INHIBITION OF CAMEL LENS ζ-CRYSTALLIN/NADPH:QUINONE OXIDOREDUCTASE ACTIVITY BY CHLOROPHENOLS

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Chlorophenols comprise a major class of environmental contaminants. They are extensively used as insecticides, fungicides, mold inhibitors, antiseptics and disinfectants. We found some of these compounds to be strong inhibitors of ζ-crystallin. This oxidoreductase enzyme was isolated from camel lens and its enzymatic activity was inhibited by the chlorophenols tested in a time-independent but concentration-dependent manner. 2,4,5-Trichlorophenol was the most potent inhibitor ($IC_{50} = 3\,\mu M$; $K_i = 3.2\,\mu M$) whereas 4-chlorophenol was the least potent ($IC_{50} = 4.1$ mM). There appeared to be a relationship between the degree of chlorination of the phenols and inhibition of ζ-crystallin activity. The position of the chlorine substituent on the phenol may also influence the potency of these compounds.

INTRODUCTION

Chlorophenols comprise a major class of widely distributed and frequently occurring environmental contaminants. They are extensively used as insecticides, fungicides, mold inhibitors, antiseptics and disinfectants.[1] Chlorophenols are potent uncouplers of mitochondrial oxidative

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phosphorylation,[2,3] inhibitors of a microsomal mixed-function oxidase,[4,5] and a heme-copper respiratory oxidase (ubiquinol-1) in *Echerichia coli*.[6] Chlorophenols are toxic substances that can cause chloracne and liver damage in human.[1] They also have adverse effects on embryonic and foetal development.[7] A series of chlorophenols were reported to inhibit sperm motion in a concentration-dependent manner.[8]

In a previous study we reported the isolation of a novel homotetramer enzyme that has NADPH : quinone oxidoreductase activity, the "ζ-crystallin" from the camel lens; this enzyme constitutes about 10% of the total protein of the camel[9,10] and or guinea pig[11] lenses. It is also present at low enzymatic levels in other tissues including liver and kidney, indicating that ζ-crystallin must have an important metabolic role. The enzyme is presumed to have a structural role in the lens.[12,13] A complete amino acid sequence of ζ-crystallin deduced from cDNA of the guinea pig showed this enzyme to be related to alcohol dehydrogenases although it lacks alcohol dehydrogenase activity and the zinc binding site.[14] It has been shown that a mutation in the ζ-crystallin gene is associated with autosomal dominant congenital cataract in guinea pig and thus a similar mutant gene could be responsible for the human congenital cataract.[12]

Much information about catalytic activity of an enzyme can be gained from inhibition studies especially if the enzyme has a limited range of substrates, as is the case of ζ-crystallin.[15] In order to elucidate the nature of the active site of ζ-crystallin a series of inhibition kinetic studies were recently carried out in our laboratory.[16-18]

In this study we investigated the structure-activity relationship of the inhibition of camel lens ζ-crystallin by chlorophenols. Results showed that modification of certain regions of the phenol molecules were essential for inhibition of oxidoreductase activity of the enzyme.

MATERIALS AND METHODS

NADPH and 9,10-phenanthrenequinone (PQ) were purchased from the Sigma Chemical Company, St. Louis, USA. Chlorophenols were purchased from BDH Chemicals, Poole, England. All other chemicals were of analytical grade.

Preparation and Assay of Camel Lens ζ-Crystallin

ζ-Crystallin was purified from camel lens as described previously.[10] The NADPH : quinone oxidoreductase activity of the ζ-crystallin was determined

according to the procedure of Rao *et al.*[15] The assays were conducted at 22°C in 0.1 M Tris-HCl pH 7.8 containing 0.2 mM EDTANa$_2$ (assay buffer), 100 μM NADPH and 25 μM PQ in a final volume of 1.0 ml. The reaction was initiated by the addition of 0.5 μg of purified enzyme and the decrease in absorbance at 340 nm was followed using a Perkin Elmer Lamda 3B dual path spectrophotometer. Blanks lacking either enzyme or substrate were run routinely. PQ was dissolved in absolute ethanol; chlorophenols not soluble in water were first dissolved in 0.5 ml alcohol then assay buffer was added as required, (final alcohol concentration was c. 5% v\v). The final concentration of alcohol in the assay mixture did not exceed 1%, a concentration that had no effect on enzyme activity. A molar extinction coefficient of 6220 M^{-1} cm^{-1} for NADPH was used for the determination of enyme activity. A unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmole of NADPH min^{-1} mg protein^{-1}.

To determine the inhibitory effect of each of the chlorophenols, ζ-crystallin was pre-incubated with and without the chlorophenol at 22°C, pH 7.8 for 5 min prior to the addition of NADPH and PQ solutions. The residual enzyme activity was determined as mentioned above.

Kinetics Studies

Kinetics analyses were determined as a function of NADPH concentration (12.5–100 μM) using three concentrations for each of the chlorophenols tested, and 25 μM PQ. The nature of the inhibition produced by each set of experiments was determined by double reciprocal plots of initial velocity versus NADPH concentration. The primary and secondary plots were obtained using the Grafit computer program.[19]

Protein Determination

The protein content was determined by the method of Bradford[20] using bovine serum albumin as standard.

RESULTS AND DISCUSSION

A number of chlorinated phenols and some related compounds were assessed for their inhibitory effect on camel lens ζ-crystallin. All the chlorophenols tested inhibited the enzyme in a concentration-dependent manner (Figure 1). The onset of the inhibition was virtually instantaneous.
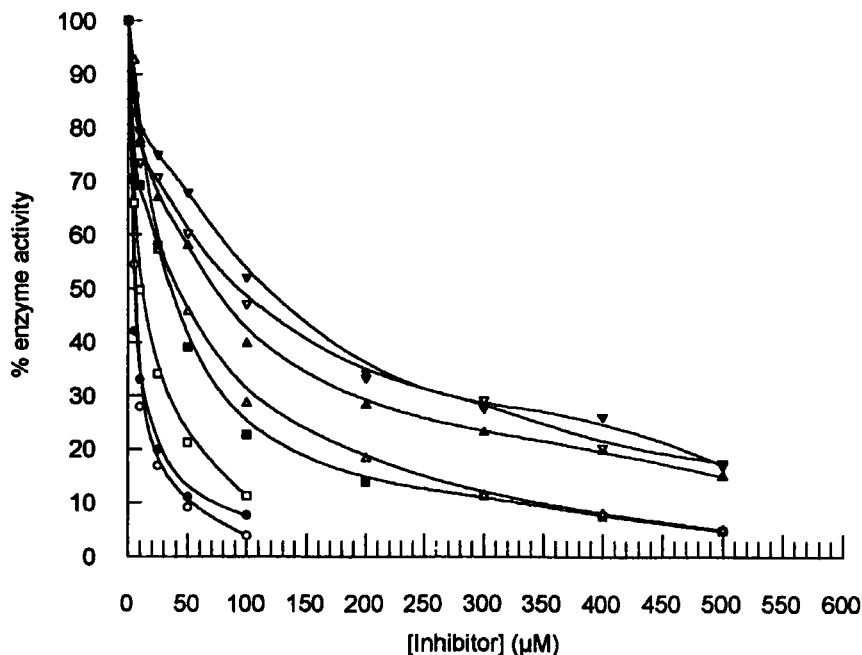
FIGURE 1   Camel lens ζ-crystallin inhibition as a function of chlorophenols concentration. The concentrations of NADPH and PQ were 100 μM and 25 μM respectively. ○ 2,4,5-Tri-chlorophenol, ● 2,3,5-Trichlorophenol, □ 2,3,6-Trichlorophenol, ■ 3,4,5-Trichlorophenol, △ 2,3,4-Trichlorophenol, ▲ 3,5-Dichlorophenol, ▽ 2,3-Dichlorophenol, ▼ 2,4-Dichlorophenol.

2,4,5-Trichlorophenol (2,4,5-TCP) and 4-chlorophenol showed the highest and least inhibition with IC_{50} values of 3.0 μM and 4.1 mM respectively. The inhibition was reversible and independent of the pre-incubation period (data not shown). Phenol and resorcinol, which have no chlorine atom on the ring, showed no inhibition even at 5 mM.

The chlorophenols were divided into three categories mono, di- and tri-chlorophenols. The most effective chlorophenol for each category was 2-chlorophenol (2-CP), 3,5-dichlorophenol (3,5-DCP) and 2,4,5-trichloro-phenol respectively, as revealed by their IC_{50} values. These were used for studying the kinetics of the inhibition as a function of NADPH (12.5–100 μM) with constant concentration of PQ (25 μM). Lineweaver–Burk plots showed typical noncompetitive inhibition (Figure 2(A), (B) and (C)). The values of K_i, a measure of the binding strength of the inhibitor-enzyme complex, were determined from plots of the slopes derived from the primary Lineweaver–Burk plots versus concentrations of the inhibitors
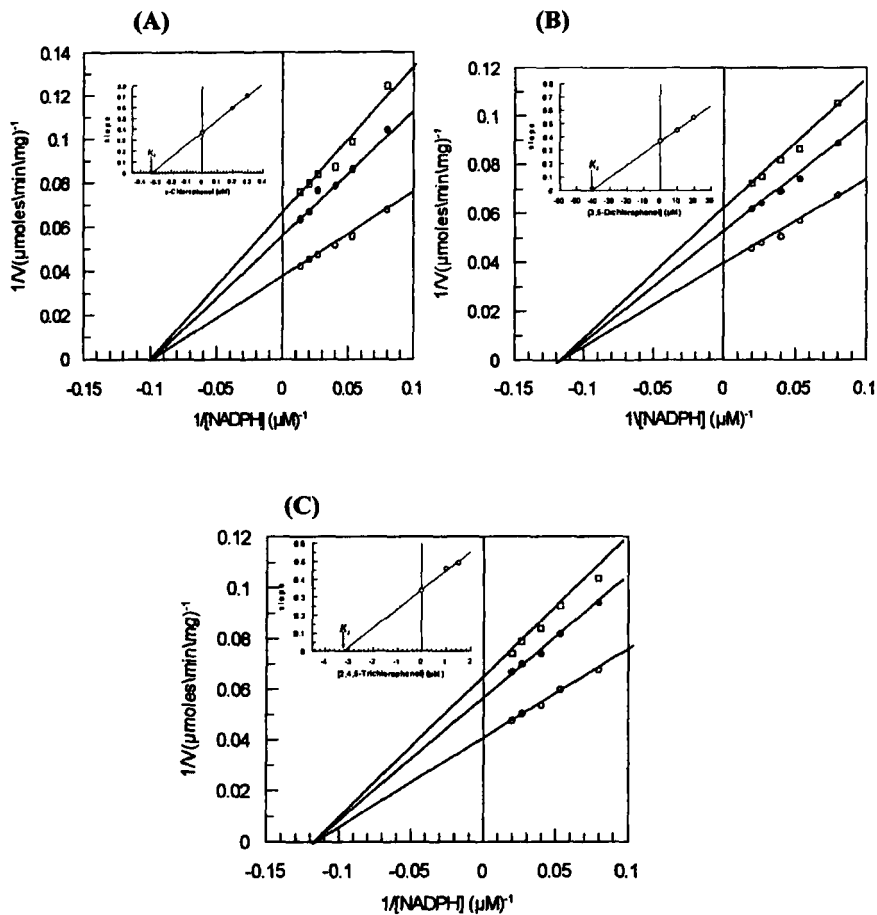
FIGURE 2 Lineweaver–Burk plot of initial velocity of camel lens ζ-crystallin activity (V^{-1}) versus [NADPH] (μM)$^{-1}$. (A) in the absence (○); presence of 0.2 mM (●) and 0.3 mM (□) 2-chlorophenol. (B) in the absence (○); presence of 10 μM (●) and 20 μM (□) 3,5-dichlorophenol. (C) in the absence (○); presence of 1.0 μM (●) and 1.5 μM (□) 2,4,5-trichlorophenol with various concentrations of NADPH (12.5–100 μM) and fixed concentration of phenanthrenequinone (25 μM). Insets show secondary replots of the slopes derived from the Lineweaver–Burk plots versus chlorophenol concentration.

(insets of Figure 2(A)–(C)). The K_i values of 2,4,5-trichlorophenol, 3,5-dichlorophenol and 2-chlorophenol were 3.2 μM, 40 μM and 320 μM respectively (Table I). These K_i values correlated well with the corresponding IC$_{50}$ values.

Neither NADPH nor PQ showed protection against the effect of chlorophenols on the enzyme (data not shown). The fact that chlorophenols showed noncompetitive inhibition with respect to NADPH as well

TABLE I   Inhibition of ζ-crystallin by Chlorophenols

Compound	IC$_{50}$	K$_i$	Type of inhibition
2-chlorophenol	500.0 μM	320.0 μM	Noncompetitive
3-chlorophenol	1.6 mM	ND	
4-chlorophenol	4.1 mM	ND	
3,5-dichlorophenol	65.0 μM	40.0 μM	Noncompetitive
2,3-dichlorophenol	92.0 μM	ND	
2,4-dichlorophenol	115.0 μM	ND	
2,4,5-trichlorophenol	3.0 μM	3.2 μM	Noncompetitive
2,3,5-trichlorophenol	4.5 μM	ND	
2,3,6-trichlorophenol	11.0 μM	7.0 μM	Noncompetitive
3,4,5-trichlorophenol	32.5 μM	ND	
2,3,4-trichlorophenol	40.0 μM	ND	
Aniline	-Nil-	—	
2-amino-4-chlorophenol	1.5 mM	ND	
4-chloro-m-cresol	4.5 mM	ND	
Phenol	-Nil-	—	
Resorcinol	-Nil-	—	

ND: Not determined.

as the lack of protection against the inhibitory effect by both NADPH and PQ, suggested that chlorophenols bind to site(s) of the enzyme other than NADPH or PQ binding sites. It appeared that a chlorinated aromatic ring and a hydroxyl group were required for the inhibition. Our earlier report[21] on the inhibition of ζ-crystallin by chloranilic acid also suggested that the presence of neither −Cl group alone nor −OH group alone was sufficient to produce inhibition. In this study, we observed a relationship between the degree of chlorination of the phenol and inhibition of the enzyme. 2,4,5-TCP was 100 times more effective than 2-CP (on the basis of K$_i$) and 12.5 times than 3,5-DCP. The position of the chlorine with respect to the hydroxyl group also appeared to be important; 2-CP was 3 and 8 times more effective than 3-CP and 4-CP respectively.

Chlorophenols are toxic substances and the major mode of action for the acute toxicity of chlorophenols involves uncoupling of oxidative phosphorylation and the inhibition of electron transport system. The effectiveness of chlorophenols in these systems is related to the number of chlorine atoms on the molecule and to a lesser extent by substituent positions on the molecule.[22] This led to several conclusions as to the importance of chlorine positions on the phenol and inhibition of enzymatic activities. In this study although the nature of inhibition of ζ-crystallin activity by the different chlorophenols tested was the same (noncompetitive), there is no consistency with regard to the position of the substituent and inhibition and, therefore, it is extremely difficult to make any general assertion. For

example with the monochlorinated phenols the order of the inhibition was ortho > meta > para, with the dichlorophenols it was meta–meta > ortho–meta > ortho–para indicating position 3 to be more effective than position 2. With the trichlorophenols, it is difficult to ascertain which position is more effective (Table I). The effect of additional functional groups on the inhibitory potency of chlorophenols was also examined. 2-amino-4-chloro-phenol is about 3 times more effective than 4-chlorophenol, whereas 4-chloro-m-cresol (4-chloro-3-methyl phenol) gave slightly lower inhibition than 4-chlorophenol. This indicates that the amino group has more influence than the methyl group on the effectiveness of the chlorophenols (Table I). Further investigations are needed to understand the mechanism by which chlorophenols inhibit ζ-crystallin.

Finally, it can be said that in addition to other toxic effects, exposure to chlorophenols may have some effect on the integrity of eye lens, at least in camel and guinea pig, since ζ-crystallin might play a very important role in the regulation of NADPH oxidation in the lens.[23]

References

[1] Roger, P.S. (Ed.) A Primer of Environmental Toxicology (1992) 98–112. Lea and Febiger Publishers, Philadelphia.
[2] Weinbach, E.C. (1954) *J. Biol. Chem.* **210**, 545–550.
[3] Loomis, W.S. (1949) *Fed. Proc.* **8**, 220.
[4] Arrhenius, E., Ronberg, L., Johansson, L. and Zetterqvist, M.A. (1977) *Chem. Biol. Interc.* **18**, 35–46.
[5] Aschmann, C., Stork, T. and Wasserman, O. (1989) *Arch-Toxicol.* **63**, 121–126.
[6] Watanabe, M.S., Mogi, T., Miyoshi, H., Iwamura, H., Matsushita, K., Adaachi, O. and Anraku, Y. (1994) *J. Biol. Chem.* **269**(46), 28899–28907.
[7] Zhao, F., Mayura, K., Hutchinson, R.W., Lewis, R.P., Burghardt, R.C. and Phillips, T.D. (1995) *Toxicol-Lett.* **78**(1), 35–42.
[8] Seibert, H., Kolossa, M. and Wassermann, O. (1989) *Cell-Biol-Toxicol.* **5**(3), 315–330.
[9] Garland, D., Rao, P.V., Delcorso, A., Mura, U. and Zigler, J.S. Jr. (1991) *Arch. Biochem. Biophys.* **285**, 134–136.
[10] Duhaiman, A.S., Rabbani, N., Aljafari, A.A. and Alhomida, A.S. (1995) *Biochem. Biophys. Res. Commun.* **215**, 632–641.
[11] Huang, Q.L., Russell, P., Stone, S.H. and Zigler, J.S. Jr. (1987) *Curr. Eye Res.* **6**, 725–732.
[12] Huang, Q.L., Du, X.Y., Stone, S.H., Amsbaugh, D.F., Datiles, M., Hu, T.S. and Zigler, J.S. Jr. (1990) *Curr. Eye Res.* **50**, 117–125.

[13] Gonzalez, P., Rao, P.V. and Zigler, J.S. Jr. (1993) *Biochem. Biophys. Res. Commun.* **191**, 902–907.
[14] Borras, T., Person, B. and Jornvall, H. (1989) *Biochemistry* **28**, 6133–6139.
[15] Rao, P.V., Krishna, C.M. and Zigler, J.S. Jr. (1992) *J. Biol. Chem.* **267**(1), 96–102.
[16] Duhaiman, A.S. (1996) *Biochem. Biophys. Res. Commun.* **218**, 648–652.
[17] Duhaiman, A.S. (1996) *J. Prot. Chem.* **15**(3), 261–264.
[18] Duhaiman, A.S. and Rabbani, N. (1996) *J. Enz. Inhib.* **11**, 13–21.
[19] Leatherbarrow, R.J. (1992) Grafit version 3.0, Erithacus Software Ltd. Staines, U.K.
[20] Bradford, M.M. (1976) *Anal. Biochem.* **72**, 248–254.
[21] Al-Hamidi, A.A.A., Shehu, R.A. and Duhaiman, A.S. (1997) *Biochem. Mol. Biol. Int.* **41**(2), 415–421.
[22] WHO-working group report (1989) in "Environmental Health Criteria" **93**, p. 208.
[23] Rao, P.V. and Zigler, J.S. Jr. (1990) *Biochem. Biophys. Res. Commun.* **167**, 1221–1228.