Differential Accessibilities of Dibasic Prohormone Processing Sites of Proenkephalin to the Aqueous Environment Revealed by H−D Exchange Mass Spectrometry†

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ABSTRACT: Proenkephalin (PE) is a prohormone containing dibasic sites that are cleaved by proteases to generate peptide neurotransmitters and hormones. Little is known about the conformational features of such protease cleavage sites within prohormone substrates. Therefore, the goal of this study was to investigate the relative accessibilities of multiple dibasic processing sites of PE by peptide amide hydrogen−deuterium exchange mass spectrometry (DXMS). DXMS demonstrated differences in the relative accessibilities of the KR, KK, and RR cleavage sites of PE to the aqueous environment. DXMS assesses relative rates of exchange of hydrogens of the polypeptide backbone of PE with deuterium atoms from D_2O (heavy water) in solvent. Analyses of peptides spanning each of the 12 dibasic PE cleavage sites illustrated differences in H−D exchange rates that reflect relative solvent accessibility. The mid-domain cleavage sites (dibasic sites 4−8) exhibited greater accessibility to the aqueous solvent compared to regions of the NH_2 and COOH domains (dibasic sites 2, 3, and 9−11, respectively). The NH_2- and COOH-terminal domains both exhibited relatively high H−D exchange rates. The hydrogen exchange rate profile of PE, as well as its circular dichroism (CD) features for secondary structure, was modified in trifluoroethanol, an organic solvent that represents a more hydrophobic environment. These findings suggest that the dibasic protease cleavage sites of the PE prohormone with differences in accessibility to the aqueous environment undergo proteolytic processing to generate active neuropeptides for cell−cell communication in neuroendocrine systems.

Proenkephalin and prohormones are inactive protein precursors that must undergo proteolytic processing to generate the smaller, active peptide neurotransmitters and hormones. The mature enkephalin neuropeptide functions in brain regulation of analgesia and behavior (*1*−*3*). Proenkephalin (PE)[1] contains multiple copies of (Met)enkephalin and related peptides (*4, 5*), which are produced by proteolytic processing of PE at multiple dibasic processing sites (*6*−*9*) that flank the enkephalin peptide sequences within PE. The processed, mature enkephalin peptides are stored within secretory vesicles and then undergo regulated secretion induced by cellular stimuli, allowing them to function as extracellular peptide neurotransmitters.

Proteolysis of proenkephalin in the regulated secretory pathway has been extensively studied and involves the cysteine protease cathepsin L combined with the subtilisin-like prohormone convertases (*6*−*9*). The prohormone convertases known as PC1/3 and PC2 represent the primary subtilisin-like proteases (*6*−*9*) that cleave at the COOH-terminal side of the dibasic residue prohormone processing sites; their actions are followed by carboxypeptidase E (CPE) that removes COOH-terminal basic residues to generate active enkephalin and related neuropeptides (*6, 10*). The more recently discovered cathepsin L protease pathway for PE processing involves preferential cleavage at the NH_2-terminal side of dibasic residue sites (*6, 11*), followed by aminopeptidase B for removal of NH_2-terminal basic residues (*12*) in the formation of mature enkephalin. Studies of protease gene knockout mice (*6*−*9, 11, 13*−*16*) have demonstrated the significant roles of secretory vesicle cathepsin L, combined with the well-known prohormone convertases, in the production of enkephalin and numerous neuropeptides functioning as key neurotransmitters and hormones.

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[1] Abbreviations: PE, proenkephalin; DXMS, hydrogen−deuterium exchange mass spectrometry; LC−MS/MS, liquid chromatography−tandem mass spectrometry; TFE, trifluoroethanol; CD, circular dichroism.

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The dibasic processing sites of PE are recognized and cleaved by the prohormone processing proteases. However, little is known about the conformational orientation and structural features of proenkephalin and prohormones at their proteolytic processing sites. For this reason, this study sought to gain knowledge of the relative accessibility of the proteolytic processing sites of proenkephalin by hydrogen−deuterium exchange mass spectrometry (DXMS) (*17*−*19*). DXMS allows evaluation of the relative rates of exchange of hydrogens of the polypeptide backbone of PE with deuterium of D_2O (heavy water) and can compare protein subdomains with respect to their relative accessibility to the aqueous solvent environment. DXMS experiments demonstrated differences in the relative accessibilities of the dibasic KR, KK, and RR cleavage sites to the aqueous environment. The mid-domain processing sites of PE exhibited higher accessibility to the aqueous solvent compared to other NH_2 and COOH domains of PE. However, the NH_2- and COOH-terminal dibasic sites exhibited relatively high rates via DXMS. In the presence of the organic solvent trifluoroethanol (TFE), PE displayed differences in DXMS properties, combined with differences in secondary structural features determined by CD (circular dichroism) (*20*−*22*). These findings suggest that under more hydrophobic conditions, such as association of PE with membranes in cells, the orientation of the dibasic processing sites with respect to the aqueous environment may differ.

The observed differences in the relative accessibility of dibasic sites within PE suggest differential conformational features among these proteolytic processing sites. These findings suggest that dibasic prohormone processing sites with differences in accessibility to the aqueous solvent undergo proteolytic processing to generate mature active enkephalin and related neuropeptides.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Human Proenkephalin. Recombinant human PE with an N-His tag was generated by expression in *Escherichia coli* and purified with a Ni^{2+} affinity column. The pET19b expression vector was used to generate recombinant human PE in Rosetta 2(DE3) *E. coli* cells (EMD, San Diego, CA). The PE cDNA, with deletion of the signal sequence and incorporation of NdeI and BamHI restriction sites at the 5′ and 3′ ends, respectively, was generated by RT-PCR of total RNA from the human striatum (Stratagene, 540135) using Taq DNA polymerase (Qiagen, Valencia, CA) and the primers 5′-AAAAACATATGGAATGCAGCCAGGATTGCGCGAC-3′ and 5′-AAAAAGGATCCTTAAAATCTCATAAATC-CTCCGTATCTTTTTTCC-3′ (Invitrogen, Carlsbad, CA). PCR mixtures contained 1 ng of cDNA as a template and 0.3 μM primers with 35 thermocycles of 60 s at 94 °C, 60 s at 45 °C, and 70 s at 72 °C. Following digestion of the amplified PE DNA with restriction enzymes NdeI and BamHI, the digested PE DNA was ligated using T4 DNA ligase (Invitrogen) to NdeI- and BamHI-digested plasmid expression vector pET19b (EMD). The 732 bp PE nucleotide sequence in the PE-pET19b vector was verified by DNA sequencing (Davis Sequencing, Inc., Davis, CA), and the DNA sequence was used to deduce the primary amino acid sequence.

PE was expressed via the PE-pET19b vector by its transformation into Rosetta 2(DE3) cells (EMD), a BL21 cell line-derived *E. coli* host. PE expression in *E. coli* cells was conducted in LB medium with 50 μg/mL carbenicillin and 30 μg/mL chloramphenicol utilizing the T7 expression system. *E. coli* cells were grown at 37 °C with agitation to an optical density of 0.6−0.8 at 600 nm and induced with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) for 3 h at 37 °C and harvested by centrifugation at 4000*g* for 15 min at 4 °C. The cells were resuspended in 100 mM Tris-HCl (pH 7.0) and lysed by sonication (6 × 20 s, power level 4, microtip, Misonix, Ultrasonic Processor XL) at 4 °C. The disrupted cells were centrifuged at 16000*g* for 15 min at 4 °C. Intact PE was localized primarily in inclusion bodies; therefore, the cell pellet was suspended in 100 mM Tris-HCl (pH 7.0) and 6 M urea, and solubilized PE was obtained as the soluble fraction after centrifugation at 20000*g*.

Purification of N-His-tagged PE was conducted by applying the solubilized PE sample to a His bind Ni^{2+} affinity column (EMD) equilibrated with 100 mM sodium phosphate, 20 mM Tris (pH 8.0), 6 M urea (buffer A), and 5 mM imidazole. After the mixture had been washed with 40 mM imidazole in buffer A, PE was eluted with 300 mM imidazole in buffer A. Urea in the PE sample was removed in steps at lower urea concentrations (5, 3, 2, 1, and 0.5 M urea) by dialysis (4 °C) into a buffer consisting of 100 mM sodium phosphate, 20 mM Tris (pH 7.5), 5 mM glutathione (GSH), and 0.5 mM oxidized glutathione (GSSG) (Calbiochem, San Diego, CA). Glutathione was removed by dialysis into 50 mM Tris (pH 7.5) for storage of recombinant PE.

Purified recombinant PE was confirmed by SDS−PAGE using a NuPAGE 12% Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA) under reducing conditions and Coomassie staining. In addition, PE was evaluated by immunoblotting with an anti-(Met)enkephalin polyclonal antibody (1:5000, Chemicon, Temecula, CA) and visualized using an ECL Plus (GE Healthcare, Piscataway, NJ) detection system. The protein concentration was estimated by absorbance measurements at 280 nm using the theoretical molar extinction coefficient of 33640 M^{-1} cm^{-1} for recombinant His-tagged PE.

Optimization of Pepsin Digestion of Proenkephalin for DXMS Studies. PE digestion by pepsin is a requisite step prior to enhanced deuterium exchange mass spectrometry (DXMS) experiments. In optimizing this process, the total number of peptides produced from pepsin digestion was evaluated under several different conditions, including concentrations of denaturant and lengths of time for reduction of disulfide bridges. For each sample test, 50 μg of PE in 9 μL of 5 mM Tris (pH 7.2) was diluted in 27 μL of 8.3 mM Tris and 50 mM NaCl (pH 7.2) (on ice), representing the dilution of the protein into D_2O-based buffers in deuterium exchange experiments. The sample was then diluted with 54 μL of a cold solution (0 °C) of 0.8% formic acid, 16.6% glycerol, 1 M tris(2-carboxyethyl)phosphine (TCEP), and guanidine hydrochloride (GuHCl) at final concentrations of 0.05, 0.5, 1.0, 2.0, and 4.0 M. This quenching step represented the reduction of hydrogen−deuterium exchange with a decrease in pH to 2.2−2.5 in addition to denaturing the protein prior to pepsin proteolysis with GuHCl and acidic conditions. The quenching process was allowed to proceed on ice for either 1 or 5 min, representing the time extremes

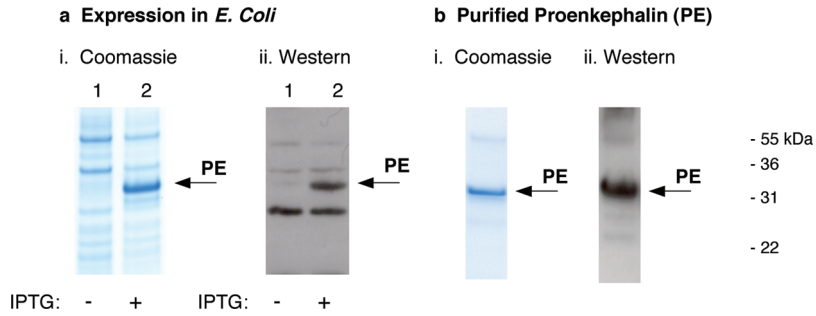

FIGURE 1: Expression and purification of recombinant human PE. (a) Expression of human PE. Expression of PE in *E. coli* was induced in the presence of IPTG (panel i, lane 2), shown by the 33 kDa PE band on SDS−PAGE stained with Coomassie blue. Cells grown in the absence of IPTG did not show PE (lane 1). The induced PE band was recognized by anti-(Met)enkephalin in Western blots (panel ii, lane 2). Controls without IPTG showed no PE induction (panel ii, lane 1). (b) Affnity purification of PE. The N-His-tagged PE was purified with a Ni^{2+} affinity column. Purified PE was illustrated by SDS−PAGE gels stained with Coomassie blue (panel I, 1 μg of purified PE per lane). The purified PE was recognized by anti-(Met)enkephalin by a Western blot (panel ii). Purification yielded 30−50 mg of recombinant PE per liter of *E. coli*.

allowed for TCEP to reduce disulfide bonds, after which the sample was frozen by being submerged in dry ice. Procedures for pepsin digestion for DXMS have been described previously (*23, 24*). Briefly, the quenched sample at 0 °C was passed over a porcine pepsin-immobilized column, and the proteolytic peptides were collected onto a C18 column (Vydac). The separated products were mass analyzed using a Thermo Finnigan LCQ mass spectrometer and determination of pepsin-generated peptide sequences from the resulting MS:MS data sets facilitated through the use of Sequest (Finnigan, Inc.).

Hydrogen−Deuterium Exchange Mass Spectrometry Studies of Proenkephalin. PE samples were prepared with three states of hydrogen−deuterium exchange in each deuterium exchange experiment, consisting of nondeuterated (ND), deuterated, and fully deuterated (FD). The nondeuterated sample was processed exactly as described in the digestion optimization described in the previous paragraph. The FD sample represents the "maximum" hydrogen−deuterium exchange for a certain time period, which in these experiments was a period of 14 h where the samples were allowed to exchange at room temperature. The deuterated samples represent different incubation times prior to the quenching of the exchange process. All samples used 50 μg of PE in 9 μL of 5 mM Tris (pH 7.2). The ND sample was diluted with 27 μL of H_2O-based 8.3 mM Tris and 50 mM NaCl (pH 7.2), while the deuterated and FD samples were diluted in a D_2O-based buffer of the same composition. The deuterated samples were allowed to exchange at 0 °C for 10, 30, 100, 300, 1000, and 3000 s, after which the exchange was quenched with 54 μL of 0.8% formic acid, 16.6% glycerol, 1 mM TCEP, and 0.5 M GuHCl, and the quenching process was allowed to proceed for 1 min at 0 °C until the sample was frozen by being submerged in dry ice. A more detailed description of sample processing for exchange experiments has been published (*23, 24*). The pepsin digestion, chromatography, and the mass spectral acquisition proceeded as described in the digestion optimization section. Data processing and reduction of hydrogen−deuterium exchange experiments utilized DXMS data reduction software (Sierra Analytics, Modesto, CA) (*17, 25, 26*).

Examination of Proenkephalin Secondary Structure by Circular Dichroism. Circular dichroism spectra of PE were recorded with an AVIV 202 circular dichroism spectrophotometer (AVIV Biomedical) in 10 mM phosphate buffer (pH 7.1) with trifluoroethanol (TFE) at 0, 5, 10, 15, 20, and 33%. Measurements of PE, at concentrations of 15 μM, were taken in a demountable quartz cuvette with a path length of 0.2 mm (Starna Cells, 20/C-Q-0.2) from 250 to 190 nm in a single scan at a bandwidth of 1 nm, in 0.5 nm increments, at 25 °C, and with an averaging of 3 s. The PE CD spectra are from two experiments under each condition except for PE at 0% TFE, which was repeated three times. CD solvent baselines were obtained for all conditions, and the baselines were subtracted from the sample spectra prior to spectral analysis and deconvolution; spectra were smoothed using a five-point Mean-Movement smoothing filter in Mathematica 6.0 (Wolfram Research). The measured ellipticities in units of millidegrees were converted to the appropriate units and wavelength range in 1 nm increments for each deconvolution software program.

The CD spectra with converted units were analyzed and deconvoluted by CDSSTR (*21, 22*) and CONTIN (*27*). Raw CD spectra were normalized to molar circular dichroism (liters per mole per centimeter) and mean residue ellipticity [θ] units (degrees square centimeter per decimole) for CDSSTR and CONTIN, respectively. The DOS software programs were downloaded from http://www2.umdnj.edu/cdrwjweb/. The database employed in the CONTIN deconvolution consisted of 20 total reference proteins, which included four denatured proteins that are models of unordered segments of proteins. The database for CDSSTR deconvolution utilized 26 reference proteins. The CD spectra of PE were compared under buffer conditions with and without TFE.

RESULTS

Recombinant PE: Optimization of Pepsin Digestion of PE for DXMS Studies. Human proenkephalin was expressed in *E. coli* to produce sufficient quantities for DXMS and CD studies. High-level expression of N-His-tagged PE resulted in approximately 80 mg of PE protein per liter of cell expression (Figure 1). IPTG induction of PE was demonstrated by production of the ~33 kDa apparent PE band on SDS−PAGE, which was recognized by anti-(Met)enkephalin in Western blots (Figure 1a). This recombinant PE possesses a calculated molecular mass of 31073 Da; thus, the relative electrophoretic mobility of PE on SDS−PAGE represents an approximation of its apparent molecular mass. After

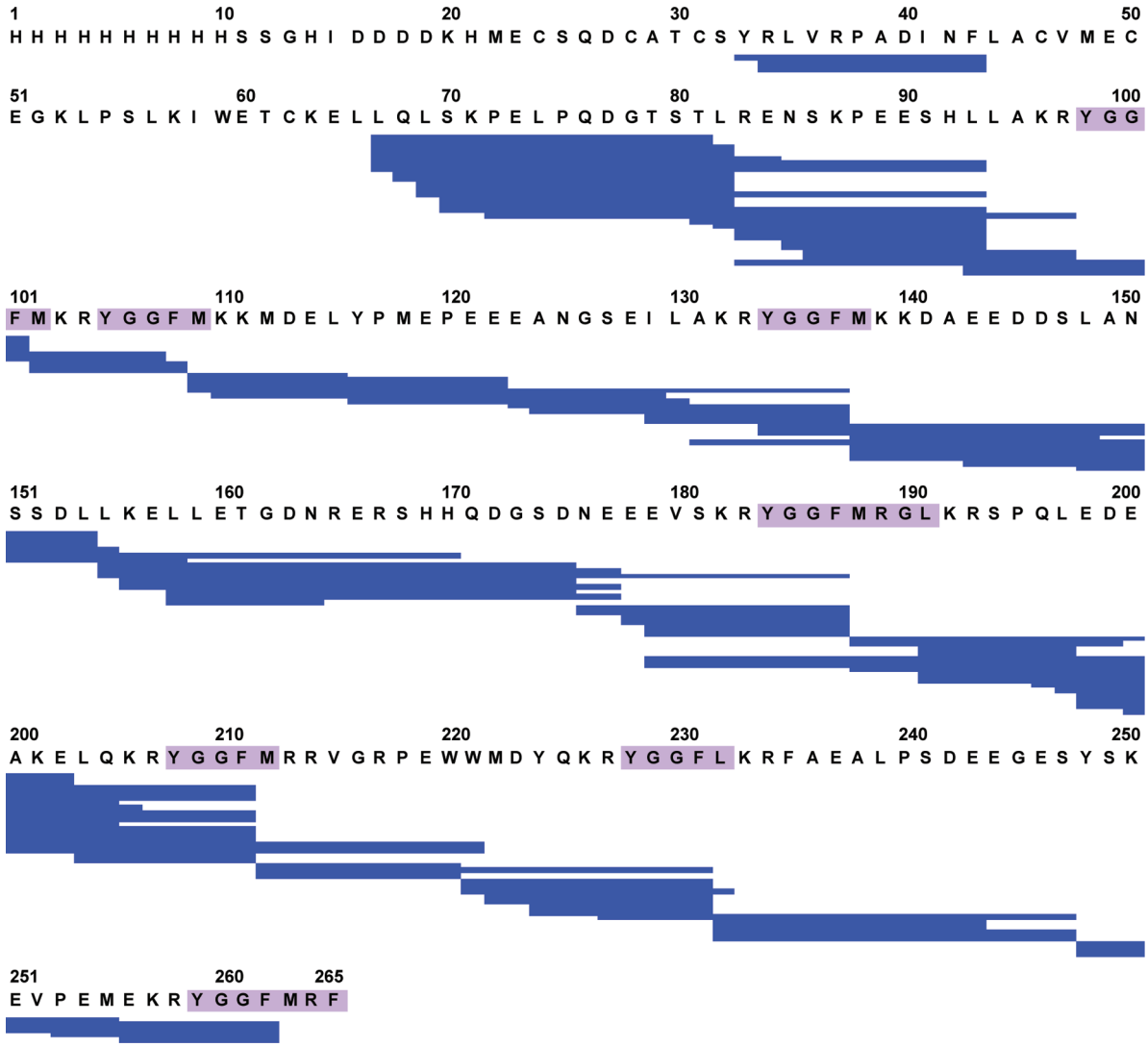

FIGURE 2: Pepsin digestion map that encompasses dibasic cleavage sites of PE. Optimization of conditions (0.5 M GuHCl and 1 M TCEP, for 1 min) for pepsin digestion of PE resulted in peptides encompassing all the dibasic cleavage sites flanking enkephalin peptides (purple) within PE. These peptides (123 peptides) provided 87% coverage of the PE sequence and included all of the dibasic sites which allowed their analyses by DXMS.

solubilization of PE from cell extracts, Ni^{2+} affinity purification resulted in a highly enriched sample of recombinant PE of the 33 kDa band on SDS−PAGE visualized by Coomassie staining and was recognized by anti-(Met)enkephalin in Western blots (Figure 1b). This recombinant PE was utilized for evaluation by DXMS and CD.

The extent of PE domains that can be studied by DXMS is determined by the peptide coverage of the protein that results from the pepsin digestion process. This important step utilizes pepsin that can function under acidic pH conditions used to quench hydrogen−deuterium exchange. Optimization of pepsin digestion of PE was assessed under several conditions, including 0.5 M GuHCl and 1 M TCEP (reducing agent) with a pepsin column, with the peptide products then undergoing LC−MS/MS analyses. PE-derived peptides produced from digestion optimization enabled 87% coverage with more than 100 overlapping peptides (Figure 2). Importantly, coverage included all the dibasic processing sites of PE. Therefore, the pepsin-generated peptides could allow evaluation of H−D exchange at regions of dibasic processing sites of PE.

DXMS Reveals Different Accessibilities of Dibasic Sites of PE to the Aqueous Environment. DXMS was utilized to compare the relative accessibilities of different domains of PE and its dibasic processing sites to the aqueous environment. The exchange between deuterium isotopes (from heavy water, D_2O) and PE amide polypeptide backbone hydrogens was rapid with extensive hydrogen−deuterium (H−D) exchange after only 10 s for the exchange period (Figure 3). H−D exchange is illustrated by peptide regions containing deuterium colored red, and regions containing hydrogen (not deuterium) are colored black (Figure 3a). During increased times for H−D exchange, the high level of deuteration was maintained (data not shown). Interestingly, some differences in H−D exchange were observed throughout the PE protein sequence.

Notably, comparison of H−D exchange at the 12 dibasic processing sites, represented by peptides that span these sites, showed differences in the relative accessibilities of the dibasic sites to the aqueous environment (Figure 3b). Dibasic sites 4−8 (dibasic sites numbered from the NH_2 to COOH domains of PE) in the midregion of PE showed greater H−D

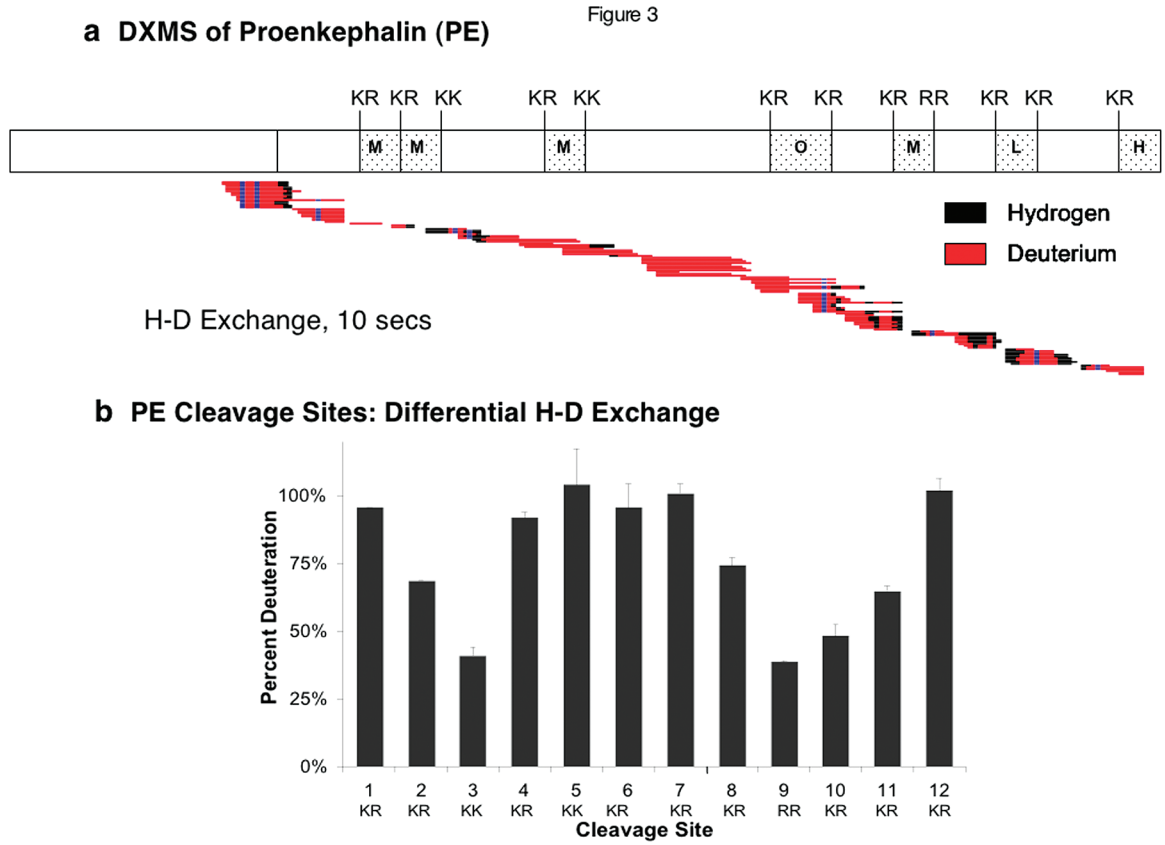

FIGURE 3: DXMS reveals differential aqueous solvent accessibilities of PE intervening domains compared to dibasic processing sites. (a) DXMS of the PE protein precursor. DXMS of PE was conducted by incubating recombinant PE in D_2O for 10 s, followed by quenching, pepsin digestion, and LC−MS/MS. Results display pepsin-generated peptide fragments containing deuterium that was exchanged for hydrogen (H−D exchange) colored red and peptide regions without H−D exchange colored black (dark blue indicates nonexchangeable prolines). The DXMS peptide map illustrates a large extent of H−D exchange throughout PE. Enkephalin-related peptide sequences within PE are indicated as (Met)enkephalin (M), (Leu)enkephalin (L), octapeptide (O, M-Arg-Gly-Leu), and heptapeptide (H, M-Arg-Phe). (b) Differential H−D exchange at the different dibasic processing sites of PE. Differences in the relative extent of H−D exchange were observed among the 12 dibasic PE cleavage sites. High levels of deuteration were observed among the dibasic sites located in the midregion of PE (sites 4−8), as well as the NH_2- and COOH-terminal sites (sites 1 and 12). Lower levels of deuteration were observed dibasic residues 2, 3, and 9−11.

exchange compared to the much lower level of deuteration at dibasic sites 2, 3, and 9−11 located in the NH_2 and COOH domains of PE. However, NH_2- and COOH-terminal dibasic sites 1 and 12 show a high level of deuteration similar to that of dibasic sites in the midregion of PE. Thus, the 12 dibasic processing sites of PE display a pattern of differential accessibility to the aqueous solvent, suggesting differential conformational orientations of these cleavage sites.

Altered Dynamics of Proenkephalin Domains and Processing Sites with Respect to the Aqueous Solvent Environment in the Presence of Trifluoroethanol (TFE). The effects of increasing the hydrophobicity of the solvent environment of PE were investigated in DXMS experiments in the presence of TFE (15%). Color representations of relative H-D exchange illustrate decreased amounts of H−D exchange in the presence of TFE for most regions of PE, and no change in a few regions of PE (Figure 4). TFE apparently restricted the overall H−D exchange of PE.

Comparison of the relative amounts of H−D exchange at dibasic processing sites of PE in the presence of TFE (15%) showed primarily a decreased level of H−D exchange compared to controls without TFE (Figure 5). Dibasic site 8 exhibited the largest decrease in the level of H−D exchange, and dibasic sites 2−7 and 10−12 exhibited more modest decreases in the level of H−D exchange. Dibasic sites 1 and 9, however, exhibited modest increases in the level of H−D exchange. These results demonstrate that TFE, an organic solvent, modified the relative accessibilities of the dibasic processing sites of PE to the aqueous solvent environment.

Secondary Structure Features of PE Examined by CD. The extent of secondary structures of PE was examined under conditions utilized for DXMS studies, in the absence and presence of TFE. CD spectra (Figure 6a) suggested extensive secondary structural features of PE consisting of α-helix (11%), β-sheet (18%), and β-turns (16%).

CD analyses of PE in the presence of TFE, an organic solvent, were also conducted to observe PE secondary structure under a more hydrophobic condition. Increasing concentrations of TFE, an organic solvent, resulted in substantial alterations in CD spectra (Figure 6b) that reflect changes in secondary structure. As the concentration of TFE increases, the percent of α-helix secondary structure was substantially increased from 11% to approximately 45% (Figure 6c). In contrast, the relative degrees of β-sheet and β-turn secondary structures were not increased with an increase in TFE concentration; rather, a modest decrease in β-sheet and β-turn structure content was observed with TFE. These findings suggest that increases in the level of α-helix structure of PE in the presence of TFE may be related to the TFE-induced restriction in overall H−D exchange of PE

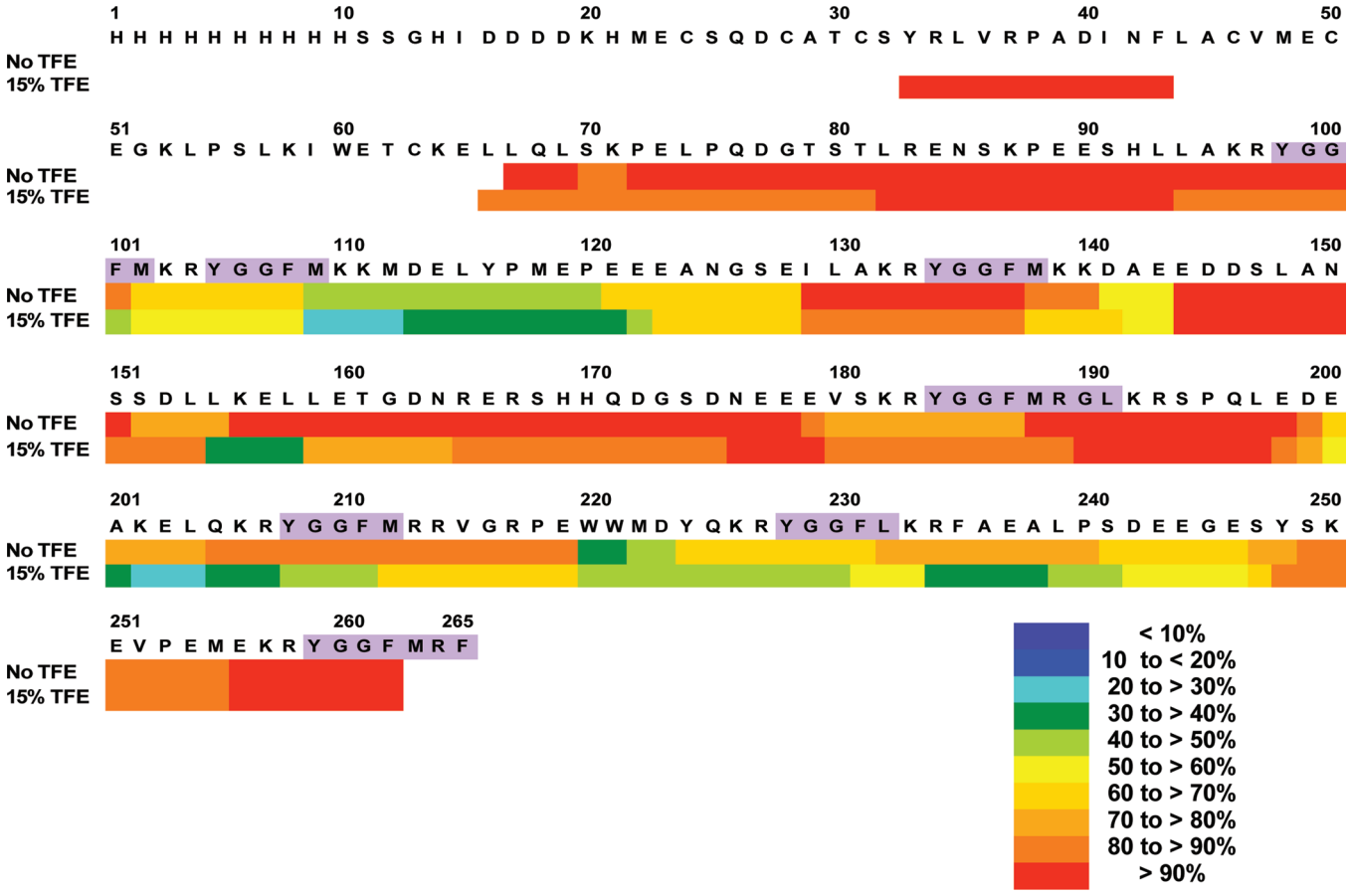

FIGURE 4: Restricted H−D exchange of PE peptide domains in the presence of TFE. H−D exchange was conducted in the presence of TFE (15%), an organic solvent, to represent a more hydrophobic environment compared to that without TFE. The H−D exchange period in D_2O was 10 s (same time as in Figure 3, without TFE). Different ranges of the percent deuteration are illustrated in a color-coded manner (see the key). Comparisons of percent deuteration of PE peptide domains in the absence and presence of TFE revealed changes in H−D exchange, represented by a reduced level of deuteration of the majority of PE domains in the presence of TFE.

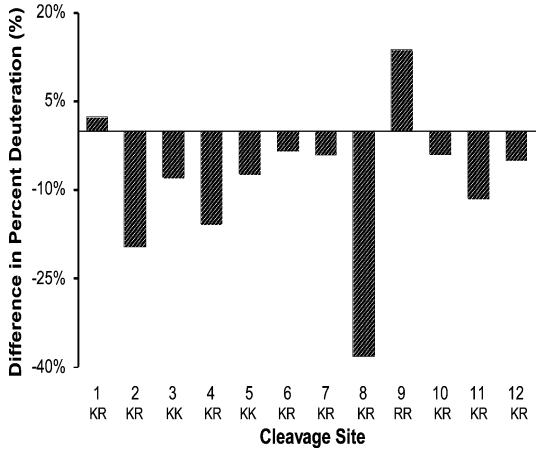

FIGURE 5: Relative accessibilities of dibasic cleavage sites to the aqueous solvent environment are altered in the presence of TFE, as revealed by H−D exchange. The average level of deuteration of each dibasic cleavage site in the presence of TFE (15%) was subtracted from that in the absence of TFE. These differences are illustrated in the bar graph plots. A reduced level of deuteration in the presence of TFE was observed for most cleavage sites, especially for sites 2 and 8. Modest increases in percent deuteration were observed for dibasic sites 1 and 9. These data illustrate differential effects of TFE in modifying relative accessibilities of PE dibasic cleavage sties to the aqueous environment.

domains. Moreover, TFE-induced alterations in H−D exchange at dibasic processing sites of PE may relate to changes in its secondary structure. Overall, DXMS and CD structural studies of PE have revealed dynamic H−D exchange properties of PE domains to the aqueous environment.

DISCUSSION

Proenkephalin possesses multiple dibasic proteolytic processing sites that require cleavage to generate the smaller, active enkephalin opioid peptide neurotransmitters. DXMS studies of PE have revealed the relative accessibilities of the dibasic processing sites of PE and have illustrated the dynamic properties of PE of highly exchangeable hydrogens (of the polypeptide backbone) for deuteriums from the D_2O aqueous environment. Comparisons among the dibasic cleavage sites of PE showed clear differences in their relative accessibility to the aqueous environment. The dibasic residue sites in the midregion of PE exhibited high relative levels of H−D exchange. However, dibasic sites near the N- and C-domains of PE, corresponding to sites 2 and 3 and sites 9−11, respectively, showed lower extents of H−D exchange. Interestingly, the dibasic sites closest to the NH_2 and COOH termini of PE showed high levels of H−D exchange. Under conditions of increased hydrophobicity with TFE (15%), marked decreases in the relative extents of H−D exchange at the majority of dibasic sites of PE were observed. CD analyses of PE showed that it possesses secondary structure features of α-helix, β-sheet, and β-turns which are present in the PE protein that displays differential accessibilities of its dibasic processing sites to the aqueous environment. It

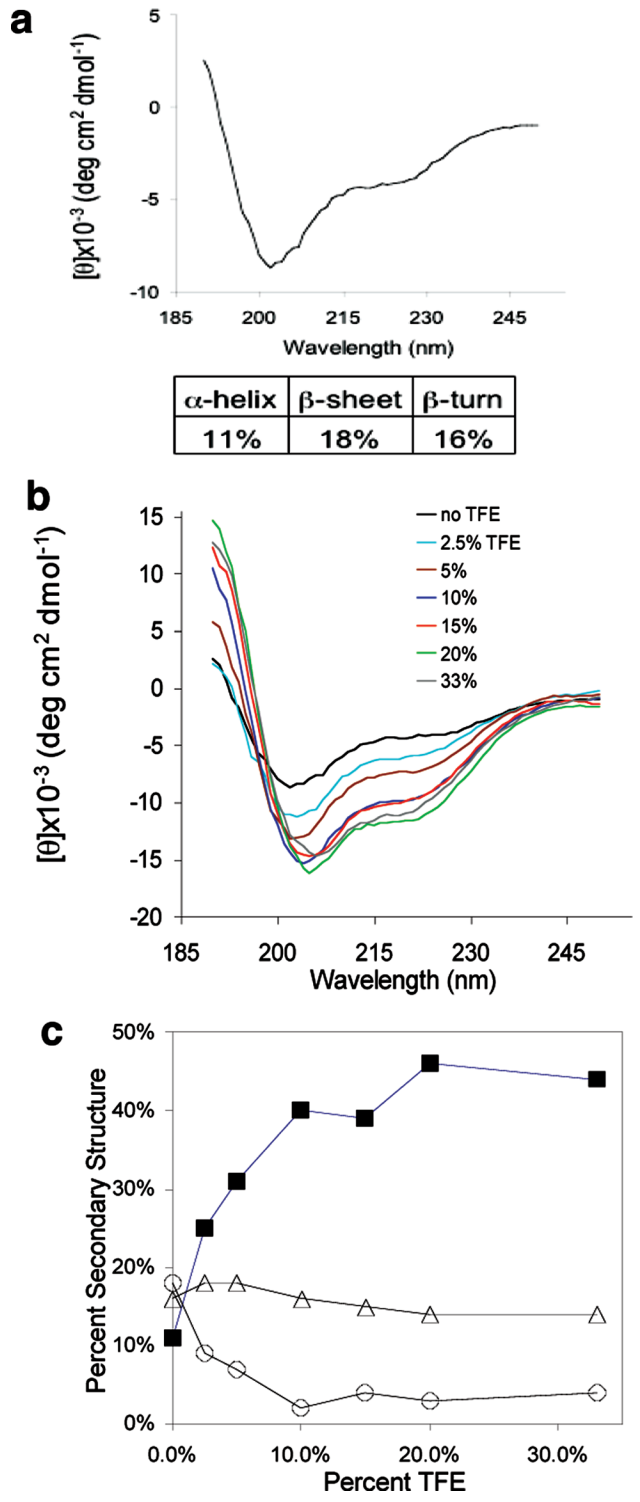

FIGURE 6: CD illustrates secondary structural features of PE in the absence and presence of TFE. (a) Analysis of the CD spectrum of PE revealed secondary structures of PE. The CD spectrum of recombinant PE was obtained and was deconvoluted using CDSSTR (*21*) to reveal secondary structures consisting of 11% α-helix, 18% *β*-sheet, 16% *β*-turn, and 55% disordered structure. (b) CD spectra of PE in the presence of TFE. CD spectra for PE were obtained at different concentrations of TFE, an organic solvent. TFE (from 0 to 33%) was found to alter the CD spectra of PE, indicating alterations in secondary structure. (c) Increase in the α-helical structure content of PE in the presence of TFE. The relative extents (percent) of changes in secondary structural features with increasing concentrations of TFE (from 0 to 33%) are illustrated for α-helix (■), *β*-sheet (○), and *β*-turn (△) features. TFE substantially increases the α-helical secondary structure content of PE.

was of interest to find that TFE substantially increased the extent of α-helical secondary structure of PE, which occurred with the more restricted H−D exchange properties of PE in the presence of TFE. The combined DXMS and CD data demonstrate the dynamic nature of the dibasic processing sites of PE with respect to differential H−D exchange with its aqueous environment, which occurs within the PE protein possessing α-helix, *β*-sheet, and *β*-turn secondary structures. These conformational features of PE differ in more hydrophobic environments (with TFE) that may represent PE associated with soluble and membrane components of cells.

Proenkephalin is synthesized at the rough endoplasmic reticulum (RER) from its mRNA and is then routed into the RER and Golgi apparatus where it is packaged into newly formed secretory vesicles. The pH at the RER and Golgi apparatus is near neutral, approximately 7.0−7.2 (*28*). Newly formed secretory vesicles bud off from the trans-Golgi network, resulting in immature secretory vesicles that initially contain an intravesicular neutral pH of ∼7.0, similar to that of the trans-Golgi. Prohormone processing has been found to occur within newly formed secretory vesicles for insulin production in clathrin-coated secretory vesicles (*29*−*31*). Proteolytic processing of proneuropeptide and prohormones by the subtilisin-like prohormone convertase enzymes, PC1/3 and PC2 (*32*), as well as by secretory vesicle cathepsin L (*33*), can occur at pH 7.0. These processing enzymes possess activity at pH 7.0 that is approximately 30−50% of their activities under more acidic conditions of pH 5.5−6.0. As the newly formed immature secretory vesicle matures, its internal pH undergoes acidification to pH 5.5−6.0 (*34, 35*).

Thus, the neutral-pH conditions for DXMS and CD studies of PE in this project reflect PE's cellular environment at the trans-Golgi apparatus and in newly formed immature secretory vesicles where initial proteolytic processing of PE occurs. Because the proneuropeptide processing enzymes possess activity at pH 7.0, results from this H−D exchange study are relevant to conditions within newly formed, immature secretory vesicles where initial PE and prohormone processing occurs.

Dibasic processing sites of PE with greater levels of accessibility to the aqueous environment may be predicted to be more readily accessible to proteases for initial processing. Indeed, analyses of the initial proenkephalin dibasic site cleaved by the secretory vesicle cathepsin L processing enzyme (previously known as "prohormone thiol protease") indicated cleavage at KR site 7 located at the COOH terminus of (Met)enkephalin-Arg-Gly-Leu (octapeptide) (*36*). Cleavage site 7 possesses one of the greatest levels of accessibility to the aqueous solvent environment shown by its H−D exchange of nearly 100% deuteration of a peptide spanning that site after exposure for 10 s to D_2O (Figure 3, 10 s). The cathepsin L protease pathway has been recently identified as a cysteine protease pathway for processing proenkephalin into active enkephalin peptide neurotransmitters (*6*).

In addition to cathepsin L in secretory vesicles, the subtilisin-like prohormone convertases participate in processing proenkephalin. Analyses of proenkephalin cleavage sites by PC1/3 and PC2 indicate sites 6 and 7 represent one of the major cleavage sites (*37*); these sites, 6 and 7, are among the most highly deuterated sites within PE (Figure 3). Furthermore, characterization of an aspartyl protease in chromaffin granules for proenkephalin processing showed

that, again, dibasic site 7 was readily cleaved (*38*). Thus, dibasic sites with high levels of H−-D exchange represent sites of cleavage by prohormone processing enzymes among studies evaluating the proenkephalin cleavage sites.

Newly formed immature secretory vesicles undergo maturation that involves acidification that brings the internal vesicular condition to pH 5.5−6.0 in mature secretory vesicles (*34, 35*). The PE and prohormone processing enzymes, i.e., the prohormone convertase and cathepsin L protease pathways, exhibit optimal activities at pH 5.5−6.0 (*32, 33*) that is consistent with the internal pH of mature secretory vesicles. Thus, PE and proneuropeptide processing occurs in mature secretory vesicles at the pH optimum of processing enzymes. It will be of interest in future studies to examine the H−D exchange and CD properties of proenkephalin under pH conditions of mature secretory vesicles at more acidic pHs of ∼5.5−6.0.

Proenkephalin in vivo is processed to several high-molecular mass intermediates of 4.5−23 kDa (*36*−*39*). Recombinant PE expressed and purified from *E. coli* has been used as substrate for in vitro prohormone processing studies (*36*), which show that recombinant PE is converted by processing proteases to their known in vivo intermediate products (4.6−22.5 kDa) via cleavage at dibasic sites. These data indicate that recombinant PE expressed in *E. coli* possesses a conformation that allows its appropriate proteolytic processing in vitro that resembles its in vivo processing. This study assessed recombinant His-tagged PE to faciliiate purification of adequate amounts of PE by nickel affinity purification; thus, H−D exchange results represent that of His-tagged PE. It will also be of interest, therefore, to investigate recombinant PE without a tag in future studies.

Among protein substrates of proteolytic enzymes, there is little knowledge of the relative accessibilities of multiple substrate cleavage sites to the aqueous environment. The results of this DXMS study of proenkephalin provide significant new knowledge of conformational H−D exchange of different protease cleavage sites within a protein substrate. The differential accessibilities of dibasic processing sites of proenkephalin suggest that a protease enzyme possesses the ability to properly interact with each dibasic site to achieve enzymatic cleavage.

DXMS showed that the PE protein displays rapid exchange of hydrogens for deuteriums. Extensive H−D exchange was observed with only 10 s as the exchange period. Longer exchange periods of ≥100 s showed nearly maximal deuteration levels of PE that were similar to the extent of H−D exchange occurring after several hours (14 h). In contrast, many other proteins, such as α-synuclein amyloid (*25*) and C-terminal Src kinase (*40*), undergo slower rates of H−D exchange for minutes and hours. The rapid rate of H−D exchange for PE suggests that the majority of its domains possess conformations that are highly accessible to the aqueous environment. These H−D exchange properties of PE occur with its secondary structures of α-helix, β-sheet, and β-turn. These secondary structural features occur with PE conformations that allow rapid H−D exchange.

In summary, DXMS studies have revealed differential accessibilities of dibasic processing sites of proenkephalin. Moreover, H−D exchange is restricted under more hydrophobic conditions, represented by inclusion of TFE in the DXMS buffer conditions. These H−D exchange properties of the dibasic sites reside within PE that possesses secondary structural features of α-helix, β-sheet, and β-turn. These findings suggest that dibasic PE prohormone processing sites with differences in accessibilty to the aqueous environment undergo proteolytic processing in the generation of active neuropeptides for cell−cell communication in the nervous and endocrine systems.

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