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Accelerated Articles

Measurement of Amide Hydrogen Exchange by MALDI-TOF Mass Spectrometry

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Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) was used to determine amide proton/deuteron (H/D) exchange rates. The method has broad application to the study of protein conformation and folding and to the study of protein−ligand interactions and requires no modifications of the instrument. Amide protons were allowed to exchange with deuterons in buffered D_2O at room temperature, pD 7.25. Exchanged deuterons were "frozen" in the exchanged state by quenching at pH 2.5, 0 °C and analyzed by MALDI-TOF MS. The matrix mixture consisted of 5 mg/mL α-cyano-4-hydroxycinnamic acid, acetonitrile, ethanol, and 0.1% TFA. The matrix was adjusted to pH 2.5, and the chilled MALDI target was rapidly dried. Deuteration of amide protons on cyclic AMP-dependent protein kinase was measured after short times of incubation in deuterium by pepsin protein digestion and MALDI-TOF MS analysis. The unseparated peptic digest was analyzed in a single spectrum of the mixture. From five spectra, H/D exchange rates were determined for some 40 peptides covering 65% of the protein sequence.

The idea that amide proton exchange rates could be an indicator of protein structure dates back to the 1950s.[1−3] Early on it was noted that side-chain protons exchange very rapidly, but protons located on peptide amide linkages have rates of exchange that are slow enough to be measured. Studies on unstructured peptides have revealed that the rate constant for proton exchange, k_{ex}, depends on the concentration of acid and base according to eq 1, where for polyalanine $k_H = 41.7\ M^{-1}\ min^{-1}$,

$$k_{ex} = k_H[H^+] + k_{OH}[OH^-] + k_w \qquad (1)$$

$k_{OH} = 1.12 \times 10^{10}\ M^{-1}\ min^{-1}$, and $k_w = 3.16 \times 10^{-2}\ min^{-1}$ at 20 °C.[4] The rate of amide proton/deuteron (H/D) exchange is highly temperature and pH dependent and is 4 orders of magnitude greater at pH 7 than at pH 2.5. At 0 °C, the half-life at the pH minimum of 2.5 ranges from 30 to 120 min.[5] Exchange rates can be measured by allowing amide protons to exchange with deuterated or tritiated H_2O at physiological temperature and pH and then quenching into cold, pH 2−3 buffer, decreasing the exchange rate by 2−3 orders of magnitude, allowing time for measurement of the amount of isotopic exchange. Advances by Englander's and Richards' groups[5−8] together with recent work using NMR and ESI MS by several groups have recently been reviewed.[9,10]

Matrix-assisted laser desorption/ionization (MALDI) coupled with time-of-flight (TOF) mass analysis is a widely used method for mass analysis of biological molecules.[11,12] Recently, attempts

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have been made to study protein folding and conformational changes by MALDI-TOF MS.[13,14] Yet MALDI-TOF has not previously been used to follow amide H/D exchange. In principle, one should be able to determine the deuterium content of a large number of peptides generated by proteolytic cleavage of a target protein from a single mass spectrum of the unseparated digest mixture. This would eliminate the HPLC separation step often required when ESI MS is used to analyze the peptides. The sensitivity of MALDI-TOF for the detection of peptides is subpicomolar, which should permit studies of proteins that are only available in limited quantities. Recent developments in MALDI-TOF instrumentation have resulted in improved mass resolution to greater than 10 000 (fwhm) over a wide mass range and mass measurement accuracy to 5 ppm or less with internal standards for peptides.[15−19] These two features make the method well suited for the measurements described here: high resolution to easily resolve the multiple isotopic peaks resulting from amide H/D exchange experiments and high mass accuracy to aid in identifying peptides resulting from the relatively nonspecific cleavage[20] by pepsin. Nevertheless, important questions remain. Is it possible to retain the deuterium content of the peptides after mixing them with a matrix solution and allowing the mixture to crystallize on the MALDI target? Once the sample is dried, how stable are the hydrogens to exchange? Can enough peptides be detected in a single mass spectrum to accurately characterize a protein−protein or protein−ligand binding site?

The data presented here answer these questions. Our results demonstrate that, with minor modifications of established MALDI-TOF sample preparation and analysis protocols, H/D exchange experiments can be easily performed. By adjusting the pH of the MALDI matrix solution to the pH minimum for amide H/D exchange, and by chilled, rapid drying of the samples on the MALDI target, minimal loss of deuterons from amide groups on peptides can be achieved. These experiments give kinetic information comparable to that obtained using ESI MS but are easier to carry out.

MATERIALS AND METHODS

Reagents. Immobilized pepsin (on cross-linked 6% agarose, 2−3 mg of pepsin/mL of gel) was obtained from Pierce Chemicals (Rockford, IL). Deuterium oxide (99.996%) was purchased from Cambridge Isotope Laboratories (Andover, MA). The matrix compound, α-cyano-4-hydroxycinnamic acid, was obtained from Aldrich Chemicals (Milwaukee, WI), and was recrystallized once from ethanol. Angiotensin I and ATP disodium salt were purchased from Sigma (St. Louis, MO). Trifluoroacetic acid (TFA; peptide synthesis grade), acetonitrile (HPLC grade), KH_2PO_4, $MgCl_2 \cdot 6H_2O$, NH_4OH, and SPECTRA/POR dialysis tubing (12 000−14 000 MW cutoff, 1.6-cm diameter) were obtained from Fisher Scientific (Pittsburgh, PA). Polypropylene 0.5-mL microcentrifuge tubes and thin-walled PCR tubes (0.5 mL) were purchased from USA/Scientific (Ocala, FL). ColorpHast pH indicator strips, pH 0−14, were obtained from EM Science (Gibbstown, NJ).

Proteins. Angiotensin I (AT), sequence DRVYIHPFHL, MH^+ (monoisotopic) mass 1296.7, was dissolved in H_2O. Portions (750 pmol) were placed into 0.5-mL microcentrifuge tubes and lyophilized. Upon resuspension in 12 μL of H_2O, the pH was approximately 7.0, as determined by pH paper.

The catalytic subunit of the cyclic AMP-dependent protein kinase (PKA) was prepared as outlined by Yonemoto et al.[21] The protein concentration was determined by A_{280}, and the concentration was adjusted to 0.3 mg/mL in 150 mM KCl, 20 mM KH_2PO_4, pH 6.85. The KCl in the protein buffer was reduced from 150 to 100, to 50 mM, and finally to 25 mM by stepwise dialysis at 4 °C. During the dialysis, the KH_2PO_4 concentration was also reduced from 20 to 15, to 10 mM, and finally to 5 mM. The resulting 11.5 mL of PKA was portioned into 0.5-mL microcentrifuge tubes each containing 750 pmol of PKA. The samples were lyophilized to dryness and stored at −20 °C. Lyophilized protein retained 100% of activity as shown by a PKA activity assay.[22] Upon addition of 12 μL of H_2O, the buffer was 50 mM KCl, 40 mM KH_2PO_4, pH 6.85, and the protein concentration was 62.5 μM.

Angiotensin I Control Experiments. Determination of the number of deuterons retained on a peptide was carried out by dissolving lyophilized AT (750 pmol/tube) in 12 μL of H_2O and adding 120 μL of 0 °C 0.1% TFA, pH 2.5. Fully deuterated samples consisted of 750 pmol of AT dissolved in 12 μL of D_2O, incubated at room temperature for 10 min, and then quenched by the addition of 120 μL of 0 °C 0.1% TFA (pH 2.5). For optimal signal-to-noise ratios, each sample was diluted 4-fold (10 μL of sample added to 30 μL of 0 °C 0.1% TFA, pH 2.5). The undiluted and diluted samples were frozen in liquid N_2 and stored at −40 °C until analysis. Some fully deuterated samples were exchanged back into H_2O by the addition of 120 μL of H_2O and incubation at 25 °C for 1−5 min before quenching by transfer into thin-walled Eppendorf tubes on ice containing a predetermined amount of 1.0% TFA, which lowered the pH of the mixture to 2.5.

Preparation of Samples for MALDI-TOF MS. The matrix used in these experiments was 5 mg/mL α-cyano-4-hydroxycinnamic acid, in a solution containing 1:1:1 acetonitrile, ethanol, and 0.1% TFA (pH 2.5). The resulting matrix was adjusted to pH 2.5 with 2% TFA, as determined by matching the color of the spot on pH paper to that of 0.1% TFA, pH 2.5. The matrix was incubated on ice and mixed by vortexing before use.

The MALDI targets were chilled on ice in a plastic case to prevent condensation of atmospheric H_2O prior to use. Frozen samples were quickly defrosted to 0 °C. Small portions, typically 5 μL, were mixed with an equal volume of matrix solution, and 1 μL was spotted onto the chilled MALDI target. The target was immediately placed in a desiccator under a moderate vacuum such that the spots would dry in 1−2 min. Slow drying in moderate vacuum was found to improve sample analysis, presumably

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because of improved crystal growth. The chilled, dried plate was transferred as quickly as possible from the desiccator to the mass spectrometer.

Protein Fragmentation by Pepsin. Pepsin digestion was carried out with immobilized pepsin at an enzyme to substrate ratio of 1:1. It was found that complete digestion of PKA occurred within 10 min at 0 °C and pH 2.5 (data not shown). Immobilized pepsin slurry (25 μL) was washed twice in a 1.5-mL Eppendorf tube with 1 mL of chilled 0.1% TFA by vortexing and subsequent centrifugation for 2 min at 7000*g* and stored on ice. Quenched protein samples (132 μL) were added to the pepsin and digested for 10 min on ice with occasional mixing. The immobilized pepsin was removed by centrifugation for 1 min at 14000*g* in a chilled Eppendorf centrifuge, and the supernatant was transferred to a 0.5-mL thin-walled microcentrifuge tube. Samples were immediately frozen in liquid N_2. Samples were stored at −40 °C for up to several hours before analysis. Samples were quenched, digested, and frozen in under 15 min.

MALDI-TOF MS. Mass spectra were acquired on a PerSeptive Biosystems Voyager DE STR. Data were acquired at a 2-GHz sampling rate, 250 000 data channels, with a 20 000-V accelerating voltage, 70% grid voltage, and 0.01% guide wire voltage and using delayed extraction with a 100-ns pulse delay. Typically, 256 scans were averaged in approximately 3 min. All reported masses are monoisotopic MH^+ masses unless otherwise noted.

Identification of Peptic Fragments of PKA. Cleavage of PKA by pepsin was found to be highly reproducible, although pepsin is a relatively nonspecific protease and cleavage sites cannot always be predicted. Accurate mass measurements in combination with sequence analysis were used to identify the individual peptides from PKA generated by pepsin cleavage. Exact masses were determined from a pepsin-digested PKA sample to which were added three internal standards, AT (mass 1296.6853), ACTH (18−39) (mass 2465.1989) and ACTH (1−17) (mass 2093.0867). Calibration was performed using a two-point calibration with the AT and ACTH (18−39) standards (PerSeptive GRAMS/ 386 software, version 3.04, Galactic Industries, Inc.). The ACTH (1−17) standard appeared to overlap with a peptide from the PKA digest and was not used in the calibration. In cases where mass searching gave more than one possibility within a 20 ppm window, peptides were identified by postsource decay (PSD) sequencing[23] or ladder sequencing with carboxypeptidase Y.[24] To be able to perform C-terminal ladder sequencing and because PSD is sometimes less effective if the ion of interest is present in a complex mixture, a separate, unlabeled peptic digest of PKA was prepared. The proteolytic peptides were separated by reversed-phase HPLC in 0.1% TFA, using a 45-min 0−50% gradient of acetonitrile on a Vydac C_{18} column (4.6 × 250 mm). Sequence analysis was then performed on the peptides contained in the collected fractions as needed.

Quantitation of the Number of Deuterons Retained on Each Peptic Fragment. The average number of deuterons present on each peptic peptide was determined by calculating the centroid of each isotopic peak cluster.[25] Briefly, spectra were calibrated according to known masses of two peptides internal to the mixture. After a two-point baseline correction had been applied, centroids were computed by integrating from the left-most edge of the monoisotopic peak to the right-most edge of the highest-mass observable isotopic peak in the envelope.[26] A program that applies this calculation to data in the Galactic SPC file format and that runs under Windows 95/NT (Microsoft, Redmond, WA) can be obtained from the authors.

Measurement of the Rate of Exchange of Deuterons onto Rapidly Exchangeable Amide Groups of PKA. Experiments were performed to demonstrate the measurement of H/D exchange rates of deuterons (from D_2O) into the amide positions of PKA. Lyophilized, buffered PKA (750 pmol) was dissolved in 12 μL of D_2O solution containing 10 mM ATP, 50 mM $MgCl_2$, pD 7.25 (all reported pD's were calculated by addition of 0.4 to the measured pH). The solution was incubated at room temperature for variable time periods ranging up to 10 min and then immediately quenched by the addition of 120 μL of 0 °C 0.1% TFA, pH 2.5, digested with pepsin, quick-frozen in liquid N_2, and stored at −40 °C. All of the samples for each kinetic experiment were defrosted at the same time, loaded on the MALDI target, and analyzed. The number of deuterons incorporated (D) was determined by taking the difference between the centroid of the isotopic peak cluster for the deuterated sample and the centroid of the undeuterated control and plotting it against time. The rate of on-exchange was determined by fitting the data to eq 2, where

$$D = B_{max}(1 - e^{-kt}) \quad (2)$$

D represents the number of deuterons on the peptide at any time and B_{max} represents the value at which the number of deuterons plateaus.

Measurement of the Rate of Back Exchange of Deuterons in the Mass Spectrometer. For highly deuterated peptides, the number of deuterons present on the peptide was found to decay slowly in the mass spectrometer during analysis despite the fact that the sample was completely dry and under high vacuum ($<10^{-6}$ Torr). To ascertain the rate constant for this decay process, experiments were performed on samples of PKA that had been deuterated for 10 min and then digested with pepsin so that several different peptides could be simultaneously studied. Several mass spectra of the PKA digest were collected at different times ranging from 2 to 70 min. Peptides in the digest were analyzed to determine an equation that best described the decay of the number of deuterons (D) over time in the mass spectrometer. For each peptide ion, the centroid of the undeuterated peptide mass envelope was subtracted from the centroid of the deuterated peptide mass envelope to determine D at each time point. The change in D over time was found to fit a single-exponential curve that decayed to a value (B_1) greater than zero (eq 3). The initial number of deuterons retained by the peptide

$$D = B_1 + B_2 e^{-kt} \quad (3)$$

($B_1 + B_2$) varied depending on the peptide sequence.

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Correction for Deuteron Back Exchange in the Mass Spectrometer. The decay of the number of deuterons present on each peptide once the sample was in the mass spectrometer depended solely on the length of time before analysis. This turned out to be important because typically four samples were simultaneously placed on the MALDI target and dried; approximately 2 min elapsed before the first sample could be analyzed and each analysis took from 3 to 5 min. To correct for the loss of deuteration during the time before a sample was analyzed, eq 4

$$D_{\text{corr}} = B_2[e^{-kt_{\text{corr}}} - e^{-kt_{\text{meas}}}] + D_{\text{meas}} \qquad (4)$$

was used to determine the number of deuterons that would have been present on the peptide (D_{corr}) at any earlier analysis time (t_{corr}), usually $t = 0$. Equation 4 incorporates the rate constant for decay as well as the number of deuterons that are susceptible to loss, B_2, that were determined from the control experiments and are unique to each peptide in the mixture (eq 3). The constant B_1 from eq 3 is incorporated into the measured number of deuterons, D_{meas}, which would be equal to B_1 at long measurement times and will always be larger than B_1 at short measurement times.

RESULTS AND DISCUSSION

Demonstration of Retention of Amide Deuterons under MALDI Analysis Conditions. Control experiments were performed to optimize the sample preparation conditions for observation of deuterated amides in peptides. Initially, conditions were devised to observe deuteration of AT, a 10-amino acid peptide with no secondary structure. The calculated centroid of the isotopic peak cluster for undeuterated AT, which represents the average mass, was 1297.497, which agrees well with the theoretical value of 1297.510. The centroid of the fully deuterated AT was 1301.068, a gain of 3.57 deuterons on average. AT is expected to have eight amide groups that could retain deuterons, since the proline and the N-terminus are not expected to retain deuterium. However, complete deuteration did not shift the centroid the full 8 expected mass units. Incubation of AT for longer times in buffered D_2O did not result in any further increases in the observed deuteration. The value of the centroid represents 45% of the maximum that would be expected for full deuteration. This observed amount of deuteration is similar to that observed in ESI MS studies[27] and is less than 100% due to exchange that occurs under quench conditions.[4] After 1 or 5 min of off-exchange by 11-fold dilution into H_2O at 25 °C, the centroids decreased to 1298.036 and 1298.087, respectively, 0.55 greater than the centroid of the undeuterated AT (data not shown). The remaining 0.55 deuteron represents an increase in the centroid of 7% over the undeuterated control, which is again somewhat less than the 9% residual deuteration expected to remain after the 11-fold dilution. The difference between a fully deuterated sample and a sample that has been off-exchanged by dilution into H_2O represents the "window" within which the variation in the amount of deuteration may be measured.

H/D Exchange Analysis of the Total Protein Sequence in a Single Spectrum. One of the advantages of MALDI-TOF MS is that with high resolution, a mixture of peptides can be easily analyzed because the MH^+ ions are usually the only ions observed for each peptic fragment. The MALDI mass spectrum of the entire peptic digest of PKA showed approximately 60 peaks, each of which had baseline resolution between adjacent peaks in the mass envelope (Figure 1). Using a combination of PSD and C-terminal ladder sequencing, as well as accurate mass determinations, 42 peaks in the spectrum were identified (Table 1). In some cases, the identification required use of a PKA digest that had been HPLC-separated. The identification of each peptide was relatively labor-intensive, but fortunately pepsin cleavage, although not completely predictable a priori, is highly reproducible. Thus, an important advantage of the MALDI-TOF method described here is that the HPLC separation need only be performed once on an undeuterated digest in order to obtain the separated peptides for identification. Once this is accomplished, kinetic information and H/D exchange information can all be obtained from mass spectra of the unseparated mixture of peptides.

After determining that a good number of peptic peptides were observed in a single spectrum of digested, unlabeled PKA, it was important to demonstrate that deuterated PKA could be digested and subsequently the amount of deuteration of each peptic peptide could be determined from the mass spectrum of the complex mixture. An expansion of the mass spectrum showing only the isotopic peak cluster corresponding to the peptide with mass

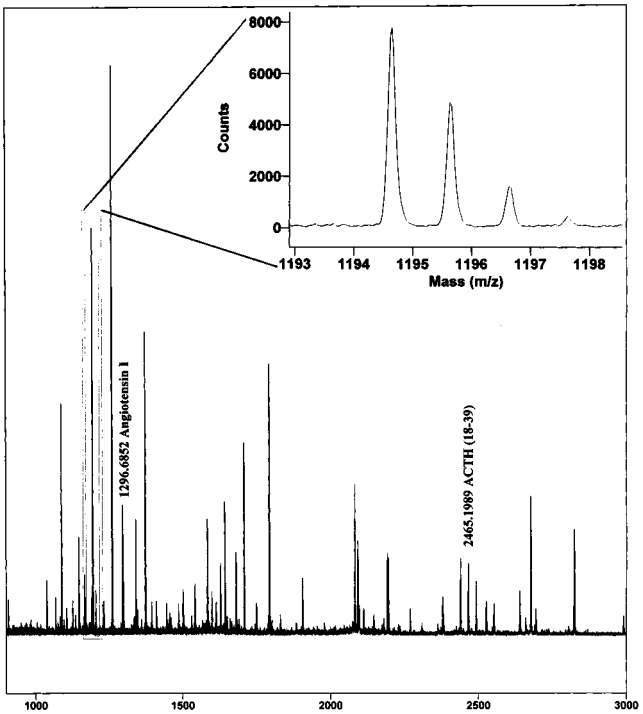

Figure 1. MALDI-TOF mass spectrum of the mixture of peptides obtained from pepsin digestion of undeuterated PKA. The PKA (750 pmol) was digested for 10 min using a 1:1 ratio of immobilized pepsin in a final volume of 132 μL of 0.1% TFA. For this and all subsequent samples, 0.7 pmol of PKA digest was spotted on the MALDI target. Three internal standards, AT (mass 1296.6853), ACTH (1−17) (mass 2093.0867), and ACTH (18−39) (mass 2465.1989) were added to the mixture before analysis; however, only the two that were used for calibration (AT and ACTH (18−39)) are labeled. The inset is an expansion of the region of the spectrum showing the isotopic peaks of the peptide of mass 1194.6463.

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Table 1. Identification of Peaks Observed in the MALDI Spectrum of the Digest Mixture after Treatment of PKA with Pepsin

mass (MH^+) (theor)[a]	mass (MH^+)[b]	fraction no.[c]	position	sequence	method of identification[d]
	1038.4074	39	231−239	K^+ adduct of MAAGYPPFF	PSD
1068.5731	1068.5621	20	18−26	FLAKAKEDF	PSD
1083.6528	1083.6476	34	268−276	LLRNLLQVD	C-term
1088.6582	1088.6507	30, 31	92−100	KRILQAVNF	PSD
	1126.6037	not found	92−100	K^+ adduct of KRILQAVNF	
1147.6113	1147.6052	12	164−172	IYRDLKPEN	PSD
1167.5799	1167.5746	19	212−221	SKGYNKAVDW	PSD
1190.6899	1190.6859	17	303−312	IAIYQRKVEA	PSD
1194.6485	1194.6463	23	44−54	DRIKTLGTGSF	PSD, C-term
	1205.5219	19	212−221	K^+ adduct of SKGYNKAVDW	
	1232.5989	not found	44−54	K^+ adduct of DRIKTLGTGSF	
1260.6954	1260.6933	21	163−172	IYRDLKPENL	PSD
1341.7169	1341.7118	29	43−54	FDRIKTLGTGSF	exact mass
1347.7499	1347.7647	14	278−289	TKRFGNLKNGVN	PSD
1373.7795	1373.7837	32	164−174	YRDLKPENLLI	PSD
	1411.7266	not found	164−174	K^+ adduct of YRDLKPENLLI	
1455.7374	1455.7218	5	129−139	FSHLRRIGRFS(p)[e]	exact mass
	1460.8690	15		not determined	
1486.8636	1486.8699	35	163−174	IYRDLKPENLLI	or 162−173, C-term
	1501.8826	28		not determined	
1530.7919	1530.7815	17	28−40	KKWETPSQNTAQL	PSD
1540.8012	1540.8205	33	104−116	VKLEFSFKDNSNL	PSD, C-term
1584.8024	1584.8087	31, 32	41−54	DQFDRIKTLGTGSF	PSD
	1599.8812	17		not determined	
	1613.9575	22		not determined	
1628.8886	1628.8799	21	133−145	RRIGRFSEPHARF	PSD, C-term
1643.8760	1643.8947	20	27−40	LKKWETPSQNTAQL	PSD, C-term
	1661.8223	23		not determined	
	1666.8505	not found	133−145	K^+ adduct of RRIGRFSEPHARF	
	1681.8462	20	27−40	K^+ adduct of LKKWETPSQNTAQL	
1708.8953	1708.8816	22	237−250	PFFADQPIQIYEKI	PSD, exact mass
1746.9211	1746.9113	not found	185−198	FGFAKRVKGRTWT(p)L[e]	exact mass
1750.9641	1750.9734	34	44−59	DRIKTLGTGSFGRVML	C-term
1793.9704	1793.9876	30	247−261	YEKIVSGKVRFPSHF	PSD
1907.0545	1907.0609	32	246−261	IYEKIVSGKVRFPSHF	PSD
2083.0613	2083.0725	25	247−264	YEKIVSGKVRFPSHFSSD	C-term
2113.2323	2113.2468	27	66−83	GNHYAMKILDKQKVVKLK	PSD
2147.1614	2147.1693	25	249−267	KIVSGKVRFPSHFSSDLKD	exact mass
2181.2147	2181.1969	34	83−100	KQIEHTLNEKRILQAVNF	C-term
2193.0984	2193.1106	28	129−145	FSHLRRIGRFS(p)EPHARF[e]	PSD
2196.1454	2196.1356	27, 32	247−265	YEKIVSGKVRFPSHFSSDL	PSD
	2269.2323	26		not determined	
2379.2464	2379.2471	27, 28	306−326	YQRKVEAPFIPKFKGPGDTSN	exact mass
2439.2673	2439.2647	26, 28	247−267	YEKIVSGKVRFPSHFSSDLKD	PSD
2492.3305	2492.3349	29	305−326	IYQRKVEAPFIPKFKGPGDTSN	PSD
2552.3514	2552.3351	29	246−267	IYEKIVSGKVRFPSHFSSDLKD	PSD
2676.4517	2676.4440	32	303−326	IAIYQRKVEAPFIPKFKGPGDTSN	C-term
	2693.5059	33		not determined	
2823.5201	2823.5239	34, 35	303−327	IAIYQRKVEAPFIPKFKGPGDTSNF	C-term
	2990.6171	35		not determined	

[a] Calculated masses (monoisotopic, MH^+) for identified peptides. All are within 12 ppm of the observed mass listed in column 2. [b] Experimentally observed peaks. [c] Fraction number refers to the collected fraction in which this peak was found in separate, off-line HPLC separation of a digest mixture of PKA treated with pepsin. [d] The method by which the peptide was identified. "Exact mass" indicates that only one possible peptide from PKA had a mass that matched the experimental mass within 20 ppm. Other peptides were identified by PSD or by enzymatic C-terminal sequencing (C-term) in addition to accurate mass measurements. [e] The presence of a phospho group is indicated by (p).

1194.6463 (sequence DRIKTLGTGSF) shows that similar results were obtained from peptides in the mixture as from pure AT. The centroid of the mass envelope for the undeuterated peptide was calculated to be 1195.341 (Figure 2A). When the PKA was incubated for 10 min in buffered D_2O (pD 7.25), digested, and analyzed, the centroid of the peptide increased to 1198.138, a gain of 2.8 deuterons (Figure 2B). The expected number of deuterated amide groups is 10 for this 11-residue peptide, and only partial deuteration (28%) was seen. Lower levels of deuteration were expected for the PKA peptides than for AT, because deuterium exchange into the full-length, folded protein was carried out for short times, and the amount of deuteration should depend on the degree of structure and solvent accessibility. After 1 min of off-exchange by 11-fold dilution in H_2O, the centroid returned to 1196.198 (Figure 2C). The residual 0.857 deuteron, 8.5%, was consistent with the 9% residual deuteration expected to result from the 11-fold dilution. Deuteration prior to PKA digestion and analysis did not appear to adversely affect the quality of the mass spectrum of the mixture, and varying degrees of deuteration were observed reproducibly for different peptides in the mixture.

Matrix Optimization. We have shown that amide deuterons can be stabilized for MALDI-TOF analysis simply by chilling the MALDI target and the matrix solution and by rapidly drying the sample in a vacuum desiccator. To optimize both deuteron detection and observation of the largest number of peptides from the mixture, several different compositions of matrixes were

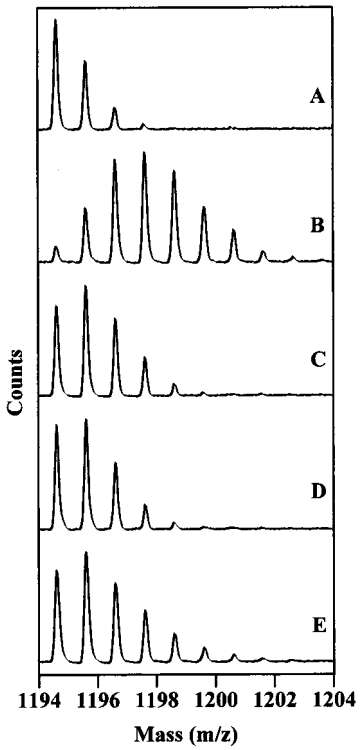

Figure 2. (A) Isotopic envelope for the peptide of mass 1194.6463 from undeuterated PKA. The amount of PKA and the digestion conditions are identical to those described in Figure 1. (B) Isotopic envelope for the same peptide as in (A) derived from PKA that was dissolved in D_2O buffered with K_2HPO_4 to pD 7.25, incubated for 10 min at 25 °C, and then diluted 11-fold into 0.1% TFA, 0 °C. (C) Isotopic envelope for the same peptide as in (A) derived from PKA that was dissolved in D_2O buffered with K_2HPO_4 to pD 7.25, incubated for 10 min at 25 °C, diluted 11-fold into H_2O at 25 °C, incubated for 1 min, and then quenched with 1% TFA to a final concentration of 0.1%. (D) Isotopic envelope for the same peptide as in (A) derived from PKA that was dissolved in D_2O buffered with K_2HPO_4 to pD 7.25, incubated for 10 min at 25 °C, and diluted 11-fold into 0.1% TFA, 0 °C. A portion (10 μL) of the sample was then mixed 1:1 with matrix solution; 1 μL of this was spotted on a MALDI target and allowed to dry slowly by evaporation at 25 °C. (E) The sample of fully deuterated peptide as in (B) that was kept in the mass spectrometer for 2.5 h before analysis.

explored. The matrix crystallization process under these new rapid-drying conditions was also investigated. Experiments with mixtures containing 2.5, 5.0, and 10.0 mg/mL α-cyano-4-hydroxycinnamic acid in 1:1:1 acetonitrile, ethanol, and 0.1% TFA, pH 2.5, showed that the largest number of peptide peaks was observed at 5.0 mg/mL. Use of 0.5% TFA instead of 0.1% TFA was also acceptable. Adjustment of the pH of the matrix solution to lower and higher pH's showed that, at pH 1.5, the loss of amide deuterons was similar to that observed at pH 2.5, while further increases in pH above 2.5 resulted in less abundant peaks for many of the peptides. Accordingly, a matrix mixture containing 5.0 mg/mL α-cyano-4-hydroxycinnamic acid in 1:1:1 acetonitrile, ethanol, and 0.1% TFA, pH 2.5, was used for the remainder of the experiments. For optimal crystallization, a moderate vacuum was applied, and drying of the chilled plate was allowed to occur over a time period of 1−2 min. Faster drying apparently did not permit adequate time for crystallization and this resulted in poorer quality mass spectra.

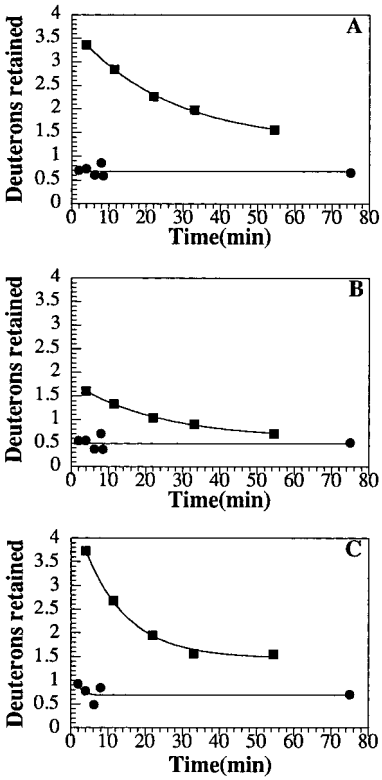

Figure 3. (A) Decrease in deuteration of the peptide of mass 1194.6463 derived from PKA over time in the mass spectrometer. (■) The PKA was deuterated for 10 min at 25 °C and quenched immediately, or (●) the PKA was deuterated for 10 min at 25 °C, off-exchanged by a 11-fold dilution into H_2O for 1 min, 25 °C before quenching. (B) Same PKA samples as in (A) but data are shown for the peptide of mass 1260.6933. (C) Same PKA samples as in (A) but data are shown for the peptide of mass 1793.9876. Data were fit to $D = B_1 + B_2e^{-kt}$ (eq 3).

Stability of Amide Deuterons during MALDI-TOF MS Analysis. To carry out kinetic experiments, it was important to characterize the stability of the amide deuterons under the conditions used in MALDI-TOF MS. Preliminary experiments demonstrated that if the MALDI target was not chilled, or if the sample was not rapidly dried, nearly complete H/D exchange occurred with the matrix solution. Retention of deuteration was achieved when the deuterated sample was mixed with matrix, spotted on a chilled MALDI target, and rapidly dried (Figure 2B). Slow drying at room temperature resulted in nearly complete loss of deuteration (Figure 2D). After 2.5 h in the mass spectrometer, the sample still retained 46% deuteration (Figure 2E) compared with the fully deuterated sample (Figure 2B). These results are consistent with the idea that, initially, exchange of amide deuterons with solvent (TFA, H_2O, and α-cyano-4-hydroxycinnamic acid) occurs in solution and that, after drying, some exchange can still occur by way of nearby carboxylic acids on the α-cyano-4-hydroxycinnamic acid and/or water of crystallization in the solid state.

By analyzing a fully deuterated sample repeatedly over several hours in the mass spectrometer, the kinetics of the solid-phase deuterium loss seen in Figure 2E were characterized. Three peptic peptides were compared to ascertain any sequence-dependent differences (Figure 3). The loss of deuteration from the fully deuterated peptides fit best to a single-exponential decay

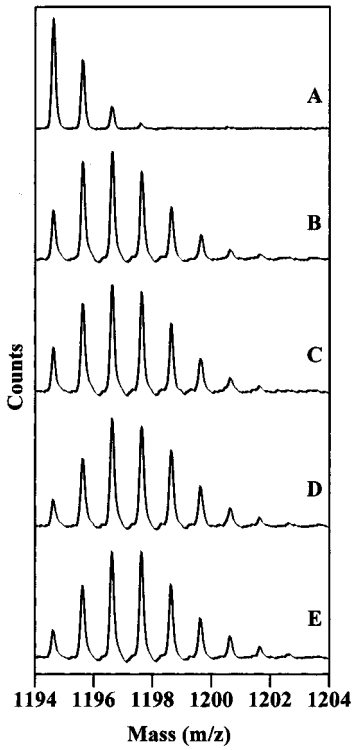

Figure 4. Kinetic experiment in which lyophilized PKA was dissolved in D_2O that was buffered with K_2HPO_4 to pD 7.25 and incubated for varying lengths of time at 25 °C before quenching the sample 11-fold into 0.1% TFA, 0 °C. The PKA was then digested with pepsin as in Figure 1. The mass spectrum of the peptic digest is expanded to show the region around the peptide of mass 1194.6463. The undeuterated spectrum is shown in (A) as a reference. Experiments B−E are for on-exchange times of 1, 2, 5, and 10 min, respectively.

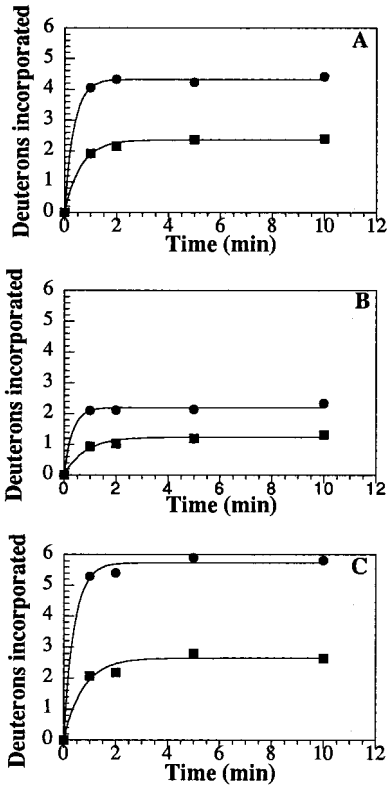

Figure 5. Rate of deuteration of three different regions of PKA as a function of time of incubation in buffered D_2O. The PKA samples were prepared as described in Figure 4. The number of deuterons was determined by calculating the centroid of each mass envelope and subtracting the centroid of the undeuterated sample (■). Correction of the data for loss during the time in the mass spectrometer according to eq 4 resulted in higher final deuteration values (●). Deuteration of residues (A) 44−54, (B) 163−172, and (C) 247−261.

that did not decay to zero ($D = B_1 + B_2e^{-kt}$, eq 3). The rate of loss of deuterons (k) varied by a factor of 2 for the different peptic fragments (approximately 0.04−0.08 min^{-1}) and was always similar to that reported by Bai et al. for rates of exchange under quench conditions (pH 2.5, 0 °C).[4] These researchers also reported sequence-dependent differences in exchange rates on the order of severalfold. The initial amount ($B_1 + B_2$) and the final amount (B_1) of deuteration differed substantially. The experiment was performed with short deuteration times, and presumably the amount of deuteration depended on solvent accessibility of the region of PKA from which the peptic fragment derived. Clearly, some of the deuterons represented by B_1 are the result of the residual deuteration present after dilution from D_2O into H_2O.

Rates of On-Exchange of Deuterons onto the Amide Positions of PKA. To demonstrate the feasibility of kinetic experiments, PKA was deuterated for 1, 2, 5, and 10 min, quenched, and digested, and mass spectra of each sample were obtained and analyzed. Figure 4 shows an expansion of the different mass spectra of the peptic digest mixtures around the peak at mass 1194.6463. The number of deuterons on the peptic fragment were determined by calculating the centroid of each mass envelope, subtracting the centroid of the corresponding undeuterated peptide mass envelope, and correcting for length of time in the mass spectrometer according to eq 4. Rapid deuteration was observed in 1−10 min (Figure 5). The peptic fragment of mass 1194.6463 was identified as residues 44−54 and is deuterated at a rate of 2.8 min^{-1} to a maximum value of 4.3 deuterons. The peptic fragment of mass 1260.6933 was identified as residues 163−172 and is deuterated at a rate of 3.0 min^{-1} to a maximum value of 2.2 deuterons. The peptic fragment of mass 1793.9876 was identified as residues 247−261 and is deuterated at a rate of 2.4 min^{-1} to a maximum value of 5.7 deuterons. The similar fast rates of deuteration probably indicate that we are observing deuteration of solvent-accessible amides, but measurements at time points less than 30 s would still require implementation of stopped flow as was demonstrated by Dharmasiri and Smith.[4,28] The MALDI-TOF method presented here could easily be interfaced with stopped-flow technology if measurement of fast amide exchange were the desired goal.

Coverage of PKA Sequence. A final important consideration in development of a method to use MALDI-TOF MS combined with pepsin digestion to study H/D exchange in proteins is how much of the sequence of a protein can be covered by peptides that are detected in a single mass spectrum of the digest mixture. Fifty peptides gave strong, identifiable peaks in the mass spectrum in the mixture of pepsin-digested PKA and of these 42 were identified (Table 1). Complete identification of the remaining eight peptides was not pursued because none of the possible identities were consistent with peptides that could give additional sequence coverage. The peptides that were identified cover 65% of the entire PKA sequence (Figure 6). The sequences that are covered

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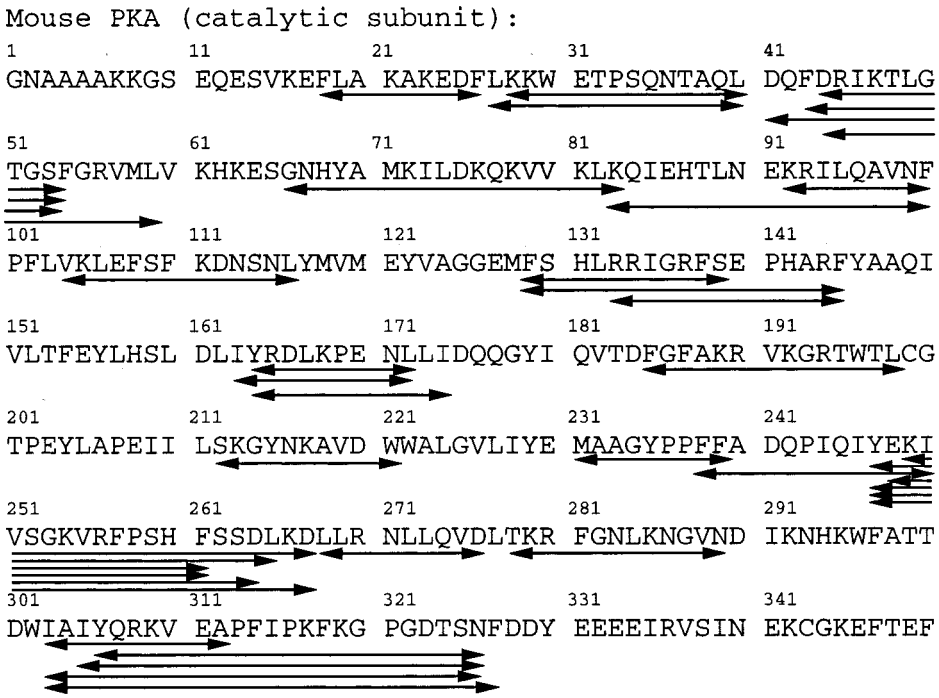

Figure 6. Amino acid sequence of PKA indicating the position of each peptic fragment that was identified in the mass spectrum of the undeuterated PKA digest.

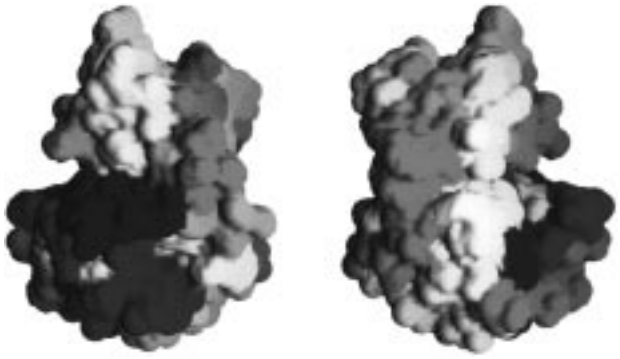

Figure 7. GRASP[30] image summarizing surface deuteration of PKA. The number of deuterons that exchanged onto various peptides from PKA were calculated by subtracting the centroid of the undeuterated peptide mass envelope from that for the peptide derived from PKA that had been incubated in buffered D_2O for 10 min, 25 °C. Three peptides that each contained more than 3 deuterons were colored red, four peptides that each contained 1.9−2.6 deuterons were colored pink, four peptides that each contained 1.4−1.6 were colored purple, and three peptides that each contained less than 1.1 deuterons were colored blue.

represent buried and surface sequences, as well as every type of secondary structure. It is known that some peptide peaks are suppressed in digest mixtures, which probably accounts for the lack of complete coverage of the protein. Indeed, some peptides were observed in the mass spectra of the HPLC fractions that were not present in the mass spectrum of the unseparated peptic digest mixture. Preliminary experiments showed that desalting the sample to remove buffer components resulted in stronger signals for some peptides, but no new peptide peaks were observed. Coverage will, of course, vary from protein to protein. Although 100% coverage of PKA is not observed, the data in a single spectrum is certainly sufficient to obtain useful data on protein folding or ligand interactions.

Analysis of all of the data showed that the number of deuterons that were on-exchanged in 10 min varied widely for the different peptides. This can be seen in the data for the three peptides shown in Figure 5. Two peptides, those of masses 1194.6463 (residues 44−54) and 1793.9876 (residues 247−261) are solvent-exposed in the structure of PKA and were highly deuterated after incubation in D_2O for 10 min. The peptide of mass 1260.6933, which is deep within the active site, was much less deuterated. Figure 7 summarizes the data for the number of deuterons that were on-exchanged in 10 min for peptides that span the PKA sequence. All of the most deuterated peptides fall on the surface and are extended strands while the lesser deuterated peptides are more buried and/or structured. It can also be seen that higher levels of deuteration in general were observed for peptides that are located in the N-terminal lobe, which may be more flexible than the C-terminal lobe.[29]

CONCLUSIONS

We have shown that MALDI-TOF MS can be used to rapidly and easily obtain kinetic data about the exchange of deuterons with amide protons on a protein. No modifications of current instrumentation are required, and the exchange data for many peptides can be obtained from a single spectrum of the digest mixture. An initial investment of time is required to identify the peptic fragments because pepsin cleavage sites cannot be predicted; however, the pattern is highly reproducible. The experiments do not require complicated handling, and H/D exchange rate information on an entire protein can be obtained from only five spectra in a matter of hours. This method should have broad utility for analyzing H/D exchange of proteins in studies of protein

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folding and protein−ligand interactions.

Abbreviations: MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; ESI, electrospray ionization; MS, mass spectrometry; fwhm, full-width at half-maximum; H/D exchange, amide proton/deuteron exchange; PSD, postsource decay; TFA, trifluoroacetic acid; PKA, catalytic subunit of the cyclic AMP-dependent protein kinase.

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