Post-translational Modifications of Aquaporin 0 (AQP0) in the Normal Human Lens: Spatial and Temporal Occurrence†

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ABSTRACT: Because of the lack of protein turnover in fiber cells of the ocular lens, Aquaporin 0 (AQP0), the most abundant membrane protein in the lens, undergoes extensive post-translational modification with fiber cell age. To map the distribution of modified forms of AQP0 within the lens, normal human lenses ranging in age from 34 to 38 were concentrically dissected into several cortical and nuclear sections. Membrane proteins still embedded in the membranes were digested with trypsin, and the resulting C-terminal peptides of AQP0 were analyzed by HPLC tandem mass spectrometry, permitting the identification of modifications and estimation of their abundance. Consistent with earlier reports, the major phosphorylation site was Ser 235, and the major sites of backbone cleavage occurred at residues 246 and 259. New findings suggest that cleavage at these sites may be a result of nonenzymatic truncation at asparagine residues. In addition, this approach revealed previously undetected sites of truncation at residues 249, 260, 261, and 262; phosphorylation at Ser 231 and to a lower extent at Ser 229; and racemization/isomerization of L-Asp 243 to D-Asp and D-iso-Asp. The spatial distribution of C-terminally modified AQP0 within the lens indicated an increase in truncation and racemization/isomerization with fiber cell age, whereas the level of Ser 235 phosphorylation increased from the outer to inner cortex but decreased in the nucleus. Furthermore, the remarkably similar pattern and distribution of truncation products from lenses from three donors suggest specific temporal mechanisms for the modification of AQP0.

The ocular lens, which transmits and focuses light onto the retina, retains cells and proteins throughout a human lifetime while maintaining its transparent nature for decades. As the lens grows, newly differentiating cells in the lens periphery compress the older fiber cells toward the center of the lens resulting in concentric layers of fiber cells that vary in age (*1*, *2*). Because of the lack of cell and protein turnover in mature fiber cells (*3*), proteins in the lens range in age from newly synthesized at the lens surface to proteins that are as old as the individual in the lens nucleus. Lens proteins are subject to age-related post-translational modifications including backbone cleavage, deamidation, isomerization, and racemization (*4*, *5*). Each appears to increase with fiber cell age. Backbone cleavage of lens proteins has been shown to occur through the proteolytic activity of enzymes (*6*, *7*) or through spontaneous, nonenzymatic truncation events (*8*). The spontaneous formation of a succinimide ring at susceptible asparagine and aspartic acid residues is a common intermediate that results in deamidation, isomerization, racemization, and backbone truncation (*9*). Within the lens, these events are not random but occur reproducibly with lens age and location within the lenses (*10*). These post-translational modifications have been shown to affect protein function and the regulation of protein activity (*11*−*14*).

Aquaporin 0 (AQP0, MIP),[1] the most abundant membrane protein in the lens, is expressed in newly differentiating fiber cells and is present in fiber cells throughout the lens. As such, AQP0 is the longest lived member of the major intrinsic protein/aquaporin family of water channels, 28 kD proteins with six transmembrane domains and intracellularly localized N and C termini. Although several different roles have been attributed to AQP0 (*15*), the ability of the protein to transport water has been demonstrated reproducibly in a number of laboratories (*16*−*18*). This raises the possibility that AQP0 is a component of the internal circulatory system in the avascular lens (*19*) contributing to lens transparency by maintaining osmotic balance. While the precise function of AQP0 in the lens is not entirely clear, its role in maintaining transparency is evidenced by the formation of a cataract in

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[1] Abbreviations: AQP0, aquaporin 0; cAMP, adenosine 3′,5′-cyclicmonophosphate; EDTA, ethylene diaamine tetraacetate; HFBA, heptaluorobutyric acid; HPLC, high-performance liquid chromatography; LC−MS, liquid chromatography−mass spectrometry; LC−ES-IMS, liquid chromatography−electrospray ionization mass spectrometry; MALDI, matrix-assisted laser desorption ionization; MALDI−TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NR, not resolved; PIMT, protein L-isoaspartate *O*-methyltransferase; PKA, protein kinase A; PKC, protein kinase C; SE, standard error; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; Tris, tris-(hydroxymethyl) aminomethane buffer.

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humans (*18*) and animals (*20*−*23*) with mutations in the AQP0 gene. Studies have also shown that a deficiency in AQP0 not only results in the formation of a cataract, but also an impaired focusing ability of the lens, a decrease in fiber cell membrane water permeability, and a nonuniform cell shape (*24*).

Post-translational modifications within the C terminus of human AQP0, a putative regulatory domain, include phosphorylation (*25*), deamidation (*25*), and extensive backbone cleavage (*25*−*28*). These modifications are more prevalent in aged fiber cells within the center or nucleus of the lens resulting in a heterogeneous distribution of post-translationally modified products of AQP0 spatially within the lens (*28*) and potentially impacting the movement of water through the lens. Decreased fiber cell membrane water permeability (*15*) and a change in membrane localization of AQP0 (*29*) both correlate with the accrual of post-translational modifications of AQP0 in older fiber cells. However, the exact molecular alterations responsible for these effects are not known.

The purpose of this study was to characterize the intralenticular distribution of post-translational modifications in the C terminus of AQP0. Three lenses, ages 34, 35, and 38, were concentrically dissected into 3−4 layers. Lens membranes were isolated from each lens section, and the C terminus of AQP0 was analyzed by mass spectrometry. Previous mass spectrometric analysis of human AQP0 from whole lens homogenates permitted the identification of many sites of C-terminal truncation, deamidation at Asn 246 and Asn 259, and phosphorylation at Ser 235 (*25*). The approach presented here revealed additional sites of truncation and phosphorylation, racemization/isomerization of aspartic acid, and facilitated estimation of the abundance of these modifications with fiber cell age. Identification of the sites of post-translational modification and their locations within the lens may provide insight into the mechanisms responsible for these modifications. In addition, knowledge of the spatial distribution of post-translationally modified products of AQP0 will aid in the understanding of AQP0 function throughout the lens and its potential contribution to the circulation of water and nutrients through the lens. Finally, this approach will permit comparisons of changes of AQP0 in normal human lenses as they occur with fiber cell age with those that occur in an age-related cataract.

EXPERIMENTAL PROCEDURES

Dissection of Human Lens and Preparation of AQP0. Human eyes were obtained from National Disease Research Interchange (Philadelphia, PA). Lenses were removed, and the Tenets of the Declaration of Helsinki for dealing with human samples were strictly followed. As previously described, lenses from male donors ages 34, 35, and 38 were dissected into 3 or 4 concentric layers consisting of the outer cortex, inner cortex, outer nucleus, and the nucleus (*10*). The lens sections were frozen and shipped on dry ice. Each lens section was homogenized with a handheld Teflon pestle homogenizer in an eppendorf tube with 10 mM NaF, 10 mM $NaHCO_3$, and 5 mM EDTA at pH 8.0 (300 μL) and centrifuged at 88000*g* for 20 min at 4 °C. Sodium fluoride was included in the homogenization buffer to inhibit endogenous phosphatases. To remove soluble and extrinsic membrane proteins, the pellet was washed sequentially with Tris buffer (1 mM $CaCl_2$, 1 mM EDTA, and 10 mM Tris base at pH 9.1), freshly made 4 and 7 M urea in Tris buffer, dH_2O, 0.1 M NaOH, and finally dH_2O. The membrane pellet was resuspended in dH_2O (200 μL), and the total protein concentration was determined by the Bradford assay (*30*).

MALDI−*MS of Intact Membrane Protein.* The membrane protein from each lens section was analyzed by MALDI−MS using sinapinic acid matrix with formic acid and hexafluoro-2-propanol (*31*). Peptide/matrix solutions (0.3 μL) were spotted and dried on a MALDI sample plate and were mass-analyzed with a Voyager DE or Voyager DE-STR TOF mass spectrometer (Applied Biosystems) following desorption with a 337-nm nitrogen laser. Thioredoxin (11 673.5 Da) and apomyoglobin (16 951.6 Da) were used as internal standards for mass calibration.

RP-HPLC−*ESI-MS Analysis of the C Terminus of AQP0.* The membrane protein (30 μg) from each lens section was digested with trypsin at a ratio of 1:50 in 10 mM ammonium bicarbonate at pH 8.0 (total volume = 200 μL) and 37 °C for 18 h. The digestion reaction was diluted with water (0.5 mL) and centrifuged at the above conditions for 20 min to pellet the membranes. The peptides released from the membrane protein were collected, dried down under vacuum, and resuspended in water (20 μL). To load approximately the same amount of sample in each LC−MS analysis, the peptide concentration was assessed by spectrophotometry using the extinction coefficient of a synthetic peptide of AQP0, Ac239−263, as a standard (Ac refers to acetylation). The amount injected was typically equivalent to the peptides released from 7 to 10 μg of total membrane protein. The peptides were separated on a 1 × 150 mm C18 Vydac column with a flow rate of 20 μL/min (Agilent 1100 Series HPLC) in-line with an ion-trap mass spectrometer (Thermo-Finnigan LCQ Classic). The HPLC gradient was 2−60% B in 120 min, 60−98% B in 30 min, and 98% B for 10 min. Solvents consisted of 0.02% heptafluorobutyric acid (HFBA) in water (A) and 0.02% HFBA in 60% acetonitrile (B). As peptides eluted from the HPLC, mass spectra were acquired using a mass range of 300−2000 *m*/*z* and tandem mass spectrometric data were automatically collected with the dynamic exclusion feature enabled. The identity of the peptides and sites of post-translational modifications were confirmed by tandem mass spectrometry. Tandem mass spectra were interpreted manually with the assistance of the Sherpa prediction algorithm for peptide fragmentation (*32*).

Relative quantitation of truncation products between residues 239−263 and phosphorylation of tryptic peptide 234−238 was based on the intensity of the signals for peptides detected in the ion-trap mass spectrometer during LC−MS, assuming equal ionization efficiencies. When the mass-to-charge ratio (*m*/*z*) of a predicted peptide was specified, the selected (or extracted) ion chromatogram was generated and the area of the peak was calculated by the Xcalibur software (Finnigan). The relative percent phosphorylation was determined from the peak area of the phosphorylated peptide compared to the sum of the peak areas for the unphosphorylated and phosphorylated peptides. The relative percent truncation observed within the tryptic peptide 239−263 was calculated from the peak area of the truncated peptide compared to the sum of total peak areas for that peptide, including the intact and all truncated forms

of the peptide, 239−*x* (where *x* is the residue at which truncation occurred). For higher molecular weight peptides, the sum of the peak areas for each charge state within the mass range 300−2000 was included in the determination of the relative abundance. Multiple analyses were performed on the three sections of the 34-year-old lens, these data are presented as the mean percent of modification ± the SE (*n* = the number of LC−MS analyses). Note that ionization efficiencies are not identical for different peptides, and thus our measurements are estimates and used to compare extents of modification between regions from a single lens.

Identification of Isomerized and Racemized Aspartic Acid. Four versions of the peptide GAKPDV, AQP0 residues 239−244, were synthesized by solid-phase peptide synthesis at the Medical University of South Carolina Biotechnology Core Facility with the aspartic acid substitutions of L-aspartic acid, L-iso-aspartic acid, D-aspartic acid, and D-iso-aspartic acid. Mixtures of the four peptides were analyzed using the same conditions for LC−ESI-MS as stated above. When the ratio of the four peptides in the mixture was varied, the elution profile was determined.

In Vitro Truncation of a C-Terminal Synthetic Peptide Mimetic. An N-terminally acetylated peptide of AQP0, Ac239−263, was synthesized as indicated above and purified. To test for the possibility of in vitro truncation of the C terminus of AQP0 in the absence of proteolytic enzymes, the synthetic peptide was incubated at 37 °C in ammonium bicarbonate buffer at pH 8.0 for 0−96 h. The incubation mixture was dried down and analyzed by LC−ESI-MS as described above.

RESULTS

Three human lenses of similar age were dissected concentrically, and fiber cell membranes were isolated from each lens section. Mass spectrometric analysis was utilized to measure the mass of AQP0 prior to trypsin digestion and the masses of tryptic peptides that were released from AQP0 following trypsin digestion from each lens section. The sites of post-translational truncation, phosphorylation, isomerization, and racemization of the C terminus of AQP0 were identified by tandem mass spectrometry. Furthermore, the relative extent of phosphorylation and backbone cleavage of the C terminus of AQP0 within different regions of the lens was determined.

Dissection of Human Lenses and Preparation of AQP0. Lens membranes containing human AQP0 were isolated from concentrically dissected regions of 34-, 35-, and 38-year-old lenses. Concentric dissection yields young fiber cells with newly synthesized proteins in the lens cortex and fiber cells as old as the individual in the lens nucleus. The 34-year-old lens was dissected into 3 sections, outer cortex (8.0−8.5 mm), inner cortex (7.0−8.0 mm), and the nucleus (0−7.0 mm), whereas the 35- and 38-year-old lenses were dissected into 4 sections, outer cortex (8.8−9.0 mm; 9.0−9.2 mm), inner cortex (8.2−8.8 mm; 8.4−9.0 mm), nucleus (7.0−8.2 mm; 7.0−8.4 mm), and inner nucleus (0−7.0 mm; 0−7.0 mm), respectively. The equatorial diameters in millimeters from the center of the lens are given in parentheses. The total amount of membrane protein in the three lens sections from the 34-year-old lens, as determined by Bradford analysis, was 65 μg (nucleus), 100 μg (inner cortex), and 39 μg (outer cortex). In the 35-year-old lens, the total amount of membrane protein in the four lens sections was 93.6 μg (inner nucleus), 84.8 μg (nucleus), 40.2 μg (inner cortex), and an estimated 4.5 μg (outer cortex). In the 38-year-old lens, the total membrane protein was 109 μg (inner nucleus), 110.2 μg (nucleus), 34.6 μg (inner cortex), and an estimated 7.5 μg (outer cortex). Because of the small sample size of the outer cortical layers, the protein concentration was estimated from the absorption of the tryptic peptides rather than measured by the Bradford assay.

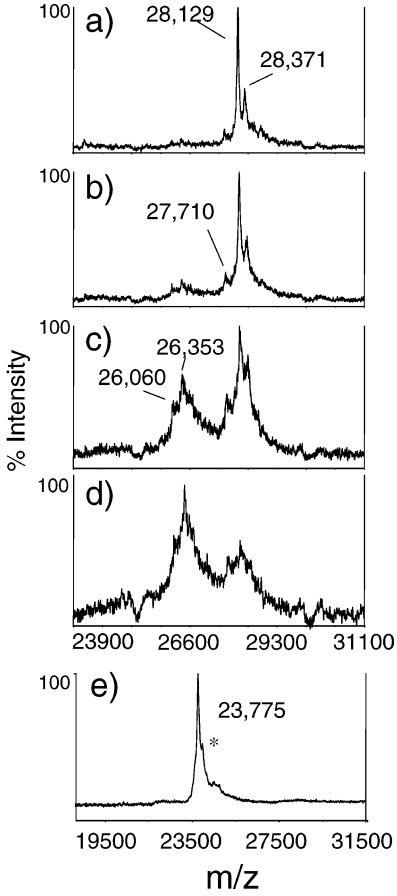

FIGURE 1: MALDI−MS of membrane protein isolated from (a) outer cortex, (b) inner cortex, (c) outer nucleus, and (d) nucleus from a 35-year-old lens. (e) Measured mass after trypsin cleavage from a 34-year-old lens sample. The average expected molecular weight of AQP0 is 28 121 kD. The expected molecular weights of the truncation products of AQP0, 1−259, 1−246, and 1−243, are 27 708, 26 357, and 26 060 Da, respectively. An internal standard was used for mass calibration. The asterisk indicates a putative sinapinic acid matrix adduct.

MALDI−MS Analysis of AQP0 Membrane Protein Isolated from Different Regions of the Lens. The membrane protein from each lens section was analyzed by MALDI−TOF MS. Figure 1 shows the mass spectra of membrane proteins isolated from the outer cortex, inner cortex, nucleus, and inner nucleus of a 35-year-old lens. In the newly differentiated fiber cells of the outer cortex, the most abundant signal in the mass spectrum corresponds to the calculated *m/z* of intact AQP0 (calculated $[M + H]^{1+}$ ion = 28 121), residues 1−263. The signals attributed to truncation correspond closely to the calculated masses of C-terminally truncated AQP0, 1−259, 1−246, and 1−243 (predicted $[M + H]^{1+}$ ions = 27 708, 26 357, and 26 060 Da, respectively). A decrease in intact AQP0 and an increased abundance of

Table 1: Mass Measurements of the Intact and Truncated Forms of AQP0 by MALDI−MS[a]

AQP0 residue numbers	calculated $[M + H]^{1+}$	34-year-old lens measured $[M + H]^{1+}$	35-year-old lens measured $[M + H]^{1+}$	38-year-old lens measured $[M + H]^{1+}$
1−263	28 121	28 141 ± 23 ($n = 4$)	28 118 ± 7 ($n = 5$)	28 122 ± 6 ($n = 6$)
1−259	27 708	NR	27 706 ± 5 ($n = 5$)	27 709 ± 6 ($n = 2$)
1−246	26 357	26 374 ± 17 ($n = 3$)	26 355 ± 15 ($n = 4$)	26 364 ± 7 ($n = 3$)
1−243	26 060	NR	26 058 ± 8 ($n = 3$)	26 061 ± 16 ($n = 2$)

[a] Masses of intact (1−263) and truncated forms of AQP0 were obtained from MALDI−MS data of lens sections from the 34-, 35-, and 38-year-old lenses. The average-measured mass ± SE is shown (n = the number of protein samples measured to obtain the molecular weight). Measurements were acquired on the Voyager DE MALDI (34-year-old lens samples) and on the Voyager DE-STR MALDI (35- and 38-year-old lens samples). NR indicates the species that were not resolved in the mass spectrum.

truncated products of AQP0 occur as a function of fiber cell age. This trend was also observed in the 34- and 38-year-old lens sections (data not shown).

Table 1 shows the measured and calculated masses of the intact and truncated forms of AQP0 observed in all sections of the three lenses. MALDI−MS analyses of the membrane protein isolated from each lens section of the 34-, 35-, and 38-year-old lenses are consistent with previous observations in whole lens homogenates that the most abundant sites of C-terminal truncation in human AQP0 are after residues 246 and 259 (*25*) and that the extent of backbone cleavage increases with fiber cell age (*28*, *33*). The higher mass shoulder at an additional 202.7 ± 4 Da (mean ± SE, n = 14), indicated by asterisks in Figure 1, is likely due to a matrix adduct of AQP0 predicted to be +206 Da (*33*). This signal was also observed for the standards, apomyoglobin and thioredoxin.

Trypsin Digestion of Lens Membrane Protein. Lens membrane proteins, while still embedded in the membrane, were digested with trypsin in a buffered aqueous solution resulting in the cleavage and release of accessible parts of the protein. To ensure that the C terminus of AQP0 was completely cleaved by trypsin, the membranes from each section of the 34-, 35-, and 38-year-old lenses were analyzed by mass spectrometry before and after digestion. Complete cleavage of the N and C termini of AQP0 1−263 to residues 6−229 by trypsin was indicated by a shift in mass from 28 131 to 23 775 Da in the MALDI mass spectrum (Figure 1e). The $[M + H]^{1+}$ ion of full-length AQP0, residues 1−263, has a calculated mass of 28 121 Da. The $[M + H]^{1+}$ ion of AQP0, residues 6−229, has an expected mass of 23 760 Da. The absence of any signal for intact AQP0 at 28 121 Da indicates that trypsin cleaves the membrane-embedded protein completely, releasing N-terminal residues 1−5 and C-terminal residues 229−263. The lack of cleavage after arginine 226 suggests that this residue may be inaccessible to trypsin. Analysis of the membrane protein from the 35- and 38-year-old lens sections demonstrated complete cleavage at residue 239 and only partial cleavage at residue 233 (data not shown). This impeded the use of the data

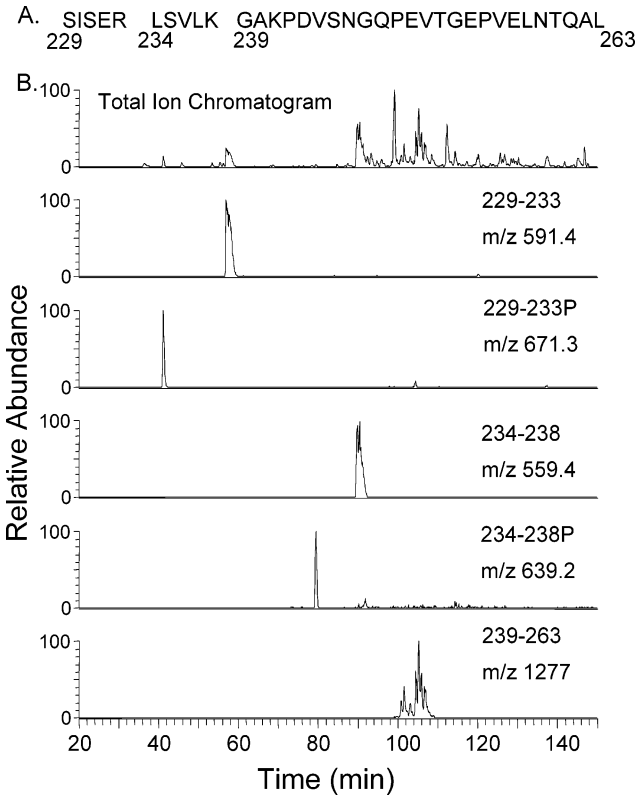

FIGURE 2: Chromatographic separation of C-terminal tryptic peptides of AQP0 from the nucleus of a 34-year-old lens. (A) Expected tryptic peptides from the C terminus of AQP0. (B) Total ion chromatogram (top panel) shows the separation of all of the tryptic peptides in the mixture. The selected ion chromatograms (lower panels) show the elution profile of the expected tryptic peptides of AQP0, 229−233, 234−238, and 239−263, and the phosphorylated peptides 229−233 and 234−238 (indicated by a P). The m/z for each peptide is shown.

obtained from the 35- and 38-year-old lens sections for the quantitative assessment of the abundance of peptides 229−233 and 234−238. Because trypsin cleavage obscures in vivo truncation that may occur on the C-terminal side of lysine and arginine residues, the use of endoproteinase Glu-C or Lys-C was explored; however, the efficiency of digestion with these enzymes was not sufficient for the quantitative analysis desired.

Identification of Post-translational Modifications in the C Terminus of AQP0 by LC−ESI-MS. Soluble C-terminal tryptic peptides were isolated by centrifugation of lens membrane components. Predicted tryptic peptides of the C terminus, 229−233, 234−238, and 239−263, and post-translationally modified forms of these peptides were enriched in the supernatant and analyzed by reverse-phase HPLC ion-trap mass spectrometry. Figure 2 shows the elution profile of several C-terminal peptides including 229−233, 234−238, 239−263, and the phosphorylated forms of 229−233 and 234−238, observed in the analysis of the nuclear section of the 34-year-old lens. The multiple peaks observed for peptide 239−263 suggest chromatographic separation of putative deamidated, racemized, or isomerized peptides because each peptide in the multiplet has the same mass ±0.5 Da and sequence related residues 239−263. Similar complex peak shapes were also observed for the truncated forms of 239−263, and the multiplicity of peaks increased with fiber cell age (data not shown).

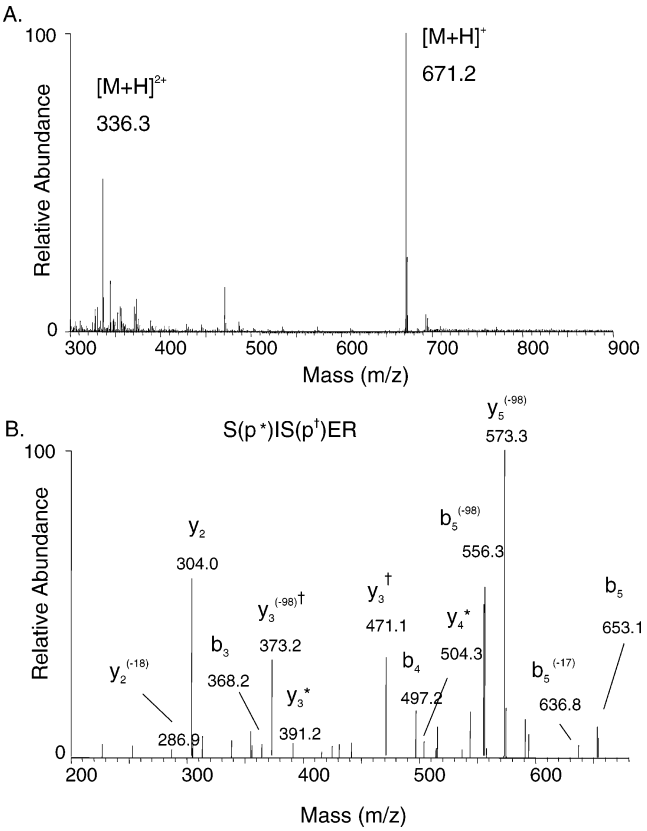

FIGURE 3: Mass spectrum and tandem mass spectrum of the phosphorylated peptide 229−233. (A) Electrospray mass spectrum of phosphopeptide 229−233, with an expected *m*/*z* of 671.2 for the singly charged ion, is shown. (B) Tandem mass spectrum is labeled with the predicted b and y ions. b and y ions consistent with phosphorylation at Ser 231 are shown with a cross, and ions consistent with phosphorylation at Ser 229 are labeled with an asterisk. Loss of 98 Da is a result of the loss of phosphoric acid upon fragmentation. The sequence of the peptide 229−233, SISER, is shown for clarity.

Phosphorylation. Phosphorylation at Ser 235 was confirmed, and new sites of phosphorylation were identified at Ser 231 and Ser 229. The phosphopeptides, 229−233 and 234−238, eluted about 20 min earlier than their unphosphorylated counterparts (Figure 2). The mass spectrum of phosphopeptide 229−233 indicates an 80 Da shift from 591.4 to 671.2, consistent with phosphorylation (Figure 3A). Tandem mass spectrometry revealed that this peptide, SISER, was phosphorylated at Ser 231 and to a much lower extent at Ser 229 (Figure 3B). Fragmentation in the ion trap, resulting in the loss of phosphoric acid (98 Da) from the precursor and product ions that contained the site of phosphorylation, was consistent with phosphorylation at Ser 231. However, on the basis of the observation of fragment ions at *m*/*z* 504.3 and 391.2, it appears that Ser 229 is also phosphorylated and that these two monophosphorylated forms of peptide 229−233 coelute. The doubly phosphorylated peptide, SISER, was not observed.

The level of phosphorylation at Ser 235 within different sections of the 34-year-old lens was estimated, and the percent of phosphorylated peptide to the total signal for the peptide is presented. The mean percent of Ser 235 phosphorylation ± the SE in each section of the 34-year-old lens was 6.4% ($n = 2$) in the outer cortex, 15.1 ± 1.8% ($n = 3$) in the inner cortex, and 7.0 ± 1.1% ($n = 3$) in the nucleus, where n= the number of LC−MS analyses. The level of Ser 235 phosphorylation in the inner cortex was significantly higher than in the nucleus as determined by the *t* test, where $p < 0.05$. Of the four lens sections in the 35- and 38-year-old lenses, the same trend was observed with the highest level of Ser 235 phosphorylation detected in the outer nuclear layer. The levels of phosphorylation reported are estimates and could be influenced by a number of factors including base hydrolysis of phosphoric acid and decreased efficiency of trypsin digestion near phosphorylated residues. To address these concerns, the data were searched for the formation of dehydroalanine following base hydrolysis of phosphoric acid and products of incomplete trypsin cleavage and, when observed, were included in the quantitative analysis. These signals when observed accounted for less than 1% of the unmodified peptide signal. In addition, the estimates of percent phosphorylation reported may be lower than in vivo levels because the ionization efficiency of phosphopeptides is generally slightly lower that their unphosphorylated counterparts (*35*).

C-Terminal Truncation. On the basis of the predicted masses of the tryptic peptide 239−263 and truncation products of this peptide, data obtained during LC−MS analyses were searched for possible truncation after each residue. Peptides truncated after residues 242−249, 251−254, and 257−262 were observed, and their sequences were confirmed by tandem mass spectrometry. Further truncation of the tryptic peptide 239−242 and truncation of tryptic peptides 229−233 and 234−238 were not observed because the masses of these products were below the mass range detected.

The level of truncation at each site within the C terminus increased with fiber cell age, and correspondingly, the amount of intact C-terminal peptide 239−263 decreased with fiber cell age. To determine if the extent of backbone cleavage of AQP0 was consistent within different regions of a normal human lens in multiple lenses of similar age, the relative levels of truncation at each site were estimated by LC−ESI-MS analysis. For simplicity, the elution profiles of all of the truncated forms of peptide 239−263 are not shown. Figure 4 shows the relative level of truncation at each site in each section of the 34-, 35-, and 38-year-old lenses. The level of truncation after residues 242, 249, and 257 were too low for quantitative assessment. Multiple analyses were performed on each section of the 34-year-old lens, and the mean percent of truncation at each site ± SE is shown (Figure 4a). Although there was an additional lens section in the 35- and 38-year-old lenses, the pattern of C-terminal truncation was remarkably similar in each lens tested (parts b and c of Figure 4). Consistent with previous studies (*25*) and the MALDI−MS analysis of the membranes (Figure 1), peptides truncated after Asn 246 and Asn 259 were the predominant signals observed. These data indicate that in different human lenses of similar age and, on the basis of gross concentric dissection, that the spatial distribution of these truncation products within the lens is fairly constant from lens to lens.

Spontaneous Backbone Cleavage at Asn 246 and Asn 259. Because the prevalent sites of truncation were also known sites of deamidation, the possibility of spontaneous backbone cleavage of a synthetic peptide of the C terminus of AQP0 was assessed. An N-terminally acetylated synthetic peptide of AQP0, Ac239−263, was purified and incubated at 37 °C

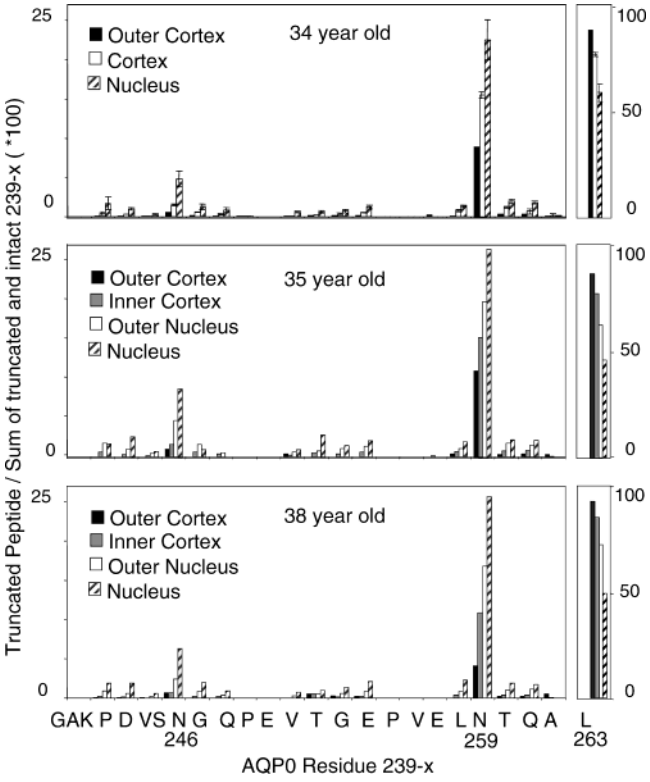

FIGURE 4: Changes in the relative abundance of truncated forms of the C-terminal peptide 239−263 with fiber cell age in 34-, 35-, and 38-year-old lenses. The percentage of truncation at each site is shown. Multiple analyses were performed on the 34-year-old lens sections (top panel), and the mean percent truncation ± SE, (n = three separate analyses) is shown. In the 34-year-old lens, the sections are labeled outer cortex (black), cortex (white), nucleus (hatched). In the 35- and 38-year-old lenses (middle and lower panels, respectively), the sections are labeled outer cortex (black), inner cortex (gray), outer nucleus (white), and nucleus (hatched).

in 10 mM ammonium bicarbonate buffer at pH 8.0. After incubation for 96 h, a low level of truncation was observed on the C-terminal side of asparagine residues that correspond to Asn 259 and Asn 246 (Figure 5). In addition, after the incubation, a single peak at time zero was transformed into two chromatographically separated peaks for the intact and truncated forms of the peptide. These peaks could result from deamidation of asparagine to aspartic acid in the intact peptide and the formation of a C-terminal asparagine and to a lesser extent aspartic acid with a C-terminal amide in the truncated peptides (*8*). The mass spectrometer settings used in this study do not easily distinguish a 1 Da shift because of deamidation in full-scan mode. When the signal intensity of the intact peptide was compared, after incubation for 96 h, ∼1% of the peptide was truncated to Ac239−259 and less than 1% was truncated to Ac239−246. These data indicate that spontaneous nonenzymatic truncation at Asn 246 and Asn 259 occurs in vitro and raise the possibility that spontaneous backbone cleavage may be the mechanism by which AQP0 is truncated at residues Asn 246 and Asn 259 in the human lens. The slow rate of in vitro truncation that occurs under these incubation conditions, which mimic the conditions used during cleavage of the protein with trypsin, also suggests that minimal spontaneous truncation occurred at these sites during the trypsin digestion for 20 h.

Isomerization of Aspartic Acid. The complex peak shape of the chromatographically separated truncated peptides of

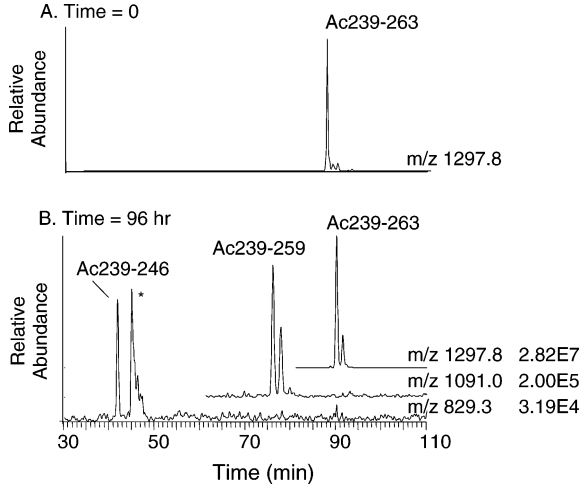

FIGURE 5: In vitro truncation of a synthetic peptide of AQP0 239−263 on the C-terminal side of Asn 246 and Asn 259. (A) Selected ion chromatogram of the synthetic peptide Ac-239−263 (m/z 1297.8) at time zero. (B) After 96 h, the peptides, Ac 239−246 (m/z 829.3), Ac 239−259 (m/z 1091.0) were observed indicating the formation of truncated products in vitro in the absence of proteolytic enzymes. The peptide sequences were confirmed by tandem mass spectrometry. The asterisk indicates a contaminant.

the C terminus led to the hypothesis that deamidation and/or isomerization/racemization of asparagine or aspartic acid occurred. In the inner cortical and nuclear sections of all three lenses tested, a new peak appeared that was chromatographically separated but had the same mass and fragmentation pattern as peptide 239−244, having the sequence GAKPDV. Figure 6a shows the elution profile of the truncated peptide 239−244, $[M + H]^{1+}$ ion 586.3 Da, in the three sections of a 34-year-old lens. The tandem mass spectra of these peptides were searched for fragment ions, y_2 −46 ion and $b_4 + H_2O$ ion, which have been shown to be specific for iso-Asp-containing peptides (Figure 6c) (*36*). The tandem mass spectrum of the first peptide that eluted with a mass of 586.3 contained y_2 and b_4 fragment ions, 187.2 and 372.3 m/z, consistent with the presence of an GAKP(iso-Asp)V. These fragment ions were absent from the tandem mass spectrum of the later eluting peptide with a mass of 586.3 (Figure 6d). Because the fragmentation pattern obtained in the tandem mass spectrum does not indicate the presence of L or D stereoisomers of the aspartic acid (*36*), peptides were synthesized incorporating L-Asp, L-iso-Asp, D-Asp, or D-iso-Asp into the peptide GAKPDV to determine if isomerization of aspartate (α-Asp) to isoaspartate (β-Asp) and/or racemization of L-Asp to D-Asp had occurred at position 243. The peptide containing the L-iso-Asp eluted first, followed 2 min later by the peptide containing D-Asp, then after 1 min D-iso-Asp, and 2.6 min later the L-Asp-containing peptide eluted (Figure 6b). Although the mixture of synthetic peptides eluted at slightly different times, the order of elution combined with their tandem mass spectra permitted the unambiguous identification of the age-related changes in Asp 243 as racemization and isomerization from L-Asp to D-Asp and to D-iso-Asp.

DISCUSSION

The experimental approach presented here permitted the analysis of post-translational modifications of the integral membrane protein, AQP0, from dissected regions of a single

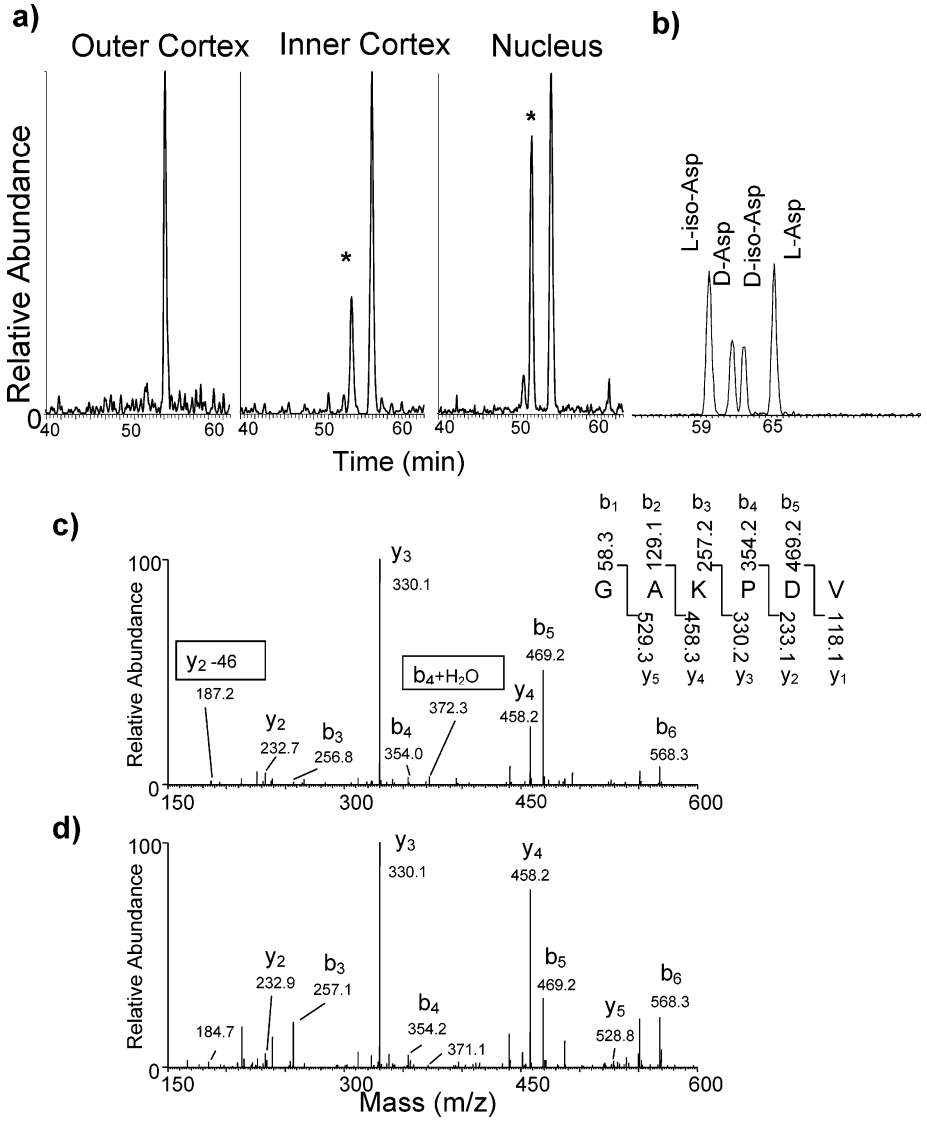

FIGURE 6: Age-related isomerization and racemization of aspartic acid 243 in the 34-year-old lens. (a) Selected ion chromatograms for the truncated peptide GAKPDV, residues 239−244 (*m*/*z* 586.3). Asterisks indicate the D-iso-aspartic acid containing peptide. (b) Effect of aspartic acid racemization and isomerization on the elution profile of synthetic peptide standards of GAKPDV, containing L-Asp, L-iso-Asp, D-Asp, or D-iso-Asp. (c) Tandem mass spectrum of the peptide GAKPDV residues 239−244 (*m*/*z* 586.3). This peptide corresponds to the early eluting peak labeled with an asterisk in a. The predicted fragments indicating the presence of iso-aspartic acid are boxed. (d) Tandem mass spectrum of the peptide GAKPDV, residues 239−244, corresponding to the unlabeled later eluting peak in a. b and y ions are labeled, and the predicted fragmentation of the peptide is shown.

human lens. Tryptic digestion of AQP0 while embedded in the membranes yielded a greatly simplified peptide mixture containing C-terminal peptides that were smaller in mass and more amenable to chromatographic separation and sequence confirmation by tandem mass spectrometry. The method required minimal sample preparation, and detailed structural data were obtained on protein isolated from as little as 5% of a lens and thus permitted analysis of AQP0 structure from dissected lens sections.

Although post-translational modification of AQP0 structure has been appreciated since early studies by Horwitz (*27*) and Takemoto (*28*), more recent mass spectrometric analysis of human AQP0 revealed molecular details such as the exact sites of truncation, phosphorylation, and deamidation (*25*). Reported in the present study, using an alternative mapping strategy, are previously undetected post-translational modifications of AQP0. These modifications include the following: additional sites of truncation at residues 249, 260, 261, and 262; phosphorylation at Ser 231 and to a lower extent at Ser 229; and racemization/isomerization of L-Asp 243 to D-Asp and D-iso-Asp. Furthermore, AQP0 modifications were quantified, and their spatial distribution within the lens was characterized.

The relative level of phosphorylation in the inner cortex is estimated to be 15% of Ser 235 compared to the nonphosphorylated form. The levels of phosphorylation at these sites were lowest in the outer cortex of the lens. This is consistent with previous observations that cAMP-induced phosphorylation of AQP0 was higher in the inner cortex and nucleus than in the outer cortex (*37*, *38*). Activators of protein kinase C (PKC), calcium and TPA (*39*), and activators of protein kinase A (PKA), cAMP and forskolin (*40*−*42*), have been shown to stimulate phosphorylation of the C terminus of AQP0. While the kinases responsible for in vivo phosphorylation of AQP0 are not known, the residues adjacent to Ser 229 and Ser 231 form consensus sequences for phosphorylation by casein kinase II and PKC, respectively. The functional role of C-terminal phosphorylation of AQP0

is not known (*43*), although it is conceivable that phosphorylation within the putative calmodulin-binding domain of AQP0 (*44*) could affect the calcium/calmodulin-mediated effects on the water permeability of AQP0 (*45*). Phosphorylation of other aquaporins has been shown to affect membrane water permeability through two different mechanisms, channel-gating and shuttling mechanisms that redistribute that protein between intracellular vesicles and the plasma membrane (*46*, *47*). Changes in the level of phosphorylation within different regions of the lens suggest that the activity of AQP0 may be regulated spatially within the lens.

Racemization and isomerization of aspartic acid residue 243 from L-aspartate (L-α-Asp) to D-aspartate (D-α-Asp) and to D-iso-aspartate (D-β-Asp), respectively, was detected in the truncated peptide 239−244 in all three lenses examined. Moreover, these modifications increased with fiber cell age. Isomerization and racemization of aspartic acid residues is commonly observed in aged proteins of the lens, teeth, erythrocytes, and brain and has been shown to impact the function of a number of proteins (*14*, *48*). D-Iso-Asp has also been detected in α-A crystallin (*5*, *49*). Isomers arise through spontaneous formation of an unstable five-membered succinimide ring when the side-chain carbonyl carbon of Asp or Asn, in the case of deamidation, is attacked by the α amino of the carboxyl residue (*9*). Hydrolysis of the succinimide ring results in four products, L-Asp, L-iso-Asp, D-Asp, and D-iso-Asp. During isomerization to iso-Asp, the peptide backbone is transferred to the side chain of the aspartic acid, thereby incorporating an additional carbon in the peptide backbone. During racemization of L-Asp to D-Asp, the configuration around the α carbon is inverted. Even though studies characterizing the rates of spontaneous racemization and isomerization of aspartate in peptides and proteins demonstrate that the primary product formed in vitro is L-iso-Asp (*9*), in the analyses of aged proteins taken directly from cells or tissues, the D-iso-Asp isomer was the primary product observed (*14*, *49*). The prevalence of the D-iso-Asp may result from the influence of the local environment and conformation (*49*) or, alternatively, from repair of the isomerized and racemized aspartate by protein L-isoaspartate *O*-methyltransferase (PIMT) (*50*). PIMT activity has been observed in the normal and cataractous human lenses (*51*). Because D-iso-Asp is not a substrate for the enzyme (*52*), in aged tissues with endogenous PIMT, the predicted products are L-Asp and D-iso-Asp. These observations are consistent with the suggestion that PIMT may be involved in the repair of proteins in the normal-aging lens (*51*).

Measured molecular weights of lens membrane proteins from each section of the lens, prior to trypsin digestion, demonstrate that the major truncation products of AQP0 in the 34−38-year-old lens are a result of cleavage on the C-terminal side of asparagine residues 246 and 259. In addition to the action of proteases, aging peptides (*9*) and proteins (*8*) are subject to spontaneous backbone cleavage at residues that can form a succinimide intermediate. Such a mechanism has been proposed for the truncation of α-A crystallin at Asn 101 (*8*). Because the prominent sites of truncation in AQP0 are also known sites of deamidation (*25*), the potential of the C terminus of AQP0 to undergo spontaneous backbone cleavage was investigated in vitro. Incubation of Ac239−263, at 37 °C for 96 h, yielded a low level of backbone cleavage at residues corresponding to Asn 246 and Asn 259. These data suggest that truncation at susceptible asparagine and aspartic acid residues in the human lens may be due to age-related succinimide formation and spontaneous backbone cleavage. On the basis of the rate of deamidation of synthetic peptides, the predicted half-times of deamidation at Asn 246 with the sequence SNG and Asn 259 with the sequence LNT would be 0.96 and 46.1 days, respectively (*53*). Studies by Geiger et al. suggest that truncation is more likely to occur at asparagine residues that have a slower rate of deamidation (*9*). Consistent with these in vitro studies, truncation at Asn 259 was the most abundant truncation product observed in the human lens.

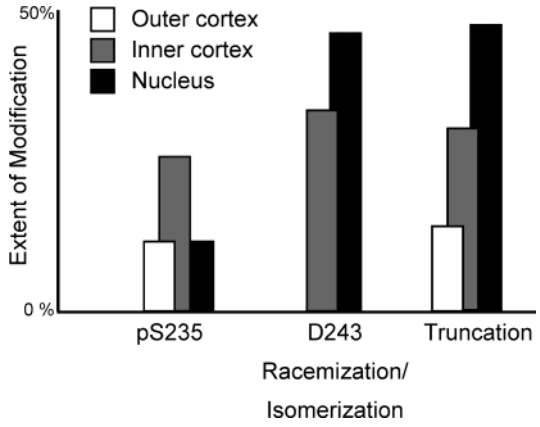

FIGURE 7: Summary schematic diagram of the distribution of AQP0 modifications in 34−38-year-old human lenses. Extents shown represent approximate values based on results from three lenses. The sum of all truncation products is shown.

Many lens proteins are subject to post-translational modifications. These modifications have been described as developmentally and spatially regulated and related to fiber cell age and lens age. When the exact sites of modification within a polypeptide are defined, putative enzymes and mechanisms responsible for the modification can be addressed. In addition, knowledge of the distribution and extent of protein modifications within the lens permits assessment of the functional consequences of these modifications. As summarized in Figure 7, each of the modifications described in this paper increased from the outer cortex containing the younger fiber cells to the nuclear older fiber cells, thus making them age-related. The exception was phosphorylation, which, after an initial increase, either remained constant or decreased in the nuclear region. Perhaps this pattern of phosphorylation is related to the differential regulation of the AQP0 function within the lens. The pattern and spatial distribution of truncation and racemization/isomerization products among these 34−38-year-old lenses were remarkably similar. This suggests the existence of nonrandom temporally well-defined mechanisms of modification. The reproducibility of these events, occurring within specific regions of the normal-aging lens, are consistent with the idea that these age-related modifications can serve as molecular clocks to alter the function or regulation of function at a given time (*53*).

The age-related modifications of the C-terminal tail of AQP0 that occur in the normal human lens include removal of parts of the protein by truncation, incorporation of a negative charge following deamidation, and the insertion of an additional carbon in the peptide backbone as a result of

isomerization of aspartate. The effects of these modifications, within a putative regulatory domain, on AQP0 function, protein−protein interactions, and regulation of function, are not known. Differential levels of phosphorylation within different regions of the lens suggest the possibility of spatial regulation of the AQP0 function. Studies addressing the effect of C-terminal truncation and phosphorylation on water permeability demonstrated that AQP0 1−243 and a S235A mutant AQP0 retain wild-type water permeability (*33*). The molecular modifications responsible for the decrease in fiber cell membrane water permeability as a function of age have not been determined (*15*, *54*). As the longest lived aquaporin and the only aquaporin in the lens fiber cells, lifetime accumulation of post-translational modifications to AQP0 may impact the internal circulation within the lens. The specific effects of these modifications on the function of AQP0 and how they pertain to maintaining lens transparency in the normal lens or to the formation of an age-related cataract remain to be elucidated.

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