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

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Camel lens ζ-crystallin/NADPH:quinone oxidoreductase activity was inhibited by Cibacron blue 3GA (CB) with 9,10-phenanthrenequinone (PQ) as an electron acceptor and NADPH as an electron donor in a time-independent and concentration dependent manner. The IC_{50} value of CB was 50 nM. The Lineweaver-Burk plots and the secondary plots indicated that the inhibition was linear mixed type (partial competitive and pure noncompetitive) with respect to NADPH and noncompetitive with respect to PQ. The estimated inhibition constant (K_i) values were 26.0 nM for NADPH and 55.0 nM for PQ respectively, suggesting that CB has high affinity towards the NADPH binding site.

The secondary plots of inhibition with respect to NADPH, also indicate a dissociation constant (K_I) value of 68.0 nM for the ζ-crystallin-NADPH-CB complex. This K_I being greater than the K_i value suggests that noncompetitive inhibition is predominant over competitive inhibition at the NADPH binding site.

KEY WORDS: Camel lens, ζ-crystallin, Cibacron blue 3GA, inhibition kinetics

INTRODUCTION

Cibacron Blue 3GA (CB) has structural analogy to nucleotides and has a combination of both aromatic (nonpolar) and sulfonate (ionic) groups which give it the special characteristic of binding strongly at coenzyme-substrate sites of several enzymes in a non-specific mode.[1] CB possesses an ability to bind to selected proteins containing a specific structure called the "dinucleotide fold".[2,3] CB is a potent inhibitor of many nucleotide-dependent dehydrogenases and kinases including NAD(P)H:(quinone-acceptor) oxidoreductases (EC 1.6.99.2).[4,5] Quinone reductases are known to have several metabolic functions such as detoxification of quinone,[6–8] electron transport[9–11] and vitamin K metabolism.[12]

ζ-crystallin a "taxon-specific" crystallin is a major soluble protein (~ 10% of total protein) in guinea-pig and camel lenses and exists as a tetramer of 35 kD polypeptides.[13,14] From its cDNA sequence, it has been shown that ζ-crystallin is distantly related to the alcohol dehydrogenase family, although it lacks alcohol dehydrogenase activity.[15]

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ζ-crystallin catalyzes the obligatory one-electron reduction of quinones requiring NADPH as an electron donor, unlike most flavin containing NAD(P)H: quinone oxidoreductases, which catalyze two-electron reduction of quinones.[16–18] Xenobiotic induction of ζ-crystallin has been reported in the lens epithelial cells.[19] ζ-crystallin has been associated with autosomal dominant congenital cataracts in guinea-pigs and thus is a candidate for human congenital cataracts.[20]

This paper reports on the inhibition of camel lens ζ-crystallin by CB. The type of inhibition and the kinetic parameters were determined, which are essential for the elucidation of the relative role of ζ-crystallin in the lens.

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MATERIALS AND METHODS

Materials

NADPH, 9,10-phenanthrenequinone and Cibacron blue 3GA were purchased from Sigma Chemical Company, St. Louis, U.S.A. All other chemicals used were of analytical grade.

Methods

Preparation and assay of camel lens ζ-crystallin ζ-crystallin was purified from camel lens as described previously.[21] The NADPH:quinone oxidoreductase activity of the ζ-crystallin was determined according to the procedure of Rao *et al.*[22] in 0.1 M Tris-HCl, pH 7.8 containing 0.2 mM $EDTANa_2$ (assay buffer) and 0.1 mM NADPH, 25 μM PQ in a final volume of 1.0 ml at 22°C. The reaction was initiated by addition of 1μg of the purified enzyme and the decrease in absorbance at 340 nm was followed using a Perkin Elmer Lamda 3B, dual path, spectrophotometer at 22°C. Blanks lacking either enzyme or substrate were run routinely. PQ was dissolved in absolute ethanol and the final concentration of alcohol in the assay mixture was 1%. Presence of 1% ethanol does not affect the enzyme activity. A molar extinction coefficient of 6220 M^{-1} cm^{-1} for NADPH was used for the determination of enzyme activity. A unit of ζ-crystallin activity was defined as the amount of enzyme catalyzing the oxidation of 1μmole of NADPH per minute per mg of protein under specified conditions.

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

Effect of preincubation

CB (10, 50 and 100 nM) was preincubated with ζ-crystallin in a final volume of 1.0 ml assay buffer for different time intervals and then 100 μM NADPH and 25 μM PQ were added and the enzyme activity was determined as mentioned above.

To determine the protective effect of NADPH or PQ, ζ-crystallin and 100 μM NADPH were incubated for 5 min in a final volume of 1.0 ml assay buffer. Various concentrations of CB (0–200 nM) were added followed by 25 μM of PQ and the activity was determined. Similarly, ζ-crystallin and 25 μM PQ were preincubated for 5 min in a final volume of

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1.0 ml assay buffer. Various concentrations of CB (0–200 nM) were added followed by 100 μM NADPH and the enzyme activity was determined as mentioned above.

Kinetics studies

Kinetics analyses were conducted using three concentrations of CB with varying NADPH concentration (10–200 μM) and the concentration of PQ was held constant at 25 μM. Similarly, the concentration of PQ was varied from 5–40 μM and NADPH concentration was held constant at 100 μM. The nature of the inhibition produced by each set of experiments was determined by analysis of double-reciprocal plots of initial velocity versus NADPH or PQ concentration. The plots and their secondary plots were obtained using the GraFit computer program.[23]

Protein determination

The protein content was determined by the method of Bradford[24] using bovine serum albumin as standard.

RESULTS AND DISCUSSION

The inhibition of ζ-crystallin by CB was time-independent at all concentrations tested. The onset of inhibition was virtually instantaneous.

NADPH has been shown to protect guinea-pig lens ζ-crystallin and other oxidoreductases against loss of activity on incubation with sulfhydryl modifying reagents and H_2O_2.[18,23] Preincubation of ζ-crystallin with NADPH (100 μM) or PQ (25 μM) did not provide any protection to the camel lens ζ-crystallin from the inhibitory effect of CB.

The inhibitory effect of CB on the activity of ζ-crystallin is presented in Figure 1. CB at various concentrations (5–200 nM) inhibited ζ-crystallin activity (12.5–75%) in a concentration-dependent manner. Maximum inhibition was found at a concentration range between 5 and 60 nM of CB. The IC_{50} value of CB was 50 nM.

Strong inhibition of ζ-crystallin activity by CB at a nanomolar range depends on its structure analogy to nucleotides and may be the combination of aromatic (nonpolar) and sulfonate (ionic) groups which impart a special characteristic to the CB molecule[1] to bind effectively at the NADPH binding site of ζ-crystallin even in the presence of PQ substrate.

A Lineweaver-Burk plot of ζ-crystallin activity in the presence of different concentrations of CB (0, 10, 50, 100 nM) with varying NADPH concentration (10–200 μM) and 25 μM of PQ showed that the inhibition of CB was of the linear mixed type (Figure 2A). The value of the inhibition constant (K_i) was determined by replotting the slope values derived from the primary Lineweaver-Burk plot versus CB concentrations. K_i was found to be 26 nM with respect to NADPH (inset of Figure 2A). The replot also resulted in a straight line which does not pass through the origin confirming that the inhibition is of partial competitive type unlike pure competitive inhibition where the straight line passes through the origin.

Decrease in V_{max} as seen in Figure 2A is an indication of a noncompetitive type of inhibition. The pure noncompetitive nature of inhibition was confirmed by a secondary replot, in which $1/V_{max}$ was plotted against CB concentrations. The plot showed a straight

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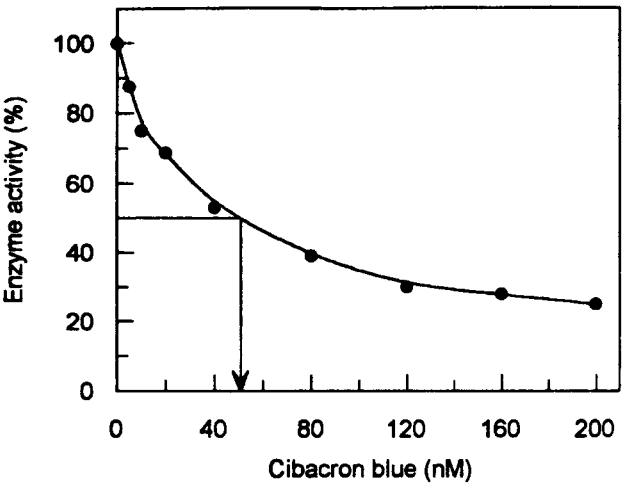

FIGURE 1 Camel lens ζ-crystallin inhibition as a function of CB concentration. The concentration of NADPH and PQ was 100 μM and 25 μM respectively. The arrow points to the IC_{50} value of CB.

line (Figure 3) and not a hyperbolic curve as expected in the case of partial noncompetitive type of inhibition. The K_I of CB was calculated from this plot. The K_I value was 68 nM, which was greater than K_i (26 nM) indicating that the noncompetitive inhibition was predominant over competitive inhibition. Therefore, the linear mixed-type of inhibition with respect to NADPH is composed of partial competitive and pure noncompetitive.[25]

Increase in the K_m values indicated that CB decreased NADPH affinity towards the NADPH binding site; this may be due to its resemblance to the NADPH structure and presence of hydrophilic and hydrophobic groups on the CB molecule which enhance its affinity towards the NADPH binding site of ζ-crystallin. The catalytic activity (V_{max}) is also affected which may be due to conformational changes in the ζ-crystallin molecule.

Whereas, with various concentrations of PQ (5–40 μM) and at 100 μM of NADPH, the Lineweaver-Burk plot showed that the inhibition of CB with respect to PQ was of pure noncompetitive type (Figure 2B). The K_i was determined from the secondary plot by replotting the slope values derived from the primary Lineweaver-Burk plot versus CB concentrations and was 55 nM for PQ binding site of ζ-crystallin (Inset of Figure 2B). CB contains an anthraquinone moiety which might resemble the quinone substrate and bind to the quinone binding site of ζ-crystallin. On the contrary, CB showed pure noncompetitive-type of inhibition with respect to PQ (K_i = 55 nM). Despite the presence of the anthraquinone moiety on CB, anthraquinone itself did not inhibit camel lens ζ-crystallin (data not shown), which may rule out any major role of this moiety in the inhibition of camel lens ζ-crystallin.

CB was more potent than dicoumarol as an inhibitor of ζ-crystallin guinea-pig lens[26] and camel lens (data not shown). However CB was second in potency to dicoumarol as an inhibitor of DT-diaphorase (NAD(P)H:quinone oxidoreductase, E.C. 1.6.99.2),[3] which indicates that camel lens ζ-crystallin is distinct from other oxidoreductases.

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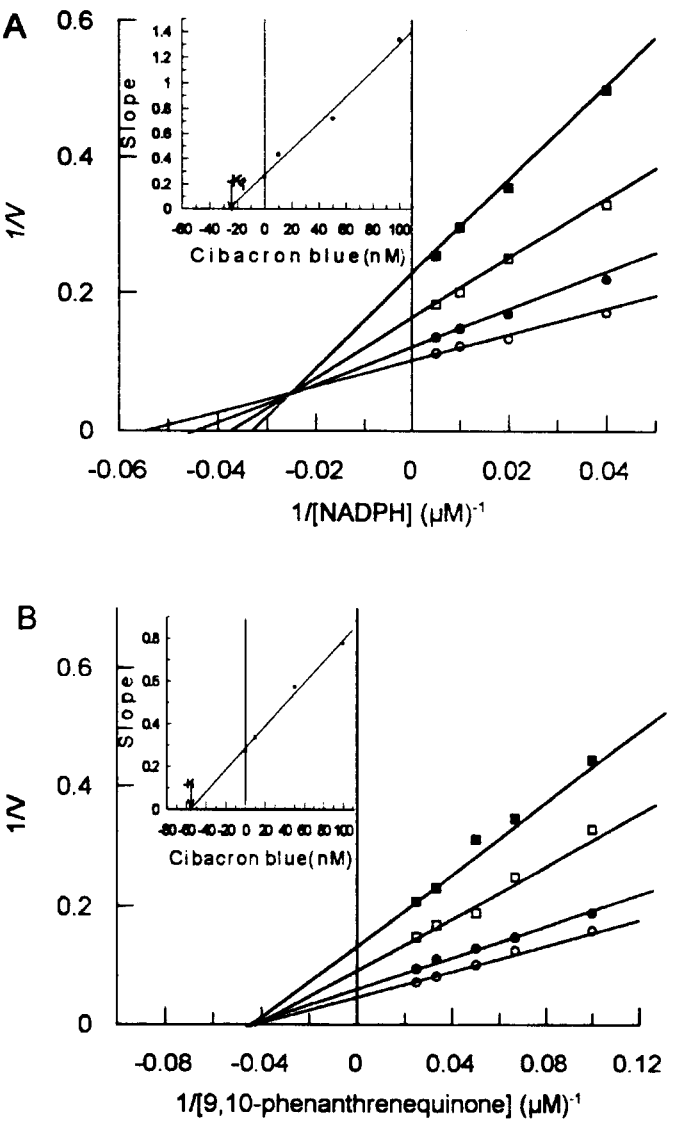

FIGURE 2 (A) Lineweaver-Burk plot of initial velocity of camel lens ζ-crystallin activity ($1/V$) versus 1/[NADPH] $(\mu M)^{-1}$ in the absence (○) and presence of 10 nM (●); 50 nM (□) and 100 nM (■) of CB with various concentrations of NADPH (10–200 μM) and fixed concentration of PQ (25 μM). Inset shows secondary replot of the slope values derived from the primary Lineweaver-Burk plot vs. CB concentrations. (B) Lineweaver-Burk plot of initial velocity of camel lens ζ-crystallin activity, ($1/V$) vs. 1/[9,10-phenanthrenequinone] $(\mu M)^{-1}$ in the absence (○) and presence of 10 nM (●); 50 nM (□) and 100 nM (■) of CB with various concentrations of PQ (5–40 μM) and a fixed concentration of NADPH (100 μM). Inset shows a secondary replot of the slope values derived from the primary Lineweaver-Burk plot vs. CB concentrations.

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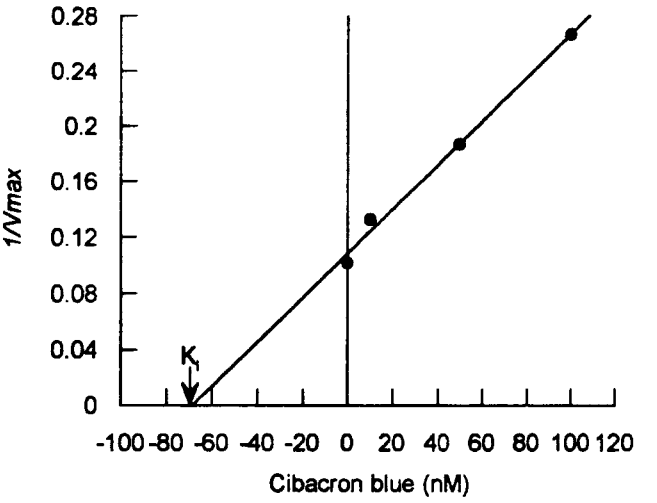

FIGURE 3 Secondary replot of $1/V_{max}$ values derived from the primary Lineweaver-Burk plot vs. CB concentrations for the determination of K_I.

The physiological significance of ζ-crystallin is not yet understood, although it might be involved in detoxification of quinones,[22] or have a role in regulation of NADPH oxidation in the lens.[27] The presence of ζ-crystallin in liver and kidney tissues in an enzyme level supports its metabolic function in these tissues.[28]

Furthermore, association of a mutation in the ζ-crystallin gene with an autosomal dominant cataract in which the mutant ζ-crystallin is unable to bind to NADPH suggests that ζ-crystallin may have an important role in the lens which may be associated with the binding and storage of NADPH apart from its quinone reductase activity.[27]

In the absence of the three-dimensional structure of ζ-crystallin, inhibition by CB should be useful in designing further studies on the structure-function relationship, particularly at the NADPH binding site of the lens ζ-crystallin.

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