Tryptophan can be oxidized in the eye lens by both enzymatic and non-enzymatic mechanisms. Oxidation products, such as kynurenines, react with proteins to form yellow-brown pigments and cause covalent cross-linking. We generated a monoclonal antibody against 3-hydroxykynurenine (3OHKYN)-modified keyhole limpet hemocyanin and characterized it using 3OHKYN-modified amino acids and proteins. This monoclonal antibody reacted with 3OHKYN-modified N\(^{\alpha}\)-acetyl lysine, N\(^{\alpha}\)-acetyl histidine, N\(^{\alpha}\)-acetyl arginine, and N\(^{\alpha}\)-acetyl cysteine. Among the several tryptophan oxidation products tested, 3OHKYN produced the highest concentration of antigen when reacted with human lens proteins. A major antigen from the reaction of 3OHKYN and N\(^{\alpha}\)-acetyl lysine was purified by reversed phase high pressure liquid chromatography, which was characterized by spectroscopy and identified as 2-amino-3-hydroxy-\(\alpha\)-((5S)-5-acetoamido-5-carboxypentyl amino)-\(\gamma\)-oxo-benzene butanoic acid. Enzyme-digested cataractous lens proteins displayed 3OHKYN-derived modifications. Immunohistochemistry revealed 3OHKYN modifications in proteins associated with the lens fiber cell plasma membrane. The low molecular products (<10,000 Da) isolated from normal lenses after reaction with glucosidase followed by incubation with proteins generated 3OHKYN-derived products. Human lens epithelial cells incubated with 3OHKYN showed intense immunoreactivity. We also investigated the effect of glycation on tryptophan oxidation and kynurenine-mediated modification of lens proteins. The results showed that glycation products failed to oxidize tryptophan or generate kynurenine modifications in proteins. Our studies indicate that 3OHKYN modifies lens proteins independent of glycation to form products that may contribute to protein aggregation and browning during cataract formation.

Crystallins are the major proteins of the lens, and they constitute 90% of the soluble proteins. A number of physicochemical changes occur in lens proteins during aging, and during cataract formation, similar changes occur at an exaggerated rate. The most striking changes in these proteins include yellowing and browning of proteins, intra- and intermolecular cross-linking, and cross-linking with fiber cell membrane proteins (1–4). Several mechanisms have been proposed for such changes, including oxidation (5, 6) and glycation (7–9). Recent studies suggest that these two processes are interrelated (10, 11) and may thus synergistically contribute to the observed lens protein modifications in aging and cataract formation.

Oxidative modifications within the lens may occur either on proteins or on protein-free constituents. Protein-free tryptophan in the lens undergoes both enzymatic (12) and non-enzymatic oxidation (13) to produce reactive kynurenines (Fig. 1). Absorption of ultraviolet light A by the lens is attributed in part to these products. The enzymatic oxidation to N-formylkynurenine (NFK)\(^1\) is initiated by indoleamine 2, 3-dioxygenase, an enzyme that is up-regulated by interferon-\(\gamma\). The next enzymatic steps produce kynurenine (KYN), 3-hydroxykynurenine (3OHKYN), 3-hydroxynanthranilic acid, quinolinic acid, and nicotinic acid (12). The kynurenines within the lens become enzymatically glycosylated to O-\(\beta\)-glucosides. Quantification of these products in the human lens shows that 3-hydroxykynurenine O-\(\beta\)-d-glucoside (3OHKYG) is the most abundant form followed by 4-(2-amino-3-hydroxyphenyl)-4-oxabutanoic acid O-\(\beta\)-d-glucoside, t-kynurenine, and 3-hydroxykynurenine (14).

The kynurenine products produced from protein-free tryptophan readily pass through cell membranes and could thus diffuse through the cortex of the lens. Kynurenines are unstable at physiological pH; they undergo side chain deamination to produce \(\alpha\), \(\beta\) unsaturated ketoaldehydes (15). Kynurenine levels decrease in the lens with age (16) and cataract formation (17), which may be due to their reaction with lens proteins. Kynurenines react with nucleophilic amino acids, such as cysteine, histidine, and lysine in lens proteins (18–20) and cysteinyl residue in GSH through Michael addition to form covalent adducts. One of the products of the reaction of 3OHKYG with lens GSH is glutathionyl-3-hydroxykynurenine glucoside (21), which accumulates during lens aging and accumulates to rel-

\(^1\) The abbreviations used are: NFK, N-formylkynurenine; MEM, minimal essential medium; AA, anthranilic acid; benzoic acid; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HBA, 4-hydroxybenzoic acid; KYN, \(\alpha\)-, \(\beta\)-kynurenine; 3OHKYN, 3-hydroxykynurenine; HLE-B3, human lens epithelial cells B3; 3OHKYN, 3-hydroxykynurenine O-\(\beta\)-d-glucoside; KLH, keyhole limpet hemocyanin; NFDM, nonfat dry milk; PBS, phosphate-buffered saline; PBST, PBS + 0.05% Tween 20; RNase A, ribonuclease A; ROS, reactive oxygen species; WS-HLP, water-soluble human lens proteins; HPLC, high pressure liquid chromatography; t-Boc, tert-butoxycarbonyl; mAb, monoclonal antibody.
Programmatically high levels in cataractous lenses (19). Vazquez et al. (22, 23) demonstrated modification of histidine and lysine residues of lens proteins by KYN and their accumulation in aging lenses, but these authors also noted that the modified products decrease in senile cataracts. Another recent study confirmed specific histidine modification in H9251 B-crystallin that was incubated with KYN; modification of histidine 83 was thought to affect its chaperone function (24). Other crystallins appear to be modified by kynurenines as well (25), suggesting that kynurenines are responsible, in part, for protein cross-linking during aging and cataract formation.

Kynurenines also play a role in reactive oxygen species (ROS)-mediated crystallin modification. For example, kynurenines such as 3OHKYN that contain a hydroxyl group can generate ROS through transition metal reduction reactions (12). Such reactions are implicated in cross-linking of crystallins. Because kynurenine-modified crystallins can generate ROS through photochemical reactions (26), weakened defenses against oxidative stress due to age or cataract formation could exacerbate lens protein modifications.

Glycation is a firmly established mechanism for protein modification during lens aging and cataract formation (7, 9, 27, 28). In this reaction, sugars, ascorbate, and dicarbonyl compounds react with amino groups of lysine and arginine residues on proteins through the formation of ketoamine adducts on proteins (10, 29). These adducts can produce ROS during their modification to advanced glycation end products (11, 30). We reasoned that glycation-derived ROS might induce the oxidation of tryptophan and contribute to lens protein modification by kynurenines.

The present study was conducted to understand the role of 3OHKYN in lens protein modification and to determine the effect of glycation on tryptophan oxidation. Using a novel monoclonal antibody raised against 3OHKYN-modified keyhole limpet hemocyanin (KLH), we demonstrate that 3OHKYN-derived products are present in human cataractous lenses, provide structure of a major antigenic product, and show that glycation products do not influence tryptophan oxidation and kynurenine-mediated modification of proteins.

EXPERIMENTAL PROCEDURES

Incubation of Proteins with 3OHKYN—Bovine serum albumin (BSA) or ribonuclease A (RNase A) at 10 mg/ml in 0.1 M sodium phosphate buffer (pH 7.4) were incubated under sterile conditions in the dark with 25 mM 3OHKYN for 3 days at 37 °C. The incubation mixture was stirred after every 24 h, and the pH was adjusted to 7.4. The incubated material was then dialyzed against 4 liters of PBS for 48 h at 4 °C.

Production of a Monoclonal Antibody against 3OHKYN-derived Modification—KLH at 10 mg/ml in 0.1 M sodium phosphate buffer (pH 7.4) was incubated with 25 mM 3OHKYN for 3 days followed by dialysis against 4 liters of PBS for 48 h at 4 °C. The antibody was prepared according to the method of Oya et al. (31). Briefly, mice were initially immunized by intraperitoneal injection with 30 g/H9262 g of 3OHKYN-modified KLH followed by three booster intraperitoneal injections with 20 g/H9262 g of the modified protein. After the final booster injection, spleen cells were collected and fused with P3/U1 murine myeloma cells using polyethylene glycol. The hybridomas were cultured in hypoxantine/aminopterin/thymidine selection medium.

An enzyme-linked immunosorbent assay (ELISA) was used to screen culture supernatants of hybridomas. Microplate wells were coated by incubation for 16 h at 4 °C with one of the following (1 µg/well) in 0.05 M sodium carbonate buffer (pH 9.7): unmodified BSA, unmodified RNase A, 3OHKYN-modified BSA, or 3OHKYN-modified RNase A. The wells were washed three times with PBS containing 0.05% Tween 20 (PBST) and blocked with 300 µl of 5% nonfat dry milk (NFDM) in PBST for 2 h at room temperature. The wells were then washed three times with PBST and incubated with 50 µl of hybridoma supernatant for 2 h at room temperature in a humid chamber. Following incubation, the
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Well's were washed three times with PBST and incubated with 50 μl of either goat anti-mouse IgG (1:15,000 dilution in PBST) or goat anti-mouse IgM (1:2,500 dilution in PBST) antibody for 1 h as described above. The wells were finally washed three times with PBST (Amersham Biosciences) and stored aliquots at –20 °C. The monoclonal antibody was determined to be of IgG₁ subclass.

Modification of Human Lens Proteins and N⁵-Acetyl Amino Acids by Tryptophan Oxidation Products—Water-soluble proteins (WS-HLP) were extracted from human lenses by homogenizing each lens in 2.0 ml of PBS followed by 2 days at 37 °C at 20,000 × g for 30 min at 4 °C. The supernatant was dialyzed against 4 liters of PBS for 24 h at 4 °C. Samples of this material (5 mg/ml) were then incubated at 37 °C for 7 days in 0.1 μM sodium phosphate buffer (pH 7.4) with one of the following: Trp, 3OHKYN, ananthanilic acid (AA) (all from Sigma), KYN, or NFK. NFK was synthesized according to Simat and Steinert (31). We also used benzoic acid and 4-hydroxybenzoic acid (HBA) (Sigma) in a competitive assay to test the specificity of the antibody to its oxidation products. The oxidation products were used at 10 times the molar concentration of the oxidation products and its oxidation products were used at 10 times the molar concentration of the antibody in this assay. We then purified the antibody against the antibody (ELISA procedure described below) in a Dynex MRX 5000 Microplate Reader. The monoclonal antibody was determined to be of IgG₁ subclass.

Preparation of Human Lenses Protein-free Filtrate from Human Lenses—Four nor-methane lenses from donors of 30 to 50 years of age were each homogenized in 2 ml of water and centrifuged at 20,000 × g for 30 min at 4 °C. The supernatant fraction was filtered through a Centricon YM-10 filter (Millipore, Bedford, MA), and the filtrate was lyophilized and reconstituted in 100 μl of water.

Incorporation of Proteins with Human Lens Protein-free Filtrate—One half of the protein-free filtrate was digested with β-glucosidase (Sigma) to obtain 3OHKYN from its glucosidase form. For this step, we used a 1% enzyme solution in 0.04 M sodium phosphate buffer (pH 5.6) and incubated the material for 2 h at 37 °C. The sample was then filtered through a Centricon YM-10 filter to remove the enzyme. The digested and non-digested protein-free filtrates were incubated for 5 days at 37 °C with 30 μg of either RNase A or WS-HLP (see above) in 1 ml of 0.1 M sodium phosphate buffer (pH 7.4). The incubated samples were then dialyzed against 2 liters of PBS at 4 °C. Finally, 30 μg of modified protein in 50 μl of PBS/well was tested for antigen using a competitive ELISA (see below).

ELISA—Microplate wells were coated with 3OHKYN-modified RNase A in 0.05 M carbonate buffer (pH 9.7) at a concentration of 1 μg/ml and incubated at 4 °C for 15 min. After washing with PBS, the wells were blocked for 2 h at room temperature with 300 μl of 5% NFDM in PBST and washed three times with PBST. The monoclonal antibody (1:200 dilution in 1% NFDM/PBS for proteins modified Trp oxidation products and 1:60 diluted for amino acids modified by 3OHKYN) was preincubated with the competitor for 2 h at 37 °C and then dispensed into specified wells and incubated at 37 °C for 60 min. The wells were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Promega Corp., Madison, WI, diluted 1:15,000) for 1 h at 37 °C as described earlier. The enzyme reaction was assessed by the addition of 100 μl of 3,3',5,5'-tetramethylbenzidine (Sigma) following the addition of 50 μl of 2% H₂O₂ and measurement of chromophore absorbance at 450 nm. Results were expressed as the ratio: B/B₀, where B is the absorbance at 450 nm and B₀ is the absorbance in the absence of competitor.

Western Blotting—WS-HLP from cataractous and normal lenses were digested with proteinase K (2% w/v) in PBS at 30 min at 37 °C, aliquots of the digest corresponding to 20 μg of protein were separated on 18% reducing gels, and the proteins were transferred electrophoretically to Immobilon P membranes (Millipore). Comparable gels were stained with BioSafe Coomassie Blue (Bio-Rad). The membranes were then blocked with 5% NFDM in PBS for 2 h and incubated overnight at 4 °C with a 1:20 dilution of the anti-KLH-3OHKYN (10.3 μg/ml) monoclonal antibody. After washing five times with 10 min with PBST, the membranes were incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (Promega Corp.) diluted 1:15,000. After repeated washing (five times for 10 min with PBST), the membranes were treated with SuperSignal West Pico chemiluminescent substrate (Pierce) for 5 min and exposed to x-ray film (Pierce, CL-XPose Film). Proteins modified by Trp oxidation products were similarly subjected to Western blotting, except that 12% gels were used and 2 μg of protein/ lane was loaded.

Immunostaining—HLE-B3 cells (from Dr. Usha Andley, Washington University, St. Louis, MO) (between passages 13 and 19) were cultured in chamber slides with minimal essential medium (MEM) containing 20% fetal bovine serum, 2 mM l-glutamine, and 50 μg/ml gentamycin. The cells were washed twice with PBS and treated with MEM containing 0% (control), 20, 50, 100, or 200 μM 3OHKYN in the absence of fetal bovine serum for 24 h. The treated cells were washed twice with PBS and then permeabilized with 4% paraformaldehyde at −20 °C for 5 min. After washing twice with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS at −20 °C for 5 min and then washed five times with PBS to remove the detergent. The next, the slides were blocked with 3% NFDM/1% BSA for 30 min at room temperature and washed twice for 5 min each time with PBS. The slides were incubated with the anti-KLH-3OHKYN antibody (10.3 μg/ml, diluted in 0.1% BSA) or non-immune mouse IgG diluted to the same concentration for 1 h at room temperature followed by 3,3',5,5'-tetramethylbenzidine (Sigma) solution. Fast atom bombardment-mass spectrometry analyses were done in the Mass Spectrometry Facility at Michigan State University, East Lansing, MI in a JEOL JX-110 double focusing mass spectrometer. UV-visible spectra were recorded using Amersham Biosciences Spectra Max 190 spectrometer.

Treatment of 3OHKYN with Ninyhdrin—The procedure was as described by Miyata and Monnier (32). 40 μM 3OHKYN was incubated with 10 mM ninyhydrin in 300 μl of ethanol/acetic acid, pH 5.0, for 10 min at 65 °C. 25 mM l-lysine was then added and incubated at 65 °C for 10 min. The mixture was dried on a Savant SpeedVac concentrator, dissolved in 200 μl of water, and analyzed by an ELISA outlined below. A control experiment in which l-lysine was used in the place of 3OHKYN was run simultaneously.
FIG. 2. Characterization of monoclonal antibody to KLH-3OHKYN. 
A, microplate wells were coated with RNase-3OHKYN, and mAb was preincubated with WS-HLP modified by NFK, Trp, KYN, benzoic acid (BA), HBA, or AA or incubated with PBS alone (control). B, electrophoresis was performed on 12% polyacrylamide gel under reducing conditions, and the gel was stained with BioSafe Coomassie Blue reagent or blotted using the monoclonal antibody for KLH-3OHKYN. Lanes are as follows: WS-HLP modified with: AA (lane 1); with benzoic acid (lane 2); HBA (lane 3); PBS (lane 4); Trp (lane 5); NFK (lane 6); KYN (lane 7); 3OHKYN (lane 8). Lane 9, 3OHKYN-modified RNase A (positive control). C, Western blotting of HLP modified by tryptophan oxidation products. Sample details are the same as in panel B.

Protein Modification by 3-Hydroxykynurenine

Immunohistochemistry—Cataractous and age-matched normal lenses from donors between the ages of 65 and 70 years were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 5-μm sections. Following dehydration, heat-induced epitope retrieval was done in 10 mM citrate buffer (pH 6.0), and the sections were treated with 3.0% hydrogen peroxide to block endogenous peroxidases. The sections were blocked with 1.5% normal horse serum and incubated for 2 h either with the anti-KLH-3OHKYN antibody (10.3 μg/ml) diluted in PBS or with non-immune mouse IgG, diluted to the same protein concentration as the primary antibody. For adsorption experiments, the antibody was preincubated overnight at 4 °C with 3OHKYN-modified RNase A in PBS (0.4 mg/ml). After washing, the slides were incubated with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) followed by ABC reagent (Vector Laboratories). They were stained initially with 3,3'-diaminobenzidine, counterstained with hematoxylin, and then permanently mounted.

Incubation of N'-Acetyl Tryptophan with Ribated Lysine-Sepharose—To determine whether glycation could catalyze the oxidation of tryptophan, we incubated N'-acetyl-tryptophan with ribated lysine-Sepharose (Amersham Biosciences). 1 g of lysine-Sepharose was washed thoroughly with 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 1 mM diethylene triamine pentaaetic acid and then incubated at 37 °C for 2 days with 500 mM ribose in the same buffer. The tubes were bubbled with argon for 15 min and sealed before incubation. The ribose-treated gel was extensively washed with PBS to remove unbound ribose. Finally, 250-mg samples of either modified or unmodified (control) gel were incubated at 37 °C with 5.0 mM N'-acetyl tryptophan in 0.1 M sodium phosphate buffer (pH 7.4) for 3, 7, and 10 days. Aliquots were subjected to C18 reversed phase HPLC using a gradient program consisting of solvents A (water with 0.01 M heptfluorobutyric acid) and B (70% acetonitrile in water and 0.01 M heptfafluorobutyric acid). The column was eluted with a linear gradient of B from 5 to 50 min at a flow rate of 1.0 ml/min, and
the column effluent was monitored for fluorescence at excitation/emission wavelengths of 290/320 nm. N-acetyl tryptophan eluted at Rt = 22 min.

We also incubated N-acetyl tryptophan during glycation; ribose (6.7 mM), lysine (0.6 mM), arginine (0.6 mM), and N-acetyl tryptophan (13.2 mM) were incubated for 5 days at 37 °C in 0.1 M sodium phosphate buffer (pH 7.4). Other incubations included N-acetyl tryptophan/ribose, N-acetyl tryptophan/arginine/ribose, N-acetyl tryptophan/lysine/ribose, all at concentrations as above. All samples were analyzed by HPLC for N-acetyl tryptophan oxidation as described above.

In another experiment, we incubated lysine-Sepharose (500 mg) with 500 mM ribose in the presence or absence of 6.7 mM N-acetyl arginine in 0.1 M sodium phosphate buffer, pH 7.4, under sterile conditions at 37 °C for 6 days. After the incubation, the gel was extensively washed with PBS. The washed gel (18 mg, glycated or non-glycated) was incubated with 16.7 mM L-tryptophan for 20 h in PBS at room temperature. After the incubation, the supernatant was incubated with 0.3 mg of RNase A in PBS for 3 days at 37 °C. The protein was then dialyzed against PBS for 16 h at 4 °C and tested in competitive ELISA for reaction with KLH-3OHKYN antibody.

To identify formation of 3OHKYN-derived products in proteins during glycation, human lens water-soluble proteins (4.3 mg/ml) were incubated under sterile conditions for 7 days at 37 °C with one of the following: 15.5 mM glucose, 15.5 mM ribose, 15.5 mM ascorbate, 7.5 mM methylglyoxal, or 7.5 mM glyoxal in 0.1 M sodium phosphate buffer (pH 7.4). We also incubated protein in the absence of glycating agents, which served as control in this experiment. After incubation, the material was dialyzed extensively against PBS at 4 °C and tested for reaction with antibody by competitive ELISA and Western blotting.

**RESULTS**

Characterization of Monoclonal Antibody for 3OHKYN Modification—The monoclonal antibody was characterized by a competitive ELISA using Trp oxidation products-modified hu-
man lens proteins. We found the strongest reaction with proteins modified by 3OHKYN (Fig. 2A), and 2 μg of 3OHKYN-modified protein blocked the antibody completely. None of the other oxidation products blocked the antibody completely at concentrations used in the assay. We did observe some reaction with proteins modified by KYN with the antibody. However, it was much less than proteins modified by 3OHKYN when tested between 1 and 10 μg of protein. At high concentrations (>10 μg), proteins incubated with NFK also displayed slight reaction. Proteins incubated with just PBS or incubated with Trp also showed reaction comparable with that of protein incubated with NFK. These observations suggest that some amount of antigen was already present in the protein isolated from young human lenses, and incubation with either NFK or Trp produced no additional antigens. Surprisingly, protein treated with the three acids, HBA, AA, and benzoic acid, all had no reactivity with the antibody, which might be due to masking of antigen as a result of conformational changes or destruction of antigen by the acid. The latter is unlikely as our incubations were done in 0.1M sodium phosphate buffer, pH 7.4, and the pH remained at 7.4 after the addition of the reactants.

Protein staining of the SDS-PAGE gel revealed that 3OH-KYN modification resulted in high molecular aggregates of lens proteins (Fig. 2B). Western blotting experiments confirmed the ELISA results. Lens proteins modified by 3OHKYN reacted with the antibody (Fig. 2C, lane 8). Among the many high molecular weight proteins, we assume that some that display immunoreactivity could be cross-linked aggregates.

To establish which amino acids are involved in kynurenine modifications, we incubated N-acetyl amino acids with kynurenines. In these incubations, we used five times higher concentrations of amino acids relative to kynurenines to complete reaction of kynurenines. In addition, since 3OHKYN is unstable in aqueous neutral solutions, we do not expect free 3OHKYN to remain after 7 days of incubations. We used the reaction products as competitors in ELISA. Our results show that 3OHKYN-modified N′-acetyl lysine, N′-acetyl cysteine, and N′-acetyl arginine reacted similarly with the antibody (Fig. 3). The reaction with 3OHKYN-modified N′-acetyl histidine was slightly stronger than other amino acids tested. Unmodified amino acids did not react with the antibody. These results indicate that the antibody recognized a structure common to all modifications.

In another experiment, we wanted to know whether the antibody could react directly with Trp and its oxidation products. 3OHKYN alone strongly reacted with our antibody, and we achieved a complete blockade of the antibody binding at 12.5 nmol of 3OHKYN (Fig. 4). Among the other products tested, we found some degree of immunoreactivity with KYN and 3-hydroxyanthranilic acid, although these were at least 50–60% lower than 3OHKYN-modified protein. The antibody was unreactive against HBA, AA, or 2-aminophenol. Our results indicate that the monoclonal antibody requires 2-amino and 3-hydroxyl groups on the benzene ring to recognize an antigen. The low reactivity with KYN as compared with 3OH-KYN suggests that the –OH group at position 3 makes 3OH-KYN the better antigen.

To determine the structure of antigenic epitope in amino acid modifications, we purified the major immunoreactive product of the reaction of N′-acetyl lysine and 3OHKYN. The mixture was separated on C18 reversed phase HPLC. Four peaks were collected by repeated injections (Fig. 5A). The product in peak at Rf = 30 min (peak 1) was the most effective inhibitor of the antibody (Fig. 5B).

**Fig. 6. Characterization of the purified antigen.** The 1H-NMR spectrum in D2O is shown. The structure of the purified product based on 1H-NMR, 13C-NMR, and fast atom bombardment-mass spectroscopy is shown in the inset.
Next, we used a C18 reversed phase cartridge for the first stage of a preparative scale purification. The purification protocol on C18 reversed phase HPLC was altered as described under "Experimental Procedures" to achieve clear separation of the peak of interest and scaled-up purification. Peak 1 in Fig. 5A eluted at ~36 min (Fig. 5C). The yield was calculated at 2.1% based on the amount of 3OHKYN used for synthesis.

Spectroscopy—The purified compound had UV absorption maxima at 270 and 370 nm (Fig. 5C, inset), which was very similar to the product isolated from the reaction of KYN and tert-butoxycarbonyl (t-Boc)-lysine. Fig. 6 shows the 1H-NMR spectrum in D2O. δH 7.44 (d, 1H, J = 8.0 Hz, H-4), 7.10 (d, 1H, J = 7.9 Hz, H-6), 7.04 (t, 1H, J = 8.0 Hz, H-5), 4.2 (dd, 1H, J = 5.0, 9.0 Hz, H-15), 4.15 (t, 1H, J = 5.0 Hz, H-9), 3.73 (m, 2H, H-8), 3.03 (m, 2H, H-11), 1.88 (s, 3H, CH3-18), 1.76 (m, 1H, H-14), 1.64 (m, H-12,12',14', H-12), 1.34 (m, 2H, J = 7.3, 14.1 Hz, H-13,13', H-13); C 199.9 (CO-7), 176.0 (CO), 172.1 (CO), 147.9 (C-2), 147.9 (C-18), 128.3 (C-3), 123.7 (C-5), 123.4 (C-4), 122.8 (C-6), 121.1 (C-1), 56.7 (C-9), 52.7 (C-15), 47.2 (C-11), 38.7 (C-8), 28.5 (C-12), 28.0 (CH3), 25.5 (C-14), 22.6 (C-13). Fast atom bombardment-mass spectroscopy spectrum showed M+H of 396.1, which is comparable with the calculated 396.2 empirical formula of C18H25N3O7. Based on these results, we determined the structure of the product shown in Fig. 6, inset.

Reactive of the Purified Product with the Monoclonal Antibody—We tested the purified product for reaction with the antibody using a competitive ELISA. The purified compound completely blocked antibody binding at 12.5 nmol (Fig. 7). The reaction of the purified product with antibody was very similar to that of 3OHKYN. Based on observations following incubation with various amino acids and the purified product, we were able to identify a core structure that is recognized by the antibody. This structure is (2-amino-3-hydroxy)-4-oxo-benzene butanoic acid (Fig. 8). Our results suggest that attachment of lysine at C-9 does not influence the antibody recognition. To confirm that the antibody recognizes the core structure, we treated 3OHKYN with ninhydrin. We expected ninhydrin to deaminate 3OHKYN to a keto acid or an aldehyde. We found that the resulting product had nearly 4-fold enhanced reactivity with the antibody (Fig. 9). Lysine treated with ninhydrin in the same way failed to react with the antibody. Taken together, these observations confirm that the antibody reacts with a core structure in modified N-acetyl lysine, N-acetyl histidine, N-acetyl arginine, and N-acetyl cysteine.

Modification of Proteins by Protein-free Filtrates from Human Lens—Protein-free filtrates of material obtained from normal human lenses were treated with β-glucosidase to remove glucose from kynurenine glucosides and then incubated at 37 °C for 5 days with either RNase A or WS-HLP. The modified proteins were then analyzed in a competitive ELISA. Controls were from incubations of proteins with protein-free filtrates that were not treated with β-glucosidase. Fig. 10 shows that 30 μg of the filtrate-modified proteins (RNase A as well as WS-HLP) inhibited binding of the antibody nearly 100% if pretreated with β-glucosidase. These results support findings that human lenses contain kynurenine glucosides and further indicate that kynurenine glucosides by themselves may not produce antibody-recognizable
products, but after removal of glucose, they may react with proteins to produce immunoreactive products.

3OHKYN Modifications in the Human Lens—To detect kynurenine modifications that had occurred in vivo, we extracted water-soluble proteins from normal and cataractous lenses and digested them mildly with proteinase K. This treatment was necessary to expose buried antigenic epitopes. The samples were then subjected to SDS-PAGE (Fig. 11A) followed by immunoblotting. We noted immunoreactivity in some cataractous lenses (lanes 2–4) but not in normal (non-cataractous) age-matched lenses (lane 1). A representative Western blot is shown in Fig. 11B.

We used immunohistochemistry to localize the kynurenine modifications in paraffin sections of normal and cataractous human lenses, and although we noted 3OHKYN modifications in the cataractous lens (Fig. 11C, panel 2), we found none in the normal lens (lane 1). The positive reactivity from a cataractous lens preparation was markedly reduced when the antibody was preincubated with 3OHKYN-modified RNase A (panel 3).

Since it appeared that the antibody reaction occurred primarily in the fiber cell plasma membrane (Fig. 11C), we used an antibody against aquaporin-0 to confirm that the 3OHKYN modifications were indeed localized in the lens fiber cell plasma membrane. Aquaporin-0, which is abundant in lens fiber cell plasma membrane, is also known as MP26. The staining pattern indicates that 3OHKYN modifications occurred primarily in proteins within the plasma membrane or proteins associated with plasma membrane (Fig. 11, panel 5).

Effect of 3OHKYN Treatment on Human Lens Epithelial Cells—HLE-B3 cells on chamber slides were treated for 24 h with either 100 or 200 μM 3OHKYN in MEM without calf serum. The cells were fixed with 4% paraformaldehyde and treated with the monoclonal antibody followed by Oregon Green-conjugated secondary antibody. Cells treated with 100 μM 3OHKYN showed an antibody reaction within the cytoplasm that was even more intense with 200 μM 3OHKYN (Fig. 12A, panels 1–3). The staining was markedly diminished when the antibody was preincubated with 3OHKYN-modified RNase A (data not shown). Non-immune mouse IgG caused no reaction (Fig. 12A, panels 4–6). Further, HLE-B3 cells that were incubated without 3OHKYN showed no immunoreactivity (Fig. 12B, panels 7–9). These results indicate that immunoreactivity is related specifically to intracellular 3OHKYN-derived products.

Tryptophan Oxidation by Glycation—we examined the effect of ribose-mediated glycation on the oxidation of protein-free N'-acetyl tryptophan. HPLC results indicated no degradation of N'-acetyl tryptophan either from incubation with ribated lysine-Sepharose (Fig. 13A) or during glycation of lysine and arginine with ribose (Fig. 13B). We also studied tryptophan oxidation and modification of proteins by glycation products.
This was achieved by incubating tryptophan with glycated lysine-Sepharose (by ribose + arginine) followed by incubation with RNase A. RNase A was then tested in a competitive ELISA for reaction with the KLH-3OHKYN antibody. The results showed the absence of antibody recognizable products in the incubated protein (Fig. 13C).

We then studied glycation of human lens proteins with sugars, ascorbate, and methylglyoxal as a possible mechanism for tryptophan oxidation and protein modification. WS-HLP was glycated with glucose, ribose, and ascorbate and tested for reaction with 3OHKYN antibody in a competitive ELISA. None of the modified proteins were immunoreactive, even when the wells were coated with 1.4 μg of modified protein in a direct ELISA. Similarly, there was no immunoreactivity in a competitive ELISA (Fig. 13D). A slight reactivity was seen in ascorbate-modified proteins, but this could not be confirmed in Western blotting. All other glycated proteins including those reacted with methylglyoxal and glyoxal were negative in Western blotting. All other glycated proteins including those reacted with methylglyoxal and glyoxal were negative in Western blotting. These results suggest that tryptophan oxidation and formation of antibody reactive products are not produced during glycation with sugars, ascorbate, and α-dicarbonyls.

**DISCUSSION**

Our monoclonal antibody recognizes kynurenine-derived structures containing a hydroxyl group on benzene ring. Michael addition at C-9 on oxidized tryptophan has been well documented, and several additive products of lysine, histidine, and cysteine have been isolated and detected in the human lens (12, 19, 22). Our antibody reacted nearly as well with several 3OHKYN modifications, including N\(^{\text{acetyl}}\) lysine, N\(^{\text{acetyl}}\) histidine, N\(^{\text{acetyl}}\) arginine, and N\(^{\text{acetyl}}\) cysteine, although the N\(^{\text{acetyl}}\) histidine modifications reacted more strongly than products of the other amino acids. The fact that unmodified acetyl amino acids react only weakly or not at all with the antibody suggests a common epitope among the modified amino acids. The most likely epitope is (2-amino-3-hydroxy)-4-oxo-benzeno butanoic acid. In fact, the structures identified by the reaction of KYN with acetyl amino acids all have a structure in common very similar to the one recognized by our antibody but lacking the 3-hydroxyl group (22).

We used sequential HPLC methods and immunoreactivity to establish the structure of the antigen formed from the reaction of N\(^{\text{acetyl}}\) lysine and 3OHKYN. We isolated and purified a major product recognized by our monoclonal antibody and found that it was very similar to N\(^{\text{acetyl}}\)-t-Boc-(L)-lysyl-L,K-lysinurine, a product of the reaction of t-Boc-lysine and KYN (22). Vazquez et al. (22) suggested that kynurenines under slightly basic conditions undergo deamination, and then nucleophilic amino acids can react with C-9 through Michael addition. We assume that similar reactions with 3OHKYN occurred under our experimental conditions, and to support this assumption, spectroscopy data indicate that N\(^{\text{acetyl}}\) lysine is linked to the aliphatic chain of 3OHKYN through the e-amino group. Mass spectrometry m/z of 396.1 (M+1) suggests that the structure is 2-amino-3-hydroxy-α-((5S)-5-acetaminoo-5-carboxypentyl amio)-γ-oxo-benzeno butanoic acid. The absorption spectrum of the purified compound has maxima at 270 and 370 nm, similar to the lysylkynurenine described by Vazquez et al. (22).

Our antibody reacted equally well with 3OHKYN and the purified product, and the removal of an amino group at C-9 further enhanced the reaction of 3OHKYN. These findings suggest that the antibody requires 3-OH and 2-NH\(_2\) groups in the benzene ring to recognize the antigen, and the removal of the positive charge on the C-9 amino group by the Michael addition of nucleophilic amino acids enhances reaction with the antibody. Our detection of antigen(s) in the modified proteins suggests that it is the 3OHKYN-derived modifications and not the free 3OHKYN that are detected in lens proteins and in vitro modified proteins. 3OHKYN is unstable at physiological conditions; it becomes converted to xanthomatin type compounds (34). Whether the compounds generated during protein and amino acid incubations are derived from these products is not known, but the purified antigen is a Michael adduct of N\(^{\text{acetyl}}\)lysine and 3OHKYN. Thus, we believe that Michael adducts are probably the major antigenic epitopes for the antibody.

Because we found 3OHKYN modification in cataractous lenses, but not in normal lenses, we suspect that these modifications contribute to cataract formation. Our immunohistochemical study showed that the products are primarily associated with plasma membrane of fiber cells, which further suggests that 3OHKYN modifies either plasma membrane proteins and/or proteins associated with plasma membrane. Binding of crystallins to the plasma membrane of fiber cells during cataract formation is a well recognized phenomenon (3). We cannot rule out modification of aquaporin-0, another component that occurs in relatively high amounts in the plasma membrane of lens fiber cells (35). In fact, experiments with appropriate antibodies and cataractous lenses showed co-localization of immunoreaction due to aquaporin-0 and 3OHKYN modifications. Because our attempts to immunoprecipitate aquaporin-0 from cataractous lenses were unsuccessful, we were unable to provide further proof for its modification by 3OHKYN.

Vazquez et al. (22) proposed that 3OHKYN might react with proteins similarly to KYN. Our own observations support this idea. We find that the major immunogenic product from the reaction of N\(^{\text{acetyl}}\)lysine and 3OHKYN is similar to the product isolated by Vazquez et al. (22) from the reaction of N\(^{\text{acetyl}}\)-t-Boc-lysine and KYN. We agree with Vazquez et al. (22) that reactions of KYN and 3OHKYN likely proceed in a similar
fashion. The same authors also thought that \( \text{o-aminophenol} \) moiety in 3OHKYN might undergo oxidation to produce reactive oxygen species. If this happens within the lens, it could exacerbate protein modification by 3OHKYN. Reduced glutathione within the lens could inhibit kynurenine modification by competing for reaction with kynurenines. Since glutathione concentration is usually reduced in the inner nucleus of aging and cataractous lenses, KYN reactions can proceed unhindered in this part of the lens. Whether this also occurs with 3OHKYNG is yet to be determined.

The occurrence of glucosides of kynurenines in human lenses is fairly well established (21, 36). Although both 3OHKYN and 3OHKYNG in the human lens decrease with age (possibly because of reaction with proteins), lenses from aged donors still contain significant amounts of these products. Combined cortical and nuclear concentrations up to \(-7\) nmol/g of lens 3OHKYN were reported for the aged human lens (16); concentrations of 3OHKYNG are much higher, with as much as 800 nmol/g of lens (16). Further evidence was provided by immunoreaction of proteins after incubation with protein-free filtrates of human lens. We observed an enormous increase in the immunoreactivity of proteins after they were exposed to protein-free filtrates of \( \beta \)-glucosidase-treated filtrates of human lens. Our results indicate that 3OHKYNG concentrations are much higher than 3OHKYN. This is in accord with the data of Bova et al. (16). The fact that human lens contains \( \beta \)-glucosidase suggests that 3OHKYNG is converted to 3OHKYN and products that are detected by our antibody. The activity of \( \beta \)-glucosidase increases in cataractous lenses (37) so that the higher immunoreactivity with our antibody in cataractous lenses may be due, in part, to enhanced formation of 3OHKYN. However, there are conflicting reports on 3OHKYNG levels in cataractous lenses. One study found no difference in 3OHKYNG concentrations between brunescent and non-brunescent human lens nuclei (38), but the investigators did not measure the total glucoside concentration in the lens. Another study reported decreased levels of 3OHKYNG in cataractous lenses (17), which could be due to reaction of this compound with lens proteins during cataractogenesis.

The intense immunopositive reaction observed when we incubated human lens epithelial cells with 3OHKYN shows that 3OHKYN can pass through plasma membrane of lens epithelial cells. If fiber cells in the lens are permeable as well, we would expect 3OHKYN modifications to occur in both cortical and nuclear regions of the lens. However, decrease in glutathione with aging and cataract formation (39, 40), espe-
cially in the nucleus, may promote 3OHKYN modifications in this region.

We anticipated that ROS generated during glycation might oxidize tryptophan and that the oxidation products of Trp would then react with proteins to chemically modify them. We were surprised to find that neither preformed glycation products nor glycation in the presence of tryptophan contributed to the oxidation of protein-free tryptophan. The monoclonal antibody against 3OHKYN-induced protein modifications failed to recognize either glycated proteins or proteins that were glycated in the presence of external tryptophan. The absence of immunoreactivity among various glycation products that we examined indicates that glycation does not lead to formation of kynurenines, although we cannot rule out an effect of glycation on the enzymatic oxidation of tryptophan. However, our antibody recognized proteins modified by 3OHKYN in the human lens. These findings imply that reactions of tryptophan oxidation products along with glycation occur in the human lens and that 3OHKYN-mediated reactions occur independently of glycation. Together these reactions are likely to contribute to cataractogenesis.

In summary, we showed that 3OHKYN-derived modifications can occur in the human lens, and we believe that these modifications may contribute to cataract formation. Our studies indicate that kynurenines are not produced during glycation of proteins. Since production of kynurenines is increased in the brains of patients with Huntington disease (41) and in the plasma of end stage renal disease patients (42), our antibody modifications may contribute to cataract formation. We thank Dr. Antonia Miller for critical reading of the manuscript.
