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INHIBITION KINETICS OF CAMEL LENS ζ-CRYSTALLIN: MULTIPLE INHIBITION STUDIES

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The inhibition of camel lens ζ-crystallin by nitrofurantoin (NF) was uncompetitive with respect to co-factor NADPH, (K_i = 90 μM) and competitive with respect to the substrate 9,10-phenanthrenequinone (PQ), (K_i = 50 μM). Inhibition at micromolar concentrations was also observed with dicoumarol, $NADP^+$ and cibacron blue (CB).

Theorell-Yonetani double-inhibition analysis showed that NF and dicoumarol were mutually exclusive inhibitors against PQ. However, analysis of NF and $NADP^+$ by a double-inhibition plot showed that they simultaneously bind to the enzyme molecule. These studies demonstrate that NF and dicoumarol share the same site so that both molecules are prevented from binding at the same time, while NF and $NADP^+$ can bind simultaneously to different sites on the enzyme.

Although CB was noncompetitive with respect to PQ, double inhibition analysis showed that CB and dicoumarol or NF were mutually exclusive inhibitors against PQ, implying a distinct mode of inhibition for CB.

Keywords: ζ-crystallin, nitrofurantoin, cibacron blue, dicoumarol, double inhibition

INTRODUCTION

Lens ζ-crystallin is a one-electron transfer NADPH:quinone oxidoreductase which lacks a flavin moiety and has a limited substrate specificity.[1] ζ-Crystallin is a major soluble lens crystallin (~10% of the total soluble protein) of camel and guinea pig lenses and is composed of four identical 35 kD subunits.[2,3] 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, besides its presumed structural role in the lens.[4,5]

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A complete amino acid sequence of ζ-crystallin deduced from the cDNA of guinea pig showed that ζ-crystallin is distantly related to alcohol dehydrogenases although it lacks alcohol dehydrogenase activity and the zinc binding site.[6] Interestingly a mutant in the ζ-crystallin gene was found to be associated with autosomal dominant congenital cataract in guinea-pigs and thus a similar mutant gene could be a candidate for human congenital cataract.[7]

A unique feature of ζ-crystallin which distinguishes it from other quinone oxidoreductases, is its inhibition by dicoumarol, a widely used oral anticoagulant. Dicoumarol usually inhibits quinone oxidoreductases competitively with respect to NADPH, whereas it inhibits ζ-crystallin competitively with respect to the quinone substrate and uncompetitively with respect to NADPH.[1,8] Similarly nitrofurantoin (NF), an antibacterial agent, was shown to inhibit guinea-pig lens ζ-crystallin with a similar mechanism.[1] Although a number of inhibitors of ζ-crystallin have been identified, their detailed kinetic analyses and interaction with the enzyme have not been reported.[1] In order to elucidate the nature of the active site of ζ-crystallin a series of inhibition kinetic studies have been recently carried out in our laboratory.[8–10] In this report we have investigated the inhibition kinetics of NF and the combined effect of this inhibitor with other inhibitors of ζ-crystallin using Theorell-Yonetani double-inhibition analysis.[11] This method is used to determine whether the two inhibitors compete for a common site or interact with different sites of the enzyme. Moreover, it gives an interaction constant (α) between two inhibitors in the enzyme-inhibitor complex.

MATERIALS AND METHODS

Dicoumarol, nitrofurantoin, cibacron blue 3GA, 9,10-phenanthrenequinone, NADPH, $NADP^+$ were purchased from Sigma Chemical Company, St. Louis, USA. Dimethylformamide was obtained from Winlab, England. All other chemicals used were of analytical grade.

Preparation of ζ-Crystallin

ζ-Crystallin was purified from camel lens to homogeneity as previously described,[3] using a Sepharose CL-6B gel filtration column and 2′,5′-ADP Sepharose 4B affinity column chromatography. The enzyme purity was estimated to be >95% and its catalytic activity was 25 unit/mg. A unit of enzyme activity is defined as 1 μmol of NADPH oxidized/min per mg protein at 22°C. Protein was determined by the method of Bradford,[12] with bovine serum albumin as standard.

Enzyme Assay

The NADPH:quinone oxidoreductase activity of ζ-crystallin was determined by measuring the initial rate of NADPH oxidation at 340 nm ($E = 6220\ M^{-1}\ cm^{-1}$) at 22°C using a Perkin Elmer Lambda 3B recording spectrophotometer. A standard reaction mixture of a final volume of 1.0 ml contained 0.1 M Tris-HCl pH 7.8, 0.2 mM $EDTANa_2$, 0.1 mM NADPH and 0.5 μg enzyme. For the determination of inhibition kinetics the enzyme was preincubated with nitrofurantoin and/or dicoumarol for 5 min followed by the addition of NADPH and PQ and then the residual enzyme activity was determined. Nitrofurantoin was dissolved in *N,N*-dimethylformamide (DMF) and the solution was diluted with the assay buffer to appropriate concentrations (final concentration of DMF in the assay mixtures was <0.06% which did not affect the enzyme activity). 9,10-Phenanthrenequinone was dissolved in absolute ethanol. Final concentration of alcohol in the assay mixture was 1% which did not affect the enzyme activity. Blanks lacking either substrate, inhibitors or enzyme were run as a routine. Triplicate measurements of the initial rate of reaction at each substrate or inhibitor concentration were made.

Inhibition Studies

Kinetics analysis of inhibition by NF was conducted using three concentrations of NF (25, 50 and 100 μM) with varying PQ (5–25 μM) and the concentration of NADPH was held constant at 100 μM. Similarly, the concentrations of NADPH varied from 5–100 μM and PQ concentration was held constant at 25 μM. The nature of the inhibition produced by NF was determined by analysis of double reciprocal plots of initial velocity versus PQ or NADPH concentrations.

Multiple inhibition experiments were performed with the following inhibitors pairs: NF/dicoumarol, NF/$NADP^+$, dicoumarol/CB. The rates of inhibition reaction were measured with varying concentration of one inhibitor (I_1) in the presence of fixed different concentrations of the second inhibitor (I_2) at constant substrate (PQ) and cofactor (NADPH) concentrations. Data were used to construct a Yonetani and Theorell plot using the GraFit computer program.[13] Analysis was performed using the models described by Yonetani and Theorell[11] and Segel.[14]

RESULTS AND DISCUSSION

NF at various concentrations (25–200 μM) inhibited ζ-crystallin activity (10–80%) in a concentration dependent manner. The IC_{50} of NF was 50 μM.

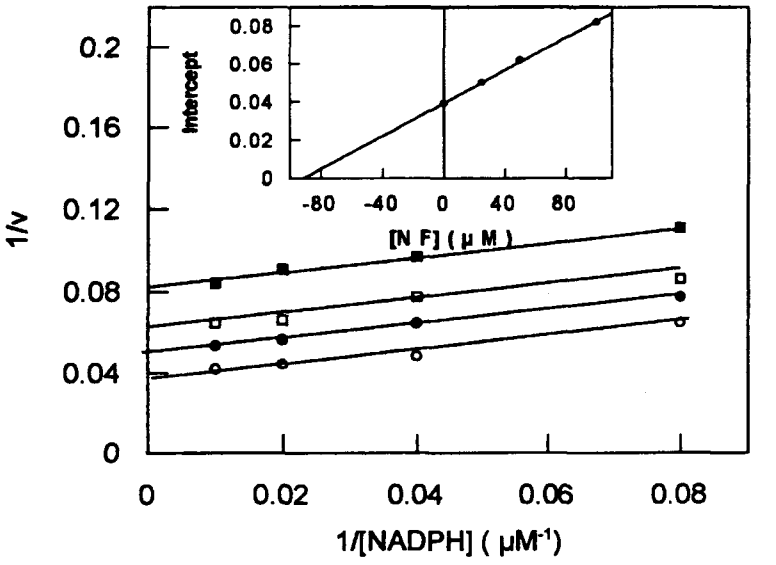

FIGURE 1 Lineweaver-Burk plot of initial velocity of camel lens ζ-crystallin activity ($1/v$) versus 1/[NADPH] (μM^{-1}) in the absence (○) and presence of 25 μM (●); 50 μM (□) and 100 μM (■) of NF with various concentrations of NADPH (5–100 μM) and fixed concentration of PQ (25 μM). Inset shows a secondary plot of the intercept values derived from the primary Lineweaver-Burk plot versus corresponding NF concentrations.

A Lineweaver-Burk plot of ζ-crystallin activity in the presence of fixed different concentrations of NF (0, 25, 50, 100 μM) with varying NADPH concentrations between 5–100 μM and at 25 μM PQ showed that the inhibition of NF was of the uncompetitive type with respect to NADPH (Figure 1). The inhibition constant (K_i) was determined by replotting the intercept ($1/V_{max}$) values derived from the primary Lineweaver-Burk plot versus corresponding NF concentrations. The K_i value was found to be 90 μM (inset of Figure 1). The replot resulted in a straight line which does not pass through the origin confirming that the inhibition of ζ-crystallin activity by NF was only of the uncompetitive type. However, with variable concentrations of PQ (5–25 μM) and fixed concentration of NADPH (100 μM), a Lineweaver-Burk plot showed that the inhibition of NF was of the competitive type with respect to PQ (Figure 2). The K_i value was determined from the secondary plot by replotting the slope values derived from the primary Lineweaver-Burk plot versus the corresponding NF concentrations which was estimated to be 50 μM with respect to PQ (inset of Figure 2).

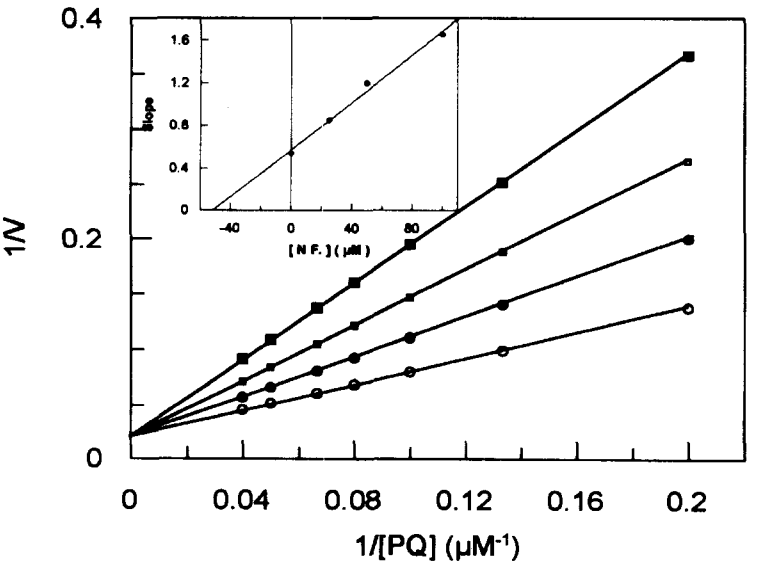

FIGURE 2 Lineweaver-Burk plot of initial velocity of camel lens ζ-crystallin activity ($1/v$) versus 1/[PQ] (μM^{-1}) in the absence (○) and presence of 25 μM (●); 50 μM (□) and 100 μM (■) of NF with various concentrations of PQ (5–25 μM) and fixed concentration of NADPH (100 μM). Inset shows a secondary plot of the slope values derived from the primary Lineweaver-Burk plot versus corresponding NF concentrations.

Therefore, NF was found to be an uncompetitive inhibitor of NADPH:quinone oxidoreductase activity of camel lens ζ-crystallin with respect to NADPH and a competitive inhibitor with respect to PQ, which is in good agreement with the earlier work of Rao and Zigler[1] where NF and dicoumarol were found to be competitive inhibitors of guinea-pig lens ζ-crystallin activity.

Multiple inhibition experiments were performed using the method of Yonetani and Theorell to investigate whether two inhibitors such as NF and dicoumarol interact with the same or with different binding sites. The equation that describes the relationship between two inhibitors and their interaction with the enzyme is given as:

$$\frac{1}{v_i} = \frac{1}{v}\left(1 + \frac{[I_1]}{K_1} + \frac{[I_2]}{K_2} + \frac{[I_1][I_2]}{\alpha K_1 K_2}\right), \tag{1}$$

where v is the velocity in the absence of inhibitor, v_i is the velocity in the presence of inhibitor, K_1 and K_2 are dissociation constants for EI_1 and EI_2 and α is an interaction constant.

According to Equation (1) both substrate PQ and cofactor NADPH are held constant and one of the inhibitors is varied with a fixed different concentration of the other inhibitor. When v_i^{-1} versus the variable inhibitor concentrations is plotted, this results in either parallel lines or intersecting lines. The parallel lines are obtained when no ternary complex of the two inhibitors and the enzyme (EI_1I_2) is formed and both inhibitors compete for the same site, while the intersecting lines are obtained when a EI_1I_2 complex is formed. The lines may intersect above the horizontal axis when I_1 combines more readily with EI_2 than with E (positive cooperativity) but when I_1 combines less readily with EI_2 than with E, the lines will intersect below the horizontal lines (negative cooperativity). Finally when I_1 combines with E as readily as with EI_2, the lines will intersect on the horizontal lines.

In the present study the concentrations of NADPH and PQ were held constant at 100 μM and 25 μM, respectively, and the concentration of the NF-dicoumarol inhibitor pairs was varied; the concentrations of NF were 0, 25, 50 and 100 μM, while the concentrations of dicoumarol were 0, 2.5, 5.0, 7.5 and 10.0 μM. The plot of v^{-1} versus NF concentration at fixed different levels of dicoumarol resulted in a family of parallel lines, where $\alpha = \infty$, indicating that NF and dicoumarol compete for a common site, which is most likely the substrate PQ site. Therefore NF and dicoumarol were mutually exclusive inhibitors of camel lens ζ-crystallin (Figure 3). The data from Figure 3 was replotted according to the procedures described by Segel[14] to obtain the inhibition constants describing the interaction of the individual inhibitor with the enzyme, which gave a K_i value of 90 μM for NF and 13 μM for dicoumarol. These values are in agreement with the K_i values obtained from inhibition studies with the individual inhibitors (data not shown).

When the NF-$NADP^+$ inhibitor pair was used in the multiple inhibition experiments, at various concentration of NF (0, 25, 50 and 100 μM) and at fixed different concentration of $NADP^+$ (0, 100, 150, 200, 300 and 400 μM), the plot of v^{-1} versus NF concentrations gave rise to a family of intersecting lines above the x-axis as shown in Figure 4, indicating that the two inhibitors interact with different sites on the enzyme (simultaneously binding to the enzyme) and a ternary complex of enzyme-NF-$NADP^+$ complex is formed, with $\infty > \alpha > 0$. The α value was calculated and found to be 0.47. Extrapolation from the point of intersection of the lines in Figure 4 to the x-axis, gave an αK_1 value of 0.28 mM for $NADP^+$, which is the dissociation constant of $NADP^+$ from the enzyme-NF-$NADP^+$ complex. The α value of 0.47 reflected a favorable interaction between NF and $NADP^+$ in the enzyme-NF-$NADP^+$ complex. This positive interaction between the two inhibitors may be attributed to a number of causes which include hydrophilic and hydrophobic interactions between the inhibitors and the enzyme in addition to conformational changes in the enzyme protein.

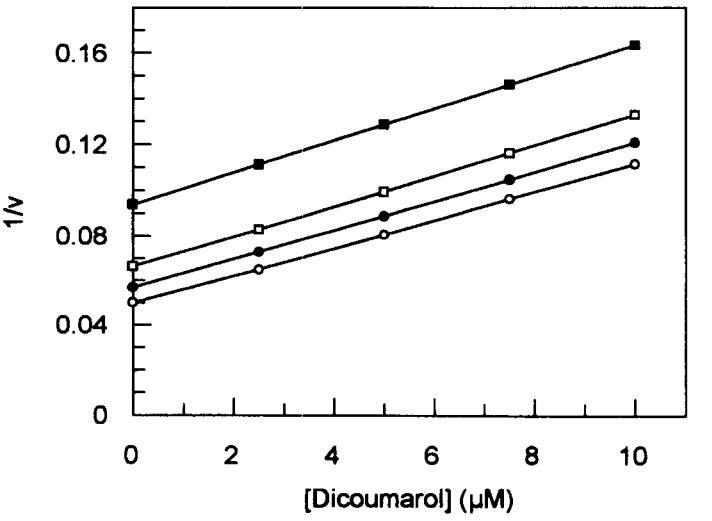

FIGURE 3 Double inhibition of camel lens ζ-crystallin activity by nitrofurantoin and dicoumarol. ζ-Crystallin was assayed at a fixed concentrations of NADPH (100 μM) and PQ (25 μM) in the assay buffer. Effect of 0 (○); 25 μM (●); 50 μM (□) and 100 μM (■) of nitrofurantoin on the inhibition by dicoumarol.

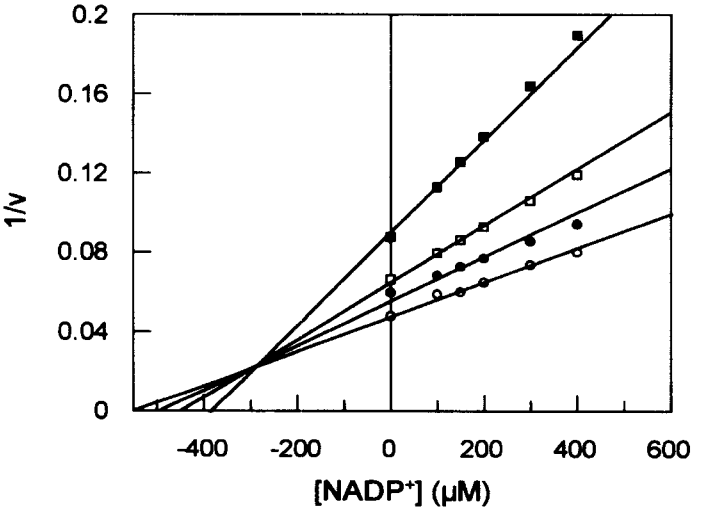

FIGURE 4 Double inhibition of camel lens ζ-crystallin activity by nitrofurantoin and $NADP^+$. ζ-Crystallin was assayed at a fixed concentrations of NADPH (100 μM) and PQ (25 μM) in the assay buffer. Effect of 0 (○); 25 μM (●); 50 μM (□) and 100 μM (■) of nitrofurantoin on the inhibition by $NADP^+$.

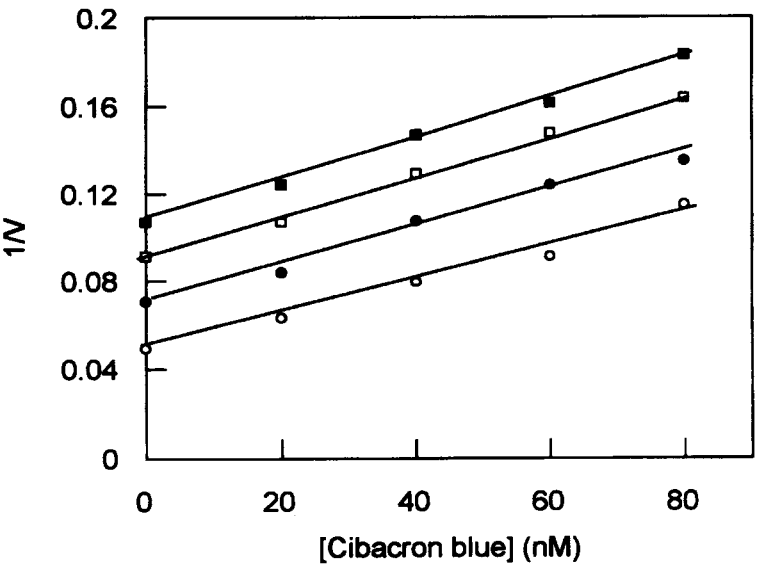

FIGURE 5 Double inhibition of camel lens ζ-crystallin activity by dicoumarol and cibacron blue. ζ-Crystallin was assayed at a fixed concentrations of NADPH (100 μM) and PQ (25 μM) in the assay buffer. Effect of 0 (○); 2.5 μM (●); 5.0 μM (□) and 7.5 μM (■) of dicoumarol on the inhibition by cibacron blue.

Previously we have shown that CB inhibited ζ-crystallin in a noncompetitive manner with respect to PQ and in a mixed inhibition manner with respect to NADPH (competitive plus noncompetitive).[9] When the dicoumarol/CB inhibitor pair was used in the multiple inhibition experiments, at various concentration of dicoumarol (0, 2.5, 5.0 and 7.5 μM) and at fixed different concentrations of CB (0, 20, 40, 60 and 80 nM), the plot of v^{-1} versus CB concentrations yielded a family of parallel lines. Inhibition by a mixture of CB and NF also yielded parallel lines for the Yonetani-Theorell plots (data not shown), indicating that CB and any of these two inhibitors may compete for a common site on ζ-crystallin (Figure 5).

From the results it is concluded that NF and dicoumarol, act as competitive inhibitors for the substrate site. The similarity in binding between these inhibitors was substantiated by multiple inhibition analyses. Inhibition by a mixture of NF and dicoumarol (substrate-competitive inhibitors) yielded parallel Yonetani-Theorell plots demonstrating that these two inhibitors compete for a common site on ζ-crystallin. Furthermore, inhibition by a mixture of NF and $NADP^+$ (Figure 4) or dicoumarol and $NADP^+$ (data not shown) gave intercepted lines above the horizontal line, pointing out that these inhibitors do not share a common site.

Since mixed-type kinetics result when an inhibitor binds at two sites on an enzyme and its interaction with one site excludes substrate binding while interaction at the second site may or may not alter binding at the first site. This may explain the obtained parallel lines when a mixture of CB and dicoumarol or CB and NF are used. In such a mechanism, CB could serve as a noncompetitive inhibitor with respect to PQ due to its binding to the NADPH site, therefore CB and NF appear to be mutually exclusive inhibitors. However, since there is no clear structural similarity between CB and NF or quinone and because of the fact that CB noncompetitively inhibits the enzyme with respect to PQ, this mode of inhibition can be explained on the basis that CB causes a mixed type of inhibition with respect to NADPH.

To understand the detailed structure of the active site of this enzyme, its three dimensional structure needs to be solved by X-ray crystallography. Nevertheless, in the absence of such data and in the light of the present investigation it is most likely that ζ-crystallin has at least two distinct sites, the substrate and the cofactor site. One can directly interpret the inhibition of ζ-crystallin by NF and dicoumarol to be mutually exclusive inhibitors competing for the quinone substrate site, whereas NF and $NADP^+$ could bind to non exclusive sites, i.e. the quinone site and the cofactor site, respectively.

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