ACADEMIC PRESS

Biochemical and Biophysical Research Communications 293 (2002) 440–445

BBRC

www.academicpress.com

ζ-Crystallin displays strong selectivity for salicylic acid over aspirin

Mohammad D. Bazzi*

Department of Biochemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia

Received 17 March 2002

Abstract

Interaction of camel lens ζ-crystallin with aspirin was investigated by activity and fluorescence measurements. Aspirin minimally inhibited the oxidoreductase activity of the enzyme and weakly quenched its fluorescence. However, significant fluorescence quenching of ζ-crystallin coincided with the appearance of a fluorescence signal characteristic of salicylic acid thereby raising the possibility that salicylic acid might have been the moiety responsible for inhibition and fluorescence quenching. Direct fluorescence measurements showed that ζ-crystallin had a much higher affinity for salicylic acid than aspirin (K_i of about 24 μM for salicylic acid versus 630 μM for aspirin). Salicylic acid was also far more effective in inhibiting ζ-crystallin than aspirin (K_i values were 23 μM versus 820 μM, respectively). Inhibition kinetics suggested that salicylic acid interacted with ζ-crystallin via a binding site that was distinct from that of NADPH. Salicylic acid also interacted with and quenched the fluorescence of camel lens α-crystallin suggesting a general mode of interaction with lens proteins. Within the normal therapeutic concentrations of salicylic acid or aspirin, only crystallin–salicylic acid interactions might be significant. These results showed that camel lens ζ- and α-crystallin exhibited remarkable selectivity for salicylic acid over aspirin, and thus, could be considered as salicylate-binding proteins.

Keywords: ζ-Crystallin; α-Crystallin; Aspirin; Salicylate-binding proteins; Cataract; Protein fluorescence

ζ-Crystallin (EC 1.6.5.5) is a taxon-specific enzyme/crystallin constituting about 10% of the soluble proteins of camel, llama or guinea pig lenses [1,2]. The enzyme exhibits an NADPH–quinone oxidoreductase activity as it catalyzes the reduction of certain quinones in the presence of NADPH [3]. Structurally, ζ-crystallin belongs to the alcohol dehydrogenase superfamily, even though it lacks the alcohol dehydrogenase activity and the zinc-binding loop [4].

The physiological significance of ζ-crystallin as a lens crystallin is not clear. It has been suggested that ζ-crystallin might function as a detoxification tool via a variety of mechanisms [3]. For example, ζ-crystallin exhibits high affinity for NADPH [5], and thus, may function as an NADPH-binding protein to keep the lens environment in a reduced form. However, the significance of the oxidoreductase activity of the enzyme remains unclear; ζ-crystallin can reduce quinones and other oxidants, but the reaction may produce oxygen radicals under aerobic conditions [3]. Thus, reagents that inhibit the enzyme may enhance its proposed function as a detoxification tool.

A number of studies suggested a possible link between ζ-crystallin and cataract formation in guinea pigs [6–8]. Several in vitro and in vivo studies have implicated aspirin as a factor in protecting against or delaying the onset of cataract [9–16], even though clinical trials have indicated that aspirin consumption had little or no benefits for cataract development in human [17–20]. Aspirin is reported to react with a variety of proteins, often by acetylating lysine residues [16,21–23], and thereby preventing more harmful posttranslational modifications [21,24,25]. Interestingly, acetylation of proteins by aspirin generates salicylic acid, which may function as a free-radical trapping agent in vivo. Selective binding of salicylic acid by ζ-crystallin would add a new dimension to its proposed role as a crystallin. To date, there are no reports of specific binding of salicylic acid to ζ-crystallin or other crystallins.

The present study was initiated to examine the interactions of camel lens crystallins with aspirin by fluorescence spectroscopy. The results suggested that ζ- as

* Fax: +966-1-467-5791.
E-mail address: mbazzi@ksu.edu.sa.

0006-291X/02/$ - see front matter
PII: S0006-291X(02)00248-6

well as α-crystallin had a much higher affinity for salicylic acid than aspirin and that salicylic acid inhibited the activity of ζ-crystallin. The great selectivity of lens crystallins for salicylic acid over aspirin suggested that these crystallins probably had a specific binding site for salicylate. These results may have significant implications for the proposed functions of aspirin in the lens.

Materials and methods

NADPH, 9, 10-phenanthrenequinone (PQ), aspirin, and salicylic acid were purchased from the Sigma Chemical, St. Louis, MO, USA. Suparose 12 HR column was purchased from Amersham-Pharmacia, Uppsala, Sweden. Other chemicals were of the highest purity available. Camel eyes were obtained from a local abattoir.

Solutions of NADPH, PQ, aspirin, and salicylic acid were prepared freshly and their concentrations were determined by absorption using the molar extinction coefficients of 6220 M^{-1} cm^{-1} at 340 nm, 31,623 M^{-1} cm^{-1} at 252 nm, 1286 M^{-1} cm^{-1} at 275 nm, and 7079 M^{-1} cm^{-1} at 236 nm, respectively [26].

Protein purification. ζ-Crystallin was purified from camel lens as described previously [27]. The enzyme was assayed in 20 mM phosphate buffer, pH 7.8, containing 0.2 mM EDTA, 100 μM NADPH, 25 μM PQ, and 0.5 μg enzyme in a final volume of 1.0 ml at 22 °C. The reaction was initiated by the addition of PQ and the decrease in absorbance at 340 nm was recorded. Blanks lacking either enzyme or substrate were run routinely.

α-Crystallin was purified from the same lenses used for ζ-crystallin purification. The αL-crystallin peak was pooled, concentrated, and applied on Suparose 12 HR gel filtration column (1 × 30 cm). The purified protein consisted of αLA- and αLB-crystallin subunits and was used as such without further fractionations.

Inhibition of ζ-crystallin. Activity of the enzyme (0.5 μg) was measured as a function of the added ligand in the presence of 100 μM NADPH and 25 μM PQ (1.0 ml final volume). The residual activity of the enzyme was determined in each case and expressed as a percentage of that obtained in the absence of the ligand. The tested ligands included aspirin, salicylic acid (2-hydroxybenzoic acid), 3-hydroxybenzoic acid, benzoic acid, and phenol.

Double reciprocal plots were used to determine the kinetics of salicylic acid inhibition as a function of NADPH. These experiments were conducted by measuring the ζ-crystallin activity as a function of NADPH (10–100 μM) at constant PQ (25 μM) and salicylic acid (0, 20, 50, or 75 μM). All measurements were carried out in duplicates. The inhibition constant (K_i) was calculated by plotting the apparent intercepts versus salicylic acid.

Fluorescence measurements. Steady-state fluorescence measurements were conducted using 1.6 ml buffer containing 20 mM sodium phosphate, pH 7.8, 0.2 mM EDTA, and 1 μM protein. Fluorescence spectra were obtained by exciting tryptophan (284 nm) and scanning the emission intensity (290–450 nm). Protein–ligand binding was measured as a function of the added ligand (aspirin or salicylic acid) at fixed protein concentration (1 μM). Control experiments lacking the protein were run simultaneously with each assay. Saturation curves of ligand–crystallin binding were determined by calculating the area of protein emission (between 290 and 350 nm) as a function of the added ligand. Binding was reported as fluorescence quenching using the following formula: $Q\% = (F_0 - F_i) \times 100/F_0$, where F_0 and F_i are the calculated areas of protein emission in the absence and presence of ligand, respectively. The contribution of the ligand to the fluorescence was considered in all cases.

All fluorescence measurements were conducted at 22 °C on Jasco FP750 spectrofluorometer attached to a PC. Data collection and manipulation were carried out using manufacturer's software.

Other methods. Protein was determined either by the Bradford method [28] using bovine serum albumin as a standard or by absorption using the extinction coefficient of 1.34 for 0.1% ζ-crystallin at 280 nm [27]. Binding and/or inhibition parameters were determined by non-linear least squares fitting of the data to the general rate equation $Y_i = Y_{max}X_i/(K_i + X_i)$, where Y_i was the magnitude of inhibition/quenching at ligand concentration X_i, Y_{max} was the maximum binding (inhibition), and K_i was the binding (inhibition) constant. All data manipulations were performed using Kaleidagraph software.

Results

Fig. 1A shows fluorescence spectra of ζ-crystallin in the absence or presence of aspirin. Increasing the concentration of aspirin appeared to slightly quench the fluorescence of ζ-crystallin. Quenching of the fluorescence (if any) was instantaneous with the addition of aspirin and did not change the position or the shape of the fluorescence suggesting a simple binding event (Fig. 1A). The magnitude of fluorescence quenching was dependent on the concentration of aspirin (Fig. 1B).

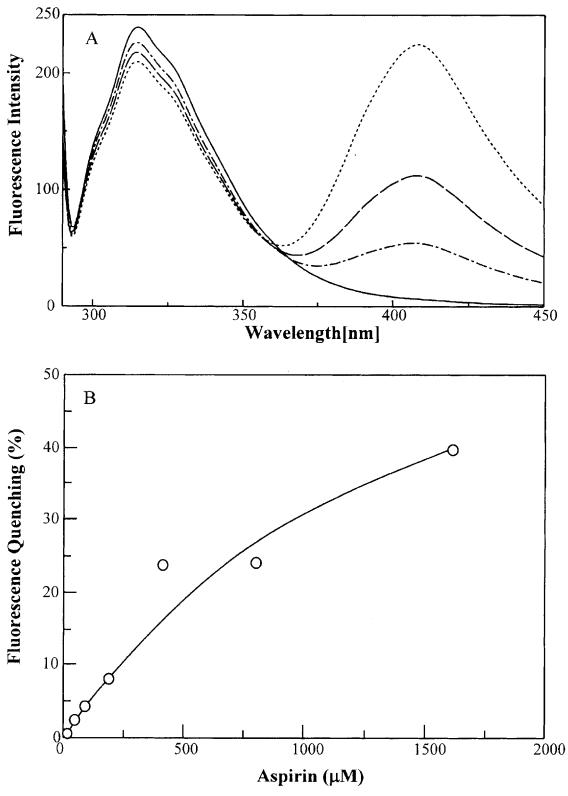

Fig. 1. Effect of aspirin on the fluorescence of ζ-crystallin. Panel A shows the emission spectra of ζ-crystallin ($\lambda_{ex} = 284$ nm) in the presence of 0 (solid line), 50 μM (dashed-dotted line), 100 μM (dashed line), or 200 μM aspirin (dotted line). Panel B shows the magnitude of fluorescence quenching as a function of aspirin. The area corresponding to protein fluorescence (295–350 nm region) was calculated and plotted as fluorescence quenching as described in the Method section. In both panels, fluorescence was measured using 1.6 ml phosphate buffer (20 mM), pH 7.8, containing 0.2 mM EDTA and 1 μM ζ-crystallin. All measurements were conducted at 22 °C.

Aspirin appeared to quench about 40% of the fluorescence signal with a K_i of about 630 μM.

Significant fluorescence quenching by aspirin coincided with the appearance of a strong fluorescence signal at 409 nm (Fig. 1A). This fluorescence signal is characteristic of salicylic acid, since aspirin does not fluoresce in this region. This raised the possibility that salicylic acid might have been the active moiety in quenching the fluorescence of ζ-crystallin. Fig. 2A shows fluorescence spectra of ζ-crystallin in the presence of selected concentrations of salicylic acid. Salicylic acid significantly quenched the fluorescence of ζ-crystallin without altering its shape or position. Fig. 2B shows quantitative analysis of fluorescence quenching as a function of salicylic acid. Salicylic acid quenched about 60% of ζ-crystallin fluorescence with a K_i of about 24 μM.

The above results suggested that ζ-crystallin had a much higher affinity for salicylate over aspirin and it was possible that the fluorescence quenching observed in Fig. 1 was induced by small amounts of salicylic acid present in aspirin preparations. Based on the magnitude of fluorescence at 409 nm, it was estimated that aspirin preparations had undergone about 1% hydrolysis. It was of concern that aspirin–crystallin interaction might have accelerated the hydrolysis of aspirin; aspirin is known to acetylate many proteins and such a reaction would generate salicylic acid. To examine this possibility, relative rates of aspirin hydrolysis in the presence or absence of lens proteins were examined by monitoring the fluorescence of salicylic acid. Surprisingly, the rate of aspirin hydrolysis in the presence of ζ-crystallin, crystallin, or lens homogenate was significantly lower than that of the control (Fig. 3). By contrast, the presence of BSA slightly increased the rate of aspirin hydrolysis. Aspirin has been reported to acetylate serum albumin [22,29] and such a reaction may account for the increased rate of aspirin hydrolysis. The mechanism by which crystallins reduced the aspirin-hydrolysis rate is unknown.

Fig. 4A compares the impact of aspirin, salicylic acid, and a number of structurally related molecules on the activity of ζ-crystallin. In all the cases examined, the onset of inhibition was virtually instantaneous with the addition of inhibitors. Both aspirin and salicylic acid appeared to inhibit the enzyme, but salicylic acid was far more potent, causing a nearly complete inhibition at

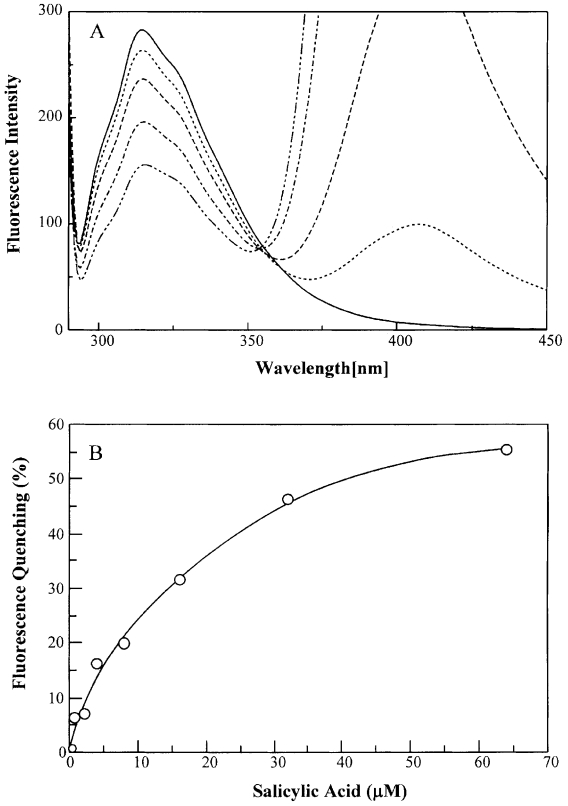

Fig. 2. Effect of salicylic acid on the fluorescence of ζ-crystallin. Panel A shows emission spectra of ζ-crystallin in the presence of 0 (solid line), 1 μM (dotted line), 4 μM (dashed line), 16 μM (dotted-dashed line), or 64 μM salicylic acid (dashed-triple dotted line). Panel B shows the magnitude of fluorescence quenching as a function of salicylic acid. All other parameters were as described in Fig. 1.

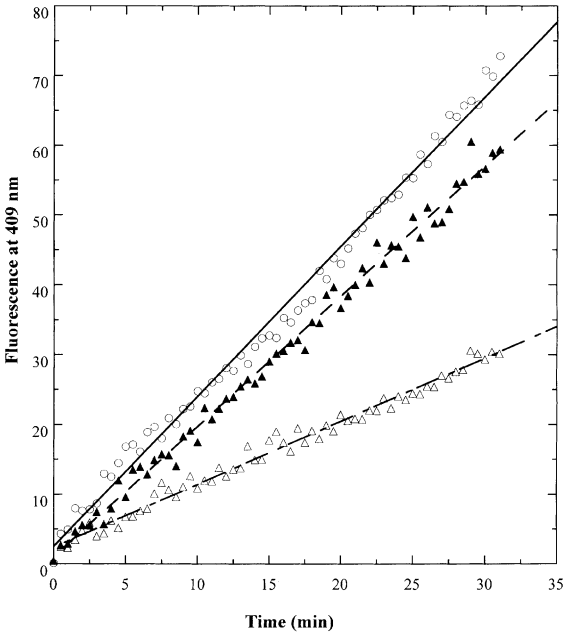

Fig. 3. Effect of lens proteins on aspirin hydrolysis. Relative rates of aspirin hydrolysis were measured by monitoring the fluorescence of salicylic acid as a function of time ($\lambda_{ex} = 294$ nm, $\lambda_{em} = 409$ nm). The fluorescence of 100 μM aspirin in 20 mM phosphate buffer, pH 7.8, was measured for samples containing aspirin alone (▲), aspirin plus 5 mg/ml BSA (○), or aspirin plus 5 mg/ml lens homogenate (△). Purified ζ- as well as α-crystallin preparations produced undistinguishable results from those of lens homogenate and were omitted for clarity. The lines were offset for the purpose of comparison and the ordinate scale corresponds to approximately 1 μM salicylic acid generated. All samples were monitored simultaneously using the parallel kinetics software package of Jasco Instruments.

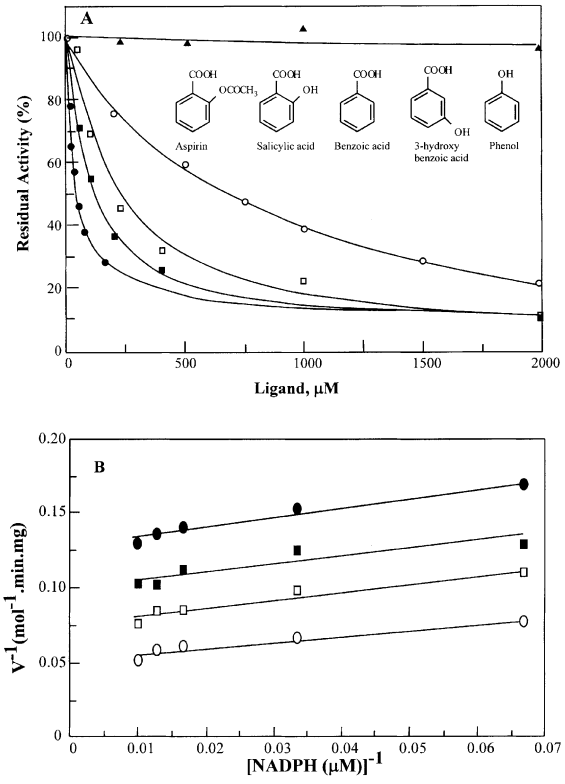

Fig. 4. Specificity of ζ-crystallin–salicylic acid interaction. Panel A shows inhibition of ζ-crystallin by salicylic acid and other structurally related ligands. Enzyme activity was measured a function of salicylic acid (●), aspirin (○), benzoic acid (□), 3-hydroxybenzoic acid (■), or phenol (▲) as described in the Method section. The residual activity was expressed as a percentage of that obtained in the absence of inhibitor. Panel B shows the Lineweaver–Burk plot of initial velocities (V^{-1}) versus 1/NADPH. The enzyme activity was measured as a function of NADPH in the presence of 0 (○), 20 μM (□), 50 μM (■), or 75 μM salicylic acid (●).

100 μM. Based on the observed K_i values for aspirin versus salicylic acid (820 μM versus 28 μM), it is possible that salicylic acid was the active moiety in both cases; hydrolysis of aspirin preparations by as little as 3% would produce enough salicylic acid to account for the inhibition pattern observed in Fig. 4. Benzoic acid was less efficient than salicylic acid ($K_i = 190$ M) in inhibiting the enzyme indicating the importance of free hydroxyl for ligand–enzyme binding. Phenol was ineffective as an inhibitor at all concentrations tested, and hence, the importance of the carboxyl group for enzyme-ligand recognition. Interestingly, salicylic acid (2-hydroxybenzoic acid) was more potent than 3-hydroxybenzoic acid (K_i values were 28 μM versus 110 μM) indicating the importance of substitution geometry. These results pointed to a specific interaction between ζ-crystallin and salicylic acid.

Inhibition kinetics of ζ-crystallin by salicylic acid was investigated (Fig. 4B). It was possible that salicylic acid interfered with NADPH binding to the enzyme. Fig. 4B shows Lineweaver–Burk plots of the enzyme activity as a function of NADPH in the presence of different concentrations of salicylic acid. Salicylic acid appeared to be an uncompetitive inhibitor with respect to NADPH, with a K_i of 60 μM; the latter was determined by plotting the intercepts of the primary plots versus salicylic acid. It should be noted that salicylic acid could not be classified as an uncompetitive inhibitor since it interacted with ζ-crystallin in the absence of NADPH (Fig. 2). The uncompetitive nature of inhibition may have arisen from the high affinity interaction between NADPH and ζ-crystallin so that virtually only NADPH–ζ-crystallin complex was available for interaction with salicylic acid [5]. Nevertheless, the uncompetitive nature of inhibition reinforced the conclusion that salicylic acid and NADPH probably had distinct binding sites.

Fig. 5 shows the interaction of α-crystallin with aspirin or salicylic acid as examined by fluorescence measurements. Aspirin (200 μM) did not alter the flu-

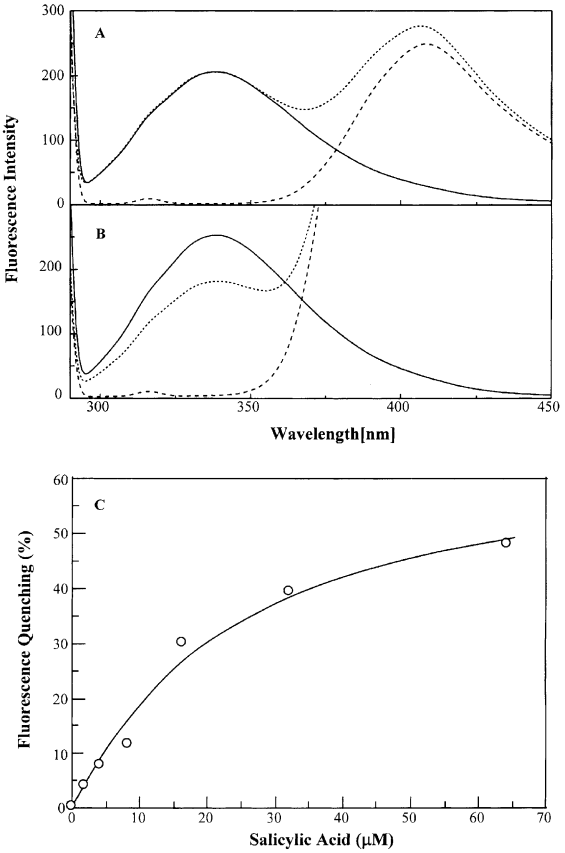

Fig. 5. Interaction of α-crystallin with salicylic acid. Panel A shows the effect of adding 200 μM aspirin on the emission spectra of 1 μM α-crystallin. Panel B shows the effect of adding 16 μM salicylic acid. In both panels, the fluorescence of protein alone (solid lines), protein plus ligand (dotted lines), and ligand alone (dashed lines) are shown. Panel C shows the magnitude of fluorescence quenching of α-crystallin as a function of salicylic acid. Other experimental conditions were as described in Fig. 1.

orescence of α-crystallin significantly, indicating weak (if any) binding event (Fig. 5A). Salicylic acid, however, significantly quenched the fluorescence of the protein (Fig. 5B). Fig. 5C quantitates fluorescence quenching of α-crystallin as a function of salicylic acid. Salicylic acid quenched about 60% of the protein fluorescence with a K_i of about 28 μM. These binding parameters were almost identical with those obtained with ζ-crystallin (compare Fig. 5C with 2B). It should be noted that the fluorescence of salicylic acid overlapped α-crystallin fluorescence (maximum at 340 nm) more than ζ-crystallin (maximum at 313 nm). Nevertheless, interaction of α-crystallin with salicylic acid was evident at low concentrations of salicylic acid, where fluorescence peaks did not overlap significantly (Fig. 5B).

Discussion

Activity and fluorescence measurements indicated that ζ-crystallin had a remarkable selectivity for salicylic acid over aspirin (Figs. 2 and 4). The selectivity for salicylic acid may actually be greater than that implicated in Figs. 2 and 4, since it is virtually impossible to have aqueous preparations of aspirin that are free of salicylate. Considering the structural similarities between aspirin and salicylic acid, the great selectivity may have risen from a specific interaction between ζ-crystallin and salicylic acid; simple charge or hydrophobic interactions could not account for such selectivity. Both the carboxyl and the hydroxyl groups of salicylic acid as well as their relative positions were important for protein–ligand recognition (Fig. 4A). A possible interpretation was that ζ-crystallin had a specific binding site for salicylic acid. α-Crystallin probably had a similar site, since it also interacted with salicylic acid preferentially over aspirin. β-Crystallin but not γ-crystallin also exhibited similar results. The affinity of crystallin–salicylic acid interaction (approximately 24 μM for both α- and ζ-crystallins; Figs. 2 and 5) is well within the therapeutic concentrations of salicylic acid (∼100–500 μM).

The ability of crystallins to bind salicylic acid may have a significant physiological impact. Salicylic acid has extensively been used as a trapping agent for hydroxyl radicals in vivo and in vitro [30–34]. Specific binding of α-, β-, and/or ζ-crystallin to salicylic acid would probably provide a major center for trapping radicals in the lens. There are reports that lens may retain and/or accumulate salicylate [35–37]. Crystallin–salicylate binding would probably concentrate or prolong the presence of salicylic acid in the lens thereby extending its usefulness as a radical-trapping agent.

The strong inhibition of ζ-crystallin by salicylic acid may actually enhance its proposed radical-trapping capability. ζ-Crystallin has been reported to catalyze the reduction of quinones via a one-electron transfer mechanism, which under aerobic conditions would generate only oxygen radical at the expense of available NADPH [3]; oxygen would reoxidize the resulting semiquinone back to quinone and generate an oxygen radical; the oxidoreductase activity of ζ-crystallin would be beneficial as a detoxification tool in anaerobic conditions only.

It is perhaps important to contrast the specificity of ζ-crystallin binding to NADPH versus salicylic acid. Like certain other enzymes, ζ-crystallin exhibits great selectivity for NADPH over NADH [3,5]. An important factor for such a selectivity is the presence of an essential lysine residue in the NADPH-binding site, even though a number of other factors may also contribute to this selectivity [38]. It is very likely that the presumed salicylic acid-binding site also had an essential lysine residue; both the hydroxyl and carboxyl groups of salicylic acid were major recognition factors (see above). Inhibition kinetics (Fig. 4B) suggested that salicylic acid was an uncompetitive inhibitor with respect to NADPH, and thus, probably had a distinct binding site. The salicylate-binding site is probably far less defined than that of NADPH; there are little structural similarities between α- and ζ-crystallins, yet both proteins bound salicylic acid with similar affinities.

Acknowledgments

I thank Dr. Ali S. Duhaiman and Dr. Nayyar Rabbani for their valuable help.

References

[1] Q.L. Huang, P. Russell, S.H. Stone, J.S. Zigler Jr., Curr. Eye Res. 6 (1987) 725–732.
[2] D. Garland, P.V. Rao, A. Del Corso, U. Mura, J.S. Zigler Jr., Arch. Biochem. Biophys. 285 (1991) 134–136.
[3] P.V. Rao, C.M. Krishna, J.S. Zigler Jr., J. Biol. Chem. 267 (1992) 96–102.
[4] T. Borras, B. Persson, H. Jornvall, Biochemistry 28 (1989) 6133–6139.
[5] M.D. Bazzi, N. Rabbani, A.S. Duhaiman, Biochim. Biophys. Acta 1544 (2001) 283–288.
[6] P.V. Rao, J.S. Zigler Jr., Exp. Eye Res. 54 (1992) 627–630.
[7] T. Borras, H. Jornvall, A. Rodokanaki, P. Gonzalez, I. Rodriguez, C. Hernandez-Calzadilla, Exp. Eye Res. 50 (1990) 729–735.
[8] Q.L. Huang, X.Y. Du, S.H. Stone, D.F. Amsbaugh, M. Datiles, T.S. Hu, J.S. Zigler Jr., Exp. Eye Res. 50 (1990) 317–325.
[9] G.V. Shastri, M. Thomas, A.J. Victoria, R. Selvakumar, A.S. Kanagasabapathy, K. Thomas, Lakshmi, Indian J. Exp. Biol. 36 (1998) 651–657.
[10] S.K. Gupta, S. Joshi, R. Tandon, P. Mathur, Indian J. Ophthalmol. 45 (1997) 221–225.
[11] A. Hasan, J.B. Smith, W. Qin, D.L. Smith, Exp. Eye Res. 57 (1993) 29–35.
[12] R. Blakytny, J.J. Harding, Exp. Eye Res. 54 (1992) 509–518.
[13] M.S. Swamy, E.C. Abraham, Inv. Ophthalmol. Vis. Sci. 30 (1989) 1120–1126.

[14] J.J. Harding, M. Egerton, R.S. Harding, Acta Ophthalmol. (Copenh) 67 (1989) 518–524.
[15] J.J. Harding, R. van Heyningen, Br. J. Ophthalmol. 72 (1988) 809–814.
[16] M. Crompton, K.C. Rixon, J.J. Harding, Exp. Eye Res. 40 (1985) 297–311.
[17] W.G. Christen, U.A. Ajani, D.A. Schaumberg, R.J. Glynn, J.E. Manson, C.H. Hennekens, Arch Ophthalmol. 119 (2001) 405–412.
[18] W.G. Christen, J.E. Manson, R.J. Glynn, U.A. Ajani, D.A. Schaumberg, R.D. Sperduto, J.E. Buring, C.H. Hennekens, Ophthalmic Epidemiol. 5 (1998) 133–142.
[19] E.Y. Chew, G.A. Williams, T.C. Burton, F.B. Barton, N.A. Remaley, F.L. Ferris III, Arch. Ophthalmol. 110 (1992) 339–342.
[20] J.M. Seddon, W.G. Christen, J.E. Manson, J.E. Buring, R.D. Sperduto, C.H. Hennekens, Arch. Ophthalmol. 109 (1991) 252–255.
[21] H.F. Bunn, K.H. Gabbay, P.M. Gallop, Science 200 (1978) 21–27.
[22] D. Hawkins, R.N. Pinckard, R.S. Farr, Science 160 (1968) 780–781.
[23] R.N. Pinchard, D. Hawkins, R.S. Farr, Nature 219 (1968) 68–69.
[24] M.M. Heath, K.C. Rixon, J.J. Harding, Biochim. Biophys. Acta 1315 (1996) 176–184.
[25] A. Stevens, J. Am. Optom. Assoc. 66 (1995) 744–749.
[26] CRC, CRC Handbook of Chemistry and Physics, CRC Press, FL.
[27] A.S. Duhaiman, N. Rabbani, A.A. AlJafari, A.S. Alhomida, Biochem. Biophys. Res. Commun. 215 (1995) 632–640.
[28] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[29] J.W. Burch, B. Blazer-Yost, Thromb. Res. 23 (1981) 447–452.
[30] C. Coudray, A. Favier, Free Radic. Biol. Med. 29 (2000) 1064–1070.
[31] A.R. Tabatabaei, F.S. Abbott, Free Radic. Biol. Med. 26 (1999) 1054–1058.
[32] A. Ghiselli, Methods Mol. Biol. 108 (1998) 89–100.
[33] T.C. Dinis, V.M. Maderia, L.M. Almeida, Arch. Biochem. Biophys. 315 (1994) 161–169.
[34] H. Kaur, B. Halliwell, Methods Enzymol. 233 (1994) 67–82.
[35] P. Valeri, L. Romanelli, B. Martinelli, A. Guglielmotti, B. Catanese, Lens Eye Toxic. Res. 6 (1989) 465–475.
[36] P. Valeri, L. Romanelli, L. De Paolis, B. Martinelli, J. Pharm. Pharmacol. 40 (1988) 823–824.
[37] E. Cotlier, Y.R. Sharma, T. Niven, M. Brescia, Am. J. Med. 74 (1983) 83–90.
[38] K.J. Edwards, J.D. Barton, J. Rossjohn, J.M. Thorn, G.L. Taylor, D.L. Ollis, Arch. Biochem. Biophys. 328 (1996) 173–183.