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SHORT COMMUNICATION

The Hydrogen–Deuterium Exchange at α-Carbon Atom in *N*,*N*,*N*-Trialkylglycine Residue: ESI-MS Studies

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Abstract

Derivatization of peptides as quaternary ammonium salts (QAS) is a known method for sensitive detection by electrospray ionization tandem mass spectrometry. Hydrogens at α-carbon atom in *N*,*N*,*N*-trialkylglycine residue can be easily exchanged by deuterons. The exchange reaction is base-catalyzed and is dramatically slow at lower pH. Introduced deuterons are stable in acidic aqueous solution and are not back-exchanged during LC-MS analysis. Increased ionization efficiency, provided by the fixed positive charge on QAS group, as well as the deuterium labeling, enables the analysis of trace amounts of peptides.

Key words: Quaternary ammonium salts, HDX, Betaine, Charge-tagged peptides

Introduction

Certain protons in peptides and proteins, like amide hydrogens, N- and C-terminal hydrogens, and those in side-chain functional groups, undergo exchange with protons from a solvent. Hydrogens bound to carbon usually are not exchangeable; however, under specific conditions, it is possible to replace protons attached to α-carbon by deuterons. A facile method for the exchange of acidic hydrogens by means of keto-enol equilibria is provided by acid/base-catalyzed H/D exchange (HDX) reactions [1]. Hydrogens at aromatic and aliphatic carbons were also found to scramble in gas phase in carboxylate and ammonium ions [2]. A mechanism for base-catalyzed HDX in substituted imidazoles involves ylide or carbanion formation [3, 4].

HDX is an important tool for determining structures and studying dynamic processes of biomolecules [5], as well as for obtaining isotopically labeled compounds as reference materials in mass spectrometry (e.g., for quantitative analysis in proteomics [6]). Moreover, MS signals derived from a mixture of isotopically labeled and nonlabeled products form characteristic doublets, distinguishing them from extraneous and background noise, which may facilitate detection of trace amounts of analyte [7]. The development of methods for accurate protein quantitation is currently one of the most challenging areas in proteomic research.

Stable, isotopically labeled tags could be prepared by conventional synthesis from commercially available precursors; however, long synthetic routes and high costs of starting materials must often be taken into account. More rapid and cost effective is the labeling by direct HDX [1].

In our previous study, we have presented a new method of peptide derivatization as QAS on solid support, which offers a novel approach to increase sensitivity in peptide analysis by electrospray ionization mass spectrometry (ESI-MS) [8].We have also analyzed the fragmentation pathways of QAS-peptides, with the aid of deuterium-labeled analogs [9]. In this article, we present results of base-catalyzed HDX study in peptides conjugated with QAS. We examine the exchange of acidic C-H hydrogen atoms in *N*,*N*,*N*-trialkyl-

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glycine residues and discuss the application of deuterium-labeled QAS analogs for quantitative analysis of peptides.

Experimental

Materials

The details of performed syntheses are given as Supplementary Data.

Isotopic Exchange

HDX was initiated by dissolving 0.1 mg of QAS-peptide derivative in 200 μL of D_2O at room temperature. After 20 min, all protons from the amide bonds and amino acid side chains were exchanged by deuterons, as judged from ESI-MS analysis. Then, 2 μL of Et_3N was added and the mixture was analyzed by ESI-MS. The acidic C-H hydrogens were substituted by deuterons after 1 min in peptides derivatized at α-amino group and after 3 h in peptides derivatized at lysine side chain amino group.

Back-Exchange of Amide Protons

The sample was afterwards lyophilized and redissolved in 200 μL of 50:50 acetonitrile-water mixture containing 0.1% HCOOH, which caused the back-exchange of labile deuterons into protons after 20 min.

Back-Exchange of Protons in N,N,N-Trialkylglycine Residue

The sample was lyophilized again and dissolved in 200 μL of H_2O containing 2 μL of Et_3N. The acidic deuterons were substituted by protons from a solvent after 1 min in peptides derivatized at α-amino group and after 3 h in peptides derivatized at lysine side chain amino group, as judged from ESI-MS analysis.

Mass Spectrometry

Mass spectrometric experiments were performed on a Bruker micrOTOF-Q mass spectrometer (Bruker Daltonics, Bremen, Germany) and on an FT-ICR (Fourier transform ion cyclotron resonance) Apex-Qe Ultra 7 T instrument (Bruker Daltonics). The instrumental settings and measurement details are described in Supplementary Data.

Results and Discussion

The aim of our work was to investigate the base-catalyzed HDX in peptide derivatives containing *N*,*N*,*N*-trialkylglycine attached to N-terminus or lysine side chain. In order to verify the influence of different QAS groups on the exchange reaction we have synthesized on solid support a series of model peptides containing trimethyl-, triethyl-, tripropyl-, tributyl-, and 1,4-diazabicyclo[2.2.2]octylammonium acetyl (TMAA, TEAA, TPAA, TBAA, and DBAA, respectively) moiety (Supplementary Data, Table 1S). To investigate the influence of various amino acid residues on the HDX, the immunosuppressive fragment of HLA-DQ molecule [10] containing the H-Asp-Val-Tyr-Thr-NH_2 sequence and series of its analogs were selected as model peptides. To eliminate labile protons from peptide backbone amide groups, a series of oligoproline peptides containing from three to six proline residues were synthesized. The QAS group was also introduced to the side chain of inner lysine residue in a model peptide H-Met-Gln-Ile-Phe-Val-Lys-Thr-OH (N-terminal ubiquitin fragment). To examine the influence of QAS on the HDX reaction, two peptides, H-Gly-Asp-Val-Tyr-Thr-NH_2 and H-Gly-Met-Gln-Ile-Phe-Val-Lys-Thr-OH, with sequences similar to the model peptides were synthesized, where the quaternary ammonium acetyl moiety was replaced by glycine. We also obtained a peptide derivative containing N^{ϵ},N^{ϵ},N^{ϵ}-triethyllysine residue to investigate the possibility of HDX at carbon atoms unable to form ammonium ylide.

We have examined the isotopic exchange of all synthesized QAS-peptide derivatives. The ESI-MS and MS/MS spectra of a representative QAS-peptide **18** (DBAA-Pro-Pro-Pro-Pro-Pro-NH_2) are presented in Figure 1. First we analyzed the sample dissolved in acetonitrile-water mixture containing 0.1% HCOOH and we observed a peak at *m/z*=752.4, corresponding to QAS peptide derivative (Figure 1a, Panel A).

In the spectrum recorded in D_2O (pD=7.4) the peak has shifted to *m/z*=754.5 (Figure 1b, Panel A), due to the exchange of two labile protons into deuterons. The analysis of ESI-MS/MS spectrum of the deuterated M^+ ion of the QAS-peptide (Figure 1b, Panel B) confirmed that the two hydrogens from C-terminal amide group were exchanged by deuterons, since the masses of all y-type ions are shifted by 2 Da compared with fragments obtained in the fragmentation of QAS-peptide dissolved in the H_2O/MeCN/HCOOH mixture (Figure 1a, Panel B). After the addition of a base (pD=12.3), the *m/z* value increased by further 2 Da (Figure 1c, Panel A), which suggests that two additional hydrogens in QAS-derivatized peptide were exchanged by deuterons. The fragmentation of M^+ ion produced a series of N-terminal fragment ions (a- and b-type) with masses higher by 2 Da, compared with the corresponding fragments obtained for QAS-peptide dissolved in D_2O (Figure 1b and c, Panel B), while the masses of the C-terminal fragments remained unchanged. These results revealed that the HDX in alkaline D_2O solution occurs at the α-carbon atom bound to QAS.

After lyophilization and redissolving the sample in the H_2O/MeCN/HCOOH mixture, the peak corresponding to the peptide **18** was shifted to the value *m/z*=754.5 (Figure 1d, Panel A) and the analysis of MS/MS spectrum indicates that in these conditions the two deuterons of the C-terminal amide group were back-exchanged by protons (Figure 1d, Panel B). The *m/z* values of peaks corresponding to b_1 and y_5 fragment ions indicate accurately that only the deuterons

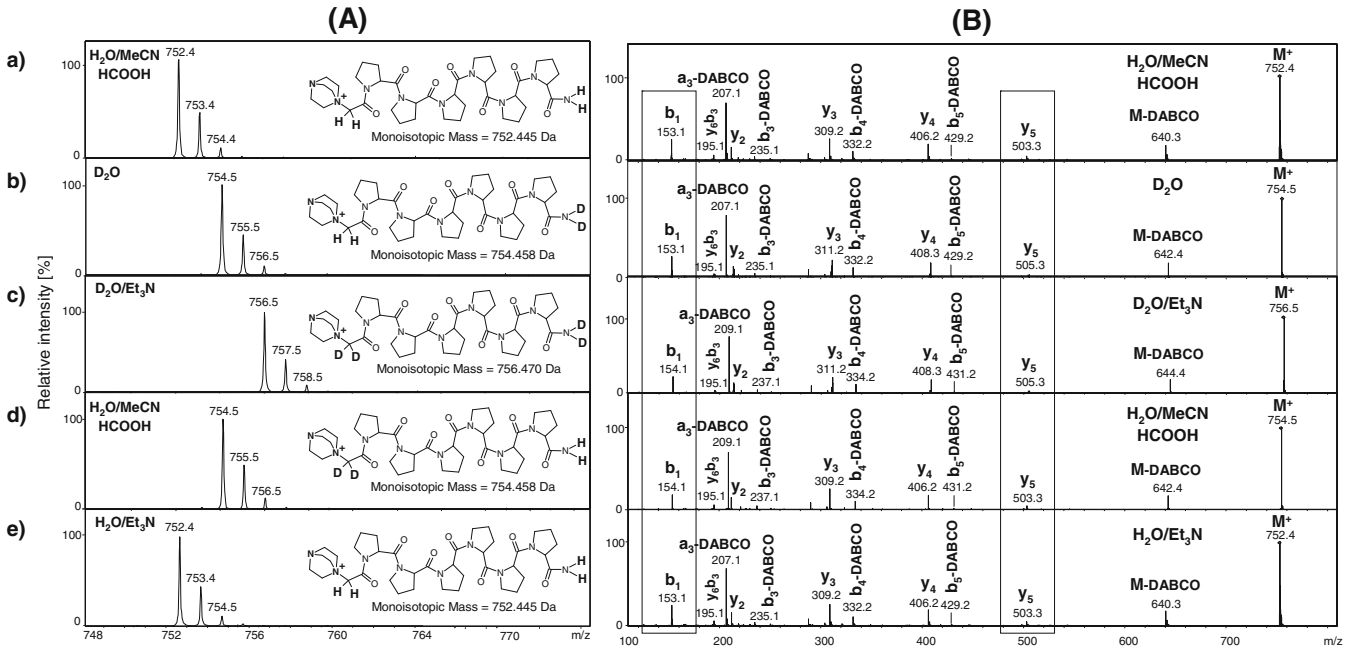

Figure 1. **(A)** ESI-MS spectra of peptide **18**, dissolved in different solutions: **(a)** acetonitrile–water mixture containing 0.1% HCOOH; **(b)** D_2O; **(c)** D_2O containing 1% Et_3N; **(d)** acetonitrile–water mixture containing 0.1% HCOOH, after lyophilization from D_2O containing 1% Et_3N; **(e)** H_2O containing 1% Et_3N, after lyophilization from acetonitrile–water mixture containing 0.1% HCOOH. **(B)** ESI-MS/MS analysis of the respective ions

located at the α-carbon atom bound to QAS remain in the QAS-peptide after it has been dissolved in the mixture of H_2O/MeCN/HCOOH. Consecutive lyophilization and redissolving in alkaline H_2O solution (pH=12) resulted in the return to the initial *m/z* value of the peak corresponding to QAS-peptide derivative, due to the back-exchange of deuterons from α-carbon by protons (Figure 1e, Panel A). Similar results were obtained for other investigated compounds (Supplementary Data, Figures 2–15S), which indicate that neither the type of QAS moiety nor the peptide sequence affect the isotope exchange reactions. It is worth noting that in these experiments, HDX was not observed for other protons connected to carbon atoms, as was confirmed by MS/MS analysis.

For QAS-peptide derivatives **24** (TEAA-Met-Gln-Ile-Phe-Val-Lys(TEAA)-Thr-OH) and **25** (DBAA-Met-Gln-Ile-Phe-Val-Lys(DBAA)-Thr-OH), containing two QAS moieties, the substitution of four protons by deuterons was observed, due to the presence of two possible exchange sites (Supplementary Data, Figure 16S). However, the exchange reaction of protons located at α-carbon atom of acetyl moiety attached to lysine side chain amino group required a longer incubation in alkaline D_2O solution and was completed after 3 h. During experiments the appearance of a new peak with *m/z* higher of 16 Da was also observed, as a consequence of methionine side chain oxidation, since sulfides easily undergo oxidation to sulfones and sulfoxides.

In order to determine the influence of the presence of QAS moiety on the H/D substitution reaction, we have investigated the unmodified peptides, H-Gly-Asp-Val-Tyr-Thr-NH_2 and H-Gly-Met-Gln-Ile-Phe-Val-Lys-Thr-OH, containing 11 and 15 labile protons, respectively. For these peptides, the additional 2 Da shift of the main signal in alkaline D_2O solution was not observed (Supplementary Data, Figures 17–18S). We have also examined the exchange reaction for peptide derivative **22** (Ac-Met-Gln-Ile-Phe-Val-Lys(Et_3N)-Thr-OH), containing N^{ϵ},N^{ϵ},N^{ϵ}-triethyllysine residue. For this peptide the additional mass shifting in alkaline D_2O solution was not observed (Supplementary Data, Figure 19S). These results suggest that the HDX occurs only at α-carbon atom of *N,N,N*-trialkylglycine residue in QAS-peptides, probably due to formation of ammonium ylide [11].

The initial experiments were carried out in alkaline D_2O solution obtained by adding NaOH. However, the presence of sodium cations resulted in analyzed compounds forming adducts during MS experiments, which complicates the analysis of mass spectrum. This problem may be solved by decreasing the amount of added NaOH, which reduces the rate of adducts formation, but then the appropriate pH of the solution cannot be reached. Sodium cations may be removed from the sample by desalting on a Sep-Pak column prior to the MS analysis; however, we found that the use of Et_3N as a base is much more convenient. This approach enables obtaining the appropriate conditions for the HDX reactions without sodium ions addition and also facilitates the removal of Et_3N from the sample by evaporation. Therefore, further experiments were performed using Et_3N instead of NaOH.

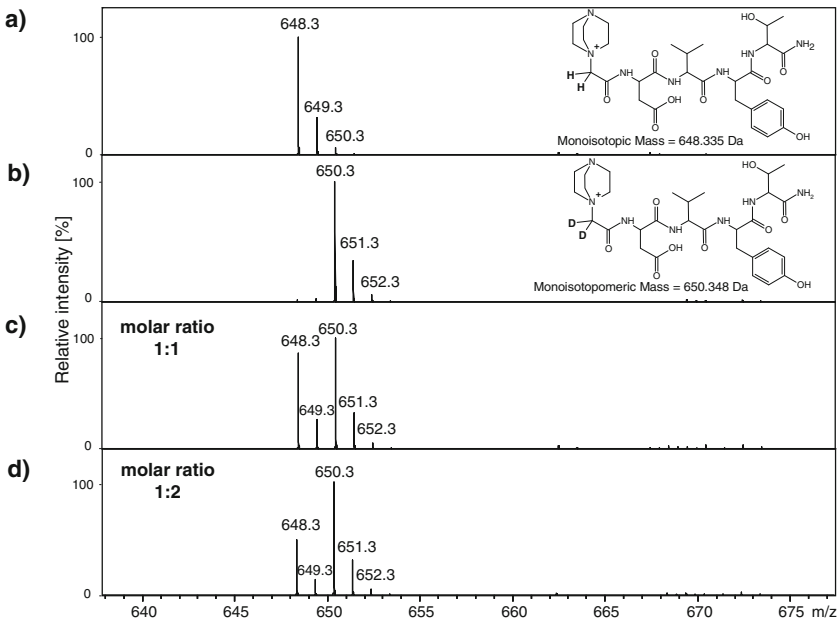

Figure 2. ESI-MS spectra of peptide **10 (a)** and its analog with two deuterons at α-carbon atom **(b)** and the mixture of these two peptides in molar ratio 1:1 **(c)** and 1:2 **(d)**

We investigated the influence of different pD on the HDX of acidic hydrogens in QAS-peptides. For this purpose we have dissolved peptides **9** (TEAA-Asp-Val-Tyr-Thr-NH_2) **10** (DBAA-Asp-Val-Tyr-Thr-NH_2), **19** (TEAA-Met-Gln-Ile-Phe-Val-Lys-Thr-OH) and **20** (DBAA-Met-Gln-Ile-Phe-Val-Lys-Thr-OH) in three solutions of D_2O, containing 0.01%, 0.1%, and 1% of Et_3N (pD 10.2, 11.4, and 12.3, respectively). The HDX reaction was examined by monitoring the isotopic pattern changes of peaks corresponding to the analyzed QAS-peptides (Supplementary Data, Figure 20, 21S). The analysis of mass spectra reveals that HDX of acidic hydrogens is completed in less than 1 min in the solution of D_2O containing 1% Et_3N (pD=12.3). In the solution of 0.1% Et_3N (pD=11.4), the H/D substitution reaction was completed after 20 min and in the third solution (0.01% Et_3N, pD=10.2) after 5 h. The obtained results indicate that the HDX reaction strongly depends on the pD of the solution and that the most convenient is pD=12.3 (1% Et_3N solution).

We have examined the back-exchange rate of peptides containing α-carbon protons selectively exchanged by deuterons. Peptides **17** (TEAA-Pro-Pro-Pro-Pro-Pro-Pro-NH_2) and **18** were incubated in the acetonitrile–water mixture containing 0.1% HCOOH (pH=3.1) for 1 month at room temperature and analyzed by ESI-MS. After the incubation no measurable changes in isotopic pattern were observed (Supplementary Data, Figure 22S), which proves that in acidic aqueous solution the back exchange of the α-carbon deuterons does not occur. A high isotopomeric stability of the deuterium labeled compounds creates a possibility of their wide application (e.g., in quantitative proteomic studies or in fragmentation pathways analysis).

One of the quantification methods in proteomics is based on the addition to analyzed mixture stable isotope labeled (^2H, ^{13}C, or ^{15}N) analogs of targeted peptides [6]. The concentration of peptide is then determined from the relative MS responses of the spiked-in compound and the target analyte. In order to verify the application of the investigated α-deuterium labeled peptides in quantitative analysis, we have prepared mixtures of peptide **10** and its deuterium analog, labeled at α-carbon atom, in 1:1 and 1:2 molar ratios in H_2O/MeCN/HCOOH solution. In MS spectrum, two signals separated by 2 Da were observed, since in the spiked-in analog there are two protons exchanged by deuterons (Figure 2). The intensity ratio of the monoisotopic signal corresponding to peptide (*m/z* 648.3) versus monoisotopomeric signal corresponding to its deuterated analog (*m/z* 650.3) were 1:1 and 1:2, which corresponds to expected values, taking into account that the peak at *m/z* 650.3, representing the $2^{13}C$ isotopomer ($C_{28}{}^{13}C_2H_{44}N_7O_9$, 650.3205), overlaps with the 2^2H isotopomer ($C_{30}H_{44}D_2N_7O_9$, 650.3477), since the mass difference is very small. The intensity ratios have been preserved in MS spectra of the mixtures recorded after 24 h incubation at room temperature.

In H/D substitution reaction monitoring a considerable back-exchange of labile deuterons during MS or LC-MS analysis is a known problem [12]. To verify the stability of the deuterium labeling in QAS peptide derivatives, we performed the LC-MS analysis of the mixture of peptides **9**, **10**, **17** and their analogs with deuterons at α-carbon atom, using standard HPLC-MS conditions (details in Supplementary Sata). We have compared the isotopic pattern of signals corresponding to peptides and their deuterated analogs obtained in MS and LC-MS analysis

(Supplementary Data, Figure 23S). The calculated ratios of isotopic signals before and after the analysis were similar in both experiments. This indicates that during the LC-MS analysis, a back-exchange of deuterons connected to α-carbon atom does not occur. The obtained results may indicate that the procedure, based on the labeling of peptides with *N*,*N*,*N*-trialkylglycine followed by base catalyzed H/D exchange, may be useful for accurate and sensitive peptide quantification (e.g., in proteomic research).

Conclusions

We have developed an efficient and straightforward method of isotopic labeling of QAS derivatized peptides by direct hydrogen/deuterium exchange. The exchange reaction is base-catalyzed, but is dramatically slow or quenched in neutral or lower pH. Deuterons, introduced at the α-carbon atom bound to QAS, are stable in acidic aqueous solution and do not undergo back-exchange during LC-MS analysis. Increased ionization efficiency, provided by fixed positive charge of the QAS group, and the possibility of isotopic labeling, make the QAS-acetyl moiety introduction a promising procedure for quantitative analysis of trace amount of peptides.

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References

1. Atzrodt, J., Derdau, V., Fey, T., Zimmermann, J.: The renaissance of H/D exchange. *Angew. Chem. Int. Ed.* **46**, 7744–7765 (2007)
2. Reed, D.R., Kass, S.R.: Hydrogen–deuterium exchange at non-labile sites: a new reaction facet with broad implications for structural and dynamic determinations. *J. Am. Soc. Mass Spectrom.* **12**, 1163–1168 (2001)
3. Takeuchi, Y., Kirk, K.L., Cohen, L.A.: Adjacent lone pair (ALP) effects in heteroaromatic systems. 2. Isotope exchange of ring hydrogens in nitro- and fluoroimidazoles. *J. Org. Chem.* **43**, 3570–3578 (1978)
4. Washabaugh, M.W., Jencks, W.P.: Thiazolium C(2)-proton exchange: general-base catalysis, direct proton transfer, and acid inhibition. *J. Am. Chem. Soc.* **111**, 674–683 (1989)
5. Englander, S.W.: Hydrogen exchange and mass spectrometry: a historical perspective. *J. Am. Soc. Mass Spectrom.* **17**, 1481–1489 (2006)
6. Elliott, M.H., Smith, D.S., Parker, C.E., Borchers, C.: Current trends in quantitative proteomics. *J. Mass Spectrom.* **44**, 1637–1660 (2009)
7. Kijewska, M.K., Stefanowicz, P., Kluczyk, A., Szewczuk, Z.: The isotopic exchange of oxygen as a tool for detection of the glycation sites in proteins. *Anal. Biochem.* **419**, 81–87 (2011)
8. Cydzik, M., Rudowska, M., Stefanowicz, P., Szewczuk, Z.: Derivatization of peptides as quaternary ammonium salts for sensitive detection by ESI-MS. *J. Pept. Sci.* **17**, 445–453 (2011)
9. Cydzik, M., Rudowska, M., Stefanowicz, P., Szewczuk, Z.: The competition of charge remote and charge directed fragmentation mechanisms in quaternary ammonium salt derivatized peptides-An isotopic exchange study. *J. Am. Soc. Mass Spectrom.* **22**, 2103–2107 (2011)
10. Szewczuk, Z., Stefanowicz, P., Wilczyński, A., Siemion, I.Z., Cierniewski, C.S., Kralisz, U., Wieczorek, Z.: The immunosuppressory and adhesive miniregion of the human major histocompatibility protein, human leukocyte antigen DQ. *Biopolymers* **40**, 571–583 (1996)
11. Clayden, J., Donnard, M., Lefranc, J., Tetlow, D.J.: Quaternary centres bearing nitrogen (α-tertiary amines) as products of molecular rearrangements. *Chem. Commun.* **47**, 4624–4639 (2011)
12. Feng, L., Orlando, R., Prestegard, J.H.: Amide proton back-exchange in deuterated peptides: applications to MS and NMR analyses. *Anal. Chem.* **78**, 6885–6892 (2006)