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Characterization and Identification of Dityrosine Cross-Linked Peptides Using Tandem Mass Spectrometry

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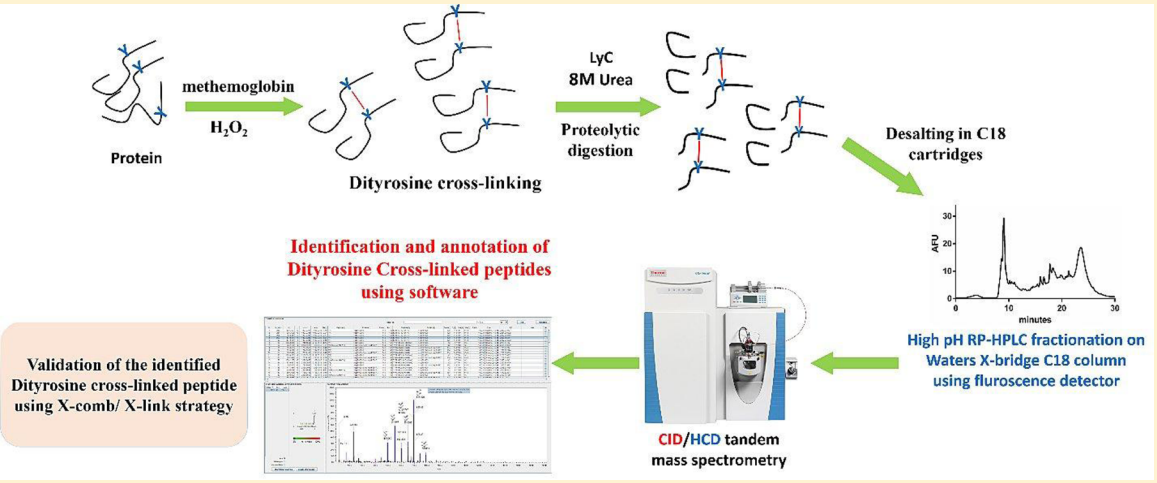
Supporting Information



ABSTRACT: The use of mass spectrometry coupled with chemical cross-linking of proteins has become a powerful tool for proteins structure and interactions studies. Unlike structural analysis of proteins using chemical reagents specific for lysine or cysteine residues, identification of gas-phase fragmentation patterns of endogenous dityrosine cross-linked peptides have not been investigated. Dityrosine cross-linking in proteins and peptides are clinical markers of oxidative stress, aging, and neurodegenerative diseases including Alzheimer's disease and Parkinson's disease. In this study, we investigated and characterized the fragmentation pattern of a synthetically prepared dityrosine cross-linked dimer of Aβ(1−16) using ESI tandem mass spectrometry. We then detailed the fragmentation pattern of dityrosine cross-linked Aβ(1−16), using collision induced dissociation (CID), higher-energy collision induced dissociation (HCD), electron transfer dissociation (ETD), and electron capture dissociation (ECD). Application of these generic fragmentation rules of dityrosine cross-linked peptides allowed for the identification of dityrosine cross-links in peptides of Aβ and α-synuclein generated in vitro by enzymatic peroxidation. We report, for the first time, the dityrosine cross-linked residues in human hemoglobin and α-synuclein under oxidative conditions. Together these tools open up the potential for automated analysis of this naturally occurring post-translation modification in neurodegenerative diseases as well as other pathological conditions.

Posttranslational modifications (PTMs) of newly synthesized proteins modulate their function, localization, enzymatic activity, and structural stability.[1] The array of modifications provides the potential to increase the number of protein products

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of a given genome.[2] Many PTMs are also dependent upon the interaction of the organism with external environmental factors. Oxidative and nitrative stress is a ubiquitous feature of aerobic metabolism and can directly modify proteins to alter structure and function.[3] Oxidative stress and endogenous mechanisms can result in the covalent cross-link between two tyrosine residues resulting in the formation of a dityrosine (DT) post translational modification.[4] Since Gross and Sizer synthesized dityrosine by enzymatic peroxidation and Anderson demonstrated that dityrosine is present in the hydrolysates of resilin, many structural protein hydrolysates have been shown to contain dityrosine.[5,6] Proteins with dityrosine cross-links have been identified in many vertebrate and invertebrate connective tissues as a product of normal PTMs contributing to their stability, elasticity, and integrity.[6−9] Dityrosine cross-linking in proteins can be promoted by oxidizing agents such as hydroxyl radical, peroxynitrite, nitrosoperoxycarbonate, and lipid hydroperoxides as well as ultraviolet and γ-irradiation.[10] Enzymatic oxidation of tyrosine (Tyr) residues in proteins by peroxidase-catalyzed mechanism has been found to promote dityrosine cross-links. In vitro, dityrosine cross-linking of proteins such as lysozyme, calmodulin, myoglobin, hemoglobin, insulin, and RNase is known to occur as a product of oxidative/nitrative stress.[11]

Dityrosine has also been identified as a clinical biomarker of oxidative stress, aging, and diverse pathological conditions. Dityrosine has been found in Alzheimer's Disease (AD),[12] Parkinson Disease (PD),[13] lipofuscin granules in the human brain,[14] and cerebrospinal fluid.[15] Enrichment of dityrosine cross-linked low density lipoproteins (LDL) have been reported in human atherosclerotic lesions and plaques.[16] Elevated levels of protein-bound dityrosine have been reported in the blood plasma of patients with chronic renal failure,[17] while diabetic patients have higher levels of free dityrosine in their urine.[18] The advantage of evaluating dityrosine as a biomarker for oxidative/nitrative stress is based upon (i) its chemical stability (unreactive to the changes in oxygen/pH), (ii) free dityrosine is not incorporated in de novo proteins synthesis, and (iii) dityrosine is released after proteolysis of oxidatively modified proteins.[4] Hence, quantitative determination of the levels of dityrosine reflects the extent of oxidative and/or nitrative stress on endogenous proteins.[2] Current quantitation of dityrosine from protein hydolysates is achieved by the measurement of a characteristic fluorescence at 420 nm upon excitation at either 315 nm (alkaline solutions) or 284 nm (acidic solutions).[19] Use of mass spectrometric quantitation of free dityrosine in biological samples has emerged as a critical analytical tool.[2] Quantitative detection of free dityrosine has been performed using isotopic dilution gas chromatography coupled to mass spectrometry (GC-MS/MS) as well as liquid chromatography coupled to ESI-MS/MS in urine and eye lens protein hydrolysates.[20,21]

Despite these advancements in quantitatively determining free dityrosine, there is a lack in our ability to determine the specific protein targets involved in the dityrosine cross-link. Routine application of LC−MS/MS-based proteomics for many PTMs such as phosphorylation and ubiquitination is quite common, but dityrosine cross-linked peptides sequences are yet to be widely analyzed.[22] Identification of dityrosine cross-linked peptides like any other cross-linked peptides poses a challenge as data complexity increases as a square of the number of proteins and potential cross-linking sites (the n^2 problem).[23] To overcome these limitations, we investigated various commonly used gas-phase fragmentation techniques to determine if there was a characteristic fragmentation for the synthetically prepared dityrosine cross-linked Aβ dimer in ESI-MS/MS that could be used to develop a characteristic set of fragmentation rules. In this report, we discuss the fragmentation pattern of dityrosine cross-linked Aβ(1−16) dimer using collision-induced dissociation (CID), higher-energy collision induced dissociation (HCD), electron transfer dissociation (ETD), and electron capture dissociation (ECD). This allowed us to propose a generic workflow for the identification of dityrosine cross-linked peptides, which led to successful identification of novel dityrosine cross-links in human hemoglobin and α-synuclein. This work flow provides the foundation for the identification of protein pairs involved in endogenous dityrosine cross-links.

MATERIALS AND METHODS

Sample Preparation. Protein concentrations were determined using UV−visible spectroscopy (for hemoglobin $\varepsilon_{405\ nm}$ = 178000 M^{-1} cm^{-1}, α-synuclein $\varepsilon_{280\ nm}$ = 5120 M^{-1} cm^{-1}, and Aβ(1−42) $\varepsilon_{214\ nm}$ = 94526 M^{-1} cm^{-1}). Hemoglobin from Sigma-Aldrich (H7379) was prepared by dissolving in phosphate buffer saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) pH 7.4. See the Supporting Information for Aβ(1−42) and α-synuclein stock solution preparation. Hydrogen peroxide (H_2O_2) solutions were freshly prepared by diluting a 30% stock (Merck) solution to an appropriate concentration; the concentration of the diluted stock was estimated using the $\varepsilon_{240\ nm}$ = 43.6 M^{-1} cm^{-1}.

In vitro Dityrosine Cross-Link Generation in Proteins. In this study, the peroxidase activity of hemoglobin was used to generate dityrosine cross-links in Aβ(1−42) and α-synuclein. Five micromolar hemoglobin was incubated with H_2O_2 (100 μM) for 10 min to produce the active catalyst (ferrylhemoglobin). To this solution, recombinant α-synuclein or Aβ(1−42) was added to a final concentration of 20 and 25 μM, respectively. Following this, each sample was then incubated with 20 μM H_2O_2 (final concentration) at room temperature for 30 min. The control samples were prepared without any Hb. The cross-linked oligomeric Aβ(1−42) or α-synuclein samples were centrifuged for 5 min and then the supernatant (100 μL) was loaded on to a Superdex 75 10/300 GL size exclusion column (GE Healthcare Life Science) at a flow rate of 0.75 mL/min. Fractions of 0.8 mL were collected using an AKTA fraction collector (Amersham Biosciences).

Data Analysis and Identification of Dityrosine Cross-Linked Peptides. Analyses of the fragment ion of the ESI-MS/MS data for the synthetic dityrosine cross-linked Aβ(1−16) dimer was performed manually in Agilent Qualitative Mass Hunter software (CID on the Q-TOF) and Xcalibur software (HCD and ETD on the LTQ Orbitrap Elite). The nomenclature for naming the product ions generated from either by single backbone cleavage or multiple cleavages during charge-remote fragmentation processes was used for annotating the MS/MS spectra. Besides manual analysis of dityrosine cross-linked peptides, a combination of software tools such as StavroX,[24] which was designed specifically for cross-linked analysis, and standard database search tools such as Mascot were used in order to gain further insight into the specific fragmentation processes of dityrosine cross-linked peptides.[25] Dityrosine cross-linked peptides were considered significant if both the standard Mascot search and Stavrox analysis were concordant. The available crystal structures from PDB (2DN2 for hemoglobin and 1XQ8 for α-synuclein) were used to visualize proteins. Modeling and analysis of proteins to generate the final models of dityrosine

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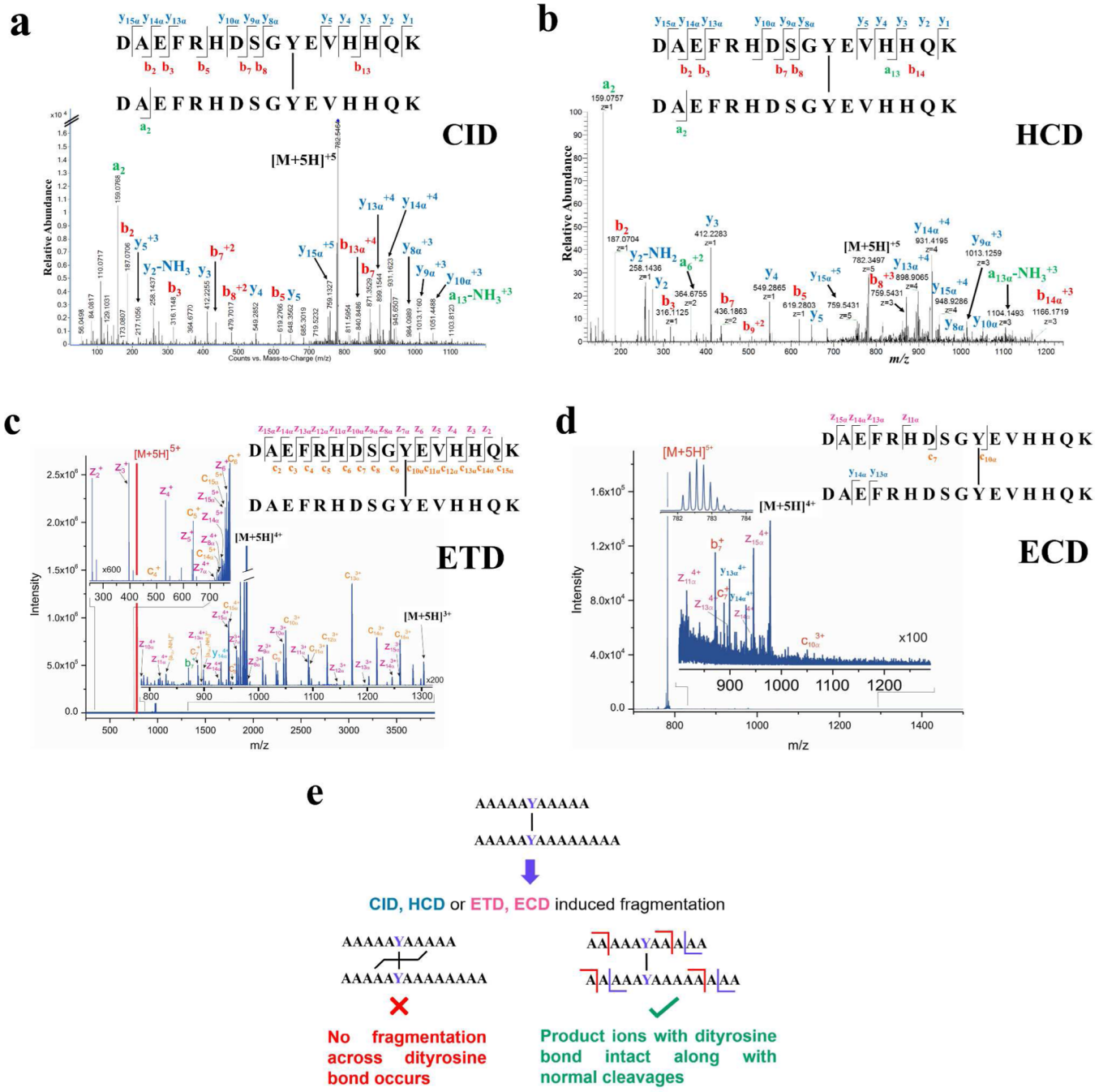

Figure 1. Characterization of gas-phase fragmentation dityrosine cross-linked peptide. (a) The annotated CID ESI-MS/MS spectra of the homodimeric dityrosine cross-linked Aβ(1−16). The precursor ion $[M+5H]^{5+}$ at m/z 782.1417 was selected for fragmentation for CID with collision energy of 23.4 eV on a 6550 Q-TOF. (b) Annotated HCD ESI-MS/MS spectrum of homodimeric dityrosine cross-linked Aβ(1−16) on LTQ-Orbitrap. The precursor ion $[M+5H]^{5+}$ at m/z 782.1417 was targeted for fragmentation with a normalized collision energy of 35 eV and the activation time of 0.1 ms. (c) Annotated ETD MS/MS spectrum of homodimeric dityrosine cross-linked Aβ(1−16) with the precursor ion $[M+5H]^{5+}$ at m/z 782.15 was targeted for fragmentation with the ETD reagent for 1 ms. (d) Annotated ECD MS/MS spectrum of homodimeric dityrosine cross-linked Aβ(1−16) with the precursor ion $[M+5H]^{5+}$ at m/z 782.15 using electrons produced from EMS cell. (e) Schematic representation of gas-phase fragmentation mechanism and feasible product ions from dityrosine cross-linked peptide. Amino acid residues of the peptide side chains of the dityrosine cross-linked peptide has been labeled as A and tyrosine has been labeled Y.

cross-link sites in proteins was performed in UCSF CHIMERA.[26] Cross-link map was generated using xiNET.[27]

RESULTS

Gas-Phase Fragmentation of Synthetic Dityrosine Cross-Linked Aβ. Peptide sequencing by tandem mass spectrometry forms the basis of bottom-up (shotgun) proteomics. The most common and informative ions are generated by fragmentation at the amide bond between amino acids in tandem mass spectrometry. For the dityrosine cross-linked peptides, we adapted a nomenclature for the possible fragments (Figure S1) that contain both the cross-link and the interpeptide, designating the longer chain as α and the smaller chain β (Figure S1, Schilling nomenclature).[28] We used a

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simplified nomenclature to label homodimeric dityrosine cross-links, where fragments containing the cross-link were designated with an α (e.g., $b_{12}\alpha$). To investigate the fragmentation pattern and formulate generic rules for dityrosine cross-linked peptides, synthetically prepared dityrosine cross-linked Aβ(1−42) with oxidized Met35 (sulfoxy methionine) was taken as a model system. High-resolution HPLC-ESI-MS of the synthetic dityrosine cross-linked Aβ(1−42) peptide displayed a charge envelope with expected $[M + 6H]^{6+}$, $[M + 7H]^{7+}$, and $[M + 8H]^{8+}$ ions with m/z 1510.5747, 1295.0597, and 1133.1810 (Figure S2a), respectively. However, this model peptide was not used subsequently due to (i) its high hydrophobicity-induced aggregation on C18 column and (ii) poor elution profile.

We next investigated the proteolytic digestion of the dityrosine cross-linked Aβ(1−42) under denaturing conditions with LysC before MS/MS analysis. The ESI-Q-TOF of the digested dityrosine cross-linked Aβ(1−42) dimer resulted in a dityrosine cross-linked Aβ(1−16) dimer with a charge envelope from z = 8−3 (Figure S2b). The CID fragmentation pattern of this peptide at precursor $[M + 5H]^{5+}$ ion with m/z 752.1471 is shown in Figure 1a. The C-terminal of the dityrosine cross-linked peptide in the MS/MS spectrum was covered by the series of y_1−y_6 ions, while the N-terminal sequence was mostly covered by the series of b_2−b_8 product ions (Figure 1a). Additionally, product ions with the dityrosine cross-link intact were also observed in the CID spectrum as indicated by the highly charged series $y_{8\alpha}$−$y_{15\alpha}$ ions, along with $b_{13\alpha}$ (Figure 1a). We did not see any cleavage of the C−C bond between aromatic rings of dityrosine yielding monomeric Aβ(1−16). High pH reversed phase HPLC of the proteolytically digested dityrosine cross-linked Aβ(1−42) using a fluorescence detector indicated the presence of an equimolar mixture of two dityrosine cross-linked peptides (Figure S3a).[29,30] While peak 1 corresponds to dityrosine cross-linked Aβ(1−16) dimer as verified from ESI-MS/MS (Figure 1a), the structure of the other LysC-digested dityrosine cross-linked Aβ dimer corresponding to peak 2 was confirmed by ESI-MS/MS to be a heterodimer of Aβ(1−16 and 1−10) resulting from incomplete peptide synthesis (Figure S3b).[30] The CID fragmentation pattern for the heterodimeric dityrosine cross-linked peptide (Figure S3b) was found to be similar to that of the homodimeric dityrosine cross-linked Aβ(1−16) peptide. Higher-energy C-trap dissociation (HCD) on a LTQ Orbitrap Elite resulted in a similar fragmentation pattern observed with CID for homodimeric dityrosine cross-linked Aβ(1−16) peptide (Figure 1b).

Electron transfer dissociation (ETD) and electron capture dissociation (ECD) by electron transfer/capture to protonated peptides causing random peptide backbone breakage are a nonergodic process (not involving intramolecular vibrational energy redistribution) unlike CID/HCD.[31] ETD/ECD is also preferred for protonated peptides with high charge states and have been used successfully for sequencing large proteins.[32] Different types of fragmentation products were observed in the ETD spectra of homodimeric dityrosine cross-linked Aβ(1−16) peptide in Orbitrap Fusion Lumos (Figure 1c and Figure S4) by targeting the $[M + 5H]^{5+}$ ion (m/z 782.1417) with increasing reaction time. ETD spectrum even with the lowest ETD reaction time of 1 ms (Figure 1c) showed complete amino acid sequence except for the lowest c_1^+ and z_1^+ fragments that were beyond the mass range used where observation of their counterpart ions $z_{15\alpha}^{3+}$ and $c_{15\alpha}^{3+}$ assisted in the assignment of the respective N- and C-termini amino acids. Registration of the $c_{n\alpha}$ and $z_{n\alpha}$ fragment ions indicates the fragmentation of one side chain across the dityrosine cross-link with the other peptide chain intact. The intensity of the c_2^+ and c_3+ ions was very weak under these experimental conditions, but application of the ETD supplemental activation (combination of ETD and HCD at 10% normalized collision energy) resulted in a marked intensity increase consistent with an activation barrier in the formation of these fragment ions. At the ETD reaction time of 1 ms, 5+ charge precursor ion reduces to 4+ and 3+ charge states. Observation of $z_{15\alpha}^{5+}$, $z_{14\alpha}^{5+}$, $c_{15\alpha}^{5+}$, and $c_{14\alpha}^{5+}$ ions was unexpected and may be due to simultaneous electron autodetachment similar to vibrational excitation of small molecules by electrons through the formation of short-lived negative ion resonances.[33] The 4+ charge reduced species fragmented via formation of two pairs of complementary ions: (i) 4+ charge fragments and neutral counterparts and (ii) 3+ and 1+ charge states fragments. Both pathways were observed in the ETD spectrum with 1 ms reaction time (Figure 1c). A third type of fragmentation from the 4+ reduced ions into two counterpart ions both with 2+ charge states were not observed. 3+ reduced ions did not fragment into doubly charged fragments either. Increasing the ETD reaction time to 5 and 10 ms resulted in further reduction of the precursor ions to 2+ charge reduced states along with the observation of doubly charged fragment ions; since their real precursor ions are 2+ reduced ions, the counterpart species for these doubly charged fragment ions are neutral fragments. Singly charged reduced ions and fragment ions with another peptide chain intact started to appear for the ETD reaction time 15 ms and longer (Figure S4). Although these spectra were obtained with $[M + 5H]^{5+}$ precursor ions, nevertheless, higher ETD reaction time spectra show ETD type fragmentation of reduced species rather than that of the selected precursor. Therefore, only ETD spectrum with 1 ms reaction time (Figure 1c) can be most closely related to the ETD spectrum of the $[M + 5H]^{5+}$ precursor ions. Similar fragmentation pattern along with reduced species of the precursor $[M + 5H]^{5+}$ ion were observed both in the ECD reaction using electrons produced by EMS cell (Figure 1d) as well as in FT-ICR-MS/MS (Figure S5), although ECD was not as detailed as ETD fragmentation.

The fragmentation features for dityrosine cross-linked homodimeric and heterodimeric peptides followed specific general rules (Figure 1e). First, the fragmentation of the peptide chains led to the cleavage on either side of the dityrosine cross-linking site similar to that observed in linear peptides. Second, fragmentations led to formation of product ions on one side chain across the DT bond, with the second peptide chain intact (Figure 1e). Thus, the structure and the sequence of the dityrosine cross-linked dimerized peptide can be determined by MS/MS fragmentation in CID, HCD, and ETD. Since, no C−C bond cleavage occurs at the aromatic ring of the dityrosine in MS/MS, we can intuitively use the information to localize the dityrosine bond in a cross-linked peptide with multiple tyrosine residues by careful evaluation of the fragment ions.

Identification of in Vitro Dityrosine Cross-Linked Peptides. In vitro dimerization of Aβ by dityrosine cross-linking is known to occur in the presence of Cu^{2+}/ascorbic acid or by peroxidases in the presence of H_2O_2.[34,35] Human hemoglobin has a pseudoperoxidase activity due to the formation of ferrylhemoglobin (Fe(IV)=O) species (Figure S6) in the presence of H_2O_2, which is similar to compound II in peroxidases. Incubation of Aβ(1−42) with hemoglobin/H_2O_2 led to the formation of covalently cross-linked Aβ oligomers (dimers and tetramers) as detected by Western blots of the fractions from size-exclusion chromatography (SEC) (Figure 2a). The Y10A mutant of Aβ(1−42) did not form such SDS-

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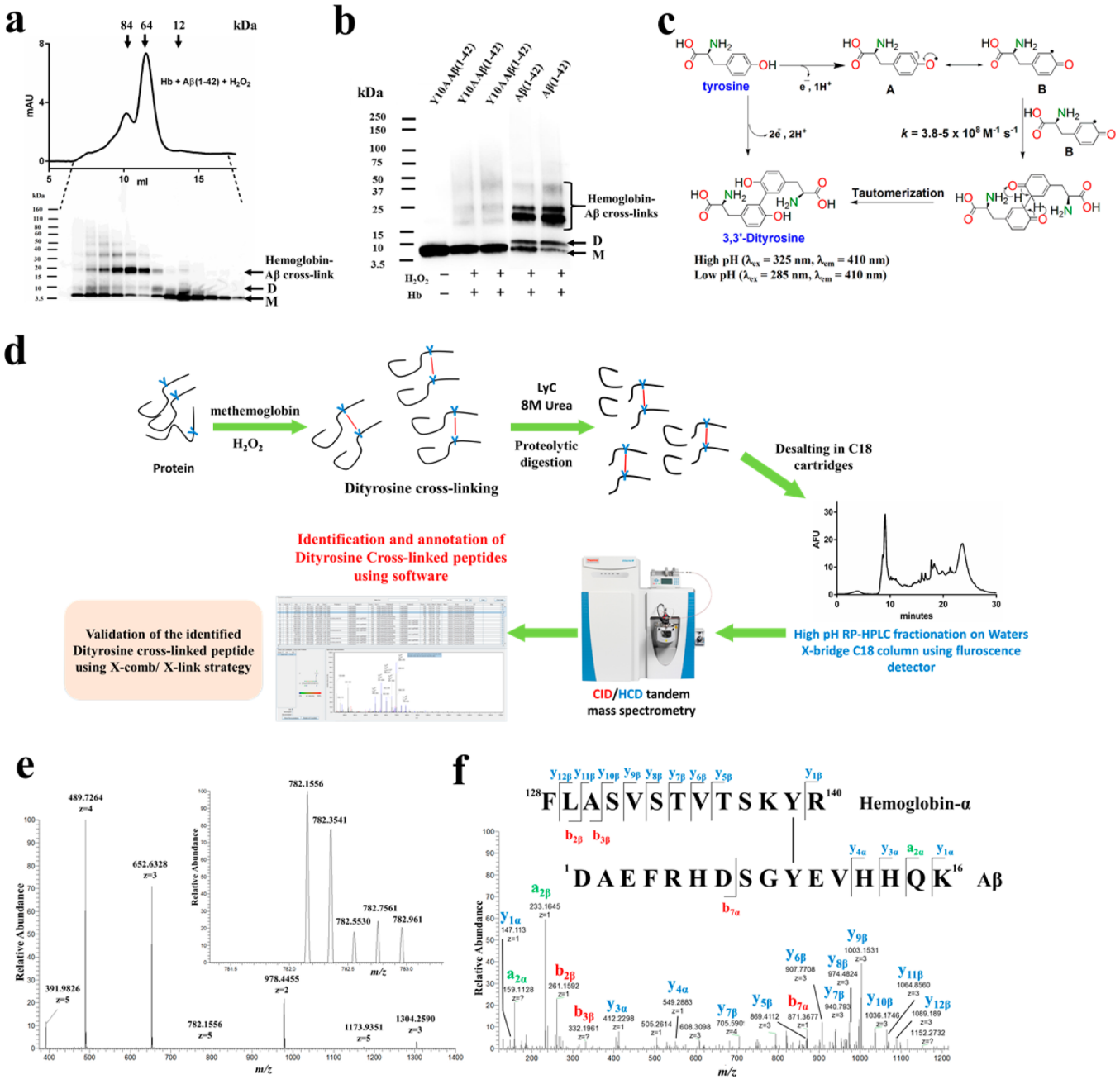

Figure 2. Catalytic peroxidation of Aβ(1−42) by hemoglobin in the presence of H_2O_2 induces dityrosine cross-linked oligomers of Aβ. (a) Western blot analysis after of the fractions with W0−2 antibody from SEC of the mixture of 20 μM Aβ(1−42) with 5 μM hemoglobin and 20 μM H_2O_2, demonstrating the formation of oligomeric species of Aβ(1−42) (M, monomers; D, dimers; hemoglobin-Aβ cross-link; and high molecular weight oligomers). (b) Mutation of Tyr10 clearly illustrates that these oligomeric species of Aβ(1−42) are dityrosine cross-linked, as oligomeric species are not observed when Y10A mutant of Aβ(1−42) with hemoglobin/H_2O_2. From left to right, 20 μM Y10A mutant of Aβ(1−42) incubated with 20 μM H_2O_2; 20 μM Y10A mutant of Aβ(1−42) incubated with 5 μM hemoglobin and 20 μM H_2O_2; 20 μM Y10A mutant of Aβ(1−42) incubated with 5 μM hemoglobin and 100 μM H_2O_2; 20 μM Aβ(1−42) incubated with 5 μM hemoglobin and 20 μM H_2O_2; 10 μM Aβ(1−42) incubated with 5 μM hemoglobin and 20 μM H_2O_2. (c) Reaction mechanism of dityrosine cross-link formation from tyrosine. (d) A schematic for bottom-up workflow for identifying dityrosine cross-links by off-line separation of proteolytically digested dityrosine cross-linked peptides and analysis of the ESI-MS/MS these peptides using StavroX software. (e) ESI-MS spectra of the dityrosine cross-linked Aβ(1−16) formed due to peroxidation by hemoglobin/H_2O_2 following LysC digestion and high pH HPLC fractionation. Inset displays the isotopic distribution of +5 charge state (m/z = 782.1556). (f) Annotated HCD-MS/MS of a dityrosine cross-link peptide between ^{128}FLASVSTVTSKYR^{140} (hemoglobin α) and ^{1}DAEFRHDSGYEVHHQK^{16} (Aβ), precursor ion $[M + 5H]^{5+}$ at m/z 705.5533 (M = 3522.7665) cross-linked between Y140 of hemoglobin α and Y10 of Aβ, respectively.

resistant oligomers (Figure 2b). These covalently cross-linked Aβ peptides were digested by LysC, and the resulting peptides were fractionated by reverse-phase HPLC at high pH (pH 10) using fluorescence detection of dityrosine (λ_{ex} = 325 nm; λ_{em} = 410 nm). The dityrosine fluorescent peaks were then analyzed by ESI-MS/MS using HCD fragmentation (Figure 2d). The major fluorescent peak at 9.4 min in the high pH HPLC chromatogram was consistent with the formation of a dityrosine cross-linked dimer (Figure S7). ESI-MS in LTQ Orbitrap confirmed it to be the dityrosine cross-linked Aβ(1−16) dimer (Figure 2e and

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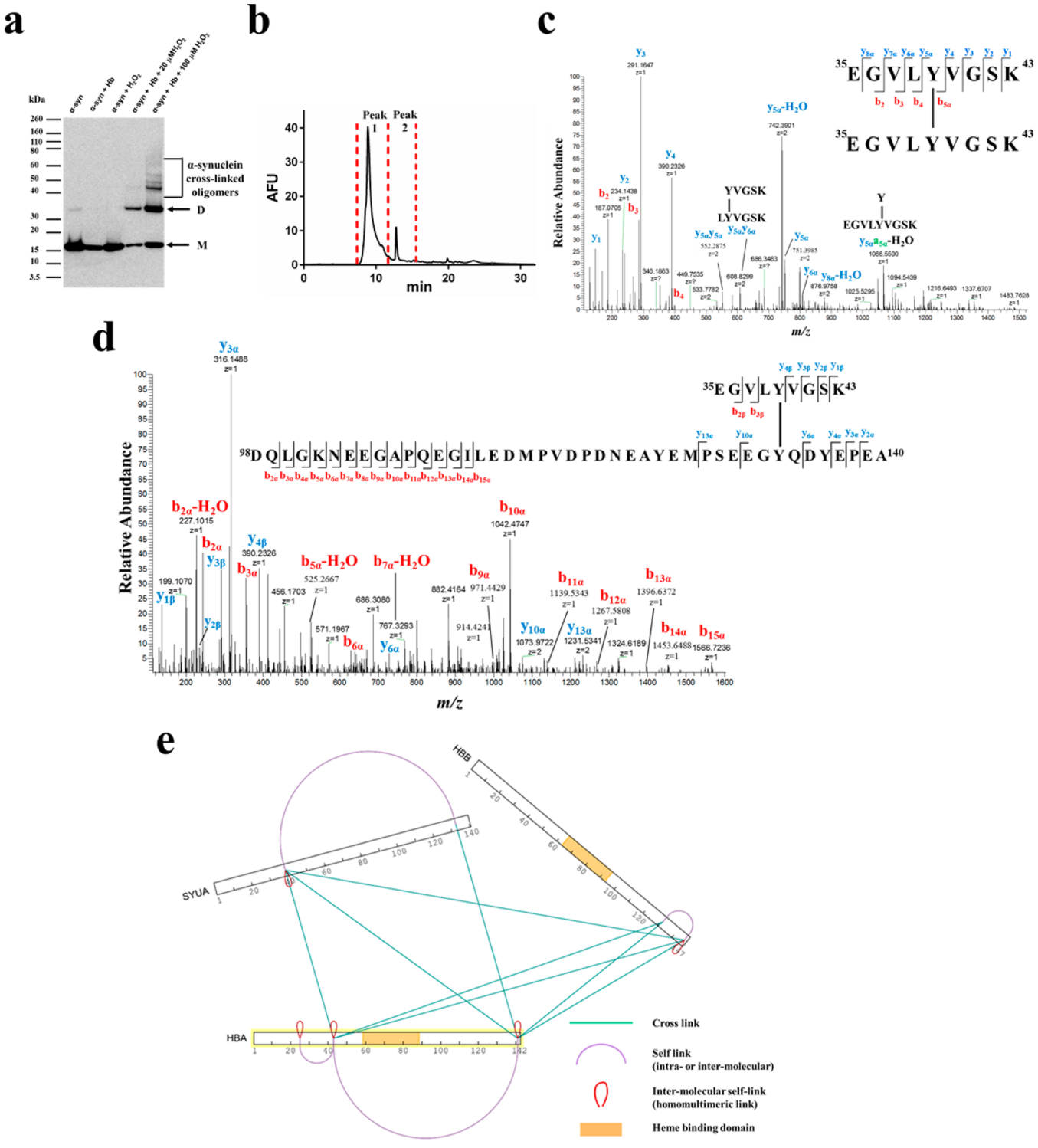

Figure 3. Catalytic peroxidation of α-synuclein by hemoglobin/H_2O_2 lead to the formation of dityrosine cross-linked oligomers of α-synuclein. (a) Western blot analysis of oligomerization of α-synuclein by hemoglobin/H_2O_2. From left to right, 20 μM α-synuclein; 20 μM α-synuclein incubated with 20 μM H_2O_2; 20 μM α-synuclein incubated with 5 μM hemoglobin; 20 μM α-synuclein incubated with 5 μM hemoglobin and 20 μM H_2O_2; 20 μM α-synuclein incubated with 5 μM hemoglobin and 50 μM H_2O_2 (M, monomers; D, dimers and cross-linked oligomers). (b) Detection of two dityrosine positive peaks of α-synuclein peptides, peak 1 and peak 2, marked in red from the lane 5 generated in vitro by ferrylhemoglobin. (c) The HCD ESI-MS/MS spectrum of the dityrosine cross-linked ^{35}EGVLYVGSK^{43} homodimer with $[M + 3H]^{3+}$ at m/z 634.0069 (M = 1899.0210) corresponding to peak 2 in (b). (d) The annotated HCD-MS/MS spectrum of the heterodimer of α-synuclein present in the peak 1 dityrosine cross-link between ^{35}EGVTYVGSK^{43} and ^{98}DQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEGYQYEPEA^{140} with $[M + 4H]^{4+}$ at m/z 1444.8856 (M = 5775.5424), cross-linked between Y39 and Y133. (e) Identification of novel dityrosine cross-links between hemoglobin α/hemoglobin β and α-synuclein and between hemoglobin α and hemoglobin β by StavroX.

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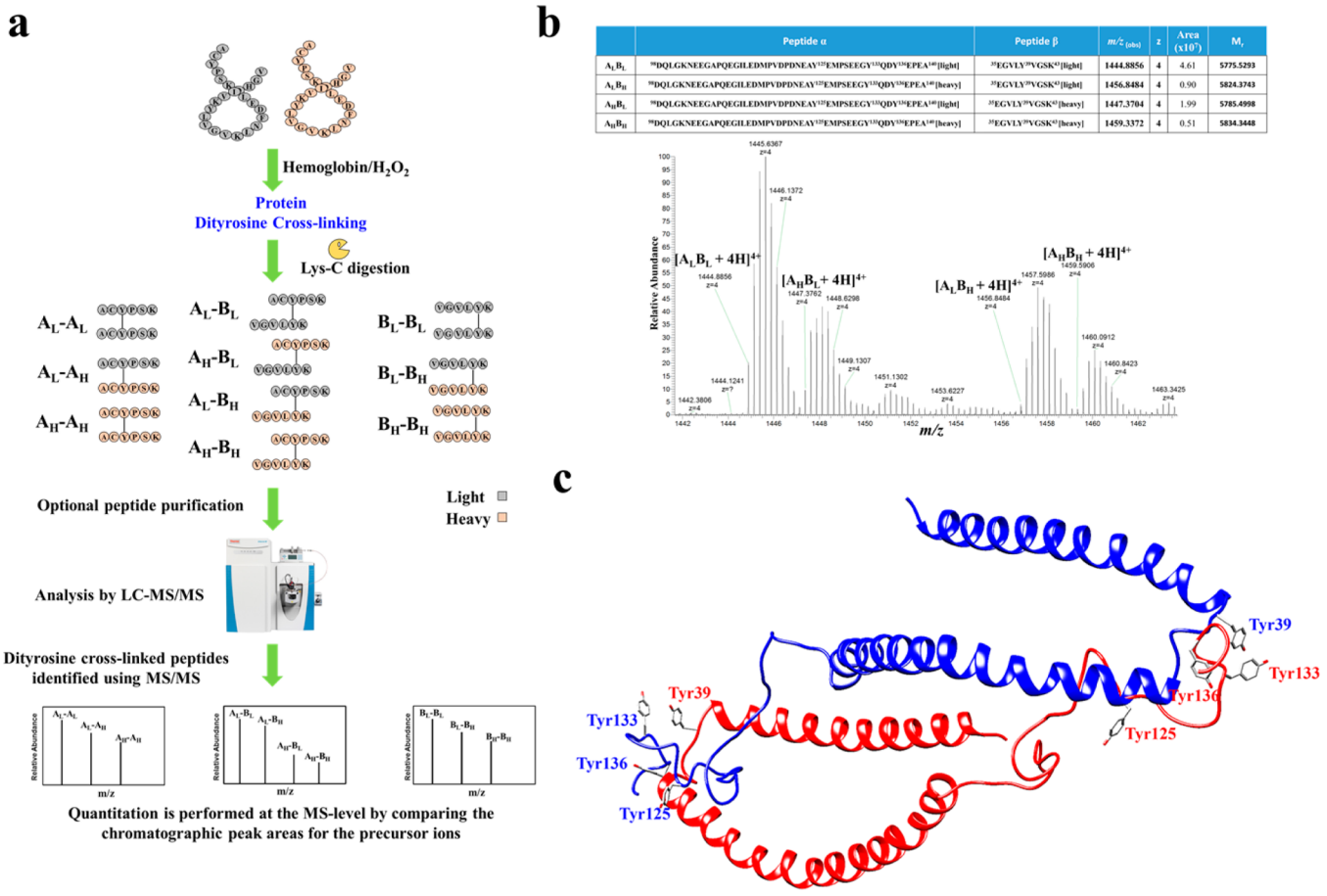

Figure 4. Identification of inter- and intramolecular cross-links using ^{15}N labeled and nonlabeled α-synuclein. (a) Schematic representation of the workflow for relative quantitation of dityrosine cross-linked peptides. A mixture of nonlabeled protein and ^{15}N labeled protein was incubated with hemoglobin/H_2O_2 system for dityrosine cross-link formation. Proteolytic digestion by endoproteinase LysC and a diagram of the possible nonlabeled and ^{15}N labeled dityrosine cross-linked peptides (i.e., A_LA_L, A_LA_H, and A_HA_H for homodimeric dityrosine cross-link; A_LB_L, A_LB_H, A_HB_L, and A_HB_H for heterodimeric dityrosine cross-link). The proteolytic peptides were fractionated by reversed-phase HPLC under high pH conditions.[29] The dityrosine positive fractions from HPLC are subjected to ESI-MS/MS for identification. Relative quantitation can be performed by comparing the chromatographic peak areas for each form of the cross-linked peptide (EIC) separately as they coelute. (b) MS spectra of normal and ^{15}N-labeled dityrosine cross-linked heterodimer between ^{35}EGVLYVGSK^{43} and ^{98}DQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEEGYQYEPEA^{140} (A_LB_L, A_LB_H, A_HB_L, and A_HB_H) used for the relative quantitation of these dityrosine cross-linked peptides. (c) Proposed heterodimeric structure of α-synuclein (PDB: 1XQ8) as determined by the cross-links observed.

Figure S8). Upon analyzing other dityrosine positive fractions (14 min-15 min), we also found a dityrosine cross-link between ^{127}FLASVSTVTSKYR^{141} (hemoglobin α) and ^{1}DAEFRHDSGYEVHHQK^{16} (Aβ) (Figure 2f), consistent with the 20 kDa apparent tetramer observed by Western blot (Figure 2, panels a and b).

Analysis of the other fractions (Figure S7a) demonstrated multiple dityrosine cross-linked peptides of hemoglobin. Hemoglobin in red blood cells has been reported to form dityrosine cross-link adducts when exposed to a constant flux of H_2O_2.[36] Hemoglobin is a tetrameric protein consisting of two pairs of hemoglobin α and hemoglobin β chain. Pure human hemoglobin (methemoglobin) incubated with H_2O_2 formed ferrylhemoglobin (Figure S6), which was then analyzed in ESI-MS/MS using HCD after in-solution proteolytic digestion. Dityrosine cross-links occurred between Tyr24, Tyr42, and Tyr140 in hemoglobin α (Figure S9) and Tyr130 and Tyr145 in hemoglobin β (Figure S9). Analysis of digested peptides of human hemoglobin treated with H_2O_2 revealed the formation of multiple dityrosine cross-linked peptides involving both hemoglobin α and hemoglobin β chains (Figure S10a-l and Table S1). However, this simple bottom-up approach could not distinguish between intramolecular and intermolecular dityrosine cross-links of the hemoglobin tetramer.

Similarly, α-synuclein is also known to form dityrosine cross-linked oligomers in vitro in the presence of Cu^{2+}/H_2O_2 or ascorbic acid as well as by enzymatic peroxidation.[37,38] However, these cross-links have not been fully characterized but are proposed to represent a neurotoxic oligomer. Western blot analysis (antibody 4D6) revealed that monomeric α-synuclein when incubated with hemoglobin/H_2O_2 forms oligomers (Figure 3a). Reversed phase HPLC with fluorescence detection of proteolytically digested α-synuclein oligomers revealed the presence of two main dityrosine peaks (Figure 3b). ESI-MS/MS with HCD of peak 2 in Figure 3b revealed the structure as homodimeric dityrosine cross-link of ^{35}EGVLYVGSK^{43} peptide (Figure 3c), confirming dimerization of α-synuclein by dityrosine cross-linking. This was further verified using complementary ETD fragmentation (Figure S11). Analysis of peak 1 in Figure 3b revealed the structure of a heterodimeric DT cross-link of α-synuclein between ^{35}EGVLYVGSK^{43} and ^{98}DQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEEGYQDY-

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EPEA[140] (one missed cleavage) peptides with possible dityrosine cross-linking between Tyr39 and Tyr125/Tyr133/Ty136 (Figure 3d). The presence of $y_{13\alpha}^{2+}$ and $y_{15\alpha}^{2+}$ product ions and a series of $y_{2\alpha}-y_{5\alpha}$ ions lead to the successful identification and assignment of the position of the cross-linking site in this heterodimeric dityrosine cross-link of α-synuclein between Tyr39 and Tyr133. On further evaluation of the peak 1 using StavroX, software designed to analyze cross-linked peptides,[24] it was possible to find dityrosine cross-linked peptides between α-synuclein and hemoglobin α/ hemoglobin β (Figure S12). This also explains the nature of the higher molecular weight bands at 48 and >64 kDa in the Western blot (Figure 3a). All identified intra- and interprotein dityrosine cross-links between α-synuclein and hemoglobin α/hemoglobin β were summarized in the form of a linkage map (Figure 3e and Table S2).

In order to determine if the dityrosine cross-links observed were intramolecular or intermolecular, the experiment was performed with a mixture of ^{15}N labeled recombinant α-synuclein and nonlabeled α-synuclein (Figure 4a and Figure S13). Intramolecular cross-linking of labeled and nonlabeled A and B peptides should generate only A_LB_L and A_HB_H dityrosine cross-linked heterodimers (Figure 4a), whereas formation of mixtures of heavy and light cross-linked peptides (A_LB_H and A_HB_L) would be an outcome of intermolecular cross-linking. Extracted ion chromatograms (EIC) (Figure 4b) as well as the MS/MS fragmentation spectra validated that the isotope scrambling was successful in both the homodimer (Figure S14, panels a–c) and heterodimer (Figure S14, panels d–g) of α-synuclein. Homodimer of α-synuclein must be intermolecular which is also confirmed by the quantitative ratio of the EIC (Figures S15 and S16). The experimental observation of A_LB_H and A_HB_L heterodimers (Figure 4b) indicate intermolecular dityrosine cross-linking (Figure S14, panels d–g). The relative quantitative ratios for the isotopically scrambled heterodimer were $A_LB_L/A_LB_H \sim 0.96$, $A_LB_L/A_HB_L \sim 2.19$, $A_LB_H/A_HB_L \sim 2.27$, $A_LB_L/A_HB_H \sim 2.02$, and $A_HB_L/A_HB_H \sim 0.92$ (Figure 4b and Figure S16), indicating an intermolecular mechanism for the heterodimer generation with negligible intramolecular dityrosine cross-linking in α-synuclein. The quantitative ^{15}N-labeled amino acid scrambling in dityrosine peptides enabled us to model the orientation of α-synuclein in solution after considering the distance constraints imposed by the formation of dityrosine (Figure 4c).

DISCUSSIONS

Dityrosine is an important naturally occurring post translational modifications (PTM). In structural proteins dityrosine cross-linking confers stability and elasticity, while in others provides resistance to proteolysis.[2] Dityrosine cross-linked peptides and proteins have been proposed to have neurotoxic roles in Alzheimer's and Parkinson's Diseases.[12,39] The study of dityrosine in proteins and peptides has been limited to measuring either derivatized or underivatized dityrosine after acid hydrolysis.[2] However, the extreme conditions required for acid hydrolysis and requirement of large sample sizes has limited our understanding of dityrosine in vivo.[2,19] The major limitation in dityrosine research is the inability to determine the location and identity of the dityrosine cross-link proteins. Here, we addressed these limitations and difficulties to identify dityrosine cross-linked peptides using mass spectroscopy in a bottom-up proteomic workflow. We then used this workflow to understand the dityrosine cross-links that can occur under oxidative conditions for two important neurological proteins, Aβ and α-synuclein. Dityrosine cross-linked oligomers of these proteins are proposed to be an important factor in the pathophysiology of Alzheimer's and Parkinson's disease, but little molecular detail is available.

We characterized the fragmentation pattern of model dityrosine Aβ peptides using CID, HCD, ETD, and ECD with the hypothesis that a unique fragmentation pattern characteristic of dityrosine-containing peptides could ensue similar to that produced by synthetic cross-linkers or disulfide linked peptides when fragmented by CID or ETD.[40−42] However, we did not find any distinguishing features that would indicate the presence of a dityrosine cross-link over a linear peptide. This means to conduct de novo searches for tyrosine (Tyr) peptides in biological samples, the search space will increase as a square of the number of Tyr-containing peptides.[23] Even though Tyr is in relatively low frequency peptide (2.92%) (http://web.expasy.org/docs/relnotes/relstat.html),[43] the potential dityrosine peptide database would be every Tyr-containing peptide squared, which for the human genome results in a searchable space on the order of 10^8 sequences. This would lead to an unacceptably high false discovery rate (FDR) for any computational search algorithm.[44] Thus, alternative approaches will be required to mine general shotgun proteomic data for dityrosine-containing peptides from biological samples such as fluorescence.

Dityrosine cross-linked forms of α-synuclein and Aβ are both implicated as the minimal oligomeric unit required for neurotoxicity. For Aβ, the only potential dityrosine cross-link is via Tyr10; however, for α-synuclein, it is not clear which of the four tyrosine residues are involved in the cross-link. We demonstrate that α-synuclein can be modified by both intermolecular and intramolecular dityrosine cross-links. We used HCD and ETD to characterize the intermolecular dityrosine cross-linked homopeptide dimer (^{35}EGVLYVGSK^{43}) (Figure 3c and Figure S11) and cross-link between Tyr39 and Tyr133 in the heteropeptide dimer (Figure 3d) of α-synuclein. Using ^{15}N labeled α-synuclein, we demonstrated that a dityrosine cross-linked heterodimer (AB) preferentially forms by intermolecular mechanism (Figure 4b and Figure S16). The distance constraints imposed by the cross-link between Tyr39 and Tyr133 indicate the formation of a head-to-tail dimer of the two protein units (Figure 4c). The formation of dityrosine cross-linked oligomers of α-synuclein could serve as a nucleus to drive oligomer formation of monomeric α-synuclein,[45,46] contributing to the prion-like activity observed.[47]

Aβ dimers isolated from the cerebral cortex of post-mortem AD brain have been reported to induce neuro/synaptotoxicity and memory disruption.[48] The dimers are chemically stable, consistent with a covalent bond like a dityrosine cross-link or a longer fragment of the amyloid precursor protein that contains the Aβ region.[49,50] Immunohistochemical detection of dityrosine have been reported in the affected regions in AD brain, enriched in the amyloid plaques.[12] Dityrosine cross-linked Aβ(1−42) is more toxic in vitro than monomeric Aβ(1−42).[30] However, dimeric Aβ species extracted from the AD brain is poorly characterized, and hence, the technique developed here provides a platform for future studies in determining both the presence as well as the extent of dityrosine cross-linked Aβ in AD.

Evaluation of additional HPLC fractions positive for dityrosine fluorescence demonstrated cross-links between Aβ with hemoglobin (Figure 2f) and α-synuclein with hemoglobin as depicted in the linkage map (Figure 3e). Since α-synuclein and hemoglobin are abundant in red blood cells[51] and neurons,[52,53] we can now examine such species in human samples, as potential

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biomarkers for Parkinson's Disease. The formation of cross-linked oligomers has some key differences to noncovalently linked oligomers, including a long lifetime due to protease resistance of the dityrosine moiety.

We have also applied the knowledge of the fragmentation feature of dityrosine cross-linked peptides to identify the possible tyrosine radical sites in ferrylhemoglobin, which form dityrosine cross-links in vitro. Dityrosine formation is an extremely fast process ($k = 3.8-5 \times 10^8\ M^{-1}\ s^{-1}$, Figure 2c),[54] and identification of dityrosine cross-linked peptides will provide the hitherto unknown knowledge of how proteins interact with their environment under oxidative and/or nitrative stress. The formation of ferrylhemoglobin from methemoglobin has been investigated by absorption, EPR spectroscopy, and resonance Raman spectroscopy.[36,55] The ferrylhemoglobin (Figure S6), Fe(IV)=O, species with a radical centered on the heme-porphyrin rapidly decays into Fe(IV)=O species with radical distributed on aromatic amino acid of the globin chain. Analysis of the dityrosine pairs of hemoglobin indicate that radical formation not only can occur at Tyr 42 of hemoglobin α chain as previously reported,[56] but radicals form at Tyr24 and Tyr140 in hemoglobin α as well as on Tyr130 and Tyr145 of hemoglobin β (Figure S9). Mildly oxidatively modified hemoglobin has an increased susceptibility to proteasomes, by partial unfolding and exposing the hydrophobic amino acid to the surface, while the highly oxidized hemoglobin is prone to aggregation, making it resistant to proteolysis.[57] Thus, understanding how these dityrosine cross-links of hemoglobin-α/hemoglobin-β alter the tetrameric structure and lead to aggregation is important for elucidating its physiological processing and may provide insight into the mechanism of blood coagulation.[58]

CONCLUSION

Oxidative stress and nitrative stress lead to alteration in every level of protein structure as well as it normal function. One of these changes is the formation of the oxidative covalent cross-linkage between two tyrosine residues known as the dityrosine bond. Immunohistochemical detection of dityrosine has been confirmed in Aβ plaques in AD brain as well as in Lewy bodies in the brains with PD. Despite advancements in quantitatively determining free dityrosine, information regarding specific protein targets and dityrosine cross-linking sites in the proteins is very limited. Here we report the development of generic rules of fragmentation for dityrosine cross-linked peptides in tandem mass spectrometry, which allowed for automated identification of dityrosine cross-links in peptides of Aβ, hemoglobin, and α-synuclein generated in vitro by enzymatic peroxidation. Oxidative stress is universal, and this methodology opens up the potential in identifying dityrosine cross-linked proteins that can serve as biomarkers in diverse pathological conditions such as AD, PD, atherosclerotic plaques, cancer, and aging. We also report that isotope scrambling during dityrosine cross-linking can be intuitively used for assessment in the structural changes in proteins undergoing oxidative stress. However, alternative methods have to be developed for using this tool for identifying and characterizing this natural PTM in peptides and proteins present in biological samples such as the human proteome.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b00941.

Supporting methods for ^{15}N-labeled recombinant α-synuclein production, synthesis of dityrosine cross-linked Aβ(1−42), detailed instrumentation for separation of dityrosine cross-linked peptides after proteolysis, Western blot procedure, instrumentation for LC−MS/MS with CID, HCD, ETD, and ECD in FT-ICR-MS, schematic representation of the modified designation of the fragment ions, ESI-MS spectra, high pH RP-HPLC, annotated CID-MS/MS spectrum, absorption spectra, detection of DT cross-linked Aβ(1-42) formed in vitro by Hb in the presence of H_2O_2, annotated spectra for $[M + 5H]^{5+}$, DT cross-link analysis in human hemoglobin, annotated HCD spectra, annotated ETD spectra, integrated EIC, annotated HCD MS/MS spectra, MS spectra, chromatographic peak areas of labelled and non-labelled homodimers and heterodimers and integrated EIC, table of all the validated dityrosine cross-linked human hemoglobin, and a list of protein tyrosine cross-linking sites (PDF)

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