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Shotgun Analysis of Integral Membrane Proteins Facilitated by Elevated Temperature

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The beneficial effects on peak selectivity and resolution of conducting liquid chromatography (LC) at elevated temperature (e.g., 30−80 °C) are generally well-known; however, its importance for peptide recovery is not nearly as well recognized. This report demonstrates that μLC analysis of membrane proteomic samples significantly benefits from the application of heat. Enriched membrane and membrane-embedded peptides (the latter obtained by membrane shaving) were analyzed by μLC-tandem mass spectrometry (MS/MS) from 20 to 60 °C using a standard reversed-phase material. Maximal protein and hydrophobic peptide recovery was obtained at 60 °C. The membrane-shaving method employed, a recently optimized version of the high pH/proteinase K protocol, provided significant integral membrane protein enrichment: 98% of identified proteins were predicted to have at least one transmembrane domain (87% to have at least three), and 68% of peptides were predicted to contain transmembrane segments. Analysis of this highly enriched sample at elevated temperature increased protein identifications by 400%, and peptide identifications by 500%, as compared to room-temperature separation. Given that most μLC-MS/MS analyses are currently conducted at room temperature, the findings described herein should be of considerable value for improving the comprehensive study of integral membrane proteins.

Integral membrane proteins (IMPs) are critical for the maintenance of biological systems and represent important targets for the treatment of disease. To have a more complete understanding of their myriad roles in patho/physiological processes, detailed proteomic information [e.g., abundance, activity, localization, structure, sequence, post-translational modifications (PTMs), and molecular interactions] is required. However, given the challenging nature of these amphipathic species, even basic characterization of IMPs lags far behind that of their soluble counterparts. For many years, the gold standard for complex proteome analysis was 2D gel electrophoresis followed by identification using mass spectrometry; however, even with recent improvements, most IMP classes remain resistant to characterization.[1] In response to the difficulties inherent to gel-based separation of intact IMPs, shotgun[2] proteomic characterization was adopted.[3−5] Shotgun proteomics combines chromatographic, electrophoretic, or affinity purification separation of complex protein digests with electrospray ionization (ESI)[6] or matrix-assisted laser desorption/ionization (MALDI),[7] commonly in the form of microcapillary liquid chromatography-tandem mass spectrometry (μLC-MS/MS).[8] However, sample preparation still presented a problem for IMPs due to their intractable solubilization in ESI-compatible detergents and subsequent incomplete digestion.[9] In response, several creative solutions were introduced, including digestion in aqueous methanol,[4,10−13] proteolytic shaving of soluble domains,[12−16] and biotin-[10,17] or lectin-mediated[18−22] affinity enrichment. As will be discussed, however, because shotgun strategies were originally optimized for soluble peptides, typical μLC separation strategies may also require

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optimization for the comprehensive study of IMPs.[9] For any proteomic analysis, it is desirable to maximize sequence coverage; however, if chromatography is skewed toward soluble domains, information regarding transmembrane domain (TMD) sequence and PTMs may be lost. Indeed, by ignoring the hydrophobic peptides, even the probability of *identifying* the protein may decrease since IMPs often have a significant percentage of their sequence embedded in the membrane. As mentioned, one approach for studying IMPs is to shave off the soluble domains from intact membranes using, for example, the high pH/proteinase K (hppK) method.[16] The soluble protease-accessible peptide fraction is then isolated by centrifugation and analyzed by μLC-MS/MS; however, analysis of the membrane-embedded peptides (MEPs) is less straightforward. MEP samples tend to be much more viscous, are harder to load onto a μLC column, and often exhibit problematic elution (unpublished results). It was observed empirically that the application of heat significantly reduced sample loading and running difficulties, and, importantly, increased peptide (particularly hydrophobic) recovery as well.

The beneficial effects of conducting LC at elevated temperatures are well recognized; higher temperature (e.g., above room temperature; typically 30−80 °C) is known to improve peak selectivity and resolution[23,24] and can be necessary for the successful elution of some very hydrophobic proteins.[25] However, an investigation of the role of temperature in the recovery of hydrophobic peptides has yet to be reported. The findings described herein should be of significant value for those in the proteomics community studying IMPs using standard μLC-MS/MS methods. For, while thermostatted LC systems are common, the column heater functionality was largely omitted during the development of μLC for interfacing with electrospray mass spectrometers; as a result, most analyses are currently conducted at room temperature. This report details the effects of heat on the analysis of enriched membrane protein and TMD samples by μLC-MS/MS using a standard reversed-phase (RP) material.

METHODS

Reagents. All solvents were obtained from Mallinckrodt Baker (Phillipsburg, NJ), and all chemicals were purchased from either Mallinckrodt Baker or Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Preparation of an Enriched Plasma Membrane Fraction. HeLa cells expressing Flag/His-tagged human dopamine transporter (cells are the subject of other ongoing experiments) were grown to confluency, harvested, and pelleted by centrifugation at 1000*g* for 5 min. Cell pellets were suspended in 100 mM potassium phosphate buffer (PB) containing 0.5 M sucrose, 5 mM $MgCl_2$, and 1:1000 dilution of protease inhibitor cocktail (Sigma-Aldrich, p8340). Cells were Dounce homogenized (30 strokes) and centrifuged at 1000*g* for 5 min to pellet unbroken cells and nuclei. The postnuclear supernatant was loaded onto a discontinuous sucrose gradient[26] [1.3 M, 0.86 M, 0.5 M (load), 0.25 M] and centrifuged at 79 000*g* (SW40Ti rotor, Beckman Coulter, Fullerton, CA) for 1 h. The SIII fraction (between 1.3 and 0.86 M) containing enriched plasma membrane was collected via transfer pipet. The sample was diluted with an equal volume of PB and centrifuged at 28 000*g* for 30 min. The resulting membrane pellet was resuspended in 0.2 M Na_2CO_3 buffer (pH 11.0) and homogenized with an insulin syringe 5× every 15 min for 1 h to open membrane vesicles and remove soluble proteins and proteins peripherally associated with the membrane bilayer.[16] Membranes were re-isolated by centrifugation at 135 000*g* for 45 min, resuspended in PB, and subject to protein quantification using the DC protein assay kit (Bio-Rad, Hercules, CA).

Trypsin Digestion of Enriched Plasma Membrane Fraction. Lipids were removed from an aliquot (800 μg) of the enriched HeLa cell plasma membrane fraction via methanol−chloroform precipitation as described[27] with slight modifications: both the aqueous methanol and chloroform layers were removed following phase separation (leaving only the interphase protein layer), precipitation was repeated twice, and, to aid subsequent dissolution, the precipitated protein was resuspended in 600 μL of methanol and sonicated (15 s, 4 W; Misonix XL2000, Farmingdale, NY) before the final centrifugation to pellet the protein. All supernatant was removed, and the pellet dissolved in 1.2 mL of 0.5 mg/mL RapiGest (Waters Corp., Milford, MA) in 50 mM NH_4HCO_3 buffer by heating to 60 °C for 15 min followed by 95 °C for 2 min. The sample was allowed to cool briefly and then reduced with 5 mM dithiothreitol at 60 °C for 30 min and alkylated with 15 mM iodoacetamide at room temperature for 30 min in the dark. The sample was then digested using sequence grade modified trypsin (Promega, Madison, WI) at 1:30 enzyme/protein concentration at 37 °C for 15 h with gentle shaking in a thermomixer (Eppendorf, Westbury, NY). The RapiGest was hydrolyzed by the addition of concentrated HCl to a final concentration of 200 mM and incubation at 37 °C for 45 min. The white particulate matter (consisting of hydrolyzed surfactant and undigested protein) was removed by centrifugation at 20 000*g* for 10 min. The supernatant was removed to a clean tube, and centrifugation was repeated twice. The final supernatant was collected for shotgun analysis.

High pH/Proteinase K−Cyanogen Bromide (HppK−CNBr) Digestion of Enriched Plasma Membrane Fraction for Membrane-Embedded Peptide (MEP) Analysis. The HppK−CNBr protocol was carried out essentially as reported elsewhere.[28] Briefly, an aliquot (6 mg) of the enriched plasma membrane fraction was pelleted by centrifugation at 20 000*g* for 45 min and resuspended in 0.2 M Na_2CO_3 buffer (pH 11.0) at a protein concentration of 1 mg/mL. The sample was homogenized with an insulin syringe 5× every 15 min for 1 h. Solid urea was added to a final concentration of ∼8 M, and the sample reduced and alkylated as described above. The sample was digested with proteinase K (Promega) at a concentration of 1:50 enzyme/substrate at 37 °C for 15 h with gentle shaking in a thermomixer. After the addition of one volume of 10% acetonitrile/water, the sample was cooled on ice for 15 min and centrifuged at 135 000*g* for 45 min. The supernatant containing the protease-accessible peptides was removed, and, in a fume hood, a solution of 0.5 mg/mL CNBr in 90% formic acid (FA) was added to the pellet,

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solublizing the MEPs for methionine-directed digestion. After incubation at room temperature for 15 h in the dark, an equal volume of methanol and 18 volumes of 100 mM Tris−HCl (pH 8.0) were added to the sample. Subsequent centrifugation at 20 000*g* for 20 min produced a compact white lipid pellet. The supernatant, containing the MEPs, was collected for proteomic analysis.

Thermostatted Microcapillary Liquid Chromatography-Tandem Mass Spectrometry (*μ*LC-MS/MS). An Agilent (Palo Alto, CA) 1100 binary HPLC pump/autosampler system and Thermo Fisher Scientific (Waltham, MA) LTQ ion trap mass spectrometer, running Xcalibur software, were used for all sample analyses. With the use of the autosampler as described,[29] protein digests [10 *μ*g of trypsin digest or 100 *μ*g (predigest) of MEP sample] were loaded onto a fused-silica (Polymicro Tech., Phoenix, AZ) capillary column (100 *μ*m i.d. with a 5 *μ*m pulled tip) packed with 12 cm of 5 *μ*m 125 Å Aqua C18 RP material (Phenomenex, Torrance, CA). A block-type column heater (Supporting Information Figure S1) was used to control the temperature of the microcapillary column, whereby the column was sandwiched in a groove between two aluminum plates (15 cm in length) outfitted with a cartridge heater (HeatTech, www.heattech.com, Belmont, CA). (Note: column heater exceeded dimensions of the column to allow for adequate temperature equilibration of the solvent.[24,30]) A T-type thermocouple (Omega, www.omega.com, Stamford, CT) provided feedback to the temperature control module (Omega). The *μ*LC was carried out using an increasing gradient of aqueous acetonitrile (MeCN) containing 0.1% FA over a 160 min run, consisting of a 25 min loading step at 5% MeCN (buffer A) followed by a gradient up to 95% MeCN (buffer B). During all runs, the flow rate through the column was maintained at 0.25 *μ*L/min—the length of the waste split was adjusted accordingly to account for decreasing solvent viscosity with increasing temperature, giving back pressures ranging from 18 bar (for 60 °C) to 26 bar (for 20 °C). Each run was followed by a 30 min wash step at the same temperature as the subsequent run, consisting of 15 min of buffer B followed by 15 min of buffer A. Mass spectra were acquired in a data-dependent mode, whereby a single full mass scan (400−1400 m/z) was followed by five tandem MS scans of the most abundant peaks. Dynamic exclusion was enabled. The column was conditioned by three initial runs at 40 °C and then allowed to cool to room temperature; runs for data analysis were then carried out in order of increasing temperature from 20 to 60 °C. (Note: same results were obtained when order was randomized, data not shown). Seven replicates were conducted for each temperature; however, only the final four were used for analysis to avoid any sample carryover. To maintain consistency, all trypsin digest runs were performed using the same column, as were all MEP runs.

Data Analysis. The MS/MS spectra generated for each run were searched against a combined NCBI human and mouse protein database (for consistency with other analyses) concatenated to a randomized decoy database[31] using a *normalized* implementation of Sequest[32] on a 96-node G5 Beowulf cluster. No proteolytic enzyme was specified during the search, and a static modification of +57 was assumed for all cysteines. For the MEP samples, a variable modification on methionine of −48 was searched to account for conversion to homoserine lactone following CNBr cleavage. The resulting peptide identifications were assembled into protein identifications using DTASelect,[33] and filters adjusted to maintain a false discovery rate (FDR, as determined by number of hits against the randomized database[34]) of <0.01% for the trypsin-digested samples and ≤5% for the MEPs. For the trypsin samples, the following thresholds were used: normalized cross-correlation score for +1, +2, +3 ions of 0.2, DeltCN value of 0.1, minimum two peptides per protein, fully tryptic peptides required, subset proteins and proteins matching uniquely to the mouse database removed. For certain analyses (Figure 3 and Supporting Information Tables S1 and S2), the four runs for each temperature were combined into one DTASelect file and refiltered using the same parameters. For the MEP samples, the following approximate DTASelect thresholds were used: normalized cross-correlation score for +1, +2, +3 ions of 0.15, DeltCN value of 0.12, minimum two peptides per protein, minimum of 30% predicted fragment ions observed, minimum sequence length of seven amino acids, subset proteins and proteins matching uniquely to the mouse database removed. For certain analyses (Figures 3 and 4, Table 1, and Supporting Information Tables S3 and S4), the four runs for each temperature were combined into one DTASelect file and refiltered using similar parameters as listed above, except the minimum peptide requirement was increased to three. Contrast[33] was used to compare DTASelect files. GRAVY (grand average of hydropathy) scores were calculated according to Kyte and Doolittle,[35] and TM helical predictions were carried out using TMHMM[36] (http://www.cbs.dtu.dk/services/TMHMM/).

RESULTS AND DISCUSSION

Sample Preparation and Shotgun Analysis. Two types of samples were prepared from an enriched HeLa cell plasma membrane fraction for evaluation of the temperature effects on *μ*LC-MS/MS analysis: a complex trypsin digest and an MEP sample. The plasma membrane proteome was obtained by sucrose gradient on HeLa cell postnuclear supernatant followed by a high pH extraction of soluble and peripherally associated membrane proteins, leaving a fraction enriched in IMPs.[16] To generate the trypsin digest sample, proteins in the enriched membrane fraction were delipidated by methanol−chloroform precipitation, solubilized in RapiGest, and digested with trypsin. The resulting sample was expected to contain peptides from both the soluble and

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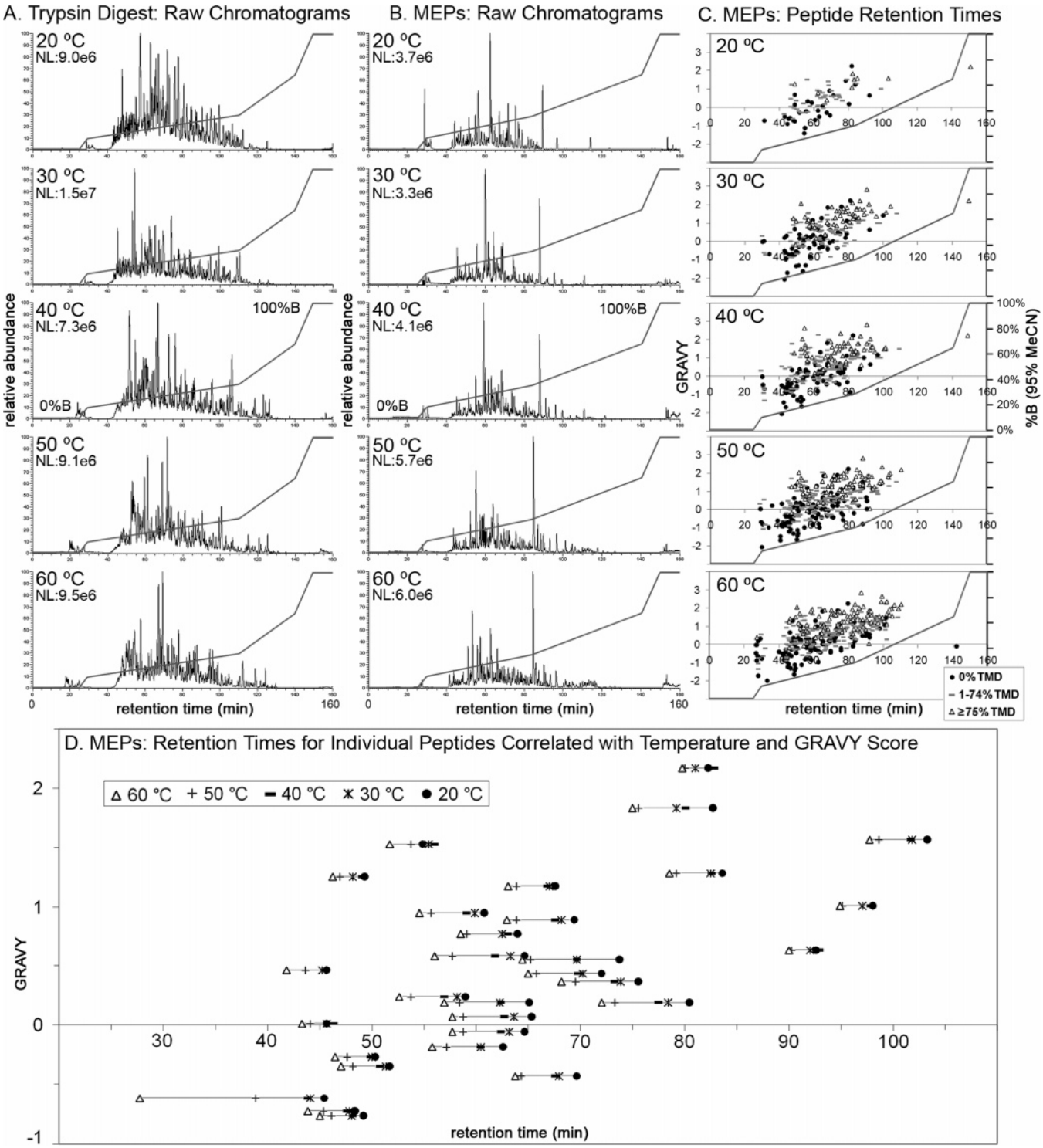

Figure 1. Chromatography. Raw μLC chromatograms for shotgun analysis of an enriched plasma membrane proteome prepared by (A) RapiGest−trypsin digest, 10 μg protein load, and (B) high pH/proteinase K−CNBr digest, 100 μg (predigest) protein load. For panels A and B, chromatograms remain largely unchanged as temperature is increased. NL = signal level, arbitrary units. (C) Peptide retention times (MEP sample) are plotted against the peptide GRAVY score; peptides with hydrophilic GRAVY scores (left *y*-axis) and no TMD coverage (filled circles) tend to elute earlier in the gradient (overlaid, scale on right *y*-axis), whereas those with hydrophobic GRAVY scores and high TMD coverage (open triangles) tend to elute later. Note: the peptide eluting at ~150 min in 20−40 °C is not identified at 50 or 60 °C; the peptide eluting at ~140 min at 60 °C is a likely false positive based on MS/MS spectrum analysis. (D) For the MEP sample, individual peptide retention times at each temperature (see legend upper left) are plotted against the peptide GRAVY score. For the majority of peptides, elevated temperature (60 °C, open triangles) decreased retention time by ~5 to 10 min as compared to room temperature (filled circles). Note: for clarity, only 30 of the 47 peptides found at all five temperatures are shown; for the complete list, see Supporting Information Table S4.

membrane-embedded domains of IMPs, along with peptides from soluble protein contaminants. Preparation of the second sample followed a more rigorous, recently optimized, high pH/proteinase K−cyanogen bromide (hppK−CNBr) protocol[28] to selectively

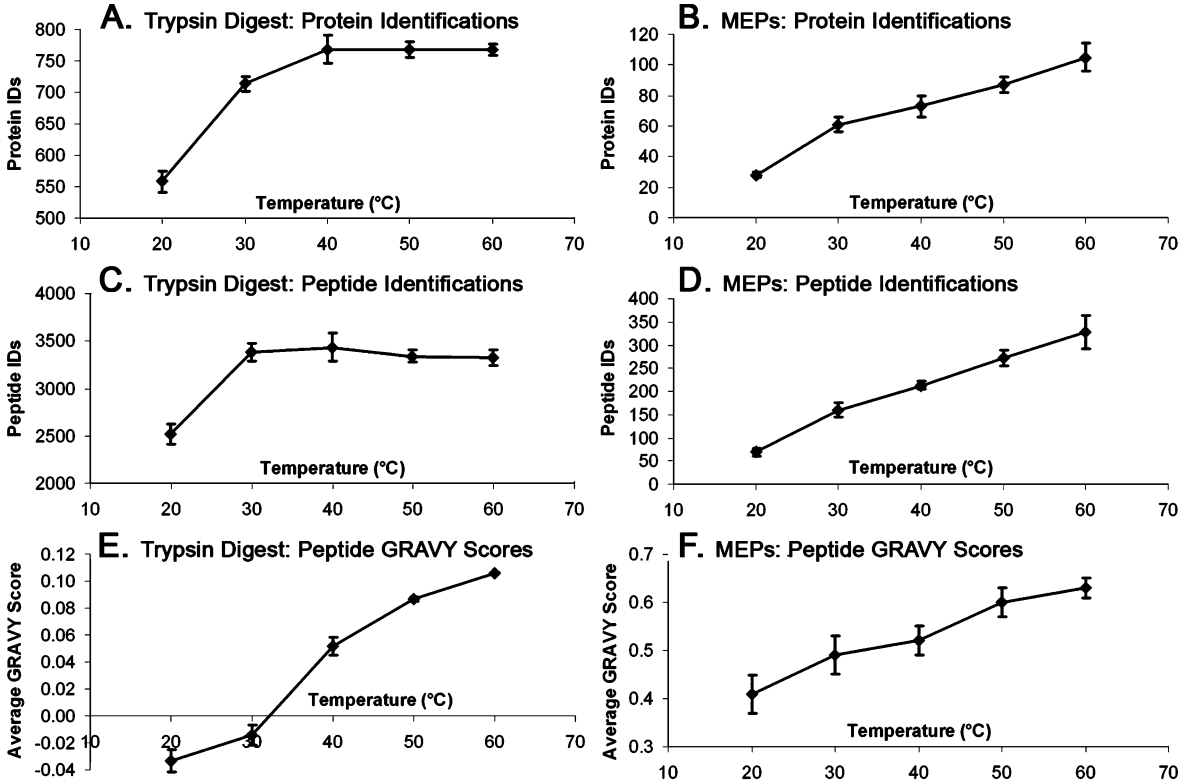

Figure 2. Characterization of protein and peptide identifications from trypsin digest (left panel) and hppK−CNBr (right panel) as a function of temperature. Elevated temperature increases the number of nonredundant protein identifications (A and B), peptide identifications (C and D), and average peptide GRAVY score (E and F). Note: false discovery rate (FDR) is <0.01% for trypsin (A) and ≤5% for MEPs (B). For all: error = ±standard deviation, $n = 4$.

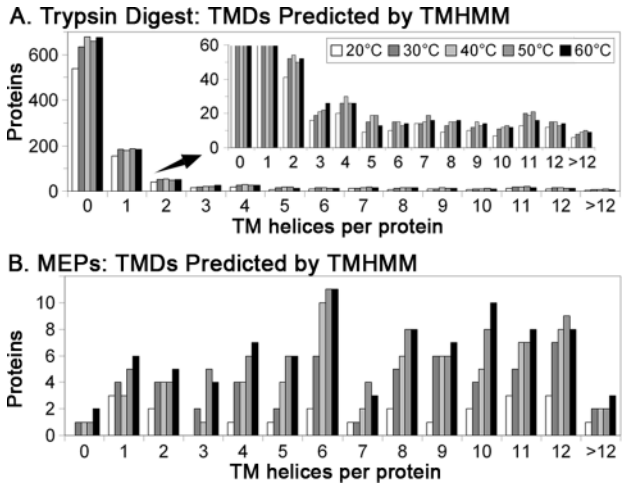

Figure 3. Predicted TMD distribution in proteins identified in trypsin digest (A) and MEP samples (B). As a percentage, integral membrane proteins are significantly more enriched in the MEP sample; however, in terms of overall numbers, more IMPs were identified using the trypsin protocol. For all categories, more proteins are identified at elevated temperature, with a pronounced increase in MEP proteins with >2 TMDs. Note: all four runs per temperature were combined for this analysis; bars are in order of increasing temperature.

obtain the membrane-embedded domains of IMPs. The enriched plasma membrane fraction was again suspended in a high pH buffer, and the exposed soluble domains were digested with a non-sequence-specific protease, proteinase K. The resulting shaved membranes were separated from the protease-accessible peptides by centrifugation and then solubilized in a solution of 90% FA and CNBr for further digestion. After dilution to 5% FA, centrifugation separated the MEPs (supernatant) from the lipid component (pellet).

Samples were analyzed using an Agilent HPLC and autosampler system interfaced with a Thermo Fisher Scientific LTQ ion trap mass spectrometer. The μLC column consisted of a 100 μm fused-silica capillary column packed with 12 cm of Aqua C18 RP material (Phenomenex). Temperature of the μLC column was controlled using an in-house-constructed block column heater (Supporting Information Figure S1). Both samples (trypsin digest and MEPs) were analyzed at five different temperatures: 20 (room temperature), 30, 40, 50, and 60 °C. A previous study indicated that the column material appears to degrade above 60 °C, giving rise to fewer protein identifications and irrevocably damaging the column (Supporting Information Figure S2). This finding is in accordance with manufacturer recommendations to not exceed 60 °C. Four replicate runs at each temperature were used for data analysis. Several criteria were used to evaluate the effects of temperature on membrane proteome analysis, including chromatography, protein/peptide identifications, peptide GRAVY scores,[35] identification of IMPs, and sequence coverage of TMDs.

Chromatography. Representative LC traces for the trypsin digest (Figure 1A) have similar elution profiles (with slight broadening at the higher temperatures) and the same gross peak shape and signal level. Significant improvement in peak shape was not observed at higher temperature, possibly due to sample complexity. The MEP raw chromatograms (Figure 1B) show similar trends. Peptide elution profiles were also analyzed, as

shown for the MEP samples in Figure 1C. Peptides with more positive (hydrophobic) GRAVY scores (*y*-axis) and higher TMD overlap (open triangles) tend to elute later than hydrophilic soluble domain peptides (closed circles); however, it is notable that, regardless of temperature, the vast majority of peptides still elute in the shallower sections of the gradient, below ~40% MeCN, where the chromatographic resolution is expected to be better. Analysis of individual peptide retention times (Figure 1D) revealed that elevated temperature (60 °C) reduces retention time by an average of 5 to 10 min as compared to room temperature (see also Supporting Information Table S4). The case was similar for the RapiGest−trypsin samples: the last peptides to elute consisted of very long, hydrophobic TMD-containing peptides, which still eluted by ~65% MeCN (~142 min; see Supporting Information Table S2-B and C).

Protein and Peptide Identifications. Temperature differences become more evident upon evaluation of the number of proteins and peptides identified at each point: for the trypsin digest, protein identifications increased 38% (558 vs 768) from 20 to 40 °C and then plateaued through 60 °C (Figure 2A). This change is mirrored by a 36% increase in the number of unique peptides identified (2510 vs 3424; Figure 2C). Protein identification overlap between any two samples was 56% (±1%) at 20 °C and 63% ± 1% for 40−60 °C; thus, run reproducibility also improved at higher temperatures.

For the MEP sample, temperature had an even more dramatic impact on the number of identified proteins and peptides: from room temperature to 60 °C, protein identifications increased 4-fold (28 vs 105; Figure 2B), and unique peptide identifications increased 5-fold (69 vs 327; Figure 2D). When the profiles of identified proteins (Figure 2, part A vs part B) and peptides (Figure 2, part C vs part D) for the trypsin digest and hppK−CNBr samples are compared, it is observed that identifications in the trypsin digest seem to plateau, whereas the MEP graphs have a more linear increase. It is possible that this is a reflection of differential temperature effects; however, it is more likely the result of analyzing samples of different complexity. Given the large number of proteins and peptides identified in the trypsin digest at the higher temperatures (~770 and ~3400, respectively) as compared to the MEP samples (105 and 327, respectively), the upper limit of identifications, as determined by the experimental set up (column length, run time, etc.) and instrument (LTQ) has probably been reached. To overcome such limitations, one could employ any or all of the following: longer microcapillary column,[37] longer run time and/or altered gradient,[38,39] different solid-phase material,[40] or high mass accuracy instrumentation.[9]

GRAVY Scores. When the average GRAVY score of identified peptides is plotted as a function of temperature (Figure 2, parts E and F), a trend toward more positive (hydrophobic) GRAVY scores with increasing temperature becomes evident (trypsin digest, −0.03 vs 0.11; hppK−CNBr, 0.41 vs 0.63). Thus, not only can the number of peptide identifications be significantly increased by running μLC at elevated temperature, but the overall population of identified peptides is actually more hydrophobic. It is of note that, over the entire temperature range, the GRAVY scores for the trypsin digest are lower than those of the MEP sample (Figure 2, part E vs part F). This observation reflects the differing compositions of the two samples; whereas the trypsin digest contains both the membrane-embedded and soluble domains of membrane proteins, the latter (more hydrophilic) domains and any soluble protein contaminants should largely have been removed in the MEP sample.

Integral Membrane Proteins. Another measure of success is the identification of IMPs, especially those with multiple TMDs. TMHMM, one of the most reliable α-helical TMD prediction algorithms,[41] was used to predict the number and location of TMDs in identified proteins. For the trypsin digest, the percentage of IMPs was predicted to be only 38% (±1%) for all temperatures, and the majority of IMPs (~58%) contained only 1 or 2 TMDs (Figure 3A). In contrast, 98% (±1%) of proteins in the MEP samples had at least one predicted TMD, and complex IMPs (>2 TMDs) were more widely represented, making up 77% of IMPs at 20 °C and 87% at 60 °C (Figure 3B). Thus, the hppK−CNBr digestion strategy provides an unprecedented enrichment of IMPs and shows no bias against complex multispanning proteins, and with regards to the latter species, elevated temperature had a pronounced impact on their successful identification. Concerning the RapiGest−trypsin method, it should be noted that even though percent enrichment was low, in terms of overall numbers, the method resulted in the identification of more IMPs than hppK−CNBr (413 vs 86) and required 10-fold less sample.

Transmembrane Domains. There was also a significant difference between the two preparation protocols when TMD sequence coverage was considered, and elevated temperature was again shown to have a positive effect. For the trypsin digest, the number of peptides overlapping predicted TMDs was exceedingly low: only 1.1% of peptides contained at least partial TMD segments at 20 °C and 2.1% at 60 °C (TMD peptides are listed in Supporting Information Table S2). Several factors may be contributing to the poor enrichment: either peptides are not generated in high frequency, are too hydrophobic to elute from the column in the temperature range tested, are too long to identify by standard ESI-MS/MS, and/or are obscured by more highly abundant species during shotgun analysis. In contrast, using the HppK−CNBr protocol, 63% of identified peptides were predicted to contain TMD regions at 20 °C and 68% at 60 °C. Importantly, peptides with high TMD overlap (≥75% of peptide sequence) also increased over the temperature gradient, from 11% at 20 °C to 27% at 60 °C (Supporting Information Table S4).

It is of note that the two preparation methods are somewhat orthogonal, as the trypsinized sample yielded more TMD-length peptides (>20 AA) with high TMD sequence coverage (≥75%) as compared to the hppK−CNBr preparation (26 vs 12 peptides, representing 17% vs 3% of all peptides with TMD overlap; Supporting Information Table S2-B vs Supporting Information Table S4-B). This observation is not surprising given that Met is significantly more common in TMDs than Lys/Arg, resulting in

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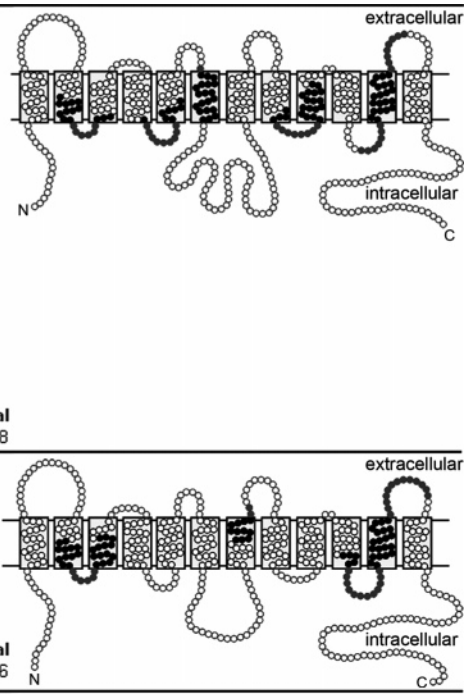
Table 1. Sequence Coverage of Two Membrane Proteins Identified in MEP Samples[a]

MCT 1	peptides	TM	*GRAVY*	20°C	30°C	40°C	50°C	60°C	
(monocarboxylic	**M.YGGGPISSILV**NKYGS**RIVM*.I** +2	2,3	*0.375*	×	×	×	×	×	
acid transporter 1)	**M.YGGGPISSILV**NKYGS**RIVM*.I** +3	2,3	*0.375*	×	×	×	×	×	
[Homo sapiens]	**M.I**GKYFYKRRP**LANGLAM*.A** +3	4,5	*-0.288*					×	
gi:115583685	W.R**GSFLILGGLLLNCCVAGALM***.R +2	6	*1.638*					×	
	M.GLVANTKPIRPR**IQYFFAASVVANGVCHM*.L**	8,9	*0.462*					×	
	M.DLVGPQR**FSSA.V** +2	11	*-0.227*	×	×	×	×	×	
	M.DLVGPQR**FSSAV.G** +2	11	*0.142*			×	×		
	M.DLVGPQR**FSSAVGL.V** +2	11	*0.364*		×	×	×	×	
	M.DLVGPQR**FSSAVGLVTIV.E** +2	11	*0.961*			×	×		
	A.VGLVTIVECCPVLLGPPLLGRLNDM*.Y +3	11	*1.172*				×	×	
	L.VTIVECCPVLLGPPLLGRLNDM*.Y +2	11	*0.986*				×	×	
	L.VTIVECCPVLLGPPLLGRLNDM*.Y +3	11	*0.986*			×	×		
	T.IVECCPVLLGPPLLGRLNDM*.Y +2	11	*0.910*		×	×	×	×	
	T.IVECCPVLLGPPLLGRLNDM*.Y +3	11	*0.910*			×	×	×	
	V.ECCPVLLGPPLLGRLNDM*.Y +2	11	*0.528*		×	×	×	×	
	V.ECCPVLLGPPLLGRLNDM*.Y +3	11	*0.528*		×	×	×		
	C.CPVLLGPPLLGRLNDM*.Y +2	11	*0.656*				×		
	C.PVLLGPPLLGRLNDM*.Y +2	11	*0.533*					×	total
	% sequence coverage:			6.2	11.0	11.2	11.0	24.6	24.8

MCT 4	peptides	TM	*GRAVY*	20°C	30°C	40°C	50°C	60°C	
(monocarboxylic	**M.LYGTGPLCSVCV**NRFGC**RPVM*.L** +3	2,3	*0.605*	×	×		×	×	
acid transporter 4)	**M.LVGGLFASLGM*.V** +2	3	*1.827*	×	×	×	×	×	
[Homo sapiens]	**M.VLGLFVPPVFV**VS.Y +2	7	*2.292*					×	
gi:4759112	**M.VGALO**FEVLM*.A +2	10	*1.510*	×				×	
	M.AIVGTHK**FSSAIGLVLLM*.E** +2	11	*1.494*		×	×	×	×	
	M.EAVAVLVGPPSGGKLLDATHVYM*.Y +2	11	*0.617*		×	×	×	×	
	M.EAVAVLVGPPSGGKLLDATHVYM*.Y +3	11	*0.617*	×	×	×	×	×	
	V.GPPSGGKLLDATHVYM*.Y +2	11	*-0.144*			×		×	total
	% sequence coverage:			14.0	15.7	11.2	15.7	20.6	20.6



[a] Predicted TMD residues are indicated in bold and underlined. ×: peptide identified for a given temperature. TMDs are numbered from left to right; filled circles correspond to identified sequences.

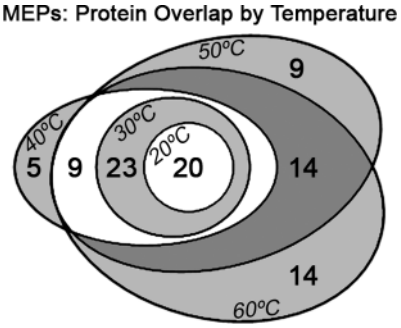


Figure 4. Overlap of proteins identified in MEP samples. The higher temperature runs are largely inclusive of proteins identified at lower temperatures. Note: all four runs per temperature were combined for this analysis; categories with ≤3 proteins were omitted for clarity but are included in Supporting Information Table S3.

shorter peptides following CNBr digestion.[42,43] Of these long, hydrophobic TMD peptides found in the trypsin samples, all but one were identified in the 60 °C runs (12 of those exclusively), and only 3 were identified (none uniquely) at room temperature. Thus, despite a clear lack of enrichment, with the aid of elevated temperature, the RapiGest−trypsin digest did result in the identification of a nontrivial number of TMD peptides (88 vs 246 for MEPs), including an important class of TM-length peptides. However, hppK−CNBr is still the method of choice for the targeted analysis of TMDs.

As a specific example of TMD coverage, two integral plasma membrane proteins identified from the MEP samples, both members of the monocarboxylic acid transporter (MCT) family, are shown in Table 1. The predicted structures shown are in accordance with Poole et al.,[44] who determined that the N and C termini, as well as the large loop between TMDs 6−7, are located in the cytoplasm. All identified peptides contain at least a portion of a predicted TMD (bold, underline), and significant coverage of a number of TM regions is observed, particularly at elevated temperature. For full list of proteins identified using hppK−CNBr digest, including TMD coverage, see Supporting Information Table S3.

Population Overlap. One remaining question is whether different populations of proteins/peptides are observed at different temperatures, or if high temperature is sufficient to capture the majority of proteomic information. The Venn diagram in Figure 4 depicts the identified protein overlap for MEPs analysis. Proteins identified at the 20 and 30 °C temperature points are nested within all higher temperatures. From 40 to 50 to 60 °C, successively more proteins were exclusively identified at each temperature. Importantly, the 60 °C temperature point encompasses 85% of all proteins identified (Supporting Information Table S3).

In terms of peptide overlap, the same trend is observed: 68% were found at 60 °C and 86% at 50 and/or 60 °C. Conversely, only 15% of peptides were found at the lowest (20 °C) temperature point and 41% at the lowest two points (20 and/or 30 °C). Similarly, whereas 42% of peptides were found *exclusively* in the 50 and/or 60 °C runs, only 4% of peptides were uniquely identified at 20 and/or 30 °C (Supporting Information Table S4). Taken together, these data clearly suggest that running μLC at higher temperature

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(60 °C) is sufficient to give a comprehensive analysis of an enriched hydrophobic peptide sample, as little additional information was gained from room temperature μLC analysis.

Rationalizing Temperature Effects. As mentioned previously, the beneficial effects of temperature on peak separation and resolution is well documented;[23,24] however, the relationship between temperature and sample recovery has been less well studied. Some understanding may be gained from examining what is known about RP LC and temperature effects on peptide retention: (1) Longer and/or more hydrophobic peptides tend to elute later than short and/or hydrophilic peptides.[24,45,46] This phenomenon is illustrated by the MEP elution profiles (Figure 1C), where peptides with higher GRAVY scores/TMD overlap tend to elute later, though still in the shallow sections of the gradient. (2) It is also known that increased temperature reduces peptide elution time due to the increased solubility of the solute in the mobile phase, decreased viscosity, and increased mass transfer between the mobile and stationary phases.[24,45,46] However, while elevated temperature is indeed observed to reduce peptide elution time by ~5 to 10 min (Figure 1D), its effect on whether not peptides elute *at all* is much more dramatic. Indeed, even a delay of up to 40 min for room temperature relative to 60 °C would have been well within the μLC run time frame for peptide observation, suggesting that simply increasing the mobile phase gradient to shift elution times will not be able to replicate the unique effect of elevated temperature. This notion that some hydrophobic peptides are so strongly retained on the stationary phase that substantial heating is required for elution is supported by the recent report[25] that cites elevated temperature (80 °C) as an essential factor for elution of highly hydrophobic intact lipid raft proteins. (3) An additional consideration for explaining selective high-temperature elution is the influence of secondary structure on the interaction of the solute with the stationary phase, as evinced by amphipathic α-helical peptides, whose retention time decreases to a greater extent than that of random coil peptides due to denaturation at elevated temperature.[46] It is of note that amphipathic α-helices are known to be present in membrane spanning regions of complex IMPs,[47] so the role of temperature as a denaturant may be a key factor in the observed increased recovery of TMD-containing peptides.

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CONCLUSION

For the shotgun analysis of membrane proteomes using a standard C18 RP material, elevated temperature is necessary and sufficient to maximize protein and peptide identifications. Importantly, the additional peptides recovered were skewed toward more hydrophobic peptides, as determined by more positive GRAVY scores and increased sequence coverage of TMDs. The somewhat linear increase in GRAVY scores and protein/peptide identifications for the MEP sample indicates that even higher temperatures (up to 90 °C) could be beneficial for hydrophobic peptide recovery, if more stable (sterically hindered) RP materials[45] are used. Finally, the block column heater employed provides a straightforward way to add thermostatting capabilities to any μLC platform, and its general use should greatly aid in the comprehensive proteomic analysis of IMPs.

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SUPPORTING INFORMATION AVAILABLE

Figure S1, illustration of column heater; Figure S2, protein identifications vs temp showing column degradation above 60 °C; Table S1, proteins/peptides identified in trypsin digest; Table S2, TMD-overlapping peptides identified in trypsin digest; Table S3, proteins/peptides identified in MEP samples, including TMD coverage; Table S4, MEP retention times; Tables S1−4 as an Excel file. This material is available free of charge via the Internet at http://pubs.acs.org.

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