Aldehyde Stress-Mediated Novel Modification of Proteins: Epimerization of the N-Terminal Amino Acid

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · DECEMBER 2013

Impact Factor: 3.53 · DOI: 10.1021/tx400354d · Source: PubMed

4 AUTHORS, INCLUDING:

Seon Hwa Lee
Tohoku University
76 PUBLICATIONS **2,465** CITATIONS
SEE PROFILE

Tomoyuki Oe
Tohoku University
80 PUBLICATIONS **1,652** CITATIONS
SEE PROFILE

Chemical Research in Toxicology

Article

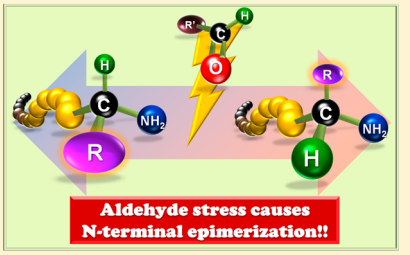
pubs.acs.org/crt

Aldehyde Stress-Mediated Novel Modification of Proteins: Epimerization of the N-Terminal Amino Acid

Ryo Kajita, Takaaki Goto, Seon Hwa Lee, and Tomoyuki Oe*

Department of Bioanalytical Chemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, Aobayama, Aoba-ku, Sendai 980-8578, Japan

ABSTRACT: Various kinds of aldehyde-mediated chemical modifications of proteins have been identified as being exclusively covalent. We report a unique noncovalent modification: the aldehyde-mediated epimerization of the N-terminal amino acid. Epimerization of amino acids is thought to cause conformational changes that alter their biological activity. However, few mechanistic studies have been performed, because epimerization of an amino acid is a miniscule change in a whole protein. Furthermore, it does not produce a mass shift, making mass spectrometric analysis difficult. Here, we have demonstrated epimerization mediated by endogenous aldehydes. A model peptide, with an N-terminal L- or D-FMRFamide, was incubated with an endogenous or synthetic aldehyde [acetaldehyde, methylglyoxal, pyridoxal 5′-phosphate (PLP), 4-oxo-2(*E*)-nonenal, 4-hydroxy-2(*E*)-nonenal, D-glucose (Glc), 4- or 2-pyridinecarboxaldehyde] under physiological conditions. Each reaction mixture was analyzed by liquid chromatography with ultraviolet detection and/or electrospray ionization mass spectrometry. Considerable epimerization occurred after incubation with some endogenous aldehydes (PLP, 40.6% after 1 day; Glc with copper ions, 6.5% after 7 days). Moreover, the epimerization also occurred in whole proteins (human serum albumin and PLP, 26.3% after 1 day). Tandem mass spectrometric studies, including deuterium labeling and sodium borohydride reduction, suggested that the epimerization results from initial Schiff base formation followed by tautomerization to ketimine that causes the chirality to be lost. This suggests that the epimerization of the N-terminal amino acid can also occur *in vivo* as a post-translational modification under a high level of aldehyde stress.



INTRODUCTION

Aldehydes are produced by a variety of endogenous metabolic processes.[1] D-Glucose (Glc) is one of the most abundant aldehydes, which undergoes glycolysis to form other aldehydes such as glyceraldehyde and methylglyoxal (MG).[1,2] 4-Oxo-2(*E*)-nonenal (ONE) and 4-hydroxy-2(*E*)-nonenal (HNE) are the major end products of lipid peroxidation.[3] Pyridoxal 5′-phosphate (PLP) and acetaldehyde (AA) are generated by the enzymes pyridoxal kinase[4] and alcohol dehydrogenase,[5] respectively. In addition, aldehydes such as acrolein (Acr) or crotonaldehyde (Cro) can be ingested from cigarette smoke and air pollution.[1] Because of its charge delocalization effect, the terminal carbonyl group of aldehydes can react with an amine group to form Schiff bases. The reactivity of aldehydes is further enhanced by α,β-unsaturation, which makes C-3 highly electron-deficient. Thus, α,β-unsaturated aldehydes readily undergo nucleophilic addition of thiols and amines. α,β-Unsaturated aldehydes can also cross-link proteins, lipids, and nucleic acids through Michael addition and Schiff base formation, acting as bifunctional electrophiles.[1,6]

Some aldehydes are cytotoxic, and the toxic effects have been attributed to their ability to modify DNA bases and the consequent mutations.[7,8] For example, ONE can modify the DNA bases 2′-deoxyguanosine (dGuo), 2′-deoxyadenosine (dAdo), and 2′-deoxycytidine (dCyd) through the formation of exocyclic heptanone-etheno (Hε) adducts, 1,N^2-HεdGuo, 1,N^6-HεdAdo, and 3,N^4-HεdCyd, respectively.[9−11] The reaction of HNE, Acr, and Cro with dGuo can give rise to exocyclic 1,N^2-propano dGuo adducts.[12] MG produces an N^2-carboxyethyl dGuo adduct upon reaction with dGuo.[13] These aldehydes are also involved in the regulation of inflammation, apoptosis, and cellular signaling through post-translational modifications (PTMs) of amino acid residues such as Cys, His, Lys, and Arg and the N-terminus.[14−17] Increased levels of MG−, ONE−, or HNE−protein adducts have been implicated in a large number of diseases, including diabetes, Parkinson's disease, Alzheimer's disease, HIV-induced dementia, and heart failure.[6,14,18] Therefore, the use of aldehyde−protein adducts as biomarkers and for dosimetry has been extensively investigated.[14−16]

In contrast to covalent modifications, epimerization of amino acids has only recently been recognized as a PTM that could cause conformational changes and alter the biological activity of proteins.[19,20] D-Amino acids arising from epimerization[19,21,22] have been detected in proteins from human and animal tissues, including tooth,[23] eye lens,[24] aorta,[25] and bone.[26] Moreover, the development of cataracts seems to coincide with the epimerization of Asp in human lenses.[27] Furthermore, higher proportions of D-Asp and D-Ser residues have been found in neuritic plaque amyloids in Alzheimer's disease.[28,29] Thus, epimerization in proteins is associated with certain diseases, although it occurs on only Asp, Ser, and Pro. Very recently, Lyons et al. reported that up to 30% of N-terminal Met was

Received: September 27, 2013

   dx.doi.org/10.1021/tx400354d | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

present as the D-form in aquaporin-0 from aged human lenses.[27] They also used NMR and liquid chromatography (LC)/UV to confirm the epimerization of N-terminal amino acids after long-term incubation under physiological conditions. This suggests that epimerization may occur at unblocked N-terminal amino acids in any protein. The proposed mechanism of epimerization is initiated by a reaction between the α-amino group at the N-terminus and the amidocabonyl on the adjacent amino acid to form an intramolecular cyclic Schiff base.[27] However, few mechanistic studies have been published,[22,30] because epimerization is a mass spectrometry (MS)-silent modification, and pairs of epimers cannot be distinguished by MS.

We have been investigating chemical modifications derived from ONE and recently reported a unique N-terminal modification of a bioactive peptide, angiotensin II (Ang II, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), as the most abundant product. The product (Ang P) contains a pyruvamide moiety at the N-terminus of Ang II, which was produced by Schiff base formation followed by decarboxylation.[31] Similarly, the formation of α-ketoamides during the reaction between aldehydes and peptides containing N-terminal amino acids other than Asp has been reported, with a mechanism initiated by Schiff base formation.[32,33] The Schiff bases formed at the N-terminal α-amino group (aldimines) undergo tautomerization to ketimines toward the α-carbon.[31,32] Because tautomerization between aldimines and ketimines causes a loss of chirality, we investigated whether the epimerization of N-terminal amino acids could be accelerated by aldehydes.

Here, we report N-terminal specific epimerization by aldehydes for the first time with detailed mechanistic studies. The epimerization of a cardioactive neuropeptide, L-FMRFamide, was examined using endogenous or synthetic aldehydes [AA, MG, PLP, ONE, HNE, Glc, and 4- or 2-pyridinecarboxaldehyde (4- or 2-PCA, respectively)]. The N-terminal amino acid epimerization mechanism was investigated by incubating L-FMRFamide with PLP in deuterated buffer. Finally, the epimerization of the N-terminal amino acid in human serum albumin (HSA) was examined after reaction with PLP, tryptic digestion, and then derivatization with dansyl chloride (Dns-Cl).

MATERIALS AND METHODS

Materials. L-FMRFamide (L-Phe^1-L-Met^2-L-Arg^3-L-Phe^4-NH_2) and D-FMRFamide (D-Phe^1-L-Met^2-L-Arg^3-L-Phe^4-NH_2) were purchased from American Peptide Co. Inc. (Sunnyvale, CA). L-DAHK (L-Asp^1-L-Ala^2-L-His^3-L-Lys^4) and D-DAHK (D-Asp^1-L-Ala^2-L-His^3-L-Lys^4) were purchased from Toray Research Center, Inc. (Tokyo, Japan). ONE and HNE were purchased from Cayman Chemical Co. (Ann Arbor, MI). PLP monohydrate, AA, MG, α-cyano-4-hydroxycinnamic acid (CHCA), sodium borohydride, and HSA [N-terminus (residues 1−24) truncated; Uniprot entry F6KPG5] were purchased from Sigma-Aldrich Co. (St. Louis, MO). 2-PCA, 4-PCA, and Dns-Cl were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Glc, ammonium bicarbonate (NH_4HCO_3), sodium dihydrogenphosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), disodium hydrogenphosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), sodium bicarbonate ($NaHCO_3$), formic acid (FA), trifluoroacetic acid (TFA), sodium hydroxide, and deuterium oxide (heavy water, D_2O; 99.9 atom % D) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Sequencing grade modified trypsin was purchased from Promega Co. (Madison, WI). Angiotensin III (Ang III) was purchased from Peptide Institute, Inc. (Osaka, Japan). ZIC-HILIC solid phase extraction cartridges (100 mg) were purchased from Merck KGaA (Darmstadt, Germany). LC grade acetonitrile and ethanol were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). ZipTipC_{18} cartridges and Amicon Ultra-0.5 centrifugal filter devices (0.5 mL, 3 kDa cutoff) were purchased from EMD Millipore Co. (Billerica, MA). OASIS HLB cartridges were obtained from Waters Corp. (Milford, MA). Purified water was prepared using a Milli-Q Integral Water Purification System (EMD Millipore Co.). Nitrogen (N_2) and helium (He) gases were purchased from Taiyo Nippon Sanso Co. (Tokyo, Japan). All other chemicals and solvents used were of reagent grade. Sodium phosphate buffer (50 mM, pH 7.4) and sodium bicarbonate buffer (0.2 M, pH 8.8) were prepared in our laboratory.

LC/UV Analyses. LC system 1, which was used for UV analyses, consisted of an L-6200 Intelligent pump and an L-6000 pump (Hitachi Co. Ltd., Tokyo, Japan) equipped with an L-4200H UV/vis detector (Hitachi Co. Ltd.). Chromato-PRO version 2.0.2 (Runtime Instruments, Sagamihara, Japan) was used for data processing. An Inertsil ODS-3 column [250 mm × 4.6 mm (inside diameter), 5 μm, 100 Å; GL Sciences Inc., Tokyo, Japan] was used for the separation. Solvent A consisted of water and acetonitrile [95:5 (v/v)] containing 0.1% (v/v) TFA, and solvent B consisted of acetonitrile and water [95:5 (v/v)] containing 0.1% (v/v) TFA. The linear gradient was as follows: 15% B at 0 min, 35% B at 30 min, 95% B at 31 min, 95% B at 40 min, 15% B at 41 min, and 15% B at 60 min. The flow rate was 1.2 mL/min, and the detection wavelength was 220 nm.

MALDI-TOF-MS Analyses. Matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS) experiments were conducted on a Voyager DE-STR MALDI-TOF mass spectrometer (AB SCIEX, Framingham, MA) at the Biomedical Research Core (School of Medicine, Tohoku University). All spectra were recorded in the positive ion mode with an accelerating voltage of 20 kV in the reflectron mode with an average of 100 shots. TOF-MS experiments were performed in the mass range of m/z 200−1000. Calibration was conducted using two internal calibrants: CHCA dimer at m/z 379.0930 (monoisotopic) and Ang III at m/z 931.5148 (monoisotopic). Data analysis was performed using Data Explorer version 4.0.0.0. (AB SCIEX).

LC/ESI-MS Analyses. LC systems 2 and 3, which were used for MS analyses, consisted of an LCQ-DECA ion trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) equipped with an ESI source in positive ion mode with an LC system, a Surveyor MS pump (separation module) equipped with a Surveyor AS autosampler, a column oven, and a PDA detector (Thermo Fisher Scientific Inc.). An Inertsil ODS-3 column [150 mm × 2.1 mm (inside diameter, 3 μm, 100 Å; GL Sciences Inc.)] was used for the separation with a flow rate of 0.2 mL/min. Solvent A consisted of water and acetonitrile [95:5 (v/v)] containing 0.1% (v/v) FA, and solvent B consisted of acetonitrile and water [95:5 (v/v)] containing 0.1% (v/v) FA. The linear gradient for LC system 2 was as follows: 5% B at 0 min, 45% B at 30 min, 95% B at 31 min, 95% B at 40 min, 5% B at 41 min, and 5% B at 60 min. The linear gradient for LC system 3 was as follows: 50% B at 0 min, 90% B at 40 min, 90% B at 45 min, 50% B at 46 min, and 50% B at 60 min. The LCQ-DECA operating conditions were as follows: heated capillary temperature, 300 °C; ion spray voltage, 5.0 kV; sheath and auxiliary gas (N_2) pressures, 90 and 15 (arbitrary units), respectively. Full scanning analyses were performed in the range of m/z 200−1500. Data-dependent scanning analyses were performed as follows: collision gas, He; default charge state, 2; default isolation width, 2.0; normalized collision energy, 35.0; activation Q, 0.250; activation time, 30.0 ms. Data analyses were performed using Xcalibur version 2.0 (Thermo Fisher Scientific Inc.).

Reaction of L-FMRFamide with an Aldehyde. L- or D-FMRFamide (0.01 μmol/10 μL of water) and an aldehyde {0.1 μmol/10 μL; PLP, AA, MG, 2-PCA, 4-PCA, Glc in water, ONE in a methyl acetate/ethanol mixture [1:1 (v/v)], or HNE in ethanol} were added to 50 mM sodium phosphate buffer (pH 7.4, 80 μL) and incubated at 37 °C. To observe the effect of Cu^{II}, copper(II) sulfate (0.001 μmol/10 μL of water) was added to the mixture of Glc (0.1 μmol/10 μL of water), L-FMRFamide (0.01 μmol/10 μL of water), and 50 mM sodium phosphate buffer (pH 7.4, 70 μL). Samples of the

dx.doi.org/10.1021/tx400354d | Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

reaction mixture (10 μL) were taken sequentially and subjected to LC/UV analysis using LC system 1 for time course analysis or LC/ESI-MS analysis using LC system 2 for structural analysis.

Sodium Borohydride Reduction of PLP-Modified FMRFamide. L-FMRFamide (0.01 μmol/10 μL of water) and PLP (0.1 μmol/10 μL of water) were added to 50 mM sodium phosphate buffer (pH 7.4, 70 μL). The mixture was incubated for 6 h at 37 °C. The reaction mixture was allowed to react with sodium borohydride (1 μmol/10 μL of 0.1 M aqueous sodium hydroxide) at 37 °C for 2 h. The mixture was allowed to cool, and the reduced products were purified by an OASIS HLB (1 mL, 10 mg) cartridge. The cartridge was conditioned with acetonitrile (1 mL) followed by 0.1% (v/v) aqueous TFA (1 mL) prior to being used. The reaction solution (100 μL) was loaded on the cartridge and washed with 0.1% (v/v) aqueous TFA (1 mL). The fraction containing the desired peptides was eluted with 75% (v/v) aqueous acetonitrile containing 0.1% (v/v) TFA (1 mL), and the eluate was evaporated *in vacuo* at room temperature. The residue was redissolved in water (100 μL). A portion of the sample (10 μL) was subjected to LC/ESI-MS analysis using LC system 2.

Epimerization Reaction in Deuterated Buffer. To prepare deuterated buffer, 50 mM sodium phosphate buffer (pH 7.4) was first evaporated *in vacuo* at room temperature and redissolved in the same volume of heavy water (D_2O). L-FMRFamide (0.01 μmol/10 μL of D_2O) and PLP (0.1 μmol/10 μL of D_2O) were added to 50 mM deuterated sodium phosphate buffer (pD ≅7.8, 80 μL). The mixture was incubated at 37 °C for 6 h. The reaction mixture was purified with a ZipTipC_{18} cartridge (0.6 μL). The cartridge was conditioned with acetonitrile (10 μL × 10) and then equilibrated with 0.1% (v/v) aqueous TFA (10 μL × 10) prior to being used. The reaction solution (30 μL) was loaded on the cartridge and washed with 0.1% (v/v) aqueous TFA (10 μL × 5). The sample was eluted with 75% (v/v) aqueous acetonitrile containing 0.1% (v/v) TFA (10 μL × 2). The eluate was evaporated *in vacuo* at room temperature. The residue was redissolved in light water (H_2O, 30 μL) and incubated at 37 °C for 6 h. A portion of the reaction mixture (10 μL) was subjected to LC/ESI-MS analysis using LC system 2.

Reaction of DAHK with PLP. L- or D-DAHK (0.02 μmol/20 μL of water) and PLP (0.2 μmol/20 μL of water) were added to 50 mM sodium phosphate buffer (pH 7.4, 160 μL) and incubated at 37 °C. Portions of the mixture (30 μL) were taken sequentially and diluted with 0.2 M sodium bicarbonate buffer (pH 8.8, 15 μL) for derivatization with Dns-Cl (5 mg/mL acetonitrile, 15 μL). After 1 h at 37 °C, the reaction was terminated by adding methylamine (50 mM in acetonitrile, 15 μL). The derivatized sample was evaporated *in vacuo* at room temperature. The residue was redissolved in 50% (v/v) aqueous acetonitrile (60 μL), and a portion of the solution (10 μL) was analyzed by LC/ESI-MS using LC system 3.

Reaction of HSA with PLP. HSA (0.01 μmol/10 μL of water) and PLP (0.1 μmol/10 μL of water) were added to 50 mM sodium phosphate buffer (pH 7.4, 80 μL). The mixture was incubated for 24 h at 37 °C. A portion of the reaction mixture (50 μL) was transferred into a filtration unit (3 kDa cutoff), and the excess reagent was eliminated by centrifugation at 11300*g* for 12 min. The remaining mixture in the filter unit was diluted with 12.5 mM aqueous NH_4HCO_3 (100 μL) and centrifuged at 11300*g* for 12 min. This step was repeated three times. The filter unit containing the HSA mixture was transferred to a new collection tube, and trypsin (0.5 μg/100 μL of 12.5 mM aqueous NH_4HCO_3) was added to the filter. After overnight incubation at 37 °C, the tryptic sample was collected by centrifugation at 11300*g* for 12 min as the filtrate. The solution was diluted with acetonitrile (200 μL) for the following cleanup. The ZIC-HILIC cartridge (100 mg) was conditioned with water (6 mL) followed by 70% (v/v) aqueous acetonitrile (6 mL) prior to being used. The diluted solution (300 μL) was then loaded on the cartridge and washed with 70% (v/v) aqueous acetonitrile (1 mL). The fraction containing DAHK was eluted with 5% (v/v) aqueous acetonitrile (2 mL), and the eluate was evaporated *in vacuo* at room temperature. The residue was redissolved in 0.2 M sodium bicarbonate buffer (pH 8.8, 15 μL) and Dns-Cl (5 mg/mL acetonitrile, 15 μL) for derivatization. The following procedure was the same as in the previous section.

RESULTS

LC/UV and MALDI-TOF-MS Analyses of the Reaction between L-FMRFamide and PLP. PLP, also known as vitamin B_6, is an important coenzyme for endogenous transamination, decarboxylation, deamination, etc.,[34] and has been used for transamination reactions *in vitro*.[32] The N-terminal D-epimer of FMRFamide, a cardioactive neuropeptide, is commercially available. Therefore, the reaction between L-FMRFamide and PLP was examined first. The baseline separation and each retention time were confirmed using authentic samples of L- and D-epimers with LC system 1, as eluted at 12.4 and 19.2 min, respectively (Figure 1A). The

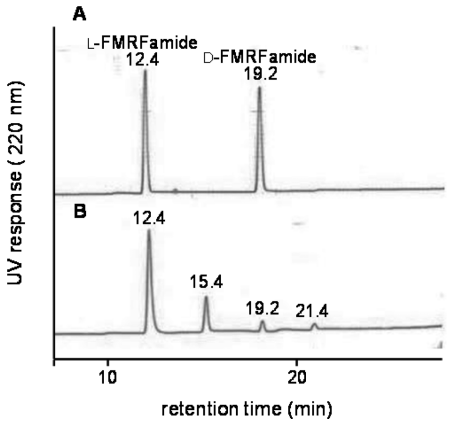


Figure 1. LC/UV (220 nm) analyses of (A) authentic L- and D-FMRFamide and (B) the reaction of L-FMRFamide with PLP at 37 °C for 2 h.

reaction of L-FMRFamide with PLP gave three products at 15.4, 19.2, and 21.4 min (Figure 1B) after incubation for 2 h at 37 °C. Because the peak at 19.2 min was eluted at the same retention time as authentic D-FMRFamide, the peak was collected and analyzed by MALDI-TOF-MS (Figure 2). The

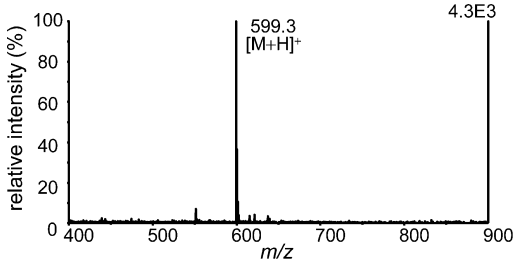


Figure 2. MALDI-TOF-MS analysis of the peak at 19.2 min in Figure 1B.

product showed an MS spectrum identical to that of the authentic sample with the protonated molecule ($[M + H]^+$) at *m/z* 599.3. This was identified as D-FMRFamide produced by the epimerization of the N-terminal Phe. The other two products were identified using LC/ESI-MS, because they were not stable enough to be analyzed by MALDI-TOF-MS.

Time course epimerization experiments were performed (Figure 3). The extent of epimerization of L-FMRFamide to the D-epimer increased with reaction time (Figure 3A). Two other products, eluted at 15.4 and 21.4 min, were formed before the D-epimer and reached a maximum within 1 h and then decreased slightly. The epimerization of D-FMRFamide to the

dx.doi.org/10.1021/tx400354d | *Chem. Res. Toxicol.* XXXX, XXX, XXX–XXX

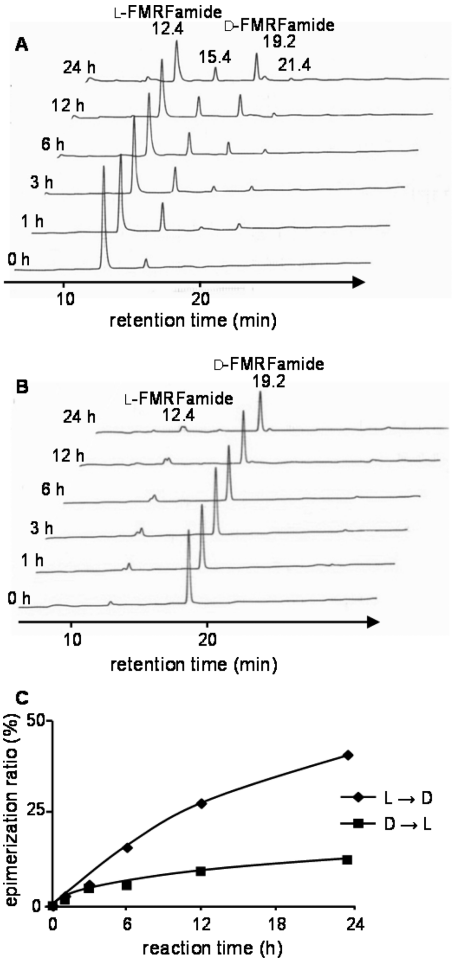


Figure 3. LC/UV (220 nm) analyses of the reaction between FMRFamide and PLP at 37 °C for 24 h: (A) L-FMRFamide and (B) D-FMRFamide. (C) Time course for the epimerization ratio. Filled triangles denote data for the L- to D-epimer conversion; filled squares denote data for the D- to L-epimer conversion.

L-epimer was also observed with retention times (12.4 min) identical to those in Figure 3A (Figure 3B). However, the product peaks for the D-epimer were much smaller than those for the L-epimer. The epimerization ratios are summarized in Figure 3C as a ratio of the D-epimer to the total L-epimer and D-epimer [epimerization ratio (%) = peak area of D-epimer/(peak area of L-epimer + peak area of D-epimer) × 100]. The epimerization ratio of the L-epimer increased gradually in a time-dependent manner and reached 40.6% after 24 h. In contrast, the epimerization ratio of the D-epimer was approximately 11.8% throughout the reaction.

LC/ESI-MS and MS^n Analysis of the Reaction between L-FMRFamide and PLP. To identify other products formed during the reaction, the reaction mixture was subjected to LC/ESI-MS analysis (Figure 4). The peaks at 13.7 and 17.1 min were identified as L-FMRFamide and D-FMRFamide, respectively, by comparison with authentic samples. The total ion chromatogram (TIC) showed an elution pattern different from the chromatogram obtained by LC/UV analyses. It indicated the presence of four products that eluted at 21.1, 22.1, 25.3, and 29.4 min (Figure 4A). The ESI mass spectra of L-FMRFamide and D-FMRFamide revealed base ion peaks at m/z 599.3 ($[M + H]^+$) (Figure 4B). Although identical base ion peaks at m/z 828.3 were observed for all four products (Figure 4C), an

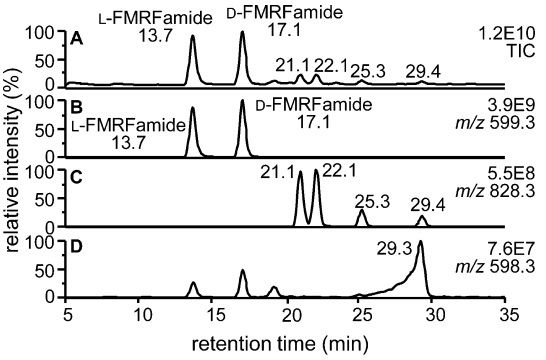


Figure 4. LC/ESI-MS analysis of the reaction between L-FMRFamide and PLP at 37 °C for 6 h. (A) TIC for m/z 200−1500. (B) EIC of m/z 599.3 ($[M + H]^+$) for L- and D-FMRFamide. (C) EIC of m/z 828.3 ($[M + H]^+$) for PLP-modified FMRFamide. (D) EIC of m/z 598.3 ($[M + H]^+$) for α-ketoamide-FMRFamide.

intense ion peak at m/z 598.3 was detected in only the ESI mass spectrum of the peak at 29.3 min (Figure 4D). Figure 4 shows the extracted ion chromatograms (EICs) of the three most intense ions, which reveals the presence of five major products in the reaction mixture.

Considering typical aldehyde reactions and the mass shift of +229 Da corresponding to the condensation of PLP, it is reasonable to suppose that these peaks were epimers of the Schiff base stabilized by the aromatic conjugation system of PLP. Tandem mass spectrometry (MS/MS) analysis of the product (22.1 min) at m/z 828.3 (Figure 5A) resulted in the formation of a single product ion at m/z 730.4 (Figure 5B), corresponding to a decrease in mass of 98 Da (− $[H_3PO_4]$). The −98 Da mass shift is often observed when PLP-modified peptides are subjected to MS/MS analysis.[35] The MS^3 analysis of the m/z 730.4 peak revealed that a modification occurred at the N-terminus of L-FMRFamide (Figure 5C). Thus, all b ions that were detected (b_2 and b_3) appeared with an increase of 131 Da (+ $[PLP - H_2O - H_3PO_4]$) when they were compared with the corresponding b ions of intact L-FMRFamide. However, the y ions (y_2 and y_3) remained unmodified. Other products eluting at 21.1, 25.3, and 29.4 min exhibited MS, MS/MS, and MS^3 spectra identical to those of the product eluting at 22.1 min. The four products (21.1, 22.1, 25.3, and 29.4 min) were then subjected to sodium borohydride reduction. After the reaction, the products were further purified and desalted. LC/ESI-MS analysis of reduced products revealed the $[M + H]^+$ peak at m/z 830.4 (Figure 6A), which corresponded to the addition of two hydrogen atoms. MS/MS analysis of the m/z 830.4 ion revealed a major product ion at m/z 732.4 (Figure 6B), corresponding to the loss of phosphoric acid (− $[H_3PO_4]$). MS^3 analysis of m/z 732.4 generated unmodified y_2 and y_3 ions, modified b_3 ions, and an internal fragment ion (F), which is consistent with a modification to Phe[1] (Figure 6C). All modified ions showed an increase in mass units of 2 Da compared with the MS^3 spectrum of PLP-modified L-FMRFamide (Figure 5C). This confirmed that two hydrogen atoms were added to the N-terminus. On the basis of these results, the four products at m/z 828.3 formed in the reaction between L-FMRFamide and PLP were identified as epimers of the Schiff base.

In the EIC of the m/z 598.3 ion (Figure 4D), the product eluting at 29.3 min exhibited a remarkable leading edge. A similar phenomenon was observed for pyruvamide-Ang II (Ang

dx.doi.org/10.1021/tx400354d | Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

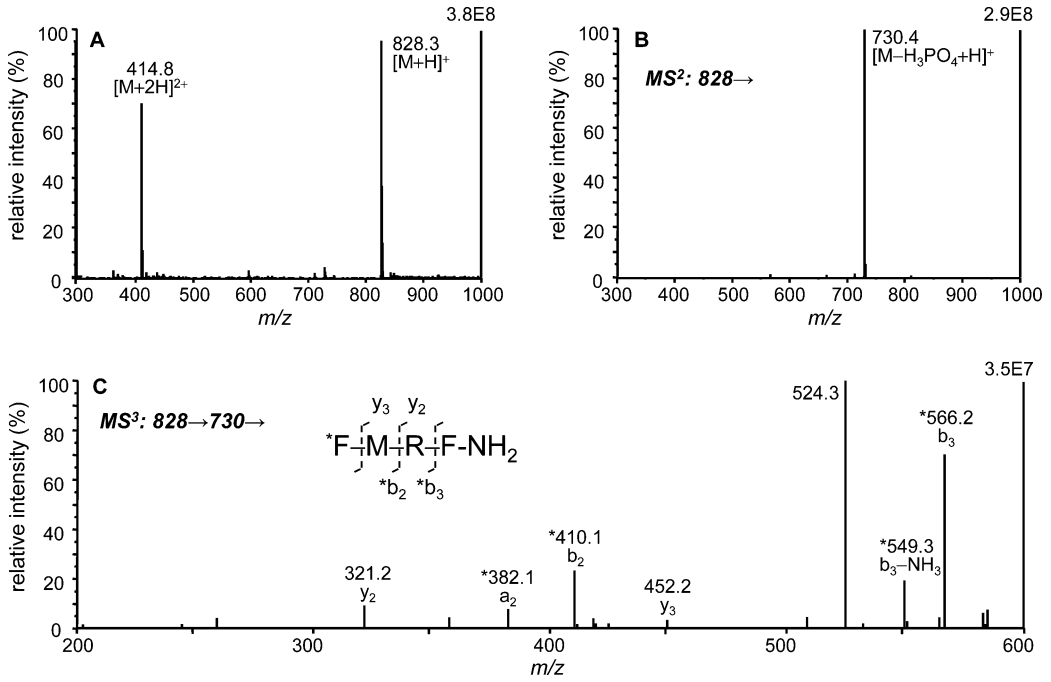


Figure 5. LC/ESI-MS/MS analysis of the product at 22.1 min in Figure 4C: (A) full scan spectrum, (B) MS^2 spectrum of m/z 828.3 (+ [PLP − H_2O]), and (C) MS^3 spectrum of m/z 730.4 (+ [PLP − H_2O − H_3PO_4]). Asterisks indicate modified ions (+131 Da).

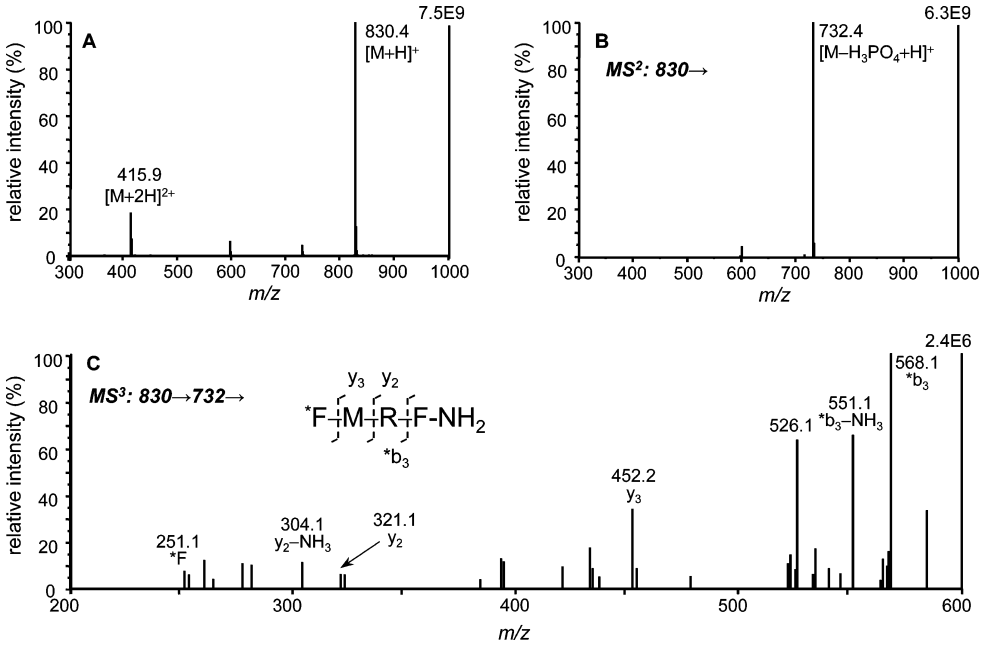


Figure 6. LC/ESI-MS/MS analysis of the product at 22.1 min in Figure 4C after sodium borohydride reduction: (A) full scan spectrum, (B) MS^2 spectrum of m/z 830.4 (+ [PLP − H_2O + 2H]), and (C) MS^3 spectrum of m/z 732.4 (+ [PLP − H_2O + 2H − H_3PO_4]). Asterisks indicate modified ions (+131 Da + 2H).

P) in our previous study.[31] A mass shift of −1 Da was typically observed when an N-terminal α-ketoamide was formed via a Schiff base intermediate, followed by deamination during the reaction of certain aldehydes with N-terminal α-amino groups.[31−33] MS/MS analysis of the m/z 598.3 ion revealed that a modification occurred at the N-terminus of L-FMRFamide (Figure 7). All a and b ions that were detected (a_2, a_3, b_2, and b_3) appeared with a decrease in mass of 1 Da from those of the corresponding a and b ions of intact L-FMRFamide. In contrast, the y ions (y_2 and y_3) remained unmodified. Therefore, the product at 29.3 min was identified as the N-terminal α-ketoamide form of L-FMRFamide.

Reaction between L-FMRFamide and PLP in Deuterated Buffer. To confirm the mechanism of epimerization, the L-FMRFamide was reacted with PLP in deuterated buffer (pD ≅7.8), where the α-hydrogen of the N-terminal amino acid could be replaced with a deuterium atom during epimerization. After incubation for 6 h, the reaction mixture was purified and redissolved in light water to exchange the α-amino group deuterium with a proton. LC/ESI-MS analysis revealed the

dx.doi.org/10.1021/tx400354d | Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

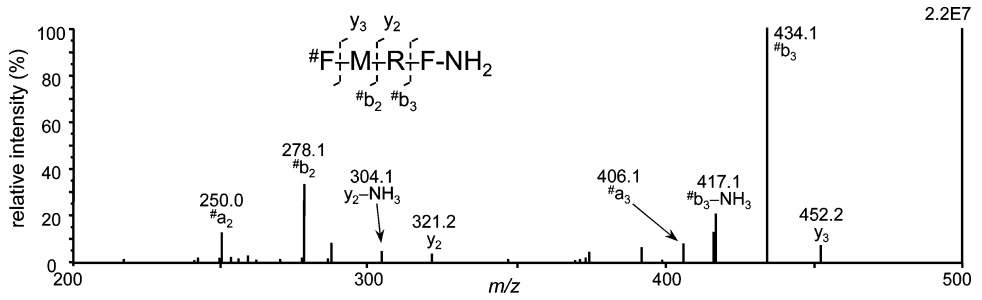


Figure 7. LC/ESI-MS/MS analysis of the product at 29.3 min in Figure 4D. MS^2 spectrum of m/z 598.3. Number signs (#) indicate modified ions (−1 Da).

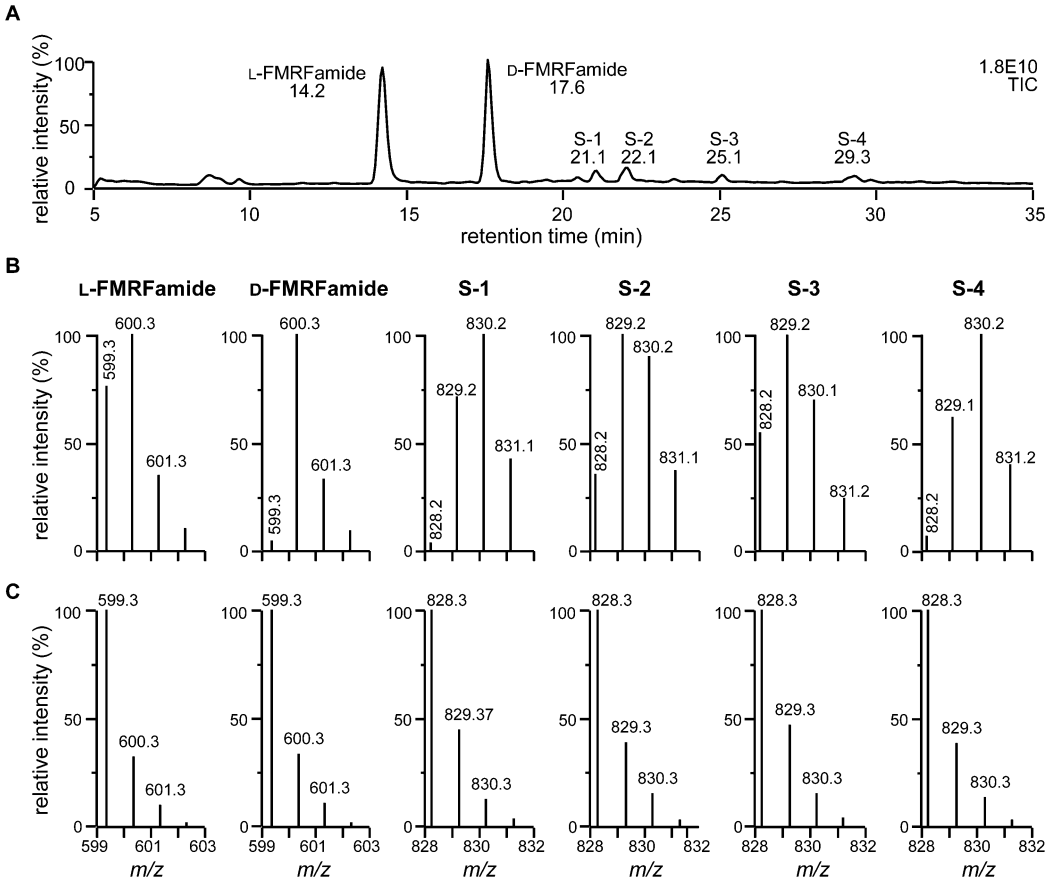


Figure 8. LC/ESI-MS analyses of the reaction between L-FMRFamide and PLP in deuterated buffer at 37 °C for 6 h. (A) TIC for m/z 200−1500. (B) Mass spectrum of each peak in panel A. (C) Mass spectrum of each corresponding peak in normal buffer (control).

presence of five products (D-FMRFamide, 17.6 min; Schiff base epimer S-1, 21.1 min; Schiff base epimer S-2, 22.1 min; Schiff base epimer S-3, 25.1 min; Schiff base epimer S-4, 29.3 min) together with residual L-FMRFamide at 14.2 min (Figure 8A). Although this TIC was quite similar to that obtained from the reaction of L-FMRFamide with PLP in normal buffer (Figure 4), the isotope peak profile of each product indicated the incorporation of deuterium. Thus, the masses of D-FMRFamide, S-1, and S-4 were increased by 1 Da (Figure 8B) compared with masses in normal buffer (Figure 8C). The isotope peak profiles of L-FMRFamide, S-2, and S-3 also shifted to a higher mass because of partial deuterium incorporation (Figure 8B,C). The isotope ratios [(M + 1)/M] of all the products, including the L-epimer found in normal and deuterated buffer, are summarized in Table 1. The values of the isotope ratio further confirmed that deuterium was added to

Table 1. Isotope Ratios [(M + 1)/M] of All Products, Including the L-Epimer, from the Reaction between L-FMRFamide and PLP at 37 °C for 6 h[a]

peak	retention time (min)	isotope ratio [(M + 1)/M]		
		theoretical value	in light water	in heavy water
L-FMRFamide	14.4	0.31	0.32	0.43
D-FMRFamide	17.7	0.31	0.32	2.70
S-1	21.4	0.40	0.41	2.80
S-2	22.3	0.40	0.39	0.60
S-3	25.4	0.40	0.42	0.61
S-4	29.4	0.40	0.39	2.06

[a]The reaction was performed with both normal and deuterated (heavy water) buffers.

dx.doi.org/10.1021/tx400354d | Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

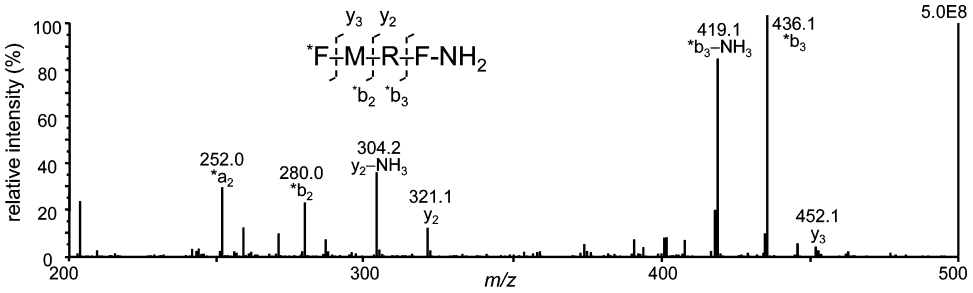


Figure 9. LC/ESI-MS/MS analysis of deuterated D-FMRFamide. MS^2 spectrum of m/z 600.3 (Figure 8B, left). Asterisks indicate modified ions (+1 Da).

all products, although the degree of deuterium incorporation differed: L-FMRFamide, S-2, and S-3 were partially deuterated, and D-FMRFamide, S-1, and S-4 were highly deuterated.

In the MS/MS spectrum of D-FMRFamide (17.6 min) at m/z 600.3 (Figure 9), all the b ions that were detected (b_2 and b_3) appeared with an increase of 1 Da compared with the masses of the corresponding b ions of intact L-FMRFamide. However, the y ions (y_2 and y_3) remained unmodified, clearly indicating that the C^{α}-H/D exchange occurred at the N-terminal amino acid.

Reaction between Other Aldehydes and L-FMRFamide. Because epimerization was observed in the PLP experiment, the following aldehydes were also examined: endogenous MG (unconjugated aldehyde), AA (α-ketoaldehyde), ONE and HNE (α,β-conjugated aldehyde), Glc (α-hydroxy aldehyde), and the synthetic aldehydes 2- and 4-PCA (PLP analogues). LC/ESI-MS analysis revealed the presence of multiple products in the reactions of L-FMRFamide with each aldehyde in sodium phosphate buffer (pH 7.4) at 37 °C for 24 h. In the case of 2-PCA, its EICs showed the presence of D-FMRFamide (Figure 10A, top) together with three other products (Figure 10A, bottom), indicating that 2-PCA-mediated epimerization occurred on the N-terminus of L-FMRFamide. Three products had an identical protonated molecule, which corresponded to an increase of 89 Da (+ [2-PCA − H_2O], Schiff base) (Figure 10A, bottom). LC/ESI-MS analyses also revealed the formation of D-FMRFamide and Schiff bases when 4-PCA, ONE, or HNE reacted with L-FMRFamide. The epimerization ratios after 24 h for 4-PCA, ONE, and HNE were 3.3, 8.8, and 1.8%, respectively (Table 2).

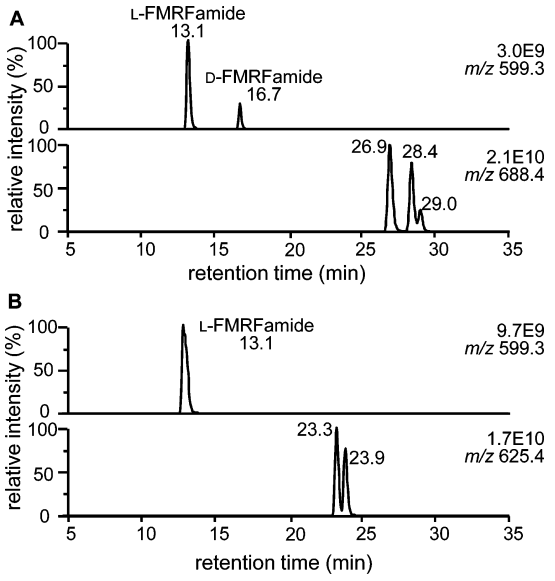
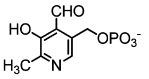
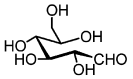
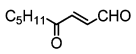
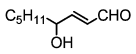


Figure 10. LC/ESI-MS analyses of the PLP-mediated epimerization at 37 °C for 24 h with (A) 2-PCA and (B) AA. The upper EICs are of m/z 599.3 ($[M + H]^+$) for FMRFamide, and the lower EICs are of the corresponding Schiff bases at m/z 688.4 and 625.4 ($[M + H]^+$).

Table 2. Epimerization Ratios of L-FMRFamide with Various Aldehydes at 37 °C for 24 h[a]

Aldehydes	Epimerization ratio (%)	Aldehydes	Epimerization ratio (%)
 PLP	40.6	H_3C-CHO AA	0
 2-PCA	4.9	 MG	0
 4-PCA	3.3	 Glc	
 ONE	8.8	Cu^{II} (−)	0 (0)*
		Cu^{II} (+)	0 (6.5)*
 HNE	1.8		

[a]Asterisks indicate prolonged incubation for 7 days.

Conversely, when AA was reacted with L-FMRFamide for 24 h, D-FMRFamide was not detected in the reaction mixture. In the EICs of the AA products (Figure 10B, bottom), two products were eluted at 23.3 and 23.9 min with an identical $[M + H]^+$ ion at m/z 625.4 that corresponded to Schiff bases (+26 Da, [+ AA − H_2O]). However, no peak was observed for D-FMRFamide in the EIC of the m/z 599.3 ion (Figure 10B, top). For MG and Glc, only Schiff bases were formed. These results suggest that the epimerization reaction with AA, MG, or Glc did not occur or was not fast enough for epimerization to be detected within 24 h, although Schiff bases were produced.

Glycation, which is a nonenzymatic PTM by carbohydrate, is slow under physiological conditions. However, even after incubation of the reaction mixture for 7 days, no epimerization

dx.doi.org/10.1021/tx400354d | Chem. Res. Toxicol. XXXX, XXX, XXX–XXX

of L-FMRFamide was observed. Recently, it has been reported that the oxidative deamination of Lys residues can occur during incubation with Glc at a very low concentration of copper ions.[36] This reaction could also occur in the N-terminal amino group. Glc was incubated with L-FMRFamide at 37 °C in the presence of 10 μM copper(II) for 7 days. An epimerization ratio of 6.5% was observed (Table 2). In the absence of Glc, copper(II) did not enhance the epimerization of the N-terminus on the peptide. In addition, the copper ions did not influence the epimerization mediated by other aldehydes.

N-Terminal Epimerization in Human Serum Albumin. The N-terminal epimerization of a whole protein incubated with aldehyde was investigated. HSA (66 kDa, 585 amino acid residues), which is the most abundant protein in serum, was used as a model protein. Because analyzing N-terminal epimerization in a whole protein makes LC separation difficult, tryptic digestion was performed before LC/ESI-MS analysis. The N-terminal tryptic tetrapeptide of HSA, D^1AHK^4, was dansylated, because it was too polar to be retained on the ODS column. During the dansylation, no epimerization was observed (data not shown). Synthetic DAHK was incubated with PLP and monitored by LC/ESI-MS after the dansylation (Figure 11A). As expected, the extent of formation of D-DAHK

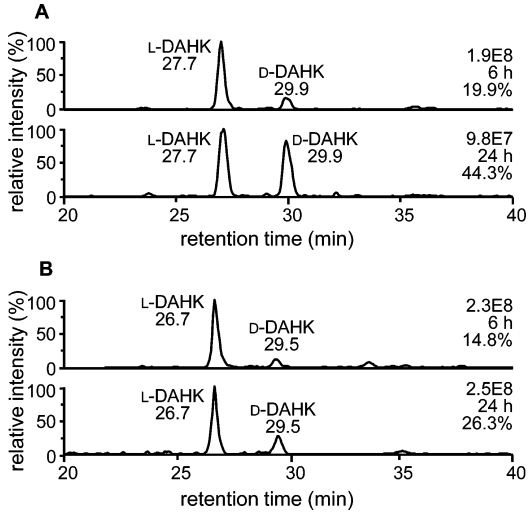


Figure 11. LC/ESI-MS analyses of dansylated DAHK (A) Reaction between L-DAHK and PLP at 37 °C. (B) Reaction between HSA and PLP at 37 °C followed by tryptic digestion. The top and bottom spectra are for 6 and 24 h reactions, respectively. The EIC was at m/z 1169.2 for tri-Dns-DAHK.

increased with incubation time, and the epimerization ratio reached 44.3% after 24 h. Whole HSA was incubated with PLP under the same reaction conditions, followed by tryptic digestion and dansylation (Figure 11B). Epimerization was also observed in the whole protein, although it was slower than that of DAHK. The epimerization ratio was calculated to be 26.3% after 24 h.

DISCUSSION

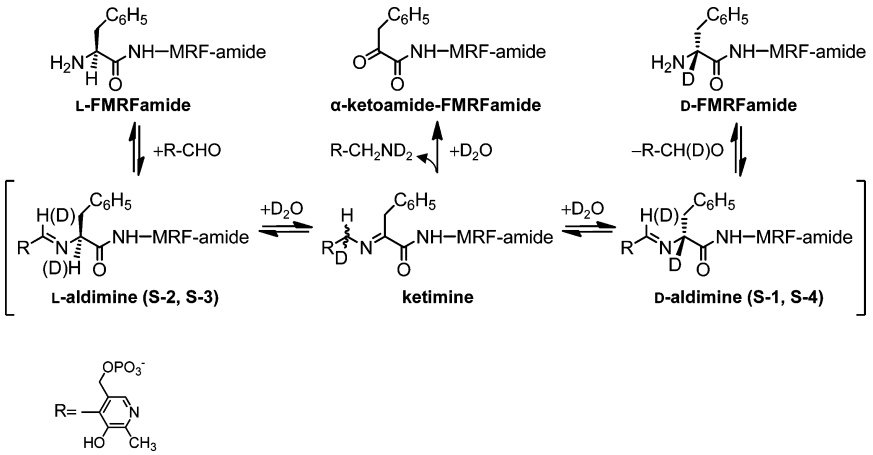
The conversion of L-amino acids to their enantiomers (D-amino acids) has been of great interest in geochemistry, food chemistry, and physiology.[37−39] This process is usually termed racemization, although racemic amino acids should contain equal amounts of L- and D-isomers, or epimerization if several chiral centers are involved. *In vivo* epimerization is a spontaneous PTM that occurs slowly during the natural aging of proteins.[37] Thus, it was initially used to determine the relative age and turnover rates of long-lived proteins.[39] Certain amino acid residues epimerize more rapidly than others: Asp/Asn > Glu/Gln > Ser > Ala.[40] The epimerization of Asp has received the most attention because it can be detected in archeological bone samples and in living human tissue.[41] However, the epimerization of N-terminal amino acids has not been thoroughly studied.

We have now shown that N-terminal amino acids can be efficiently epimerized in the presence of endogenous aldehydes under physiological conditions. In the reaction of L-FMRFamide with PLP, the formation of D-FMRFamide was observed at a retention time identical to that of the authentic standard (Figures 1 and 4). The identity of D-FMRFamide was further confirmed by MALDI-TOF-MS and LC/ESI-MS analyses. The level of conversion of L-FMRFamide to the D-epimer increased in a time-dependent manner, and the epimerization ratio reached 40.6% after incubation for 24 h. This is a remarkable epimerization ratio, particularly as a recent study found the ratio of N-terminal epimerization in the PFHSPSY peptide to be approximately 5% after 10 weeks at 37 °C in HEPES or TES buffers (100 mM, pH 7.4).[27] Compared with the L-epimer, D-FMRFamide was epimerized at a slower rate (11.8%). The discrepancy in epimerization ratio between the D- and L-amino acids has also been observed in peptides containing N-terminal Pro or Met, although the reason for this is unclear.[27] In addition to D-FMRFamide, five other products were detected from the reaction between L-FMRFamide and PLP (Figure 4). LC/ESI-MS analyses of the four products eluting at 21.1, 22.1, 25.3, and 29.4 min (Figure 4C) showed an identical $[M + H]^+$ ion at m/z 828.3. They were identified as epimers of the Schiff base formed at the N-terminus, based on the LC/ESI-MS^n analyses before and after sodium borohydride reduction. The product that eluted last (29.3 min) with its $[M + H]^+$ ion at m/z 598.3 (Figure 4D) was identified as α-ketoamide-FMRFamide by LC/ESI-MS/MS analysis.

The mechanism of N-terminal epimerization was clarified using the reaction of L-FMRFamide with PLP in deuterated buffer (pD ≅7.8). Figure 8 and Table 1 show that at least one deuterium atom was incorporated into all products, including L-FMRFamide. Mass spectra of L- and D-FMRFamide (Figure 8B) indicated the addition of one deuterium atom. In contrast, epimers of S-1−S-4 gained more than one deuterium atom. The products were divided into two groups according to their isotope ratios (Table 1): partially deuterated, L-FMRFamide, S-2, and S-3; highly deuterated, D-FMRFamide, S-1, and S-4. On the basis of these results, we propose a mechanism in which the initial Schiff base (aldimine) formation is followed by tautomerization to the ketimine, and the chirality is lost (Scheme 1). Thus, the reaction of the α-amino group on the N-terminus of L-FMRFamide with the carbonyl group of PLP formed a Schiff base (L-aldimine). The L-aldimine would be converted to the D-aldimine through tautomerization to a ketimine, which has a prochiral α-carbon. The ketimine can undergo either hydrolysis to form α-ketoamide-FMRFamide or protonation to yield the D-aldimine because it occurs in a nonstereospecific manner. In the deuterated buffer, the L-aldimine can add deuterium at the imine carbon to form a ketimine, which incorporates a further deuterium at the α-carbon. This results in the addition of more than one deuterium (S-1−S-4). Both the L- and D-aldimine are then hydrolyzed, yielding L- and D-FMRFamide, respectively, which should

dx.doi.org/10.1021/tx400354d | Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

Scheme 1. Proposed Mechanism for the PLP-Mediated Epimerization of L-FMRFamide



incorporate one deuterium atom. The initial formation of the L-aldimine (S-2 and S-3) and its hydrolysis back to L-FMRFamide can lead to partial incorporation of deuterium because they occur before the epimerization of the α-carbon. The highly deuterated D-aldimine (S-1 and S-4) and D-FMRFamide confirmed their formation through the ketimine. The generation of the ketimine was further supported by the detection of α-ketoamide-FMRFamide.

The reaction of L-FMRFamide with other aldehydes showed that the epimerization of the N-terminal amino acid may depend on the stability of the Schiff base intermediate. PLP or ONE can stabilize the Schiff base with longer conjugation systems, such as the aromatic pyridyl group or α,β-unsaturated ketone moiety, respectively. The stabilized Schiff base (aldimine) can then be converted to its enantiomer via ketimine formation. In contrast, aldehydes without an extended conjugation system (AA, MG, and Glc) did not undergo N-terminal epimerization. α-Ketoamide-FMRFamide was not detected in the reaction with AA, MG, or Glc, indicating that the ketimine was not produced from unstable aldimines. When copper ions were added to the reaction with Glc, D-FMRFamide was generated with a 6.5% epimerization ratio after 7 days. This is because the copper ions stabilized the Schiff base intermediate through the formation of a coordination complex as reported previously.[36] The observation of Glc-mediated epimerization in the presence of copper ions may have implications for Type 2 diabetes because individuals with this disease have been reported to have a significantly elevated level of serum copper.[42]

Finally, we examined the N-terminal epimerization of HSA, which is believed to be the main target of chemical stress during physiological events such as an increased level of oxidative stress from degenerative aging diseases, and a high level of glucose stress in diabetes mellitus.[43] From the reaction of PLP with DAHK (N-terminal tryptic tetrapeptide of HSA) or whole HSA, we detected the epimerization of the N-terminal amino acid with a ratio of 44.3 or 26.3%, respectively, which strongly suggests that this type of PTM can occur in biological systems.

In summary, we have demonstrated the aldehyde-mediated epimerization of N-terminal amino acids. Because the epimerization of proteins may have been overlooked by conventional MS-based proteomics strategies, analyzing N-terminal epimerization could be an alternative approach to identifying aldehyde stress markers. α-Dicarbonyls are formed through the autoxidation of glucose and the degradation of Amadori products or Schiff base adducts by metal ion catalysis.[44] Furthermore, the formation and accumulation of advanced glycation end products are associated with aging and the long-term complications of diabetes.[45] Therefore, we believe that epimerization of N-terminal amino acids can also occur *in vivo* as a PTM under higher levels of aldehyde stress, such as hyperglycemia in diabetes.

AUTHOR INFORMATION

Corresponding Author

*Department of Bioanalytical Chemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aramaki-Aoba, Aoba-ku, Sendai 980-8578, Japan. Telephone: +81-22-795-6817. Fax: +81-22-795-6816. E-mail: t-oe@mail.pharm.tohoku.ac.jp.

Funding

This work was supported in part by a Grant-in-Aid for Challenging Exploratory Research (to T.O., 21659035 for 2009−2010) and Grants-in-Aid for Scientific Research (C) (to T.G., 40344684 for 2012−2014; to S.H.L., 22590130 for 2010−2012) from the Japan Society for the Promotion of Science (JSPS).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Prof. Ian A. Blair (University of Pennsylvania, Philadelphia, PA) for kindly donating the LCQ-DECA. We also thank the Biomedical Research Core, School of Medicine, Tohoku University, for the use of the MALDI-TOF-MS instrument.

ABBREVIATIONS

AA, acetaldehyde; Acr, acrolein; Ang, angiotensin; Ang P, pyruvamide-angiotensin II; CHCA, α-cyano-4-hydroxycinnamic acid; Cro, crotonaldehyde; dAdo, 2′-deoxyadenosine; dCyd, 2′-deoxycytidine; dGuo, 2′-deoxyguanosine; D-DAHK, D-Asp^1-L-Ala^2-L-His^3-L-Lys^4; L-DAHK, L-Asp^1-L-Ala^2-L-His^3-L-Lys^4; Dns-Cl, dansyl chloride; EIC, extracted ion chromatogram; ESI, electrospray ionization; FA, formic acid; D-FMRFamide, D-Phe^1-L-Met^2-L-Arg^3-L-Phe^4-NH_2; L-FMRFamide, L-Phe^1-L-Met^2-L-Arg^3-L-Phe^4-NH_2; Glc, D-glucose; Hε, heptanone-etheno; HNE, 4-hydroxy-2(*E*)-nonenal; HSA, human serum albumin; LC, liquid chromatography; MALDI, matrix-assisted laser

desorption ionization; MG, methylglyoxal; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MS^n, multiple-stage mass spectrometry; ONE, 4-oxo-2(*E*)-nonenal; PCA, pyridinecarboxaldehyde; PLP, pyridoxal 5′-phosphate; PTM, post-translational modification; TFA, trifluoroacetic acid; TIC, total ion chromatogram; TOF, time-of-flight

REFERENCES

(1) O'Brien, P. J., Siraki, A. G., and Shangari, N. (2005) Aldehyde sources, metabolism, molecular toxicity mechanisms, and possible effects on human health. *Crit. Rev. Toxicol. 35*, 609−662.

(2) Wolff, S. P., Jiang, Z. Y., and Hunt, J. V. (1991) Protein glycation and oxidative stress in diabetes mellitus and ageing. *Free Radical Biol. Med. 10*, 339−352.

(3) Lee, S. H., Oe, T., and Blair, I. A. (2001) Vitamin C-induced decomposition of lipid hydroperoxides to endogenous genotoxins. *Science 292*, 2083−2086.

(4) McCormick, D. B., Gregory, M. E., and Snell, E. E. (1961) Pyridoxal phosphokinases. I. Assay, distribution, purification, and properties. *J. Biol. Chem. 236*, 2076−2084.

(5) Sultatos, L. G., Pastino, G. M., Rosenfeld, C. A., and Flynn, E. J. (2004) Incorporation of the genetic control of alcohol dehydrogenase into a physiologically based pharmacokinetic model for ethanol in humans. *Toxicol. Sci. 78*, 20−31.

(6) Conklin, D., Prough, R., and Bhatanagar, A. (2007) Aldehyde metabolism in the cardiovascular system. *Mol. BioSyst. 3*, 136−150.

(7) Marnett, L. J., and Plastaras, J. P. (2001) Endogenous DNA damage and mutation. *Trends Genet. 17*, 214−221.

(8) Lee, S. H., and Blair, I. A. (2011) Lipid peroxide-DNA adducts. In *Chemical Carcinogenesis* (Penning, T. M., Ed.) pp 227−244, Humana Press, c/o Springer Science+Business Media LLC, New York.

(9) Lee, S. H., Rindgen, D., Bible, R. H., Jr., Hajdu, E., and Blair, I. A. (2000) Characterization of 2′-deoxyadenosine adducts derived from 4-oxo-2-nonenal, a novel product of lipid peroxidation. *Chem. Res. Toxicol. 13*, 565−574.

(10) Lee, S. H., Williams, M. V., Dubois, R. N., and Blair, I. A. (2005) Cyclooxygenase-2-mediated DNA damage. *J. Biol. Chem. 280*, 28337−28346.

(11) Pollack, M., Oe, T., Lee, S. H., Silva Elipe, M. V., Arison, B. H., and Blair, I. A. (2003) Characterization of 2′-deoxycytidine adducts derived from 4-oxo-2-nonenal, a novel lipid peroxidation product. *Chem. Res. Toxicol. 16*, 893−900.

(12) Minko, I. G., Kozekov, I. D., Harris, T. M., Rizzo, C. J., Lloyd, R. S., and Stone, M. P. (2009) Chemistry and biology of DNA containing 1,N^2-deoxyguanosine adducts of the α,β-unsaturated aldehydes acrolein, crotonaldehyde, and 4-hydroxynonenal. *Chem. Res. Toxicol. 22*, 759−778.

(13) Synold, T., Xi, B., Wuenschell, G. E., Tamae, D., Figarola, J. L., Rahbar, S., and Termini, J. (2008) Advanced glycation end products of DNA: Quantification of N^2-(1-Carboxyethyl)-2′-deoxyguanosine in biological samples by liquid chromatography electrospray ionization tandem mass spectrometry. *Chem. Res. Toxicol. 21*, 2148−2155.

(14) Sayre, L. M., Lin, D., Yuan, Q., Zhu, X., and Tang, X. (2006) Protein adducts generated from products of lipid oxidation: Focus on HNE and ONE. *Drug Metab. Rev. 38*, 651−675.

(15) Oe, T., Arora, J. S., Lee, S. H., and Blair, I. A. (2003) A novel lipid hydroperoxide-derived cyclic covalent modification to histone H4. *J. Biol. Chem. 278*, 42098−42105.

(16) Yocum, A. K., Oe, T., Yergey, A. L., and Blair, I. A. (2005) Novel lipid hydroperoxide-derived hemoglobin histidine adducts as biomarkers of oxidative stress. *J. Mass Spectrom. 40*, 754−764.

(17) Oe, T., Lee, S. H., Silva Elipe, M. V., Arison, B. H., and Blair, I. A. (2003) A novel lipid hydroperoxide-derived modification to arginine. *Chem. Res. Toxicol. 16*, 1598−1605.

(18) Oya, T., Hattori, N., Mizuno, Y., Miyata, S., Maeda, S., Osawa, T., and Uchida, K. (1999) Methylglyoxal modification of protein. Chemical and immunochemical characterization of methylglyoxal-arginine adducts. *J. Biol. Chem. 274*, 18492−18502.

(19) Kreil, G. (1994) Conversion of L- to D-amino acids: A posttranslational reaction. *Science 266*, 996−997.

(20) Kamatani, Y., Minakata, H., Iwashita, T., Nomoto, K., In, Y., Doi, M., and Ishida, T. (1990) Molecular conformation of achatin-I, an endogenous neuropeptide containing D-amino acid residue. *FEBS Lett. 276*, 95−97.

(21) Kreil, G. (1994) Peptides containing a D-amino acid from frogs and molluscs. *J. Biol. Chem. 269*, 10967−10970.

(22) Fujii, N. (2005) D-Amino acid in elderly tissues. *Biol. Pharm. Bull. 28*, 1585−1589.

(23) Masters, P. M. (1983) Stereochemically altered noncollagenous protein from human dentin. *Calcif. Tissue Int. 35*, 43−47.

(24) Fujii, N., Ishibashi, Y., Satoh, K., Fujino, M., and Harada, K. (1994) Simultaneous racemization and isomerization at specific aspartic acid residues in αB-crystallin from the aged human lens. *Biochim. Biophys. Acta 1204*, 157−163.

(25) Powell, J. T., Vine, N., and Crossman, M. (1992) On the accumulation of D-aspartate in elastin and other proteins of the ageing aorta. *Atherosclerosis 97*, 201−208.

(26) Ohtani, S., Yamamoto, T., Matsushima, Y., and Kobayashi, Y. (1998) Changes in the amount of D-aspartic acid in the human femur with age. *Growth, Dev. Aging 62*, 141−148.

(27) Lyons, B., Kwan, A. H., Jamie, J., and Truscott, R. J. W. (2013) Age-dependent modification of proteins: N-terminal racemization. *FEBS J. 280*, 1980−1990.

(28) Shapira, R., Austin, G. E., and Mirra, S. S. (1988) Neuritic plaque amyloid in Alzheimer's disease is highly racemized. *J. Neurochem. 50*, 69−74.

(29) Roher, A. E., Lowenson, J. D., Clarke, S., Wolkow, C., Wang, R., Cotter, R. J., Reardon, I. M., Zurcher-Neely, H. A., Heinrikson, R. I., and Ball, M. J. (1993) Structural alterations in the peptide backbone of β-amyloid core protein may account for its deposition and stability in Alzheimer's disease. *J. Biol. Chem. 268*, 3072−3083.

(30) Amano, M., Hasegawa, J., Kobayashi, N., Kishi, N., Nakazawa, T., Uchiyama, S., and Fukui, K. (2011) Specific racemization of heavy-chain cysteine-220 in the hinge region of immunoglobulin γ1 as a possible cause of degradation during storage. *Anal. Chem. 83*, 3857−3864.

(31) Lee, S. H., Goto, T., and Oe, T. (2008) A novel 4-oxo-2(*E*)-nonenal-derived modification to angiotensin II: Oxidative decarboxylation of N-terminal aspartic acid. *Chem. Res. Toxicol. 21*, 2237−2244.

(32) Gilmore, J. M., Scheck, R. A., Esser-Kahn, A. P., Joshi, N. S., and Francis, M. B. (2006) N-Terminal protein modification through a biomimetic transamination reaction. *Angew. Chem., Int. Ed. 45*, 5307−5311.

(33) Scheck, R. A., Dedeo, M. T., Iavarone, A. T., and Francis, M. B. (2008) Optimization of a biomimetic transamination reaction. *J. Am. Chem. Soc. 130*, 11762−11770.

(34) Eliot, A. C., and Kirsch, J. F. (2004) Pyridoxal phosphate enzymes: Mechanistic, structural, and evolutionary considerations. *Annu. Rev. Biochem. 73*, 383−415.

(35) Simon, E. S., and Allison, J. (2009) Determination of pyridoxal-5′-phosphate (PLP)-bonding sites in proteins: A peptide mass fingerprinting approach based on diagnostic tandem mass spectral features of PLP-modified peptides. *Rapid Commun. Mass Spectrom. 23*, 3401−3408.

(36) Akagawa, M., Sasaki, T., and Suyama, K. (2002) Oxidative deamination of lysine residue in plasma protein of diabetic rats. *Eur. J. Biochem. 269*, 5451−5458.

(37) Bada, J. L. (1975) Amino acid racemization reactions and their geochemical implications. *Naturwissenschaften 62*, 71−79.

(38) Friedman, M. (1999) Chemistry, nutrition, and microbiology of D-amino acids. *J. Agric. Food Chem. 47*, 3457−3479.

(39) McCudden, C. R., and Kraus, V. B. (2006) Biochemistry of amino acid racemization and clinical application to musculoskeletal disease. *Clin. Biochem. 39*, 1112−1130.

(40) Cloos, P. A. C., and Christgau, S. (2002) Non-enzymatic covalent modifications of proteins: Mechanisms, physiological consequences and clinical applications. *Matrix Biol. 21*, 39−52.

(41) Ritz-Timme, S., and Collins, M. J. (2002) Racemization of aspartic acid in human proteins. *Ageing Res. Rev. 1*, 43−59.
(42) Viktorínová, A., Tošerová, E., Križko, M., and Ďuračková, Z. (2009) Altered metabolism of copper, zinc, and magnesium is associated with increased levels of glycated hemoglobin in patients with diabetes mellitus. *Metabolism 58*, 1477−1482.
(43) Goto, T., Murata, K., Lee, S. H., and Oe, T. (2013) Complete amino acid sequencing and immunoaffinity clean-up can facilitate screening of various chemical modifications on human serum albumin. *Anal. Bioanal. Chem. 405*, 7383−7395.
(44) Thornalley, P. J., Langborg, A., and Minhas, H. S. (1999) Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem. J. 344*, 109−116.
(45) Singh, R., Barden, A., Mori, T., and Beilin, L. (2001) Advanced glycation end-products: A review. *Diabetologia 44*, 129−146.