Selective Isotope Labeling Demonstrates That Hydrogen Exchange at Individual Peptide Amide Linkages Can Be Determined by Collision-Induced Dissociation Mass Spectrometry

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The rate at which hydrogens located at peptide amide linkages in proteins undergo isotopic exchange has become an important tool for detecting changes in the structures and dynamics of proteins. Hydrogen exchange has played a particularly important role in advancing our understanding of the mechanisms through which proteins fold.[1−4] Although mass spectrometry (MS) offers several significant advantages over nuclear magnetic resonance (NMR) for hydrogen-exchange measurements,[5] NMR has previously been the only practical method for determining deuterium levels at individual peptide linkages.

Collision-induced dissociation (CID) MS/MS is an obvious approach for determining deuterium levels at individual peptide linkages. However, its feasibility has not been established because the extent to which amide hydrogens undergo intramolecular scrambling has not been definitively determined. The facile exchange of these hydrogens in solution and the high energy required to cleave the backbones of peptide ions suggest that scrambling may occur during CID MS/MS. Anderegg et al., who used CID MS/MS to examine hydrogen exchange in helix-forming peptides, found higher levels of deuterium near the ends of the helices.[6] This result implied that CID MS/MS could be used to estimate deuterium levels at individual peptide amide linkages. However, other evidence suggests that amide hydrogens become scrambled during the fragmentation process.[7−9] The CID MS/MS approach for locating deuterium along peptide backbones may be validated using peptide standards displaying specific patterns of completely deuterated amide linkages. In the present study, such standards were prepared from selectively labeled cytochrome *c*, a protein for which exchange rates of all but the most rapidly exchanging amide hydrogens have been determined by NMR.[10] The general strategy used in the present study was to expose folded cytochrome *c* to D_2O for a specific time, fragment the protein into peptides with pepsin, and analyze the doubly charged fragments by CID MS/MS to determine the deuterium levels at individual peptide linkages. The deuterium levels found were compared with the deuterium levels expected, based on rate constants determined by NMR for isotopic exchange at these linkages.

Table 1. Deuterium Levels Found at Individual Peptide Amide Linkages in Five Peptic Fragments of Selectively Labeled Cytochrome *c* Analyzed by CID MS/MS[a]

Residues 1-10 (total D:4.3)

Residue	NMR	b	y"
D2	F	0.9	1.1
V3	F	0.9	⌉
E4	F	⌉	1.6
K5	F	2.0	⌉
G6	F	⌉	0.1
K7	S	0.3	1.1
K8	S	0.0	0.0
I9	S	0.1	⌉
F10	S	0.1	0.1
Sum		4.3	4.0

Residues 68-80 (total D:4.2)

Residue	NMR	b	y"
E69	S	0.1	0.0
N70	S	0.0	0.0
P71			
K72	F	0.9	?
K73	S	1.0	0.0
Y74	S	0.0	0.9
I75	S	0.0	0.0
P76			
G77	F	1.0	⌉
T78	F	1.1	2.2
K79	S	0.0	0.0
M80	S	0.0	0.0
Sum		4.1	3.1

Residues 95-104 (total D:2.1)

Residue	NMR	b	y"
A96	S	0.0	0.1
Y97	S	0.1	0.1
L98	S	0.0	0.0
K99	S	0.1	0.0
K100	S	0.0	0.0
A101	S	0.0	1.1
T102	S	0.1	0.0
N103	F	1.0	⌉
E104	F	1.0	1.1
Sum		2.3	2.4

Residues 22-36 (total D:6.3)

Residue	NMR	b	y"
G23	F	1.1	⌉
G24	F	0.8	0.4
K25	F	⌉	⌉
H26	F	1.9	1.0
K27	F	⌉	⌉
T28	F	⌉	2.7
G29	S	2.1	⌉
P30		⌉	1.1
N31	S	0.0	0.0
L32	S	0.0	?
H33	S	0.0	?
G34	F	0.0	⌉
L35	S	0.0	1.1
F36	S	0.0	⌉
Sum		5.9	6.3

Residues 83-94 (total D:4.3)

Residue	NMR	b	y"
G84	F	1.1	0.2
I85	S	0.0	0.0
K86	F	0.8	0.1
K87	F	1.3	0.9
K88	F	0.8	1.2
T89	F	0.3	⌉
E90	S	0.0	0.0
R91	S	0.0	⌉
E92	S	⌉	1.1
D93	S	0.0	⌉
L94	S	⌉	1.0
Sum		0.0	⌉
		4.3	4.5

[a] Hydrogen exchange at these amide linkage is indicated as fast (F) or slow (S), based on NMR analysis of cytochrome *c* selectively labeled in a similar manner.[8] The total deuterium in each peptide was determined in a separate MS analysis of the doubly charged ion.

Equine cytochrome *c* (2 mM in H_2O/phosphate buffer, 5 mM, pH 7.0) was diluted 20-fold into D_2O/phosphate buffer (5 mM, pD 7.0) and incubated for 10 s to selectively label peptide amide linkages. Following incubation, the samples were acidified to quench isotope exchange, digested with pepsin, and analyzed by LC−MS (2 nmole injected), as described previously.[5] Both MS and CID MS/MS analyses were performed with a Finnigan LCQ ion trap mass spectrometer. Analysis of peptic fragments from cytochrome *c* reference samples completely exchanged in D_2O indicated that only 10% of the amide deuterium was lost during digestion and HPLC and MS analysis when the HPLC flow rate was 100 μL/min and the temperature of the ESI capillary was 150 °C. Adjustments for deuterium loss during analysis were made as described previously.[11]

Results for five representative peptic fragments derived from selectively labeled cytochrome *c* are presented in Table 1.

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Hydrogen exchange at each of these peptide amide linkages is designated as fast (F) or slow (S), depending on whether the exchange rate constant was sufficiently slow ($k < 30$ h^{-1}) to be determined by NMR. For the 10 s exposure to D_2O used in this study, the deuterium levels at individual amide linkages labeled S are expected to be less than 0.08. Deuterium levels expected for amide linkages labeled F cannot be predicted because exchange at these linkages was too fast to be measured by NMR. The deuterium levels at individual peptide amide linkages, determined from differences in the mass-to-charge (*m*/*z*) of CID fragments, are presented in columns labeled b and y″. Nearly all of the ions comprising the b and y″ series of CID fragment ions of these peptides were sufficiently intense for accurate determination of their *m*/*z*. Brackets indicate regions in which one or two fragment ions were not found. For example, the bracket in the b-series of the peptic fragment including residues E4−G6 indicates that there is a total of 2.0 deuteriums in these three peptide linkages. Since labile deuterium located in the side chains was replaced with protium during HPLC, the deuterium detected by ESIMS was located at peptide amide linkages.[12]

Results for the peptic fragment including residues 83−94 clearly demonstrate that deuterium levels at individual peptide amide linkages can be determined from the *m*/*z* of the b ions. All of the linkages designated S were found by CID MS/MS to have less than 0.1 deuterium. Since MS analysis of the intact peptide showed that this fragment contained 4.3 ± 0.1 deuteriums, it follows that intramolecular migration of deuterium from deuterated linkages to nondeuterated linkages was negligible. The sum of deuterium levels at each amide linkage also indicated that this fragment contained a total of 4.3 ± 0.1 deuterium, thus satisfying the requirement for internal consistency. Although similar analysis of the y″ ions often yields deuterium levels consistent with levels derived from b ions and NMR, there are several discrepancies, suggesting that H/D transfers occur during CID fragmentation to give y″ ions. These fragmentation processes have been discussed previously.[6,13,14] Such scrambling precludes use of the y″ ions for hydrogen-exchange studies until their mechanisms of formation are understood better. The ability to prepare peptide reference standards with specific amide linkages labeled with deuterium, as described in the present study, will facilitate more detailed studies of these and related peptide fragmentation mechanisms.

Similar excellent correlation between deuterium levels expected, based on NMR, and deuterium levels found in b ions was found for the other four peptic fragments of cytochrome *c*. The only significant difference in deuterium levels found at S linkages by CID MS/MS and NMR is at Lys 73. Since the exchange rate at this residue is at the upper limit that could be measured for the NMR experimental conditions and since the NMR cross-peak for this residue was unresolved from that for Lys 8, the uncertainty in the exchange rate at Lys 73 measured by NMR may be larger than expected.[15] Although misassignment of peaks in the CID MS/MS spectrum is also possible, it would not explain this discrepancy since the total deuterium in the entire peptic fragment was 4.2 ± 0.1 and the total number of fast exchanging linkages found by NMR was only 3. It follows that exchange at one of the linkages must be considerably faster than that indicated by NMR.

While MS and NMR are complementary for measuring hydrogen-exchange rates, MS has definite advantages in sensitivity, applications to large proteins of moderate solubility, measuring exchange of the most rapidly exchanging amide hydrogens, and detection of correlated hydrogen exchange.[5] Results of the present study show that the extent of hydrogen exchange at peptide amide linkages in proteins can be determined reliably from the b-series of CID fragment ions. The present results link the advantages of mass spectrometry with the ability to detect highly localized structural changes in proteins through changes in isotope exchange rates at individual peptide amide linkages.

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Supporting Information Available: Figures and additional experimental data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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