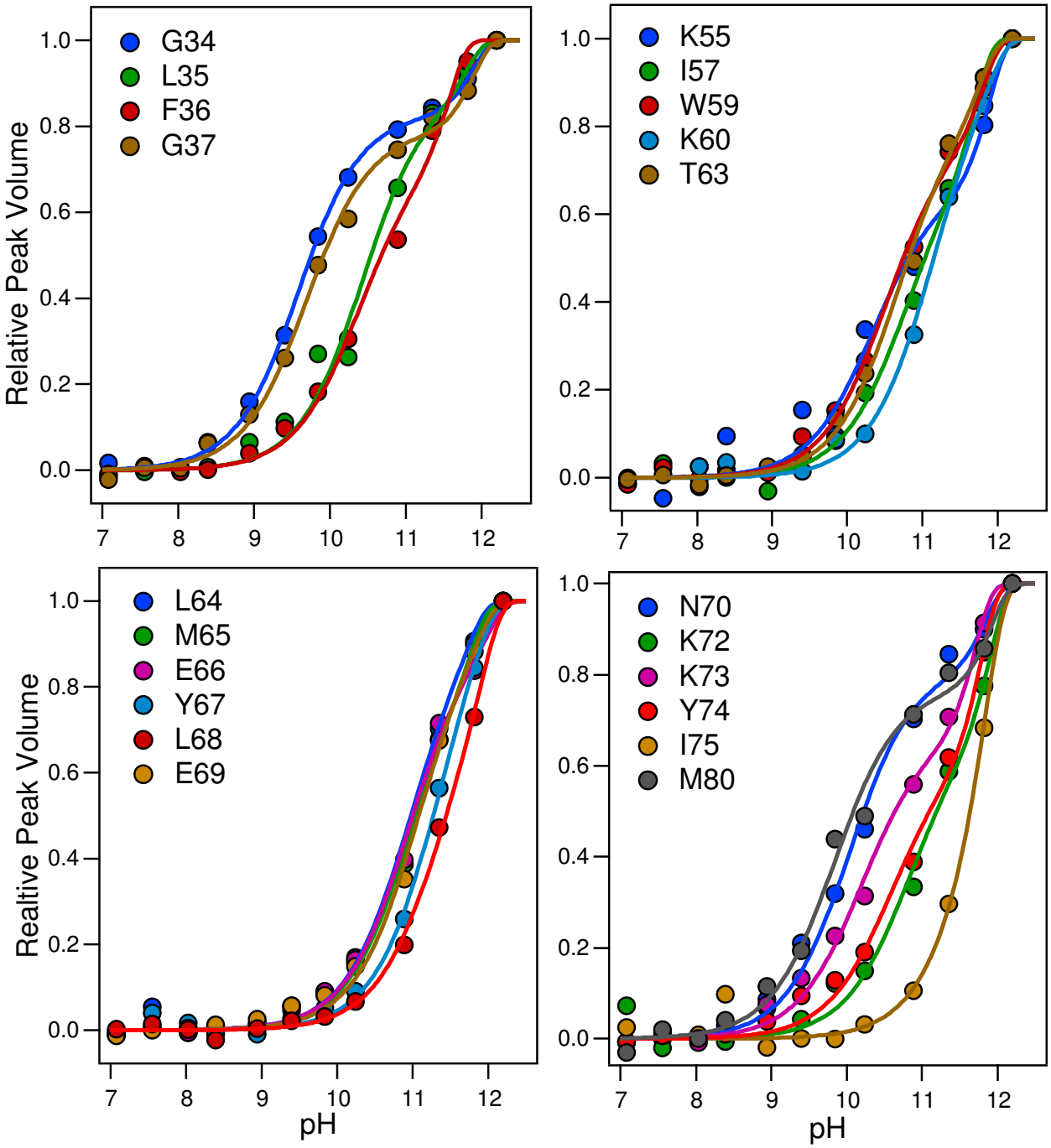
Figure S1. Competition between D-H exchange and folding at a competition time of 140 µs as a function of labeling pH (22 °C) for the complete set of amide protons in cyt *c* observable in 2D HSQC NMR spectra (panels **A** through **C**). Solid lines represent least-squares fits of Eq. 1.

A



B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L35
F36
G37
K55
I57
W59
K60
T63
L64
M65
E66
Y67
L68
E69
N70
K72
K73
Y74
I75
M80
Relative Peak Volume
Realtive Peak Volume
pH
pH

C

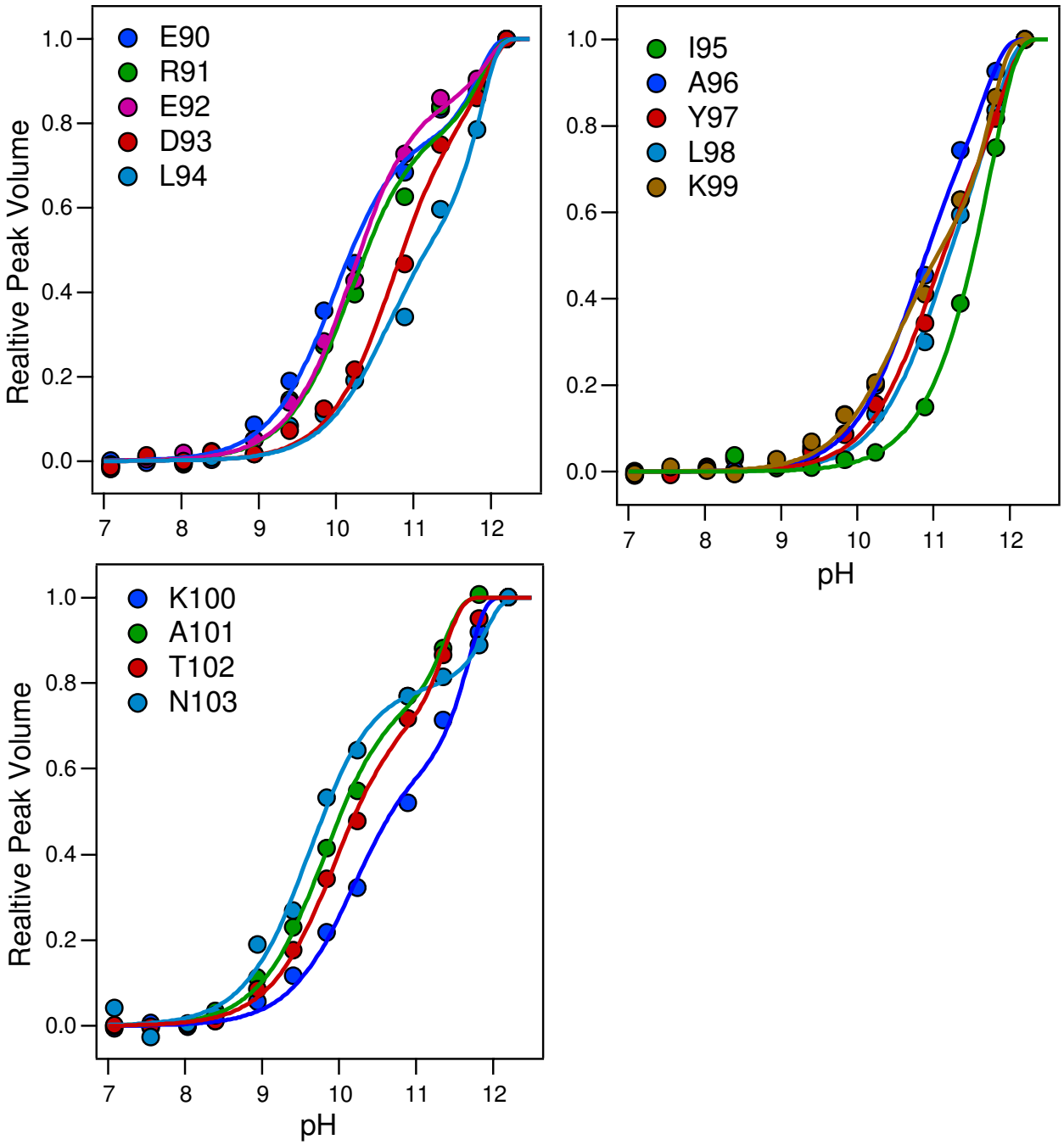

E90
R91
E92
D93
L94
I95
A96
Y97
L98
K99
K100
A101
T102
N103
Realtive Peak Volume
pH
1.0
0.8
0.6
0.4
0.2
0.0
7
8
9
10
11
12

Figure S2. **Folding kinetics of horse cyt *c* at alkaline pH.**

Panels **A** and **B** show kinetic traces for refolding of acid-unfolded cyt *c* (pH 2) at different pH values (4.9 to 12) measured by continuous-flow fluorescence at 22 ^{o}C, using the heme-induced quenching of Trp59 fluorescence as a probe to observe chain condensation. Double-exponential fits of the data in panel **A** yield rate constants of 2,700 ± 700 s^{-1} and 33,000 ± 4,000 s^{-1} with no systematic dependence on pH. The rate of the fast phase, which is dominant above pH 10 (panel **B**) increases slightly at alkaline pH and levels off at ~50,000 s^{-1} above pH 11. In panel **C** the amplitude of the initial collapse phase is plotted vs. pH over the range between 11 and 13 (circles). The loss in amplitude of this pH-independent process (τ = 20 μs) is attributed to alkaline unfolding of the *I*-state, which follows a cooperative transition with an apparent pK_a of 12.1 and a Hill coefficient of 2 (solid green line). The dashed blue line shows the pH-dependent rate constant for unfolding of the *I*-state calculated from the equilibrium constant K_{UI} and the rate constant k_{UI} = 50,000 s^{-1}.

At alkaline pH oxidized cytochrome c is known to undergo a conformational change from the native structure to a native-like alkaline state in which the Met80 heme ligand is replaced by a deprotonated lysine side chain (Lys72, 73 and 79 are the most likely candidates).[1-4] Depending on conditions the pK_a of the alkaline transition lies between 8.5 and 10.5. Our observation that the kinetics of folding is essentially independent of pH over the range from 7.8 to 10.8 indicates that any formation of a non-native lysine ligand has no measurable effect on early stages of folding.

(1) Theorell, H.; Akesson, A. J. Am. Chem. Soc. 1941, **63**, 1818.
(2) Pearce, L. L.; Gartner, A. L.; Smith, M.; Mauk, A. G. Biochemistry 1989, **28**, 3152.
(3) Pollock, W. B. R.; Rosell, F. I.; Twitchett, M. B.; Dumont, M. E.; Mauk, A. G. Biochemistry 1998, **37**, 6124.
(4) Assfalg, M.; Bertini, I.; Dolfi, A.; Turano, P.; Mauk, A. G.; Rosell, F. I.; Gray, H. B. J. Am. Chem. Soc. 2003, **125**, 2913.

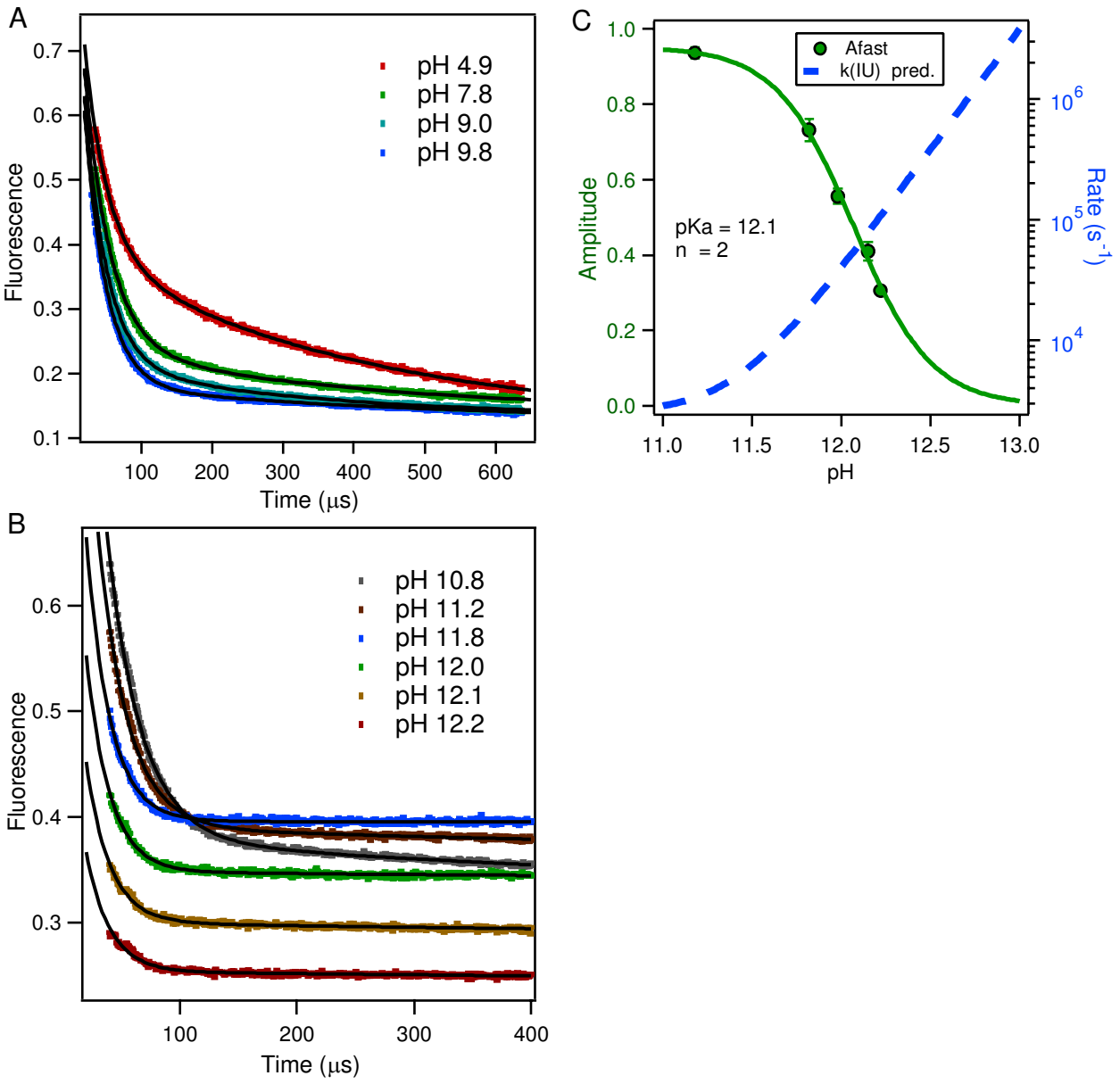
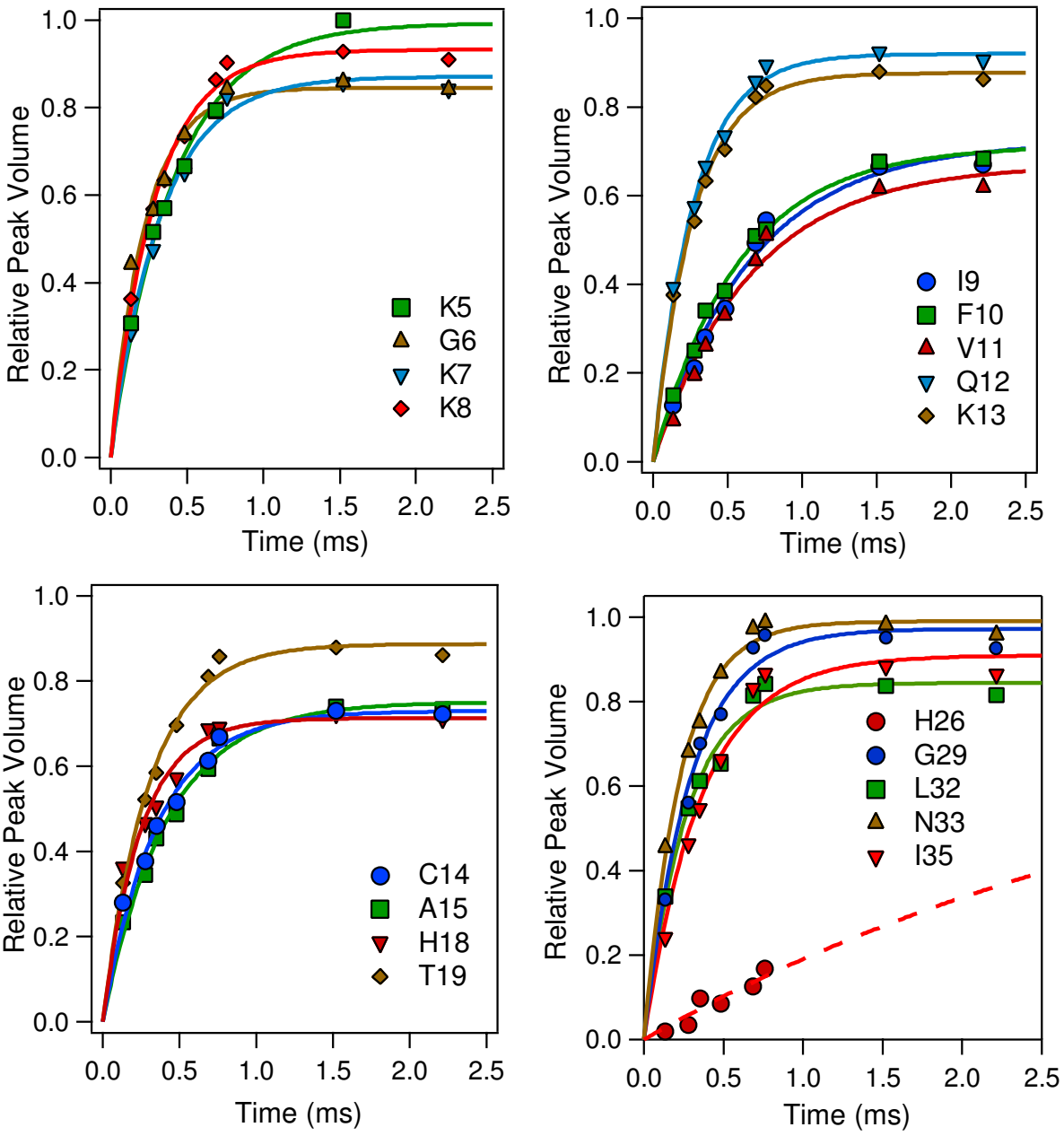
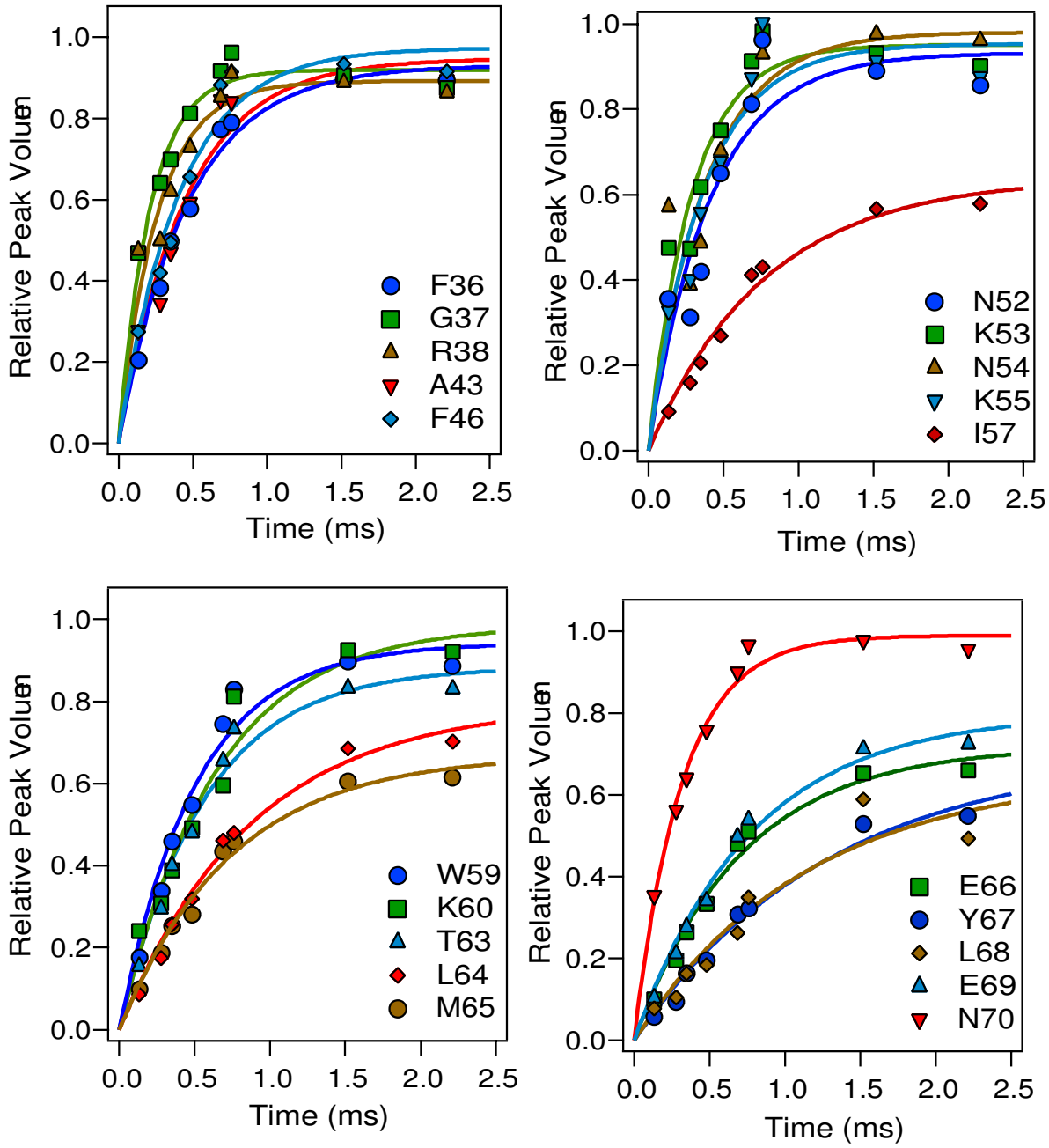

A
Fluorescence
Time (μs)
pH 4.9
pH 7.8
pH 9.0
pH 9.8
B
Fluorescence
Time (μs)
pH 10.8
pH 11.2
pH 11.8
pH 12.0
pH 12.1
pH 12.2
C
Amplitude
Rate (s-1)
pH
Afast
k(IU) pred.
pKa = 12.1
n = 2

Figure S3. Competition between D-H exchange and folding of cyt *c* as a function of competition time at pH 9.8, 22 °C, for the complete set of amide protons in cyt *c* observable in 2D HSQC NMR spectra (panels **A** through **C**). Solid lines represent least-squares fits of Eq. 2.

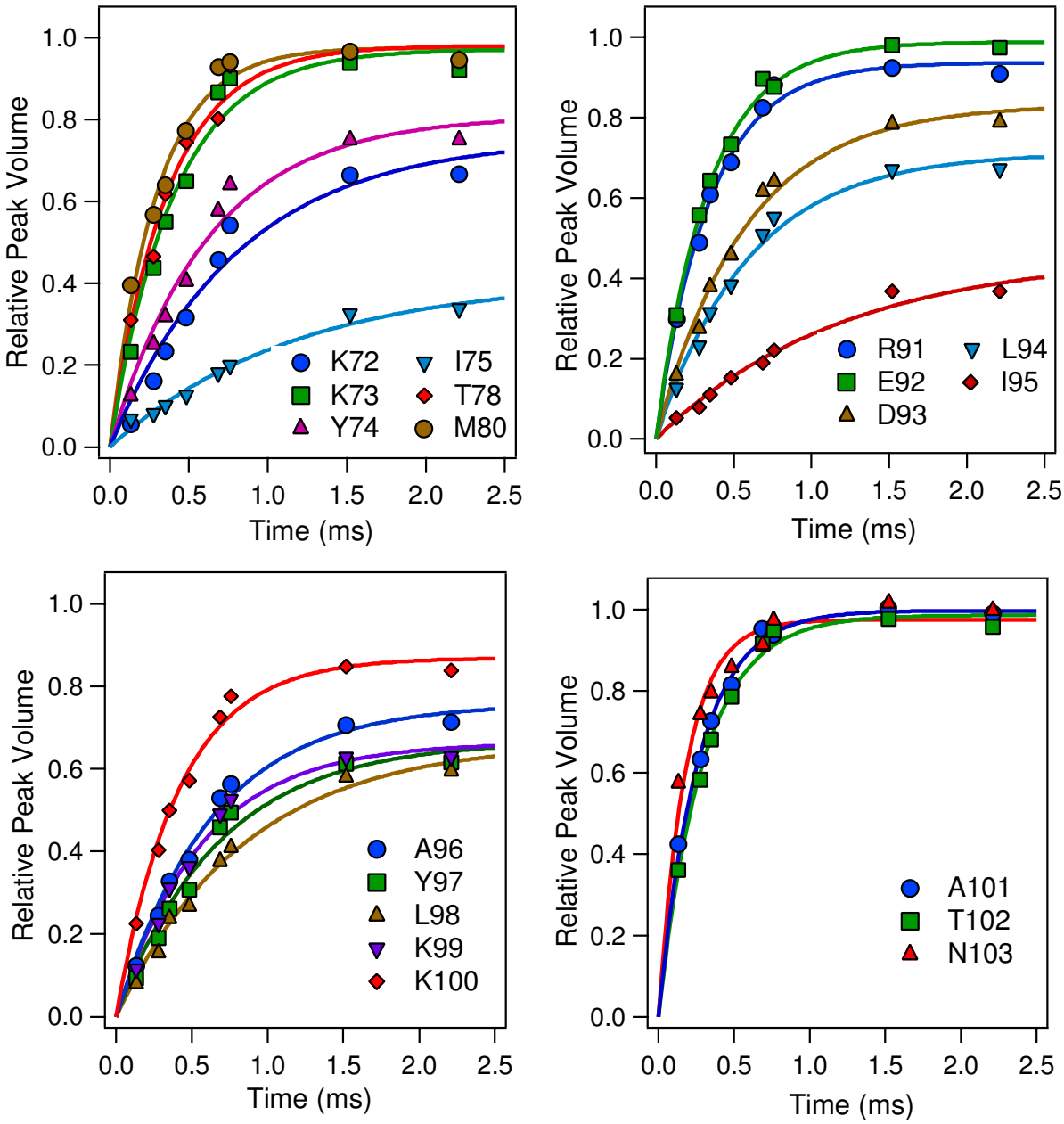
A



B


Relative Peak Volume
Time (ms)
F36
G37
R38
A43
F46
N52
K53
N54
K55
I57
W59
K60
T63
L64
M65
E66
Y67
L68
E69
N70

C


Relative Peak Volume
Time (ms)
K72
K73
Y74
I75
T78
M80
R91
E92
D93
L94
I95
A96
Y97
L98
K99
K100
A101
T102
N103