Schiff's base formation in the lens protein γ-crystallin

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Uniquely among the soluble lens-specific proteins, γ-crystallin is capable of binding the strongly chromophoric aldehyde retinal. A role for γ-crystallin in protecting lens components from toxic aldehydes resulting from membrane oxidation is proposed and a molecular model of the probable interaction site is presented. The sequence of a tetrapeptide at this site is identical to that of the retinal binding site of bacteriorhodopsin.

Cataract *Eye-lens* *γ-Crystallin* *Retinal* *Schiff's base* *Structural model*

1. INTRODUCTION

Oxidation is one of the major threats to the transparency of the ocular lens [1]. Among its effects is the release of toxic molecules including aliphatic aldehydes from lens membranes [2]. It is possible that such oxidation products are involved in the processes of cataractogenesis and aging, including protein aggregation [3] and the accumulation of unusual chromophores [1]. The tissue-specific structural proteins α-, β- and γ-crystallins are the major molecular components of mammalian lenses [3]. These proteins are extremely stable, surviving without turnover for years in the ennucleated lens fibre cells. We have examined the interaction of lens proteins with aldehydes and have found that only one fraction, γ-crystallin, binds the model compound retinal.

2. METHODS AND RESULTS

α-Crystallin, β-crystallin and γ-II and γ-III crystallins were extracted from calf lenses [4, 5]. As an analogue of the aliphatic aldehydes that are oxidatively liberated from membrane, the strong chromophore retinal (vitamin A aldehyde) was used. Equimolar quantities of protein subunits and retinal were mixed in cuvettes and difference spectra measured against retinal in a Cary 15 spectrophotometer. For both all-*trans* and 11-*cis* stereoisomers of retinal there was no reaction observed with α- or β-crystallins at pH 7. However, γ-II and γ-III crystallins, which have closely related amino-acid sequences [6,7], reacted with both stereoisomers identically. Difference absorption peaks were observed around 445 nm (fig.1a) with troughs at 380 nm corresponding to the absorption maximum of free retinal. The absorption at 445 nm was abolished within 20 min by addition of the reducing agent sodium cyanoborohydride [8] to both cuvettes. After extraction of free retinal with hexane, direct spectroscopy of the protein sample showed a small absorption at 320 nm (fig. 1b).

These results are characteristic of the formation and reduction of a protonates Schiff's base. At pH 6 and pH 8 there was no observable reaction.

The experiments were repeated with two other strongly chromophoric aldehydes pyrixodal phosphate and *o*-phthalaldehyde. These cyclic molecules gave no observable reaction, suggesting a degree of shape specificity in the binding of aldehydes to the protein.

The nature of the Schiff's base binding site was

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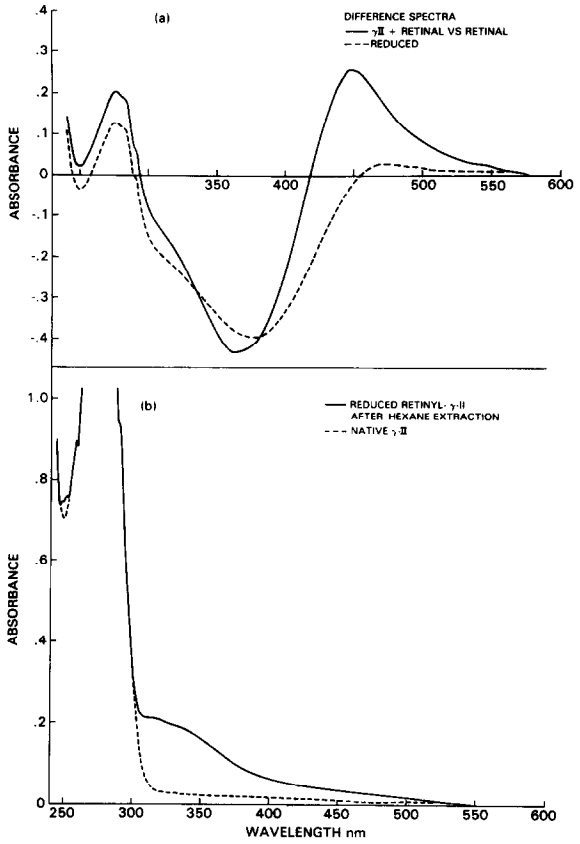

Fig. 1. Spectroscopy of the reaction between γ-II and retinal. (a) Solid line shows the difference spectrum of γ-II (5×10^{-5} M) + all-*trans* retinal (5×10^{-5} M) against all-*trans* retinal (5×10^{-5} M). Solutions were in 60 mM phosphate buffer (pH 7.1). Retinal (1 mM in ethanol) was mixed with buffer and spectra recorded at once. Dotted line shows the difference spectrum of the same sample 20 min after addition of sodium cyanoborohydride (10^{-2} M) to both cuvettes. The trough represents free retinal which has absorption in the protein region, hence the apparent decline in the absorption of the protein. Identical results were obtained with 11-*cis* retinal and with calf γ-III. (b) Solid line shows the spectrum of retinyl-γ-II extracted 3 times with hexane to remove free retinal. Dotted line shows the spectrum of native γ-II.

further investigated using γ-II crystallin for which a complete amino acid sequence and three-dimensional structure are known [9–11]. The reaction with γ-II was repeated using tritium-labelled all-*trans* retinal (a gift from the National Cancer Institute, manufactured by SRI International) substituting sodium borohydride as reducing agent. In SDS–urea–20% PAGE [12] label comigrated with the γ-II protein band at 20 kDa under these dissociating conditions. Total hydrolysis of the [^{3}H]retinyl-γ-II using 2.5 M NaOH yielded a ninhydrin-positive, labelled component with the same mobility as retinyl-lysine in thin-layer chromatography on silica gel H using a butanol–acetic acid–water system [13]. Lysine has been shown to be the active residue for retinal binding in both bacteriorhodopsin [14] and rhodopsin [13] and it is also responsible for pyridoxal phosphate binding in numerous enzymes [15]. γ-II crystallin has only two lysine residues, Lys 2 and Lys 154.

The sequence of calf γ-II shows that Lys 2 is contained in a cyanogen bromide fragment of at least 5.1 kDa while Lys 154 is in a much smaller peptide of only 1.1 kDa (1.4 kDa if modified with retinal).

[^{3}H]Retinyl-γ-II was subjected to CNBr cleavage [16] and the peptides were separated by SDS–urea–PAGE [12] a system capable of resolving peptides down to a size of 3 kDa. A major peptide band was apparent at about 5 kDa with other bands at lower M_r-values. Autoradiography showed that there was no label associated with the 5-kDa band. However, a species of 3 kDa but migrating more slowly than free retinal was strongly labelled. These results suggest that retinal binds to Lys 154.

3. DISCUSSION

One role of γ-crystallin may be to buffer the lens against oxidation by the safe accumulation of intramolecular disulphide bonds [10,11]. The results presented here further suggest that the protein is capable of another protective role in scavenging linear aldehydes, similar to retinal, released from damaged membranes. Modified and unmodified Schiff's bases resulting from these reactions would contribute to the coloured and fluorescent moieties observed in aging and cataractous lenses. Consequent upon membrane breakdown, or the generation of aldehydes from other sources by free radicals, γ-crystallin could bind the aldehydes, protecting glutathione, enzymes and other lens components [17]. Under normal conditions the labile aldehyde binding sites would probably be regenerated and the aldehydes reduced.

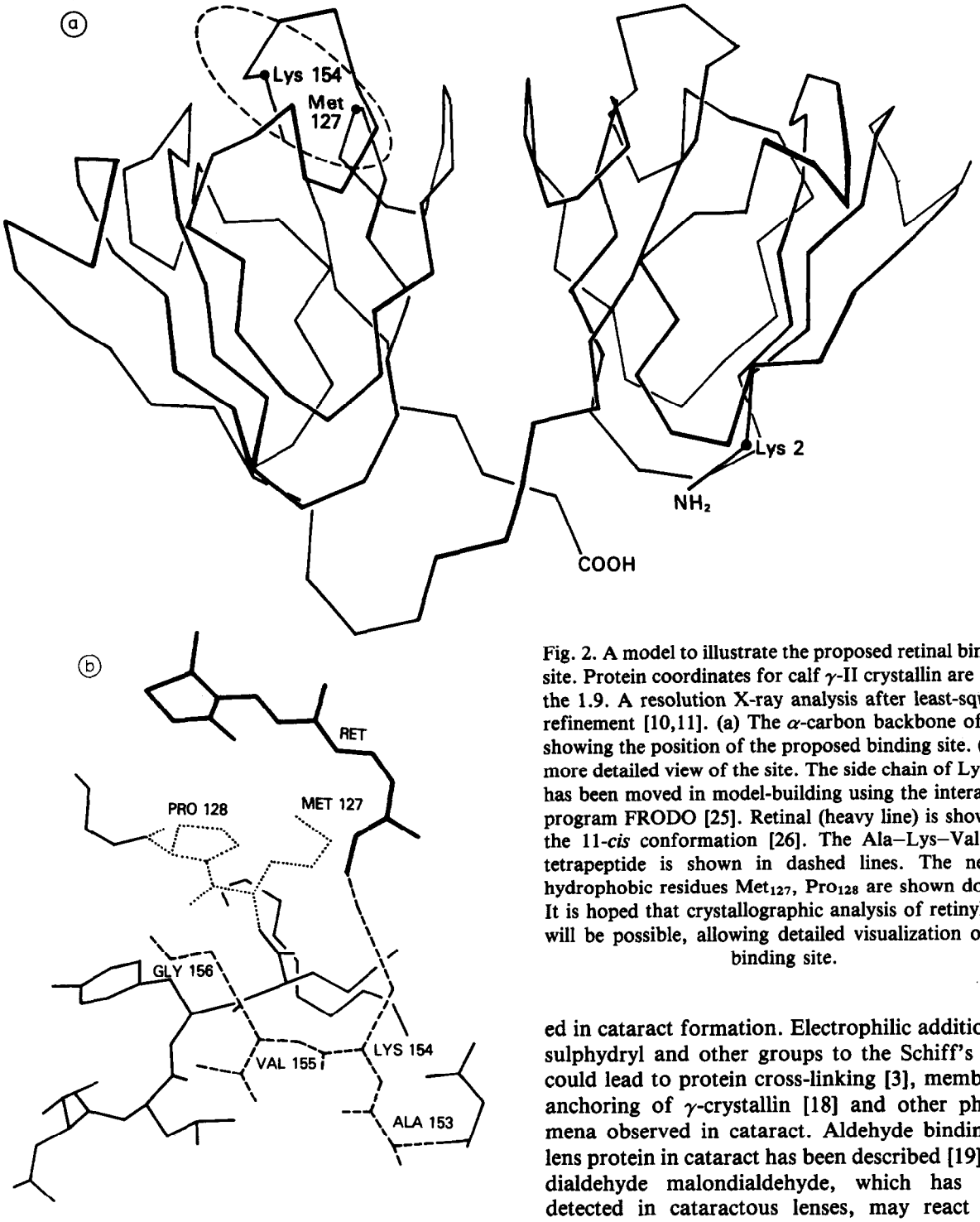

Fig. 2. A model to illustrate the proposed retinal binding site. Protein coordinates for calf γ-II crystallin are from the 1.9. A resolution X-ray analysis after least-squares refinement [10,11]. (a) The α-carbon backbone of γ-II showing the position of the proposed binding site. (b) A more detailed view of the site. The side chain of Lys 154 has been moved in model-building using the interactive program FRODO [25]. Retinal (heavy line) is shown in the 11-*cis* conformation [26]. The Ala–Lys–Val–Gly tetrapeptide is shown in dashed lines. The nearby hydrophobic residues Met_{127}, Pro_{128} are shown dotted. It is hoped that crystallographic analysis of retinyl-γ-II will be possible, allowing detailed visualization of the binding site.

However, in more extreme circumstances γ-crystallin bound to aldehyde could become involved in cataract formation. Electrophilic addition of sulphydryl and other groups to the Schiff's base could lead to protein cross-linking [3], membrane anchoring of γ-crystallin [18] and other phenomena observed in cataract. Aldehyde binding to lens protein in cataract has been described [19]; the dialdehyde malondialdehyde, which has been detected in cataractous lenses, may react with crystallins leading to precipitation [20]. In the crystallographic analysis of γ-II crystallin glutaraldehyde was used to crosslink crystals and

this produced a yellow colour (unpublished), reminiscent of the yellowing of lenses with age.

Finally, it is interesting to note that Lys_{154} is contained in a tetrapeptide Ala–Lys–Val–Gly identical to the retinal binding site in bacteriorhodopsin [21]. γ-II has only 2 alanine and 2 lysine residues [6] so the homology has added significance. There does not seem to be any obvious reason why this sequence in itself should contribute to non-enzyme mediated retinal binding. In bovine and ovine opsins the 'binding sequences' differ, although both have Ala–Lys followed by two small residues [22–24]. It would seem more likely that tertiary structure is responsible for the formation of a binding site.

Starting with the known structure of γ-II, [9–11] computer graphics model building was used to investigate the proposed Lys_{154} aldehyde binding site. Close by are two solvent-exposed, non-polar residues Met_{127} and Pro_{128}. These could form a hydrophobic patch, stabilizing retinal binding by the elimination of entropically unfavourable contacts with water. The close proximity of the lysyl amino group and aldehyde would then favour reaction. Fig. 2 shows a model of this region of γ-II with retinal bound, demonstrating the disposition of residues and the relative size of the ligand. It is unlikely that retinal itself ever reaches high levels in the lens, except in cases where degeneration of the retina occurs. Shorter aliphatic aldehydes are the likely physiological ligands for the site.

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