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Development of Structural Marker Peptides for Cystic Fibrosis Transmembrane Conductance Regulator in Cell Plasma Membrane by Reversed-Footprinting Mass Spectrometry

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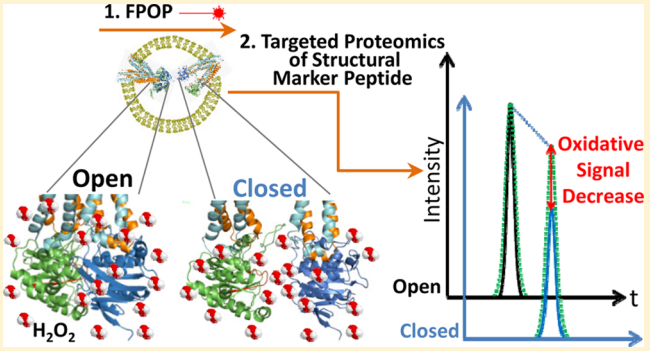
Supporting Information

ABSTRACT: A targeted mass spectrometry-based method is presented that adopts the fast photochemical oxidation of proteins (FPOP) for footprinting of cystic fibrosis transmembrane conductance regulator (CFTR) membrane transporter at its original plasma membrane location. Two analytical imperatives were sought: (1) overall simplification in data acquisition and analysis and (2) lower quantitation limits, which enabled direct analysis of intrinsically low-abundance transmembrane proteins. These goals were achieved by using a reversed-footprinting technique that monitored the unoxidized peptides remaining after the FPOP treatment. In searching for structurally informative peptides, a workflow was designed for accurate and precise quantitation of CFTR peptides produced from proteolytically digesting the plasma membrane subproteome of cells. This sample preparation strategy mitigated the need for challenging purification of large quantities of structurally intact CFTR. On the basis of the interrogated peptides, it was proposed a concept of the structural marker peptide that could report CFTR structure and function in cells. The reversed-footprinting mass spectrometry extends the FPOP technology to study conformation and interaction changes of low-abundance proteins directly in their endogenous cellular locations.



Membrane transporter proteins maintain intracellular homeostasis and intercellular communications with the peripheral environment. They are important targets for drug development. However, their structural studies have been in large part limited, especially for those proteins with high molecular weights and multidomain features.[1] Maintaining the fidelity of high-order structures of membrane proteins, from the cell membrane to solution during purification, is challenging due to the lack of a lipid bilayer for stabilization.[2,3] Both the expression and purification of large membrane transporters, in a structurally stable form, are crucial for conventional X-ray diffraction.[4] New structural biology technologies such as hard X-ray pulsed free-electron lasers and rotationally aligned solid-state NMR spectroscopy[5,6] reduce sample preparation challenges by using smaller amounts of purified membrane proteins.[7] Structural analysis of membrane transporters directly in cells can provide a route to mitigate the sample preparation problems, which can facilitate studies of function and interaction of the proteins as well as the development of new drugs.

Fast photochemical oxidation of protein (FPOP) in combination with mass spectrometry (MS) provides an emerging technology for analyzing protein structure and adds an intriguing option to the structural biology and proteomics toolbox. Shared with diverse covalent labeling strategies in the structural analysis of proteins and hydrogen exchange experiments,[8−10] the fundamental principle of the FPOP-MS technology rests on the probe accessibility to surface residues on the protein.[11,12] Most importantly, the time scale of the protein footprinting[13] in FPOP is faster than that for protein (un)folding. An excimer laser is used to generate hydroxyl radicals at an optimal wavelength which is maximally absorbed by H_2O_2 but negligibly by protein and water.[9,11,12] Other advantages include the use of time-tunable, photochemical generation of "sufficiently small" hydroxyl radicals as probes and their subsequent fast reaction rates on the side-chains of amino acid residues.[11,14−16] FPOP-MS has been applied to analyze the structure of a variety of purified proteins, in solution, using flow systems.[17−20] However, its application for analyzing membrane transporters has been hampered by their difficult preparation and structural complications.[21]

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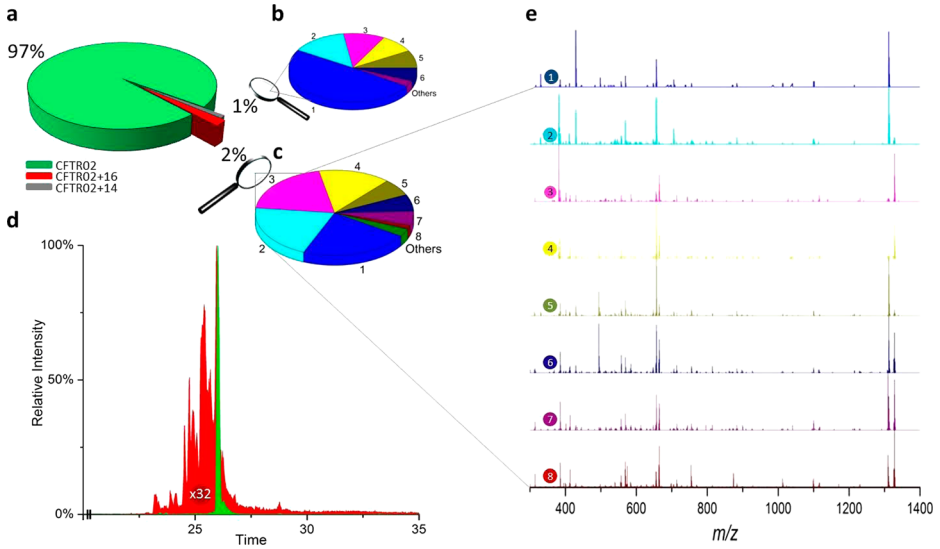

Figure 1. Quantifiability of unoxidized peptide CFTR02 vs the oxidized. (a) Populations of the oxidized and unoxidized CFTR02 peptides. (b) Different species of oxidized peptide with + 14 Da increase. (c) Different species of oxidized peptide with + 16 Da increase. (d) Extracted ion chromatograms of the native (green) and oxidized (+ 16) peptides (red, magnified by 32 times). (e) MS/MS spectra of oxidation (+ 16) products.

A physiologically important example of membrane transporters is cystic fibrosis transmembrane conductance regulator (CFTR). CFTR predominantly mediates the chloride ion flux across the cell membrane in the apical surface of epithelial cells found in the lung, pancreas, intestine, liver, and glands. Certain known mutations result in a total loss of the ion channel activity of the protein, leading to the inheritable disease of cystic fibrosis (CF).[22] CFTR mutants as the fundamental molecular defects of CF currently are the primary targets for the development of modulating drugs. These drugs are being developed to restore and rescue the channel activity in CF patients. Effective drug development relies largely on understanding drug action mechanisms and structural features that dictate the correct channeling state of CFTR.

Herein, we describe a new method development of the oxidative footprinting to analyze the endogenous structure of CFTR in the cell plasma membrane. We used targeted quantitative proteomics to sensitively and accurately measure the CFTR peptides that remained unoxidized after FPOP treatment of CFTR expressing cells as a proof-of-concept for a method that we termed as reversed-footprinting MS of proteins. In comparison, a majority of FPOP studies used semiquantitative approaches to measure both the oxidized (reacted) and the unoxidized (remaining, unreacted) peptides.[5,12,17,23] Some of these approaches have the advantage of probing protein structure with a resolution approaching the residue level. On the other hand they often demand extensive data mining and validation,[17,24] due to the existence of multiple oxidation states of residues and multiple oxidation sites on a particular peptide. In addition, an assumption is made that the unoxidized and oxidized peptide pairs have the same ionization efficiency in order to quantify the oxidation degree of a peptide. Another complication for quantitation based on oxidized peptides arises from the compromised tryptic digestion effectiveness when arginine or lysine is oxidized.[25,26] In striking contrast, the reversed-footprinting approach reported here solely measures the remaining unoxidized peptides, using isotopically labeled CFTR as the protein-level internal quantitation reference. This subtractive quantitation strategy circumvents the complications associated with analysis of oxidized peptides. Another outstanding feature of the proposed approach leverages the feasibility of targeting preselected peptides from proteins of interest, even when the proteins exist in complex proteomic samples. The targeted MS quantitation also affords the reversed-footprinting approach with low quantitation limits, which are essential to analysis of low-abundance membrane transporters.

Quantifiability Comparison of Oxidized versus Unoxidized Peptides. In the current practice of FPOP in flow systems, it is typical that large amounts of purified proteins are available for oxidative footprinting.[11,14,16,18,27−29] Therefore, determination of the major oxidative conversions of the peptides is possible. In contrast, transporter proteins like CFTR exist in small amounts in the cellular plasma membrane, which makes the application of current FPOP-MS practices very challenging, if even possible. We used three strategies in combination to overcome the quantitation challenges for low-abundance membrane proteins treated with FPOP: (1) targeting the remaining, unoxidized peptides for relative quantitation, (2) using highly sensitive and specific multiple reaction monitoring (MRM) MS for targeted quantitation,[30−32] and (3) adding the internal protein standard at an early stage of the sample preparation for high quantitation precision and accuracy.[31,32] Unoxidized peptides are advantageous analytes, versus oxidized peptides, because each oxidized peptide often represents only a tiny fraction of the oxidation product pool. Considering the following schematic oxidation reaction (Reaction 1) on peptide A:

$$\mathrm{A(total)} \xrightarrow{\mathrm{FPOP}} \mathrm{A(unox)} + \sum_{i=1}^{n} \mathrm{A(ox)}_i \qquad (1)$$

where A(total) is the initial peptide, A(unox) is the unoxidized species after an oxidation reaction, and $\mathrm{A(ox)}_i$ is a particular oxidation product. Because of multiple potential sites and different levels of oxidation, numerous reaction products are generated. Quantitation difficulties for the oxidized products are even more exacerbated when the starting amount of A is low. Therefore, FPOP footprinting of low-abundance membrane proteins is only realistic through quantitation of

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unoxidized peptides, i.e., reversed-footprinting. On the other hand this change in the quantification subject, from the individual oxidized peptide to the unoxidized peptide, results in a loss of resolution in the solvent accessibility assessment.

A comparison in the quantifiability between unoxidized and oxidized peptides was performed using the MS/MS data from a model peptide LSLVPDSEQGEAILPR (CFTR02) (for experimental details, see S1 in the Supporting Information).[32] The chromatogram acquired by data dependent analysis (DDA) observed two major classes of oxidation products, CFTR02 + 16 Da (addition of an oxygen atom) and CFTR02 + 14 Da (e.g., addition of one oxygen and deletion of two H atoms to form a C=O bond), as well as the remaining unoxidized CFTR02 (Figure S-1 in the Supporting Information). MS/MS spectra were recorded and relative quantities of each of the peptides were calculated from extracted ion chromatograms, which were demonstrated in a pie chart (Figure 1a). Only about 1% of the total peptides were identified as CFTR02 + 14 Da (Figure 1b) and about 2% as CFTR02 + 16 Da (Figure 1c). Within the 1% for CFTR02 + 14 Da, 6 major different subsets were identified according to their elution time and MS/MS profiles, in addition to many weak ones ("others" in Figure 1b). Within the 2% for CFTR02 + 16 Da (Figure 1c), there were multiple components with overlapping elution profiles (solid red chromatogram in Figure 1d). We grouped eight major elution windows based on the observed apexes on the extracted ion chromatogram (Figure 1d) and the resulting MS/MS spectra were shown (Figure 1e), enumerated from 1 to 8 according to the elution time. As shown in the MS/MS spectra (Figure S-1 in the Supporting Information), pairs of unoxidized and oxidized fragments were observed (e.g., y12 at m/z 1311.6 and y12 + O at m/z 1327.6) and their relative intensities varied for each of the 8 grouped peptide populations (Figure 1e). In striking contrast and as expected, the unoxidized CFTR02 eluted as a single sharp peak with an intensity to be 32 times higher than the strongest oxidized peptide (extracted ion chromatogram or XIC, solid green in Figure 1d). It is clearly seen that quantitation of the unoxidized peptide (green XIC) is a much easier route than that of individual oxidized products.

Photochemical Oxidation for Reversed-Footprinting of CFTR. Oxidative reversed-footprinting on wild-type CFTR, expressed on the plasma membrane of baby hamster kidney (BHK-wtCFTR) host cells, was investigated. Analyzing the protein in its physiological location in the cell can produce directly relevant results for function and interaction. CFTR in the plasma membrane, where it functions as a chloride channel, was targeted for the reversed-footprinting analysis in this work. Direct FPOP treatment of CFTR in the plasma membrane circumvents protein aggregation and unfolding problems, which are widely noted to be associated with membrane protein purification. We used three sets of identical cell samples and prepared CFTR in the plasma membrane for MS quantitation,[32] shown in Figure S-2 in the Supporting Information. Briefly cultured cells (S3 in the Supporting Information) were surface-biotinylated (S4 in the Supporting Information). Then samples, except for the control, were mildly permeabilized with a 0.002% w/v saponin buffer (S4 in the Supporting Information).[33] Saponin is among the mild detergents that can permeabilize mammalian cell plasma membrane but keep its integrity (Figure S-3 in the Supporting Information).[34,35] Permeabilization of the plasma membrane by saponin was intended to facilitate the influx of the footprinting reaction mixture into the cytosolic region of cells. A one-fifth volume of each sample of homogeneous suspension of permeabilized cells was set aside for separate measurements of the total CFTR in the sample (marked with dashed lines in the Figure S-2 in the Supporting Information), and the results were used for quantitation normalization later. The other four-fifths of the cell sample was added with the FPOP reaction reagents and was then subjected to FPOP by excimer laser radiation except for the control sample (S5 in the Supporting Information). The tryptic digest of membrane CFTR was prepared by a dual enrichment procedure.[32] A lysate of stable isotope labeling by amino acids in cell culture (SILAC) BHK-wtCFTR cells was used as the internal standard (IS) for quantitation (S6 and S7 in the Supporting Information).[32] Also a Western blot analysis was conducted before addition of SILAC-IS, for confirming the biotinylated CFTR enrichment quality (S8 in the Supporting Information). CFTR peptides, which remained unoxidized after FPOP, were quantified by nanoflow LC–MRM MS (S9 in the Supporting Information). The results for each sample were normalized against the total CFTR amount measured for the above-mentioned one-fifth of cell aliquots; this normalization was crucial to quantitative comparison of the amount of unoxidized CFTR peptides in each sample (S10 in the Supporting Information).

Among the identified peptide pool, nine peptides were quantifiable across all three samples (Table S-2, Figure S-8 in the Supporting Information and Figure 3). These peptides are unique to CFTR, blasted against a mammalian proteome database (http://www.uniprot.org/blast/); the peptide uniqueness is important because the analyzed digests are from a mixture of large plasma membrane proteins.[32] These peptides are shown on a depiction of CFTR PDB file (Figure 2), which

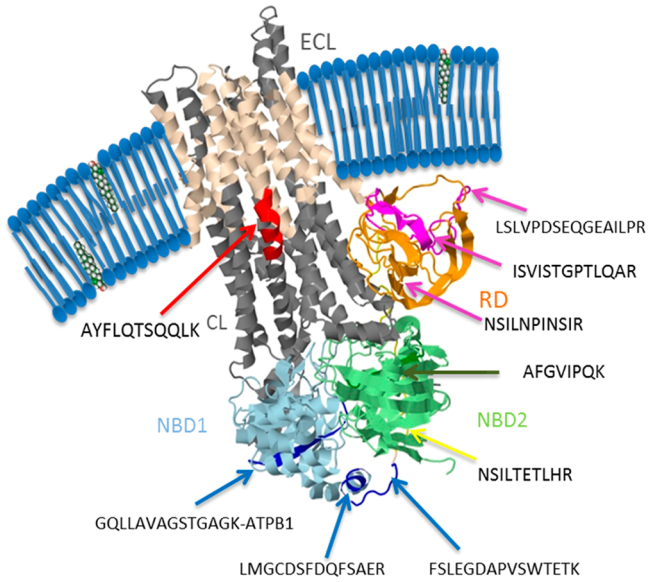

Figure 2. Reversed-footprinting of nine CFTR peptides by FPOP and MRM MS. (Protein was drawn by the PyMOL Molecular Graphics System).

is derived from fusion of the sequence of nuclear-binding domain (NBD) 1 to other domains that are constructed from Sav1866 as a known structural homologue to CFTR.[36] Among the nine peptides, AYFLQTSQQLK is in the proximity of a transmembrane domain; peptides GQLLAVAGSTGAGK and LMGCDSFDQFSAER are in NBD1; peptide AFGVIPQK is in NBD2; peptides LSLVPDSEQGEAILPR and ISVISTGPTL-

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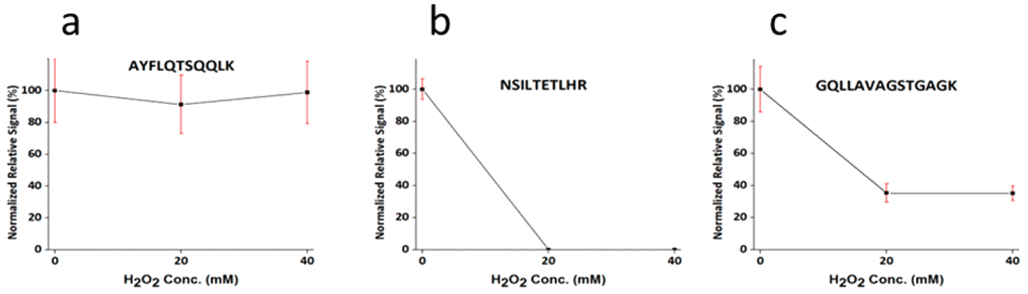

Figure 3. Oxidation dependence of selected peptides on H_2O_2 concentration: (a) peptide AYFLQTSQQLK at the proximity of the TMD, (b) Peptide NSILTETLHR at the flexible loop, and (c) peptide GQLLAVAGSTGAGK at the ATP binding pocket region of the NBD1 domain.

QAR are in the regulatory (R) domain; three peptides FSLEGDAPVSWTETK, NSILTETLHR, and NSILNPINSIR are in the loop region that links the NBD1 and R domains.

In Search for Structural Marker Peptides of CFTR in the Plasma Membrane. The concentration dependence of the hydroxyl radical accessibility to the CFTR surface was studied. The relative peptide quantity was plotted against the H_2O_2 concentrations (0, 20, and 40 mM) for each peptide. Different degrees of peptide oxidation were observed, suggesting varying hydroxyl radical accessibilities of the analyzed peptides (Figure 3 and Figures S-8 in the Supporting Information) with an assumption that the peptide oxidation is the major cause of changes measured for the unoxidized peptides, made by in-gel digestion.[25,26] These differences in oxidation could be attributed to two main factors: (1) the intrinsic reactivity of the amino acid constituents of individual peptides[9] and (2) the probe accessibility that is governed by the local and topological environment of a peptide. Peptides LMGCDSFDQFSAER, AFGVIPQK, LSLVPDSEQGEAILPR, and ISVISTGPTLQAR are in the NBD1, NBD2, or R domains, according to the model (Figure S-8 in the Supporting Information). Their moderate oxidation could be attributed not only to the folding of CFTR itself but also to intermolecular interactions of the plasma membrane CFTR with other biomolecules. For example, peptide LMGCDSFDQFSAER with a methionine residue, which is highly reactive to hydroxyl radical, survives from the complete oxidative conversion (Figure S-8 in the Supporting Information). A structural implication of this observation is that the reactive methionine residue is protected.

The amount of unoxidized peptide AYFLQTSQQLK does not substantially change with the addition of H_2O_2 (Figure 3a). According to the CFTR structure model,[36] this peptide is part of a transmembrane helix.

Peptides FSLEGDAPVSWTETK, NSILTETLHR, and NSILNPINSIR are in the same loop that connects the NBD1 and R domains. They had different oxidation behaviors. Peptide NSILTETLHR was fully oxidized upon the photolysis of 20 mM H_2O_2 (Figure 3b). This peptide is at the center of the flexible loop while peptide FSLEGDAPVSWTETK comes from the NBD1 domain and peptide NSILNPINSIR leads to the R domain (Figure S-9 in Supporting Information). Previously, our lab identified peptide NSILTETLHR as a surrogate peptide for quantification of CFTR, which produced high MS signal; this could due in part to the high yield of the peptide generation upon proteolytic digestion of the flexible, hydrophilic loop.[30]

Peptide GQLLAVAGSTGAGK is particularly interesting. This peptide is located in an ATP binding pocket on the NBD1 domain, according to the model[37,38] and its solvent accessibility significantly changes with the channel states (Figure 4 and Figure S-10 in Supporting Information). ATP binding and conformational changes of NBD1 are essential to the channel functioning of CFTR.[39] As shown in Figure 3c, 20 mM of H_2O_2 allowed for a saturated degree of oxidation at about 60%, measured by the unoxidized peptide. No further oxidation of the peptide was observed with an increase to 40 mM in the H_2O_2 concentration. It is tempting to speculate on the cause of this result by relating to reported CFTR models of different gating states.[37,38] One explanation of the observed "oxidation saturation" is the presence of two populations of CFTR, one with the channel open and the other closed (Figure 4), although strict biological and control experiments are needed to evaluate this proposal.

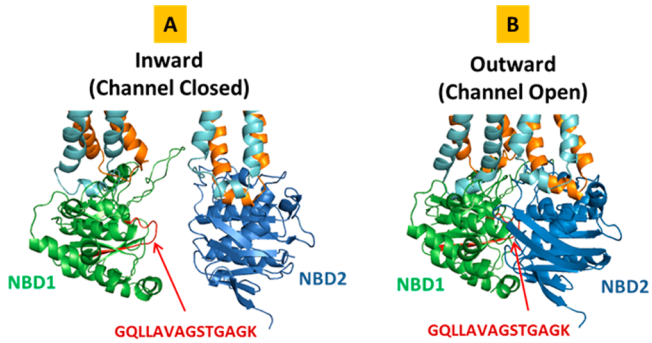

Figure 4. Illustration of the surface accessibility of peptide GQLLAVAGSTGAGK in the open and closed conformation of CFTR. Peptide GQLLAVAGSTGAGK is located at the contact surface of NBD1-NBD2.

CONCLUSION

An analytical workflow for reversed-footprinting of the solvent-accessible surface of CFTR in its native plasma membrane is presented, combining the use of quantitative targeted proteomics and FPOP. Comparative quantitation of the degree of oxidation of a particular peptide in the endogenous cellular environment opens a new path to apply the FPOP technology for analysis of proteins in cells. Employing structural marker peptides as signatures which indicate changes in protein folding and interaction can facilitate development of new drugs and investigation of the interactome.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b01962.

Experimental sections and further information, sections S1−S10; supporting tables, Tables S-1 and S-2; and supporting figures Figures S-1−S-9 (PDF).

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Notes

The authors declare no competing financial interest.

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