Various lines of evidence have shown that ALDH3A1 (aldehyde dehydrogenase 3A1) plays a critical and multifaceted role in protecting the cornea from UV-induced oxidative stress. ALDH3A1 is a corneal crystallin, which is defined as a protein recruited into the cornea for structural purposes without losing its primary function (i.e. metabolism). Although the primary role of ALDH3A1 in the metabolism of toxic aldehydes has been clearly demonstrated, including the detoxification of aldehydes produced during UV-induced lipid peroxidation, the structural role of ALDH3A1 in the cornea remains elusive. We therefore examined the potential contribution of ALDH3A1 in maintaining the optical integrity of the cornea by suppressing the aggregation and/or inactivation of other proteins through chaperone-like activity and other protective mechanisms. We found that ALDH3A1 underwent a structural transition near physiological temperatures to form a partially unfolded conformation that is suggestive of chaperone activity. Although this structural transition alone did not correlate with any protection, ALDH3A1 substantially reduced the inactivation of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal and malondialdehyde when co-incubated with NADP⁺, reinforcing the importance of the metabolic function of this corneal enzyme in the detoxification of toxic aldehydes. A large excess of ALDH3A1 also protected glucose-6-phosphate dehydrogenase from inactivation because of direct exposure to UVB light, which suggests that ALDH3A1 may shield other proteins from damaging UV rays. Collectively, these data demonstrate that ALDH3A1 can reduce protein inactivation and/or aggregation not only by detoxification of reactive aldehydes but also by directly absorbing UV energy. This study provides for the first time mechanistic evidence supporting the structural role of the corneal crystallin ALDH3A1 as a UV-absorbing constituent of the cornea.

The cornea is an avascular tissue located at the anterior portion of the eye and serves as the first defense against environmental stress to internal ocular tissues. ALDH3A1 (aldehyde dehydrogenase 3A1), a major constituent in the corneal epithelium, plays a critical and multifunctional role against UV-induced oxidative stress in the cornea (1–3). ALDH3A1 has been found to represent from 5 to 50% of the water-soluble protein fraction in the mammalian corneal epithelium depending on the species (4–6). ALDH3A1 is responsible for the metabolism of endogenous and exogenous aldehydes through NAD(P)⁺-dependent oxidation (7), including the detoxification of various aldehydes produced during UV-induced peroxidation of cellular lipids (8, 9). For example, it has been shown previously that cells expressing high levels of ALDH3A1 are substantially more resistant to UV-induced apoptosis as well as apoptosis arising from exposure to 4-hydroxy-2-nonenal (4-HNE), one of the most cytotoxic aldehyde by-products of lipid peroxidation (3). Studies with recombinant human ALDH3A1 revealed that the enzyme catalyzed the oxidation of not only 4-HNE ($K_m \sim 45 \mu M$) but a variety of other aldehydes, including hexanal ($K_m \sim 18 \mu M$), benzaldehyde ($K_m \sim 200 \mu M$), and malondialdehyde (MDA; $K_m \sim 6.6 \mu M$) as well (9). In contrast to many of the other members of the aldehyde dehydrogenase superfamily of proteins, the broad range of substrate specificity suggests that ALDH3A1 is highly versatile in its metabolic properties and can detoxify a wide range of toxic aldehydic compounds (7).

With levels of ALDH3A1 expression exceeding that needed for metabolism alone (10), it is likely that ALDH3A1 plays additional roles in the cornea, including those related to its function as a corneal crystallin. With parallels to the lens crystallins, corneal crystallins represent a group of highly expressed corneal proteins that have been recruited to serve as structural elements without losing their primary function (5, 11). In the case of ALDH3A1, the importance of primary function (i.e. metabolism) has been clearly demonstrated, whereas any structural role is speculative at best. Abedinia et al. (12) proposed that ALDH3A1 could directly absorb UV light and thereby protect other ocular elements from the deleterious effects of UVR, including protein and DNA damage. This was substantiated by the observation that the water-soluble fraction of the bovine cornea accounts for only 17% of the total protein but for nearly 50% of the total absorption of UV light (290–300 nm) (13). Additionally, high concentrations of ALDH3A1 in the cornea may simply represent a target for damaging compounds, such as 4-HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde; G6PD, glucose-6-phosphate dehydrogenase; UVR, ultraviolet radiation; LDH, l-lactic dehydrogenase; BSA, bovine serum albumin; SE-HPLC, size-exclusion high performance liquid chromatography; 2DUV, second derivative ultraviolet absorbance spectroscopy. * This work was supported by NEI Grants EY11490 (to V. V.) and EY13987 (to J. M. P.) from the National Institutes of Health, an American Foundation of Pharmaceutical Education predoctoral fellowship, and a National Institutes of Health training grant (to T. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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² The abbreviations used are: 4-HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde; G6PD, glucose-6-phosphate dehydrogenase; UVR, ultraviolet radiation; LDH, l-lactic dehydrogenase; BSA, bovine serum albumin; SE-HPLC, size-exclusion high performance liquid chromatography; 2DUV, second derivative ultraviolet absorbance spectroscopy.
as aldehydes and reactive oxygen species, so that other elements remain intact (14). It has also been suggested that ALDH3A1 may function as a chaperone to prevent non-native aggregation of damaged and/or inactivated proteins under conditions of oxidative stress (15, 16). Not unlike α-crystallin in the lens, it is reasonable to hypothesize that the chaperone activity of ALDH3A1 could help to maintain the optical qualities of the cornea by reducing non-native protein aggregation. As the cornea is a transparent tissue, the accumulation of aggregated proteins could increase light scattering and negatively impact vision. Thus, ALDH3A1 may represent an important component in maintaining the structural integrity of the cornea.

We therefore sought to investigate the potential of ALDH3A1 to protect other (i.e. target) proteins against non-native aggregation and/or inactivation in vitro through three different mechanisms related to chaperone activity as follows: the prevention of the thermally induced aggregation of target proteins, the reduction of aldehyde-induced inactivation, and the protection against UVR-induced damage. First, we examined the thermal stability of ALDH3A1 by far-UV CD as well as second derivative absorption spectroscopy (2DUV). We also evaluated the potential for ALDH3A1 to suppress the non-native aggregation of the target protein 1-lactic dehydrogenase (LDH) during isothermal incubation studies. The UV- and aldehyde-induced inactivation of the target protein glucose-6-phosphate dehydrogenase (G6PD) was then characterized by enzymatic and spectroscopic methods, followed by examining the ability of ALDH3A1 to protect against such damage.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following reagents of analytical grade were purchased from Sigma-Aldrich: sodium chloride, magnesium chloride, potassium chloride, Tris-HCl, potassium and sodium phosphate (mono- and di-basic), sodium sulfate, sodium azide, NAD(P)+, β-mercaptoethanol, EDTA, Triton X-100, Tween 20 (polyisorbate 20), glucose 6-phosphate, and 1,1,3,3-tetramethoxypropane (MDA precursor). Bovine serum albumin, glucose-6-phosphate dehydrogenase, and L-lactic dehydrogenase (LDH) during isothermal incubation studies. The α-helix structural content of ALDH3A1 was followed at 222 nm as the sample was heated from 4 to 80 °C at a rate of 1 °C/min. The resulting unfolding curve (fraction folded protein versus temperature), which was highly cooperative and fit to a two-state mechanism, was used to determine the apparent Tm as a consequence of the irreversible aggregation of the protein as described previously (18). Additionally, ALDH3A1 aggregation arising from thermal stress was assessed by following the increase in light scattering of the protein solution at 350 nm as a function of temperature using a Hewlett-Packard 8543 UV-visible spectrophotometer. ALDH3A1 was prepared at 0.1 mg/ml in 20 mM Tris-HCl, 100 mM KCl (pH 7.4) in a 1-mm path length quartz cuvette. The temperature was increased from 15 to 80 °C at a rate of 2 °C/min with a 2-min equilibration interval at each temperature step.

**Ultraviolet Absorbance Spectroscopy**—Absorbance spectra of ALDH3A1 were collected as a function of temperature using a Hewlett-Packard UV-visible spectrophotometer equipped with a Peltier temperature controller. ALDH3A1 was prepared at 0.1 mg/ml in 20 mM Tris-HCl, 100 mM KCl (pH 7.4) in a 200-μl quartz cuvette with a 1-cm path length cell. Absorbance scans were collected at 2 °C steps, with a 2-min equilibration time at each step, from 15 to 80 °C. Spectra were collected from 200–500 nm at 1-nm intervals using an integration time of 5 s. In some cases, the raw absorbance data were used to evaluate the turbidity of the solution at 350 nm. The absorbance data were transformed to second derivative spectra using Chemstation™ software as described previously (19).

Glucose-6-phosphate dehydrogenase (G6PD, 0.1 mg/ml) was incubated with either 4-HNE or MDA as described below. Immediately following the incubation, the excess 4-HNE or MDA was removed from the protein solution by dialysis against 100 mM KPO4 (pH 7.4) overnight at 4 °C using a mini dialysis unit following the manufacturer’s instructions. The protein solution (~0.1 mg/ml) was transferred to a 1-cm path length quartz cuvette, and absorbance spectra were collected at 25 °C and transformed as described above. For UVB-exposed G6PD,
the protein was irradiated at 0.1 mg/ml as described below, and absorbance data were collected immediately after exposure.

Thermal Aggregation Assays with the Target Protein i-Lactic Dehydrogenase (LDH)—LDH was prepared in 20 mM Tris-HCl, 100 mM KCl (pH 7.4) at 0.5 mg/ml. Aggregation of LDH at 37°C was monitored by light scattering at 350 nm using a DU-640 spectrophotometer with a circulating water bath. Temperature within the cells was confirmed using a type T thermocouple (Omega Engineering Inc., Stamford, CT). To evaluate the chaperone-like activity of ALDH3A1 under such conditions, LDH (0.5 mg/ml) was prepared with 0.1 and 0.25 mg/ml ALDH3A1 and the experiment repeated. ALDH3A1 was also incubated at 37°C alone at both concentrations as a control. All experiments were performed in triplicate.

Irradiation of G6PD with UVB Light—G6PD was prepared at 25 units/ml (0.1 mg/ml) or 5 units/ml (0.02 mg/ml) in 100 mM potassium phosphate (pH 7.4) and irradiated in a 1-cm path length quartz cuvette with UVB light (295 nm) at 37°C with gentle stirring using an AVIV spectrofluorometer. The photon flux of light incident on the sample during irradiation was determined to be 10³⁴ photons/s by ferrioxalate actinometry (20). Aliquots were removed from the solution at specific time points (0–30 min), placed on ice, and immediately assayed for G6PD enzymatic activity or subjected to other analyses. The experiment was repeated in the absence of the UVB light source for a dark control at each time point. The inactivation of G6PD by UVB light was also investigated in the presence of ALDH3A1 at 0.1 mg/ml (5-fold ratio) and 1.0 mg/ml (50-fold ratio) as well as bovine serum albumin (BSA) at the same concentrations. All experiments were performed in triplicate.

G6PD Incubation with 4-HNE and MDA—G6PD was prepared at 25 or 5 units/ml in 100 mM potassium phosphate (pH 7.4) and exposed to various concentrations of 4-HNE up to 2 mM for 2 h at 37°C. Controls were also performed in which G6PD was incubated at 37°C for 2 h without 4-HNE. The inactivation of G6PD was repeated in the presence of a 10-fold excess of ALDH3A1 (0.2 mg/ml), 1 mM NADP⁺, and both a 10-fold excess of ALDH3A1 and 1 mM NADP⁺. Additionally, G6PD inactivation studies were also performed in the presence of 10-fold BSA.

For MDA inactivation studies, MDA was first synthesized following the method described by Esterbauer et al. (21). Briefly, 1,1,3,3-tetramethoxypropane was acidified with 1 N HCl and dissolved under mild heating. The reaction was quenched with a large volume of 100 mM potassium phosphate (pH 7.5) and stored on ice and under foil. The concentration of the MDA stock solution was determined spectrophotometrically at 266 nm (ε = 31,500 m⁻¹ cm⁻¹). MDA was synthesized fresh daily. G6PD inactivation experiments with MDA were performed in a similar manner as described for 4-HNE above.

G6PD Enzymatic Activity Assay—The enzymatic activity of G6PD was determined by monitoring the reduction of NADP⁺ to NADPH spectrophotometrically at 340 nm at 25°C. Reactions were performed in a 1-ml quartz cuvette with a 1-cm path length. For each assay, ~0.2 µg of G6PD was added into reaction buffer (100 mM Tris-HCl, 10 mM MgCl₂, pH 8.0) that contained 2.5 mM NADP⁺. The enzymatic reaction was initiated by the addition of glucose 6-phosphate substrate. The linear portion of the reaction rates were fit to a linear slope and expressed as a specific activity with units of nmol of NADPH/min/mg of protein.

The loss of G6PD enzyme activity because of UV light, 4-HNE, and MDA was expressed as a percent of the respective control and the data used to generate inactivation curves. UVB-induced inactivation curves were fit to a simple exponential decay model using SigmaPlot software, which allowed for the calculation of the exposure time (min) required to reduce the activity of G6PD to 50% of the control. For 4-HNE-incubated protein, inactivation curves were fit to a 3-parameter sigmoid curve using SigmaPlot software to calculate the IC₅₀ value (concentration at which 50% of the protein is inactivated). MDA inactivation curves were fit to a simple exponential decay model using SigmaPlot software to calculate the IC₅₀ value.

Size Exclusion High Performance Liquid Chromatography (SE-HPLC) Analysis of G6PD—A Hewlett-Packard 1090 HPLC (250-µl injection syringe and diode array UV detection), equipped with a TSK-Gel 2000SW XL column (Tooshiba Biosciences LLC, Montgomeryville, PA), was used for SE-HPLC analysis. The mobile phase was 100 mM sodium phosphate (pH 7.2), 100 mM sodium sulfate, and 0.02% (w/v) sodium azide (22), which was filtered and degassed prior to use. Protein samples were centrifuged (10,000 x g) prior to analysis to remove insoluble material. The flow rate during analysis was 0.6 ml/min, and protein was detected at 220 nm. G6PD (0.1 mg/ml) was treated with 4-HNE and MDA or exposed to UVB light as described above, and samples were analyzed immediately after treatment. To quantify loss in soluble G6PD, chromatogram peak areas were determined by integration using Chemstation™ software.

SDS-PAGE and Western Blot Analysis—G6PD (0.1 mg/ml) was treated with 4-HNE and MDA or exposed to UVB light as described above. Protein samples containing up to 0.5 µg of G6PD (for Western blot) or 10 µg of G6PD (for Coomassie Blue staining) were electrophoresed in a 10% SDS-polyacrylamide gel under reducing conditions. Gels were either stained with Coomassie Blue stain or transferred to polyvinylidene difluoride membranes for Western blotting. Western blot membranes were blocked overnight at 4°C in 5% nonfat milk in TBST (50 mM Tris-HCl, 150 mM NaCl, pH 7.5, containing 0.1% Tween 20). The membranes were then probed with an anti-4-HNE antibody at 1:500 (kindly provided by Dr. Dennis Petersen, University of Colorado Health Sciences Center, Denver) for 1 h at room temperature in 5% nonfat milk/TBST. Following substantial washing in TBST, a horseradish peroxidase-conjugated secondary antibody was applied for 30 min at room temperature in 5% nonfat milk/TBST followed by washing in TBST. Protein bands were then visualized using chemiluminescence.

RESULTS

Thermal Unfolding and Thermal Stability of ALDH3A1—The chaperone activity of α-crystallin in the lens has been shown to be influenced by temperature, which may be related to temperature-induced structural transitions that accommo-
Thermal stability of ALDH3A1—The ability of ALDH3A1 to suppress the thermal aggregation of other proteins was investigated with the target protein LDH. LDH aggregates under physiological conditions (37 °C) (26), thus making it a suitable target protein to test the chaperone activity of ALDH3A1, which adopts a partially unfolded structure at this temperature (Fig. 2). We detected an increase in the light scattering of the LDH solution (0.5 mg/ml) at 350 nm during incubation at 37 °C (Fig. 3), indicating that this target protein has formed large, insoluble aggregates. Co-incubation of LDH with 0.1 mg/ml ALDH3A1 produced unexpected results; the addition of ALDH3A1 to the LDH solution resulted in a greater extent of light scattering than observed with LDH alone. This trend was further demonstrated by the addition of 0.25 mg/ml ALDH3A1 to the LDH incubation solution, which led to rapid and substantial solution turbidity. In contrast, incubation of ALDH3A1 alone at these two concentrations produced negligible increases in light scattering during the incubation period.

Characterization of UVB-induced Changes to G6PD—UVR is a considerable source of oxidative stress for the corneal epithelium because this cell layer absorbs the majority of all UV wavelengths (27, 28). G6PD (0.1 mg/ml) was irradiated with UVB light at a representative wavelength of 295 nm at 37 °C. We found that UVB exposure led to the inactivation of G6PD as seen by the loss of specific enzyme activity (Fig. 4A). We did not detect any changes in G6PD molecular weight (i.e. cross-linking) by SDS-PAGE (Fig. 4B), and UVB light did not induce any soluble or insoluble aggregation of G6PD by SE-HPLC or turbidity measurements (data not shown). 2DUV analysis, however, showed a small blue shift in Trp residues because of UVB exposure (Fig. 4C), suggesting that exposure to UVB light results in a slight unfolding of G6PD tertiary structure.

ALDH3A1 Protects G6PD against UVB-induced Inactivation—The UVB-induced inactivation of G6PD (5 units/ml) at 37 °C was reduced when carried out in the presence of an excess of ALDH3A1 (Fig. 5A). In addition, the extent of protection was proportional to the ALDH3A1 concentration during the irradiation experiments. We found that a 5-fold mass excess of ALDH3A1 afforded a small amount of protection against UVB-induced inactivation whereas a 50-fold mass excess of ALDH3A1 more than doubled the amount of UVB exposure time needed to reduce G6PD activity to 50% of the control (Table 1). To evaluate whether the observed protection is specific to ALDH3A1, the experiments were repeated using BSA as a control protein (29) at the same excess ratios. BSA provided a
Characterization of 4-HNE-induced Changes to G6PD—Incubation of G6PD (0.1 mg/ml) with 4-HNE for 2 h at 37 °C led to a dose-dependent inactivation of the enzyme represented by a progressive loss in specific activity (Fig. 6A). SDS-PAGE analysis showed that exposure to 4-HNE did not lead to any detectable cross-linking of G6PD (Fig. 6B, top panel); however, 4-HNE adduct formation with G6PD during the incubation was confirmed by Western blot (Fig. 6B, bottom panel). Preparative centrifugation for SE-HPLC analysis revealed that incubation with 4-HNE produced insoluble G6PD aggregates, which formed a small white pellet upon centrifugation (data not shown). This was also seen in the SE-HPLC chromatograms as a progressive loss of soluble G6PD protein (Fig. 6C). We also detected 4-HNE-induced changes in G6PD tertiary structure using 2DUV. Shifts in peak position for Trp, Tyr, and Phe residues were all apparent, although each displayed a different trend. The peak corresponding to Trp residues (Fig. 6D) showed an initial blue shift in position although red-shifted at 500 μM 4-HNE. These results suggest that the Trp residues are initially more solvent-exposed, likely due to partial unfolding of the protein, but become buried at higher concentrations of 4-HNE, which could be the consequence of substantial protein aggregation as detected by SE-HPLC. Tyr residues (Fig. 6E) progressively blue-shift as a function of 4-HNE exposure, whereas in contrast Phe residues (Fig. 6F) increasingly red-shift. Taken together, 4-HNE adduction causes perturbations in the tertiary structure of G6PD and leads to extensive noncovalent aggregation and eventual precipitation of the protein.

ALDH3A1 Protects G6PD against 4-HNE Inactivation—We then investigated the ability of ALDH3A1 to protect against 4-HNE-induced inactivation of G6PD. G6PD (5 units/ml) was incubated with various concentrations of 4-HNE for 2 h at 37 °C, after which G6PD-specific activity was immediately assayed. The resulting inactivation curve (Fig. 5C) was fit to a three-parameter sigmoid to calculate the IC50 value, the concentration of 4-HNE needed to inactivate G6PD by 50%. Coincubation of G6PD with a 10-fold mass excess of either ALDH3A1 or BSA did not result in any protection against 4-HNE inactivation. However, the inclusion of both ALDH3A1 and 1 mM NADP⁺ in the incubation solution resulted in a considerable increase in the IC50 value from 194.3 ± 40.1 μM 4-HNE (G6PD alone) to 923.3 ± 12.9 μM 4-HNE (Table 2). This effect was not attributed to NADP⁺ because NADP⁺ alone did not have a substantial impact on G6PD inactivation (Table 2).

Western blot analysis was used to determine whether ALDH3A1, either alone or in combination with NADP⁺, could reduce the extent of 4-HNE adduction to G6PD. A 10-fold excess of ALDH3A1 significantly lessened the G6PD modification by 4-HNE when compared with G6PD incubated with 500 μM 4-HNE alone (Fig. 7). The extent of 4-HNE adduct formation was further reduced in the presence of ALDH3A1 and 1 mM NADP⁺. It is worth noting that 4-HNE adducts to ALDH3A1 were also less substantial when NADP⁺ was included in the incubation mixture.

Characterization of MDA-induced Changes to G6PD—The incubation of G6PD (0.1 mg/ml) with MDA for 2 h at 37 °C also

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FIGURE 2. Second derivative absorbance spectra of ALDH3A1 as a function of temperature. A. ALDH3A1 was prepared at 0.1 mg/ml in 20 mM Tris, 100 mM KCl (pH 7.4), and data were collected at 15 °C (solid line), 25 °C (dotted line), 37 °C (dashed line), and 43 °C (dot-dashed line). Trends in the change in peak positions are indicated by arrows. Each spectrum is an average of three individual samples. B–D, the effect of temperature on the second derivative absorbance spectra peak positions of ALDH3A1. Peak positions corresponding to Trp (B), both Trp/Tyr (C), and Phe (D) are shown as a function of temperature. Values represent mean ± S.E. (n = 3).

FIGURE 3. LDH thermal aggregation assays at 37 °C. LDH was prepared at 0.5 mg/ml in 20 mM Tris, 100 mM KCl (pH 7.4) and incubated for 30 min at 37 °C (closed circles). The turbidity of the solution was monitored at 350 nm. LDH was then co-incubated with ALDH3A1 at 0.1 mg/ml (open triangles) and 0.25 mg/ml (closed squares). ALDH3A1 was also incubated alone at 0.1 mg/ml (open circles) and 0.25 mg/ml (closed triangles) for a control. Representative curves are shown in each case.

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similar extent of protection against UVB-induced inactivation (Fig. 5B; Table 1).

Characterization of 4-HNE-induced Changes to G6PD—Incubation of G6PD (0.1 mg/ml) with 4-HNE for 2 h at 37 °C led to a dose-dependent inactivation of the enzyme represented by a progressive loss in specific activity (Fig. 6A). SDS-PAGE analysis showed that exposure to 4-HNE did not lead to any detectable cross-linking of G6PD (Fig. 6B, top panel); however, 4-HNE adduct formation with G6PD during the incubation was confirmed by Western blot (Fig. 6B, bottom panel). Preparative centrifugation for SE-HPLC analysis revealed that incubation
Protective and Structural Roles of Corneal ALDH3A1

ALDH3A1 is a highly expressed protein in the corneal epithelium, accounting for up to 50% of the water-soluble protein fraction in mammalian species and the first enzyme to be categorized as a “corneal crystallin” (30). Corneal crystallins represent a group of abundant corneal proteins with many similarities with the lens crystallins. Both groups are composed of diverse, water-soluble, and cytoplasmic proteins with metabolic functions when expressed at lower levels in other tissues. The corneal and lens crystallins are also characterized by the accumulation in transparent and refractive tissues, taxon specificity across various species, and expression in excess over what is anticipated for metabolism alone (30, 31). Corneal crystallins are likely to have been recruited into the tissue to serve as structural elements without losing their primary role (5, 10, 32). The primary function of ALDH3A1 is as one of the principal enzymes responsible for the detoxification of aldehydes, including those associated with lipid peroxidation products such as 4-HNE and MDA (9, 33). Therefore, ALDH3A1 can be considered an important element in the arsenal of the cornea against oxidative stress (6). It remains unclear, however, what structural purpose if any ALDH3A1 has in the cornea as suggested by its status as a corneal crystallin.

A variety of potential structural roles have been speculated for ALDH3A1 (6). It has been proposed that the high expression of ALDH3A1 in the cornea represents a substantial fraction of the absorptive properties of the tissue (12), which is in agreement with the observation that the majority of UV light is absorbed by the epithelium at wavelengths below 310 nm (34, 35). Termed “absorbin” (13), ALDH3A1 may effectively function as a filter to absorb penetrating UVR from the environment (12). The abundant expression of transketolase, which is also considered a corneal crystallin, in rabbit keratocytes has been linked to proper light refraction and minimization of light scattering on the cellular level (10, 36), and this property may also extend to ALDH3A1. The contribution of ALDH3A1 as a structural element may include indirect functions as well. As the cornea is a transparent tissue, and this optical property is necessary for normal vision, the accumulation of damaged and denatured proteins is detrimental because such species can lead to light scattering and/or corneal cloudiness. The proposed function of ALDH3A1 as a molecular chaperone could prevent...
Protective and Structural Roles of Corneal ALDH3A1

FIGURE 5. Enzymatic inactivation of G6PD by UVB, 4-HNE, and MDA. A, UVB-induced inactivation of G6PD alone (5 units/ml; solid circles) and in the presence of 0.1 mg/ml (5-fold; open circles) and 1.0 mg/ml ALDH3A1 (50-fold; closed triangles). B, UVB-induced inactivation of G6PD alone (5 units/ml; solid circles) and in the presence of 0.1 mg/ml (5-fold; open circles) and 1.0 mg/ml BSA (50-fold; closed triangles). C, G6PD (5 units/ml) was incubated with up to 2 mM 4-HNE after which G6PD enzymatic activity was immediately assayed (solid circles). Experiments were repeated in the presence of 10-fold ALDH3A1 (0.2 mg/ml) (open circles) and 10-fold ALDH3A1 with 1 mM NADP⁺ (closed triangles). Data were fit to a three-parameter sigmoid using SigmaPlot software to generate inactivation curves. D, G6PD (5 units/ml) was incubated with up to 2 mM MDA after which G6PD enzymatic activity was immediately assayed (closed circles). Experiments were repeated in the presence of 10-fold ALDH3A1 (0.2 mg/ml) (closed triangles) and 10-fold ALDH3A1 with 1 mM NADP⁺ (open triangles). Data were fit to an exponential decay model using SigmaPlot software to generate inactivation curves. All incubations were performed at 37 °C. Values represent mean ± S.E. (n = 3), and error bars may be hidden by the data symbols in some cases.

TABLE 1

<table>
<thead>
<tr>
<th>Sample composition</th>
<th>Exposure time (min) to reduce activity by 50%</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<tr>
<td>G6PD alone</td>
<td>7.6 ± 0.35</td>
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<tr>
<td>G6PD + 5x ALDH3A1</td>
<td>9.2 ± 0.17</td>
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<tr>
<td>G6PD + 5x BSA</td>
<td>8.3 ± 0.06</td>
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<tr>
<td>G6PD + 50x ALDH3A1</td>
<td>19.1 ± 0.44</td>
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<tr>
<td>G6PD + 50x BSA</td>
<td>17.3 ± 0.73</td>
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*UVB exposure time needed to reduce the activity of G6PD by 50% was calculated using the inactivation curves shown in Fig. 5. A and B. Values represent mean ± S.E. (n = 3).*

the non-native aggregation of proteins damaged by oxidative stress (15, 16). ALDH3A1 may thus indirectly contribute to the maintenance of the transparent properties of the cornea.

The function of ALDH3A1 as a chaperone is not without precedent considering the multifunctional role of α-crystallin in the lens (37). α-Crystallin is found at exceptionally high concentrations in lens fiber cells and prevents non-native protein aggregation under a variety of stresses, including temperature (26, 37), chemical inactivation (38), and damage from direct UVR absorption (39) presumably through chaperone-like activity. Although the precise molecular mechanism of chaperone activity is still under debate, it has been hypothesized that α-crystallin, like other small heat shock proteins, undergoes a structural transition to expose previously buried hydrophobic regions to the solvent (24, 40). These hydrophobic regions can then interact with partially unfolded protein molecules, and thus α-crystallin intervenes with the formation of complexes containing non-native substrate proteins. Such structural transitions have been observed for α-crystallin at ~30 °C as well as at 65 °C (24), as the former is associated with chaperone activity under physiological conditions and the latter represents a potential basis for the heat shock properties of α-crystallin at elevated temperatures. Such protein-protein interactions may also explain the protection afforded by α-crystallin against chemical and UVR inactivation because these stresses may lead to the partial unfolding of the affected protein molecules (38).

We have shown in this study that ALDH3A1 also undergoes a structural transition at physiologic temperature, and this transition is characterized by loss in native tertiary structure because of partial unfolding (Fig. 2). It was therefore reasonable to hypothesize that the observed structural transition of ALDH3A1 at 37 °C may provide a structural basis for chaperone activity and thus an opportunity to reduce the non-native aggregation of target proteins. The temperature-induced structural transition of ALDH3A1, however, did not prevent the thermal aggregation of the target protein LDH. In contrast to the observed effect of α-crystallin under similar conditions (26, 37), the partial unfolding of ALDH3A1 at 37 °C actually exacerbated the non-native aggregation of LDH and led to the formation of insoluble aggregates (Fig. 3). The dramatic increase in insoluble protein aggregation observed with co-incubation of ALDH3A1 and LDH at 37 °C suggests that the interaction between the two partially unfolded protein species leads to insoluble non-native aggregation rather than the formation of a stable, soluble complex. It is important to note, however, that these negative in vitro results do not necessarily infer a lack of ALDH3A1 chaperone activity in vivo. Many cellular components for chaperone activity are not present in our in vitro samples. For example, the lack of an appropriate cofactor and/or a heat shock protein to refold the denatured proteins could lead to incomplete chaperone activity on the part of ALDH3A1.

Because of the physiological location of the cornea, UVR is one of the dominant sources of environmental stress for this tissue (6). UV radiation can damage ocular tissues and lead to photokeratoconjunctivitis after both long and short term expo-
It has been proposed that the direct absorption of UVR by ALDH3A1 provides a filter-like effect to reduce UV-induced damage to other corneal proteins and minimize corneal clouding (12, 13). To test this hypothesis, we first examined the effect of direct UV exposure on the activity of G6PD, an enzyme involved in the pentose phosphate pathway and the primary source of NADP⁺ production in the cornea (see Ref. 49 and references therein). Exposure of G6PD to UVB light at 37 °C led to a loss in the specific activity of the enzyme accompanied by a slight loss in native tertiary structure, although non-native aggregation was not detected by either SE-HPLC (data not shown), turbidity measurements (data not shown), or SDS-PAGE (Fig. 4). We therefore investigated the potential for ALDH3A1 to protect G6PD from UV light inactivation at 37 °C through the following two pathways. (i) Low concentrations of ALDH3A1 relative to G6PD were used to test the chaperone-like activity of the protein. (ii) High concentrations relative to G6PD were used to determine whether ALDH3A1 could prevent G6PD inactivation simply by absorbing the UV energy. It was found that a large excess (50-fold) of ALDH3A1 was required to reduce the inactivation of G6PD by UVB light (Fig. 5A). In addition, a similar extent of protection was observed with BSA (Fig. 5B) indicating that the protection was not specific to ALDH3A1 and likely not the result of chaperone-like activity. Rather, both proteins afford protection to G6PD against UV inactivation simply through the direct absorption of UV energy. Overall, these findings suggest that ALDH3A1 protection against UV-induced inactivation is due to the competitive absorption of UV light energy rather than chaperone activity. This is further supported by the observation that low ratios of α-crystallin can substantially reduce the UV-induced inactivation of G6PD under similar experimental conditions (39), whereas a large excess of ALDH3A1 is needed to exert a protective effect in this study.

One consequence of UV-induced oxidative stress in the cornea is the peroxidation of cellular lipids, specifically α-6 polyunsaturated fatty acids such as arachidonic and linoleic acids, which eventually results in the production of toxic aldehyde by-products, including 4-HNE (21). Like other α,β-unsaturated aldehydes, 4-HNE is a potent electrophile that adducts to nucleophilic sites on protein and DNA molecules and may lead to the disruption of cellular function and apoptosis (3, 21). We characterized the effect of 4-HNE adduction on the structure...
and function of G6PD at 37 °C. In addition to a loss in G6PD-specific activity (Fig. 6A), 4-HNE adduction also resulted in a loss of native tertiary structure and insoluble G6PD aggregation (Fig. 6, C–F). Such conformational changes in G6PD and subsequent aggregation suggest that 4-HNE exposure rendered the protein essentially a “substrate” for chaperone activity. Therefore, the ability of ALDH3A1 to interact with the 4-HNE-induced unfolded G6PD molecules and prevent non-native subsequent aggregation was examined. The presence of even a 10-fold excess of ALDH3A1 did not protect G6PD from inactivation and aggregation by 4-HNE at 37 °C (Fig. 5C), providing further evidence that the partially unfolded conformation of ALDH3A1 does not stabilize damaged proteins against non-native aggregation. However, co-incubation of G6PD with both ALDH3A1 and NADP+ demonstrated a substantial reduction in 4-HNE-induced G6PD inactivation. Furthermore, the metabolism of 4-HNE by ALDH3A1 also prevented the addition of 4-HNE to G6PD by Western blot (Fig. 7). Previous work has shown that recombinant ALDH3A1 can efficiently catalyze the oxidation of 4-HNE (3, 9), and our data clearly show that ALDH3A1 metabolism is an important mechanism that protects against the 4-HNE-induced inactivation and insoluble aggregation of proteins in vitro. ALDH3A1 metabolism in vivo may be critical in maintaining the optical properties of the cornea as insoluble aggregation of proteins may be problematic because of an increase in light scattering and consequential impact on corneal transparency. In addition, we have demonstrated that an excess of ALDH3A1 can reduce 4-HNE adduction to G6PD even in the absence of NADP+ (Fig. 7), suggesting that ALDH3A1 molecules alone can sequester reactive species and protect other proteins from modification. It is likely that the high expression of ALDH3A1 in the corneal epithelium creates a situation in which ALDH3A1 molecules are targeted for modification simply as a consequence of their abundance, thus providing a passive protective effect to other proteins.

UV-induced lipid peroxidation can also lead to the production of MDA (21), and earlier studies have shown that αA-crystallin can protect G6PD against MDA-induced inactivation presumably through chaperone activity (38). In this investigation, MDA inactivated G6PD and resulted in the formation of intermolecular covalent cross-links (Fig. 8A), although no structural perturbations indicative of protein unfolding were detected. The addition of an excess of ALDH3A1 alone had a small impact on MDA-induced inactivation of G6PD, whereas the inclusion of both ALDH3A1 and NADP+ afforded greater protection to

![Figure 7](https://example.com/fig7.png)

**FIGURE 7.** 4-HNE adduct formation in the presence of ALDH3A1 detected by Western blot analysis. G6PD (0.02 mg/ml) was incubated with 500 μM 4-HNE for 2 h at 37 °C alone, with a 10-fold excess of ALDH3A1 (0.1 mg/ml), and with an excess of both ALDH3A1 and 1 mM NADP+. Samples were probed with an anti-4-HNE antibody to detect modifications to both G6PD and ALDH3A1 (top panel). The change in G6PD band density was quantitated by densitometry; bands were normalized to the density of 4-HNE-adducted G6PD band from the G6PD only incubation sample (bottom panel).

![Figure 8](https://example.com/fig8.png)

**FIGURE 8.** Characterization of MDA-induced inactivation of G6PD. A, loss of G6PD specific activity with the substrate glucose 6-phosphate because of incubation with MDA for 2 h at 37 °C. B, MDA-induced cross-linking of G6PD detected by SDS-PAGE analysis. Each lane represents 10 μg of G6PD, and protein bands were visualized with Coomassie Blue staining. Molecular masses (kDa) are indicated on the left. C and D, effect of MDA incubation on the structure of G6PD by 2DUV analysis. Changes in 2DUV peak position for Trp (C) and Tyr (D) are shown as a function of MDA concentration during incubation. Values represent mean ± S.E. (n = 3), and error bars may be hidden by the data symbols in some cases.
G6PD (Fig. 5D). The extent of protection, however, was not as sizeable as that observed with 4-HNE. The notable difference in the metabolic protection of ALDH3A1 between the two aldehydes may arise from the preference of ALDH3A1 for 4-HNE as a substrate. Studies with recombinant ALDH3A1 found that the apparent $K_v$ value and catalytic efficiency ($k_{cat}$) for 4-HNE were 45 $\mu M$ and 1.7 s$^{-1}$, respectively. Parallel kinetic studies with MDA revealed an apparent $K_v$ of 6.6 $\mu M$ and a $k_{cat}$ of 1.2 s$^{-1}$ (9). These data suggest that ALDH3A1 prefers the substrate 4-HNE when compared with MDA, which could account for the considerable protection of G6PD against 4-HNE-induced inactivation.

Although we have shown herein that ALDH3A1 protects against UV-induced protein damage in vitro, the mechanisms presented in this study are not without experimental support in vivo systems. The transfection of human ALDH3A1 cDNA into human corneal epithelium cells greatly increased the resistance of these cells against UV-induced apoptosis when compared with the mock-transfected cells (3). Indeed, ALDH3A1 provided protection against both the direct exposure to UV light as well as against exposure to 4-HNE in this cell line. Additionally, UV irradiation of mice led to a severe reduction in ALDH3A1 activity (~85%), whereas the activity of other corneal enzymes remained undisrupted (50). It is presumed that the observed protection is primarily a consequence of the metabolism of aldehydes arising from the peroxidation of cellular lipids. However, the extent of protection attributed to direct UV absorption is still unclear. To better address the relative contributions of the metabolic and absorptive properties of ALDH3A1 in the protection of the cornea, we are currently working to develop mice that express high levels of inactive ALDH3A1 through site-directed mutations of the active site Cys. Experiments with these mice are anticipated to elucidate the respective importance of both mechanisms in the protection against UV-induced damage by ALDH3A1.

In summary, we present mechanistic studies that demonstrate the versatility of ALDH3A1 as a protective element of the mammalian cornea in response to oxidative stress. ALDH3A1 can protect other proteins against UV-induced damage by at least two mechanisms in vitro, detoxification of reactive aldehydic compounds and competitive absorption of UVR (Scheme 1). ALDH3A1 efficiently metabolizes various aldehydes associated with the peroxidation of cellular membranes, namely 4-HNE and MDA, which can prevent the inactivation and aggregation of the target protein G6PD. Additionally, ALDH3A1 reduces G6PD inactivation through the direct absorption of UVR, which supports the structural role of this corneal crystallin as a UV filter. The abundance of ALDH3A1 relative to the expression of other proteins could reduce the extent of UV-induced inactivation and aggregation through these two mechanisms in vivo. We anticipate that such functions would be of great importance in the maintenance of corneal transparency and may even extend to protecting other ocular elements such as the lens from UV-induced oxidative damage.

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Protective and Structural Roles of Corneal ALDH3A1

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