

Letter

Native Mass Spectrometry and Surface Induced Dissociation Provide Insight into the Post-Translational Modifications of Tetrameric AQP0 Isolated from Bovine Eye Lens

Sophie R. Harvey, Carla O'Neale, Kevin L. Schey, and Vicki H. Wysocki*



bovine eye lens, existing as multiple forms due to the different modification states naturally present.

quaporins (AQPs) are a family of channel proteins that form pores in the membranes of cells, facilitating the transport of water through cell membranes. There are 13 AQPs identified in mammals, which are expressed and regulated in a tissue-dependent manner.^{1,2} Aquaporin-0 (AQP0) is the most abundant membrane protein in the eye lens, comprising >50% of the membrane protein content in the lens.³ AQP0 is an important component of the water microcirculation system⁴ in the lens and also serves as a cell adhesion protein.⁵ Water transport through AQP0 is regulated by pH and through its interaction with calmodulin, which binds to the hydrophobic side of a short amphipathic helix located in the C-terminus.^{6–8} A number of studies have shown that AQP0 phosphorylation can play a role in calmodulin-dependent gating.^{8,9} In one such study, human AQP0 C-terminal peptides were synthesized with and without serine phosphorylation on two sites (S231 and S235), and it was found that binding of calmodulin is impaired when either site, or both, is phosphorylated, suggesting that C-terminal phosphorylation has a role in regulation of function.⁹ Hence, understanding what influence, if any, phosphorylation has on structure is of interest, along with defining the phosphorylation at the monomer and tetramer level.

Interestingly, there is little to no cellular turnover once mature lens fiber cells are formed;¹⁰ hence, age-related protein modifications accumulate with time, which may affect protein function. Therefore, lens tissue is a valuable resource to study the impact of age-related modifications on protein structure,

stability, and function. The post-translational modifications of AQP0 from bovine, rabbit, and human eye lens have been elucidated with bottom-up proteomics and MALDI imaging of lens tissue.¹¹⁻¹³ While these techniques are undoubtedly useful, they disrupt the tetrameric form of the complex, and hence, information on how proteoforms can coexist is lost. Here, we use native mass spectrometry (nMS) to study the post-translational modifications on each monomer within the tetrameric complex and how these monomers are arranged in the tetramer. Native mass spectrometry (nMS) has become a powerful structural biology tool that allows non-covalent complexes to be transferred intact into the gas-phase, and when performed on high-resolution instrumentation, even small mass differences can be resolved, e.g., ligand binding and post-translational modifications.¹⁴ Membrane proteins can be challenging to study, as they have to be solubilized within a mimetic for the membrane environment; however, in recent years, multiple mimetics have been presented that are also compatible with nMS, including detergent micelles,¹ bicelles,¹⁶ nanodiscs,¹⁷ and amphiphols.¹⁸ Once the proteincontaining mimetic is introduced into the gas-phase, collisional

Received:October 5, 2021Accepted:December 21, 2021Published:January 11, 2022







Figure 1. Full mass spectrum of bovine AQP0 isolated from bovine cortex, acquired on a Q Exactive UHMR spectrometer with -95 V in-source trapping: A) full charge state distribution of tetrameric AQP0 and B) zoom in of the 19+ charge state, highlighting the three selection windows (gray m/z 5835–5859, blue m/z 5919–5940, and green m/z 5940–5968) used for proteoform identification.

activation is used to release the intact protein complex from the mimetic. After the intact complex is released, additional activation can be used to dissociate the complex into its subunits. The most commonly used method of activation is collision-induced dissociation (CID). In CID, the ions of interest undergo multiple collisions with the collision gas (typically nitrogen or argon). This is a stepwise energy deposition process and can result in restructuring or unfolding and for protein complexes typically results in the production of highly charged monomer and the complementary N-1mer. An alternative method of dissociation involves colliding the analytes of interest with a surface and is known as surfaceinduced dissociation (SID). SID is a rapid energy deposition process, and dissociation can produce structurally informative subunits.¹⁹⁻²¹ Recent studies have also shown the use of SID to identify proteoforms in overexpressed and purified soluble protein complexes;²² here, we expand this approach to study a membrane protein complex isolated from lens tissue, which can exist in multiple modified forms.

AQP0 was purified from the cortex of bovine eye lens using offline anion exchange chromatography in n-octyl- β -Dglucopyranoside (OG) detergent. Initially, after anion exchange chromatography, fractions were pooled, concentrated, and exchanged into 200 mM ammonium acetate (AmAc) plus 2 critical micelle concentrations of tetraethylene glycol monooctyl ether (C8E4). It should be noted that incomplete removal of OG may have resulted in mixed micelles in this analysis. High-resolution nMS experiments were performed on an in-house modified Q Exactive UHMR spectrometer, modified to include an SID device between the quadrupole and C-trap.^{23,24} After transfer into the gas-phase, intact tetrameric AQP0 was released from the detergent micelle using in-source trapping. From the full mass spectrum of the intact tetramer, it can clearly be observed that there are a number of related species present, likely due to different proteoforms and combinations of proteoforms (Figure 1). The most abundant species is a species at 112, 900 \pm 1 Da, which is consistent with four copies of the full-length (AA1-263) AQP0 (expected mass 112, 892 Da). In addition, we observe singly $(+80 \pm 1 \text{ Da})$ and doubly phosphorylated $(+161 \pm 2 \text{ Da})$

tetramers and potential oleoylation (+265 \pm 2 Da), consistent with previous bottom-up studies. 11,25 In addition, truncated forms of thenon-covalent tetramer are also detected, which we have characterized as minor (up to -412 ± 2 Da mass lost from the tetramer) and moderate (up to -1976 ± 3 Da mass lost from the tetramer) truncations, as highlighted in blue and gray, respectively, in Figure 1B. In order to identify the truncations, and how they are arranged in the tetrameric complex, we used the quadrupole and selected three wide isolation windows around the 19+ charge state of the tetramer, highlighted with the different colored boxes in Figure 1B. We first performed collision-induced dissociation (referred to as HCD by the manufacturer) to produce high intensity monomer enabling accurate mass determination, as shown in Figure S1. For the selection of the tetrameric complex containing only full-length monomers (selection represented by the green box in Figure 1B), we observe nonphosphorylated and singly phosphorylated monomer products, along with an oleoylated monomer. Interestingly, despite seeing a doubly phosphorylated tetramer, CID shows no evidence of a doubly phosphorylated monomer product, suggesting instead the doubly phosphorylated tetramer contains two singly phosphorylated monomers. However, as phosphorylation is a labile modification and can be lost in collisional activation, we wanted to further probe this using SID, as discussed in more detail below. We next considered the tetramer with minor truncations (selection represented by the blue box in Figure 1B); following CID we observed primarily full-length monomer products including phosphorylated and oleated forms (accounting for ~93% relative intensity of the monomer, as determined from UniDec²⁶), as expected as the mass lost as measured on the tetramer (up to -412 Da) was not significant enough for all monomers to be truncated and suggests instead that the tetramer consists of both full-length and truncated monomers. However, two different truncated monomers were identified corresponding to AA1-259 and AA1-261, the expected and observed masses of which are given in Table S1. Finally, we considered the moderate truncated fraction (selection represented with the gray box in Figure 1B) which could either be due to more extensive truncation at the



Figure 2. AQP0 was liberated from detergent micelles using -95 V in-source trapping. Surface-induced dissociation of 19+ tetrameric AQP0 was then performed at 62 V (1178 eV), acquired for each of the isolation windows (highlighted in Figure 1) representing A) full-length AQP0, B) minor truncations, and C) moderate truncations. The left-hand panel is full mass spectrum, and the right-hand panel shows zoom in of the dimeric and trimeric species of different lengths.

monomer level or the presence of multiple truncated monomers within the tetramer. Following CID, the major species observed (~87% relative intensity, as determined from UniDec²⁶) was again the full-length monomer, including the phosphorylated full-length form. However, two additional truncations were observed in this fraction corresponding to AA1-246 and AA1-244 (see Table S1) not observed in the previous selection windows, indicating that the moderate truncations do indeed arise from a single monomer having larger truncations as opposed to multiple copies of minor truncations have been observed in MALDI imaging experiments of rabbit and human eye lens^{12,13} but have not been correlated with particular tetramer populations.

In addition to CID, we also performed SID on the three isolation windows. SID is complementary to CID for structural studies in that it enables information on substructure and subunit connectivity to be obtained in addition to proteoform information. It should be noted that, at the energies applied here, only non-covalent complex fragmentation is observed for both CID and SID, and sequence ions are not observed. If sequencing of the monomers was desired, a pseudo-MS³ approach could instead be employed.²⁷ By SID, we observe that full-length AQP0 dissociates to monomer + trimer and dimers (dimers are discussed in more detail below), consistent with the ring-like structure expected for this water channel. The monomers observed from SID were either non- or singly phosphorylated, again suggesting (as in CID) that the doubly phosphorylated tetramer contains two singly phosphorylated monomers, as opposed to one monomer that is doubly

phosphorylated, despite having multiple potential phosphorylation sites per monomer. 11,28,29 SID on the minor and major truncated species shows similar dissociation behavior to the full-length tetramer, demonstrating that the truncations do not signifcantly alter subunit structure or connectivity, as would be expected for these minor C-terminal truncations (Figure 2), which should not influence the protein-protein interfaces. Previous studies have sought to determine the functional effect of minor C-terminal truncations and have found that the water permeability of full-length and some truncated proteins is indistinguishable.³⁰ However, if truncations were to include the proposed calmodulin-binding domain (residues 227-237), modulation of water permeability by calmodulin would be lost. While the protein retains water permeability, C-terminal truncation may affect regulation of channel activity, localization of the protein, or structural properties that are not distinguished by the SID studies presented here. However, by SID, due to the high intensity of the dimer subproducts, we can start to observe subunit connectivity, for example, dimers composed of one full-length monomer and a truncated monomer or a full-length phosphorylated monomer plus a truncated monomer, even when these contributions are of low relative intensity. This could therefore be a promising approach in more aged samples, in which more extensive truncation would be expected, and when tetramers could be composed of multiple truncated forms.

The previous section focused on the analysis of samples pooled after anion exchange chromatography, which enabled the different truncations to be observed and studied with gasphase dissociation. However, in addition to the pooled

Letter



Figure 3. AQP0 was liberated from detergent micelles using -70 V in-source trapping. Surface-induced dissociation of 19+ doubly phosphorylated tetrameric AQP0 was then performed at 62 V (1178 eV): A) full mass spectrum, B) zoom in of 6+ monomer and 12+ dimer, C) zoom in of 9+ dimer, and D) zoom in of 13+ trimer.

samples, we studied the individual fractions from anion exchange chromatography. Offline anion exchange chromatography proved very effective at separating the different phosphoforms of tetrameric AQP0, enabling fractionation and hence relative enrichment of the different phosphoforms. The later eluting fractions contained the greatest extent of phosphorylation increasing to a maximum of two phosphorylations per tetramer (Figure S2). This is of note as there are multiple potential phosphorylation sites per monomer, as discussed above. Enriching for the different phosphorylated forms through fractionation allows greater insight into the phosphorylation patterns and subunit arrangements, as it becomes possible to have a sufficient signal to select primarily the doubly phosphorylated 19+ tetramer, with minor contamination from the singly phosphorylated tetramer (Figure S3). SID of the doubly phosphorylated tetramer once again highlighted that monomers were only singly phosphorylated, not doubly phosphorylated (Figure 3). While it is known that binding of calmodulin is impaired when either or both site(s) on the monomer is phosphorylated, those studies were performed at the peptide level, so the effect of multiple tetramer phosphorylations on binding and hence function is not known. Hence, the ability to enrich for the different phosphoforms using offline anion exchange could

prove beneficial in functional or structural studies, such as investigating the binding of calmodulin or determining the effect of phosphorylation on protein structure/dynamics.

We demonstrate here that, in a single experiment, SID can provide structural information, along with proteoform information, for a membrane protein complex isolated from eye lens existing in multiple different naturally occurring forms. We observed minor and moderate monomer truncations for bovine AQP0, which do not affect the SID behavior. This is as expected as SID behavior is known to be influenced by the strength of the protein:protein interfaces, and these truncations occur at the C-terminus as opposed to modifications occurring at the protein interfaces. Interestingly, in these samples, we see little cross talk of truncations-with the tetramer existing mostly with a single truncated form as opposed to containing multiple truncated forms. However, the AQP0 studied here was isolated from relatively young eye lens samples, 12-30 months, and age-related modifications are known to accumulate with time; therefore, it is expected that AQP0 isolated from older eye lens, or different organisms, will show more extensive, and perhaps more complicated, modification combinations. We believe that the coupling of CID, SID, and high-resolution MS has the potential to provide insight into the identity and connectivity of the truncations and other postpubs.acs.org/ac

Letter

translational modifications in more complex AQP0 samples, as well as for other post-translationally modified endogenous protein complexes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c04322.

Additional experimental details, materials, and methods; Table S1, identity of truncated monomers of AQP0; Figure S1 full mass spectrum and zoom in for CID experiments on the three isolation windows; Figure S2 full mass spectrum and zoom in of the various anion exchange fractions studied; and Figure S3 zoom in of the mess spectrum for selection with no activation of doubly phosphorylated AQP0 19+ tetramer (PDF)

AUTHOR INFORMATION

Corresponding Author

Vicki H. Wysocki – Department of Chemistry and Biochemistry and Resource for Native Mass Spectrometry Guided Structural Biology, The Ohio State University, Columbus, Ohio 43210, United States; o orcid.org/0000-0003-0495-2538; Email: wysocki.11@osu.edu

Authors

- Sophie R. Harvey Department of Chemistry and Biochemistry and Resource for Native Mass Spectrometry Guided Structural Biology, The Ohio State University, Columbus, Ohio 43210, United States; orcid.org/0000-0003-0763-8173
- **Carla O'Neale** Department of Biochemistry and Mass Spectrometry Research Center, Vanderbilt University, Nashville, Tennessee 37240, United States
- Kevin L. Schey Department of Biochemistry and Mass Spectrometry Research Center, Vanderbilt University, Nashville, Tennessee 37240, United States

Complete contact information is available at:

https://pubs.acs.org/10.1021/acs.analchem.1c04322

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors would like to thank Yue Ju, Wendy White, and Erin M. Panczyk for their help in the early days of this project. This work was supported via funding from the National Institutes of Health P41GM128577 and R01GM113658 awarded to V.H.W. and R01EY013462 awarded to K.L.S.

REFERENCES

(1) King, L. S.; Kozono, D.; Agre, P. Nat. Rev. Mol. Cell Biol. 2004, 5 (9), 687–698.

- (2) Morishita, Y.; Sakube, Y.; Sasaki, S.; Ishibashi, K. J. Pharmacol. Sci. 2004, 96 (3), 276–279.
- (3) Alcala, J.; Lieska, N.; Maisel, H. *Exp. Eye Res.* **1975**, *21* (6), 581–595.
- (4) Mathias, R. T.; Rae, J. L.; Baldo, G. J. *Physiol. Rev.* **1997**, 77 (1), 21–50.

(5) Kumari, S. S.; Varadaraj, K. Biochem. Biophys. Res. Commun. 2009, 390 (3), 1034–1039.

(6) Girsch, S. J.; Peracchia, C. J. Membr. Biol. 1985, 83 (3), 217–225.

(7) Girsch, S. J.; Peracchia, C. *Curr. Eye Res.* **1991**, *10* (9), 839–849.
(8) Reichow, S. L.; Clemens, D. M.; Freites, J. A.; Németh-Cahalan, K. L.; Heyden, M.; Tobias, D. J.; Hall, J. E.; Gonen, T. *Nat. Struct. Mol. Biol.* **2013**, *20* (9), 1085–1092.

- (9) Lindsey Rose, K. M.; Wang, Z.; Magrath, G. N.; Hazard, E. S.; Hildebrandt, J. D.; Schey, K. L. *Biochemistry* **2008**, 47 (1), 339–347.
- (10) Lynnerup, N.; Kjeldsen, H.; Heegaard, S.; Jacobsen, C.; Heinemeier, J. *PLoS One* **2008**, 3 (1), No. e1529.

(11) Ball, L. E.; Garland, D. L.; Crouch, R. K.; Schey, K. L. Biochemistry 2004, 43 (30), 9856–9865.

(12) Grey, A. C.; Chaurand, P.; Caprioli, R. M.; Schey, K. L. J. Proteome Res. 2009, 8 (7), 3278-3283.

(13) Thibault, D. B.; Gillam, C. J.; Grey, A. C.; Han, J.; Schey, K. L. J. Am. Soc. Mass Spectrom. 2008, 19 (6), 814-822.

- (14) Tamara, S.; den Boer, M. A.; Heck, A. J. R. Chem. Rev. 2021, DOI: 10.1021/acs.chemrev.1c00212.
- (15) Barrera, N. P.; Di Bartolo, N.; Booth, P. J.; Robinson, C. V. Science **2008**, 321 (5886), 243–246.
- (16) Hopper, J. T. S.; Yu, Y. T.-C.; Li, D.; Raymond, A.; Bostock, M.; Liko, I.; Mikhailov, V.; Laganowsky, A.; Benesch, J. L. P.; Caffrey, M.; Nietlispach, D.; Robinson, C. V. *Nat. Methods* **2013**, *10* (12), 1206–1208.
- (17) Marty, M. T.; Hoi, K. K.; Gault, J.; Robinson, C. V. Angew. Chem., Int. Ed. 2016, 55 (2), 550-554.
- (18) Leney, A. C.; McMorran, L. M.; Radford, S. E.; Ashcroft, A. E. *Anal. Chem.* **2012**, *84* (22), 9841–9847.
- (19) Stiving, A. Q.; VanAernum, Z. L.; Busch, F.; Harvey, S. R.; Sarni, S. H.; Wysocki, V. H. *Anal. Chem.* **2019**, *91* (1), 190–209.

(20) Harvey, S. R.; Seffernick, J. T.; Quintyn, R. S.; Song, Y.; Ju, Y.; Yan, J.; Sahasrabuddhe, A. N.; Norris, A.; Zhou, M.; Behrman, E. J.; Lindert, S.; Wysocki, V. H. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116* (17), 8143–8148.

- (21) Zhou, M.; Wysocki, V. H. Acc. Chem. Res. 2014, 47 (4), 1010–1018.
- (22) Harvey, S.; VanAernum, Z.; Wysocki, V. J. Am. Chem. Soc. 2021, 143, 7698.
- (23) VanAernum, Z. L.; Gilbert, J. D.; Belov, M. E.; Makarov, A. A.;
 Horning, S. R.; Wysocki, V. H. Anal. Chem. 2019, 91 (5), 3611–3618.
 (24) Harvey, S. R.; VanAernum, Z. L.; Kostelic, M. M.; Marty, M.
- T.; Wysocki, V. H. Chem. Commun. 2020, 56, 15651.
- (25) Schey, K. L.; Gutierrez, D. B.; Wang, Z.; Wei, J.; Grey, A. C. *Biochemistry* **2010**, *49* (45), 9858.
- (26) Marty, M. T.; Baldwin, A. J.; Marklund, E. G.; Hochberg, G. K. A.; Benesch, J. L. P.; Robinson, C. V. Anal. Chem. **2015**, 87 (8), 4370–4376.
- (27) Belov, M. E.; Damoc, E.; Denisov, E.; Compton, P. D.; Horning, S.; Makarov, A. A.; Kelleher, N. L. Anal. Chem. 2013, 85 (23), 11163–11173.
- (28) Wang, Z.; Han, J.; David, L. L.; Schey, K. L. Invest. Ophthalmol. Vis. Sci. 2013, 54 (2), 1135–1143.
- (29) Schey, K. L.; Little, M.; Fowler, J. G.; Crouch, R. K. Invest. Ophthalmol. Vis. Sci. 2000, 41 (1), 175-182.
- (30) Ball, L. E.; Little, M.; Nowak, M. W.; Garland, D. L.; Crouch, R. K.; Schey, K. L. *Invest. Ophthalmol. Vis. Sci.* **2003**, *44* (11), 4820–4828.