CHAPTER V

ALDEHYDE DEHYDROGENASE
AND ITS POSSIBLE ROLE
IN THE CORNEA
5. INTRODUCTION

Aldehyde dehydrogenase (ALDH), being the major soluble protein of the cornea is thought to play a role in maintaining corneal transparency. However, its exact role in the tissue is not clear, though it is thought to play a structural role (Cooper et al., 1991; Rabaey and Segers, 1981). In this chapter, the effect of UV radiation on the structure and activity of the enzyme is described. We have also studied the possible protective role of ALDH against hydroxyl radical (·OH) mediated damage of proteins, using ribonuclease A (RNase A) as a model protein, as it is known to undergo covalent modification to yield high molecular weight forms which can be visualized electrophoretically on a SDS-PAGE.

5.1. MATERIALS AND METHODS

RNase A and bovine serum albumin (BSA) were obtained from Sigma Chemicals Co., USA. Bis(hydroperoxy)naphthaldiimide, a ·OH generating compound was obtained as a gift from Prof. I. Saito, Kyoto University, Japan. This compound was coated on to Kiselgel beads as described earlier (Guptasarma, 1992).

5.1.1. Purification of ALDH

ALDH was isolated and purified from the corneal epithelium by the method of Konishi and Mimura (1992). The corneal epithelial layer was extracted in 20 mM Tris, pH 8.3. The extract was centrifuged at 10,000 rpm for 30 min and the supernatant loaded on a Sephacryl S-200 gel filtration column. The major peak was identified to contain ALDH by monitoring the activity as described earlier (Lindahl, 1977). The protein fraction was further purified on a DEAE-cellulose ion exchange column equilibrated with 20 mM Tris, pH 8.3, and the enzyme eluted with 50 mM NaCl. The fraction which yielded single bands purity on SDS-PAGE were dialysed against millipore water, concentrated by ultrafiltration and the protein concentration determined by Lowry’s method.

5.1.2. Assay for ALDH activity

ALDH activity was assayed by monitoring the change in absorbance at 340 nm or by fluorescence at 460 nm (excitation, 339 nm) in a time drive mode, caused by the production
of NADPH accompanying the oxidation of benzaldehyde used as a substrate, at 25 °C (Lindahl, 1977).

5.1.3. Fluorescence spectroscopy

Fluorescence emission spectra, synchronous excitation spectra and irradiation were carried out as described in Chapter II. In synchronous excitation spectra, a wavelength difference (Δλ) value of 60 nm was used between the excitation and emission wavelengths.

Studies of the binding of the hydrophobic fluorescent probe 8-anilinonaphthalene 1-sulphonate (ANS) were carried out by adding a stock solution of ANS to a 0.2 mg/ml protein solution to a final concentration of 10 μM. The sample was excited at 350 nm, and the emission in the 460-520 nm region monitored.

5.1.4. Α-Crystallin photoaggregation

Bovine Α-crystallin was a gift from Ms. Sivakama Sundari, CCMB, Hyderabad. 200 μl of 2.5 mg/ml Α-crystallin was taken either in 800 μl buffer or buffer containing 0.5 M D-mannitol. In case of Α-crystallin with ALDH, 800 μl of ALDH solution was added such that the ratio of crystallin to ALDH was 100:1. Photoaggregation of Α-crystallin was initiated by irradiation at 295 nm for a period of 90 min, and the loss in Trp fluorescence with time monitored as an indicator of photodamage to these residues. The photoaggregation of crystallin was monitored by following the optical scattering at 450 nm.

5.1.5. Circular dichroism spectroscopy

CD was recorded as described in Chapter III. A cuvette of pathlength 0.05 cm was used to record the far UV CD, with the protein concentration being 0.45 mg/ml. The secondary structure of the protein was estimated by using the program LINCOMB, as described by Perczel et al. (1992).

5.1.6. Generation of hydroxyl radical

•OH were generated using the "photo-Fenton" reagent I synthesized by Saito et al. (1990). The structure of I is shown in Scheme 5.1. Since I is insoluble in water as also its photoproduct, it was found to be convenient to coat I on Kieselgel beads, so that its removal
Scheme 5.1: Generation of \( \cdot \text{OH} \) by photoreaction of the photo-Fenton reagent (I) upon irradiation at 366 nm (Saito et al., 1990)
from the reaction mixture is easy and complete (Guptasarma et al., 1992). 1 mg of these beads were taken for every 1 ml of the protein solution and irradiated at 366 nm in a Hitachi F-4000 spectrofluorimeter, with constant stirring, for 90 min. The coated compound (I) undergoes photolysis upon irradiation at 366 nm, yielding methyl ester (II) and releasing pure •OH in high yield into the solution (Saito et al., 1990). Control experiments were carried out using protein solution without the Kieselgel beads, under similar conditions.

•OH mediated damage of RNase A was carried out in millipore water as buffers tend to affect •OH-mediated reactions (Davies, 1987). All experiments were carried out using 500 µl of 1 mg/ml RNase A and 500 µl of 0.1 mg/ml of ALDH or BSA. The concentration of RNase to ALDH was 10:1. In the experiment with DTT, a final concentration of 10 mM DTT was used for 1 mg/ml solution of RNase A.

5.1.7. Modification of sulphydryl groups

The sulphydryl group of ALDH was modified using p-chloromercuribenzoic acid (PCMB), which is a specific reagent for the chemical modification of sulphydryls (Means and Feeney, 1971). PCMB was added to a solution of ALDH (2 µM) in 100 molar excess under acidic conditions using acetate buffer of pH 4.6, and incubated for a period of 1 h. The unreacted PCMB was dialysed out.

5.1.8. Electrophoresis

ALDH before and after irradiation for different periods of time was subjected to SDS-PAGE on a 10% gel to check for the formation of any high molecular weight forms with irradiation. Intermolecular non-disulphide crosslinking of RNase A following •OH treatment was monitored by 10% SDS-PAGE. Samples were boiled in the presence of β-mercaptoethanol and SDS and equal amounts of the protein loaded on the gel. The gels were silver-stained to enable easy visualization of high molecular weight forms.

5.2. RESULTS AND DISCUSSION

5.2.1. Purification of corneal aldehyde dehydrogenase

The corneal epithelial extract following separation on a Sephacryl S-200 gel filtration column gave four peaks with most of the proteins eluting in the void volume fraction (Figure
Figure 5.1 A: Gel permeation chromatography of bovine corneal epithelial extract on a Sephacryl S-200 column equilibrated with 20 mM Tris, pH 8.3. The protein content of the fractions was monitored at 280 nm (---); the ALDH peak by its activity (—). B: Ion exchange chromatography of the partially purified ALDH (peak 1 of Sephacryl S-200 column) on a DEAE Cellulose column equilibrated in 20 mM Tris, pH 8.3. ALDH was eluted using 50 mM NaCl in the equilibration buffer.
Figure 5.2: 10% SDS-PAGE profile of peak fractions obtained during the purification procedure of ALDH by gel filtration and ion-exchange column.
5.1A). The peak fractions were assayed for ALDH activity which was found to elute in the void volume. These fractions were pooled and loaded on a DEAE cellulose ion exchange column. The bound ALDH was eluted using 50 mM NaCl (Figure 5.1B). The fractions so obtained were assayed for ALDH activity and their purity checked on a 10% SDS gel. Figure 5.2 shows the various fraction obtained during purification. Fractions, which yielded single band on the gel upon silver staining were used for further experiments.

5.2.2. Protective role of ALDH

Figure 5.3A shows the SDS-PAGE profile of RNase A exposed to ·OH in the absence and presence of ALDH. Lane 1 shows control RNase A. The protein moves very close to the dye front, as it has a molecular weight of 14 kDa. Upon exposure to ·OH, two distinct bands of higher molecular weight are seen, formed by the covalent crosslinking of RNase A due to free radical reaction (lane 2). These higher molecular weight products were not formed when RNase A was exposed to ·OH in the presence of ALDH (lane 4). This indicates that ALDH protects RNase A from the ·OH-mediated changes. Interestingly, ALDH itself does not undergo ·OH mediated modifications when exposed for the same period of time (Figure 5.3A, lane 5 vs lane 6).

Cysteines are known to quench free radicals. Since ALDH has three cysteine residues with one Cys-SH (Cooper et al., 1991), the protection of RNase A by ALDH seems to be due to the quenching or scavenging of ·OH by free -SH groups. As anticipated, the thiol reagent DTT, which is also known to quench free radicals, is able to inhibit the formation of higher molecular weight products of RNase by ·OH radicals (Figure 5.3B). This protection of RNase A is specific to ALDH, since the treatment of RNase A with ·OH in the presence of BSA, which has -SH groups, has no effect on the formation of high molecular weight aggregates (Figure 5.3B). BSA itself is seen to succumb to ·OH action, resulting in the formation of higher molecular weight products. This differential behaviour of ALDH and BSA is most likely due to differences in the accessibility of the -SH groups in the two proteins. Such differential susceptibility of proteins to oxidative reactions has been documented earlier (Davies, 1987).

If the protection of proteins from radical-induced changes is conferred by cysteine residues in ALDH, the modification of -SH groups in ALDH should abolish this effect. Figure
Figure 5.3: 10% SDS-PAGE profiles of RNase and ALDH, stained with silver. **A:** lane 1, control RNase A; lane 2, RNase A treated with ·OH for 90 min; lane 3, control RNase A and ALDH present in 10:1 mg ratio; lane 4, RNase A exposed to ·OH for a period of 90 min in the presence of ALDH; lane 5, control ALDH; lane 6, ALDH treated with ·OH for 90 min. **B:** lane 1, RNase A treated with ·OH for 90 min in the presence of 10 mM DTT; lane 2: control RNase A and BSA present in 10:1 mg ratio; lane 3: RNase A treated with ·OH for a period of 90 min in the presence of BSA. **C:** lane 1: control RNase A and SH-modified ALDH; lane 2: RNase A treated with OH radical for a period of 90 min in the presence of SH-modified ALDH.
5.3C shows the loss of the protective ability of ALDH upon -SH modification, towards \textit{\textsuperscript{\textbullet}OH}-induced crosslinking of RNase A. Thus, only native ALDH can prevent oxidative damage to proteins like RNase A, and its -SH groups appear to be important in this protective action.

The mechanism of protection may be by quenching or scavenging of \textit{\textsuperscript{\textbullet}OH} by -SH groups of ALDH (similar to GSH-mediated quenching of \textit{\textsuperscript{\textbullet}OH}). Though the reactions of singlet oxygen and \textit{\textsuperscript{\textbullet}OH} with the free -SH group have not been well characterized, they are thought to form both disulphides (R-S-S-R) and sulphonic acids (R-SO\textsubscript{3}H) (Halliwell and Gutteridge, 1985). Our results indicate that corneal ALDH plays a role in preventing oxidative damage to other proteins in the cytoplasm. This protective role of ALDH is well suited to a tissue like the cornea in which light-mediated generation of radicals and reactive oxygen species might be expected to occur.

5.2.3. Effect of UV irradiation on ALDH

As ALDH is thought to prevent damage to the cornea by absorbing the UV radiation, we studied the effect of UVB on the activity and structure of ALDH and its possible role in the cornea under such conditions.

ALDH was subjected to UV irradiation at 295 nm for 30, 60 and 90 min. Figure 5.4 shows the fall in the relative intensity of Trp fluorescence with irradiation. A consequent increase in the fluorescence intensity at 420 nm upon excitation at 350 nm is seen. The fluorescence band is characteristic of the Trp photoproduct, N-formylkynurenine, though it is likely that other products arise from further photoreaction (Walrant and Santus, 1974).

The far-UV CD spectrum shows a broad negative band centred at 217 nm (Figure55). Secondary structural analysis of this curve indicates that the protein has a significant amount of \textbeta-sheet (30 \%) and \textbeta-turn (38 \%) motifs. The CD spectra do not seem to change significantly following irradiation and the CD curves of ALDH before and after irradiation overlay each other (Figure 5.5). Thus, there appears to be no significant change in the secondary structure of ALDH before and after 90 min of irradiation (Table 1).

The change in intensity of fluorescence of the coenzyme NADH at 460 nm (upon excitation at 339 nm) at the end of five minutes was taken as an index of the activity of the
Figure 5.4: Changes in the relative fluorescence intensity (F/F_0) of 0.45 mg/ml ALDH as a function of irradiation time, decrease in the Trp fluorescence (---, 295 nm excitation), and increase in non-Trp fluorescence at 420 nm (---, 350 nm excitation).
Figure 5.5: Far-UV CD spectra of ALDH in 20 mM Tris, pH 8.3, before irradiation (---) and after 30 min (...), 60 min (---), and 90 min (-.-.-) of irradiation at 295 nm. CD spectra were recorded in a 0.05 cm pathlength cell. The protein concentration was 0.45 mg/ml.
Table 5.1: Changes in the secondary structure of ALDH upon irradiation at 295 nm.

The far-UV CD obtained was analysed using the program LINCOMB as described by Perzel et al., 1992.
enzyme. Figure 5.6 shows such an assay of enzymatic activity as a function of the period of irradiation. The activity of the enzyme decreases to almost 50% at the end of 90 min of irradiation. During UV-irradiation of proteins, other residues besides Trp such as Met, Cys and His are also modified (Rao et al., 1990; Guptasarma et al., 1992). These residues are not modified by direct photolysis but through photosensitized processes (Dillon et al., 1982). The decrease in the activity of ALDH could be due to the loss of Cys residues, which are essential for the activity of the enzyme and/or due to changes in the tertiary and quaternary structure of the molecule, leading to an alteration in its active site geometry.

As Trp residues, which are the major contributors to the near-UV CD signals of proteins, get photolyzed, a drop in the magnitude of the CD signal in this region is difficult to interpret as being unambiguously due to tertiary structural changes. We thus concentrate on monitoring quaternary structural alterations in the enzyme, using two independent approaches. One is the ANS binding assay, which utilizes the fact that this fluorescent probe binds to hydrophobic surfaces and displays both a blue shift and intensification in its emission as a consequence (Cardamone and Puri, 1991). The other is a hydrodynamic assay of the protein before and after irradiation, involving gel filtration on a Superose 12 column. Figure 5.7 reveals an increase in the intensity of ANS fluorescence when it is added to a solution of irradiated ALDH. With increased irradiation of ALDH, the intensity of the added ANS too increases. This suggests an increased exposure of the hydrophobic surface of the protein upon irradiation. As the secondary structure is largely intact upon photolysis of the protein (Figure 5.5), this exposure of the hydrophobic surfaces would be due to alterations in the tertiary and quaternary structure of ALDH upon irradiation.

Figure 5.8 shows the Superose 12 gel filtration chromatogram of corneal ALDH before and after 30, 60 and 90 minutes of irradiation with 295 nm light. The native protein elutes as three major bands with Ve values of 9, 10.8 and 11.8 ml, corresponding to molecular weights (based on standards) 320, 100 and 54 kDa respectively. These represent the hexamer, dimer and monomer species of corneal ALDH. Upon irradiation, a new peak with Ve 7.9 ml appears while the hexamer peak now elutes at 8.7 ml. There is no significant change in the elution volumes of the dimer and the monomer peaks. The slight change, of 0.3 ml, in the elution volume of the hexamer most likely represents a slight loosening in the packing of the aggregate. Also, while the proportions of the dimer and the monomer are not affected
Figure 5.6: Activity of ALDH with increasing time of irradiation. The activity of the enzyme was monitored by change in fluorescence intensity of NADPH (Ex 339 nm; Em 460 nm) 5 min after the addition of substrate. The change in fluorescence intensity (ΔF) is plotted against the time of irradiation.
Figure 5.7: Emission spectra of ANS binding to ALDH (...) with 30 min (---), 60 min (-.-.-), and 90 min (--.--.) of irradiation at 295 nm. Solid line shows the spectrum of ANS in buffer. Protein solution (0.2 mg/ml) in 20 mM Tris, pH 8.3, was taken and ANS added to a final concentration of 10 μM. The sample was excited at 350 nm.
Figure 5.8: High performance gel permeation chromatogram of ALDH (A) and 30 min (B), 60 min (C) and 90 min (D) irradiated samples.
significantly upon irradiation, there is a marked reduction in the percentage of the hexamer population and a concomitant appearance of a high-molecular weight form. Irradiation of corneal ALDH seems to preferentially lead to aggregates. As the change in the enzymatically active dimer form is minimal, we surmise that much of the decline in the activity of ALDH following irradiation is a result of direct damage to the essential sulphydryl groups of the Cys residues.

The high molecular weight forms of corneal ALDH, formed upon irradiating the molecule, are visualized in SDS-PAGE profiles shown in Figure 5.9. Since these runs were made in the presence of β-mercaptoethanol, the high molecular weight bands represent non-disulphide covalent crosslinks, of the type seen when the proteins are photolyzed. Prominent among these is a band of approximate molecule weight 100 kDa (dimer), while the higher homologues appear in smaller amounts. It would thus appear that the 7.9 ml elution peaks seen in the FPLC runs of Figure 5.8 represent non-covalent aggregates of this non-disulphide covalent dimer of the protein (The band that appears just below the parent band in lanes 2, 3 and 4 of Figure 5.6 seems to suggest that irradiation also leads to some degradation of ALDH).

One role ascribed to corneal ALDH is that of a UV-protectant of the eye. We attempted to assess this property of the molecule by studying its ability to inhibit the light-induced aggregation and precipitation of the lens protein Γ-crystallin. This protein is known to form water-insoluble aggregates upon irradiation with 300 nm light (Mandal et al., 1988). Figure 5.10 shows the relative light scattering of a solution of Γ-crystallin in buffer, and in the presence of ALDH, and of the radical-scavenger D-mannitol. It is clear that ALDH is able to offer protection at levels of efficiency comparable to that of mannitol. Mandal et al. (1988) have found that the sulphydryl reagent dithiothreitol also protects Γ-crystallin from light-induced precipitation. In earlier experiments, we have found corneal ALDH to protect hydroxyl radical-mediated damage to other proteins (e.g. RNase A), while ALDH whose -SH groups are modified (with p-chloromercuri benzoate) does not offer this protection (Figure 5.3). These results, taken together, suggest that the protective action of corneal ALDH may be in part due to its properly disposed -SH groups which are accessible enough to react with and quench reactive radicals generated during photolysis. In addition, the slight conformational modification that ALDH itself undergoes upon irradiation exposes hydrophobic surfaces; this
Figure 5.9: 10% SDS-PAGE of ALDH before (lane 1) or after 30, 60 and 90 min (lanes 2, 3 and 4) of irradiation at 295 nm. Equal concentrations of the protein were loaded and the gel stained with silver.
Figure 5.10: Photoaggregation of \(\Gamma\)-crystallin from bovine eye lens monitored in a spectrofluorimeter by setting both the excitation and emission wavelengths at 450 nm in the absence (open circles) or presence of ALDH (filled circles) and in the presence of 0.5 M D-mannitol (filled squares). A 0.5 mg/ml solution of \(\Gamma\)-crystallin was used. The ratio of crystallin to ALDH was 1:100.
exposure would offer a binding site to the "guest protein", \( \Gamma \)-crystallin, and help in preventing its homo-aggregation and precipitation; this would be akin to what has been seen with the 'chaperone' \( \alpha \)-crystallin, which also exposes a hydrophobic receptor surfaces upon mild conformational readjustment (Raman et al., 1995).

Our results suggest that the ALDH molecule undergoes photodamage, but the secondary structure does not seem to alter significantly. The surface hydrophobicity and hydrodynamic properties change, indicating altered tertiary structure, especially in its oligomeric states. The formation of covalent, non-disulphide crosslinking and a fall in the activity of the enzyme are observed. Under these conditions, the ALDH molecule continues to act as a protectant, both through its accessible -SH groups and by offering its hydrophobic sites for binding, thus preventing aggregation and consequent precipitation of other proteins.