THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 267, No. 1, Issue of January 5, pp. 96-102, 1992
Printed in U.S.A.

Identification and Characterization of the Enzymatic Activity of ζ-Crystallin from Guinea Pig Lens

A NOVEL NADPH:QUINONE OXIDOREDUCTASE*

(Received for publication, January 7, 1991)

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ζ-Crystallin is a major protein in the lens of certain mammals. In guinea pigs it comprises 10% of the total lens protein, and it has been shown that a mutation in the ζ-crystallin gene is associated with autosomal dominant congenital cataract. As with several other lens crystallins of limited phylogenetic distribution, ζ-crystallin has been characterized as an "enzyme/crystallin" based on its ability to reduce catalytically the electron acceptor 2,6-dichlorophenolindophenol. We report here that certain naturally occurring quinones are good substrates for the enzymatic activity of ζ-crystallin. Among the various quinones tested, the orthoquinones 1,2-naphthoquinone and 9,10-phenanthrenequinone were the best substrates whereas menadione, ubiquinone, 9,10-anthraquinone, vitamins K_1 and K_2 were inactive as substrates. This quinone reductase activity was NADPH specific and exhibited typical Michaelis-Menten kinetics. Activity was sensitive to heat and sulfhydryl reagents but was very stable on freezing. Dicumarol (K_i = 1.3 × 10^{-5} M) and nitrofurantoin (K_i = 1.4 × 10^{-5} M) inhibited the activity competitively with respect to the electron acceptor, quinone. NADPH protected the enzyme against inactivation caused by heat, *N*-ethylmaleimide, or H_2O_2. Electron paramagnetic resonance spectroscopy of the reaction products showed formation of a semiquinone radical. The enzyme activity was associated with O_2 consumption, generation of O_2^- and H_2O_2, and reduction of ferricytochrome *c*. These properties indicate that the enzyme acts through a one-electron transfer process. The substrate specificity, reaction characteristics, and physicochemical properties of ζ-crystallin demonstrate that it is an active NADPH:quinone oxidoreductase distinct from quinone reductases described previously.

The ocular lens is a transparent, protein-rich organ which focuses incident light on the retina. Crystallins, the structural proteins of the lens, account for as much as 90% of the total soluble protein (1). Apart from the ubiquitous α- and β,γ-crystallin families, there are other "taxon-specific" crystallins which exist as major proteins in certain species or phylogenetic groups. Interestingly, each of the taxon-specific crystallins has been shown to be identical to, or related to, a metabolic enzyme (2). Examples include the ε-, ρ-, δ-, τ-, and λ-crystallins which show sequence relatedness to lactate dehydrogenase 4B, aldose reductase and prostaglandin F synthetase, argininosuccinate lyase, α-enolase, and hydroxyacyl-CoA dehydrogenase, respectively. Some of these enzyme/crystallins are enzymatically active whereas others have either low or no activity (2–4).

ζ-Crystallin is a major protein (~10% of total protein) in guinea pig and camel lenses and exists as a homotetramer of 35-kDa polypeptides (5, 6). This protein, unlike the α- and β,γ-crystallins, possesses a significant amount (~40%) of α-helical structure. It has been shown to be present in small amounts in certain nonlenticular tissues (7). From its cDNA sequence (8) ζ-crystallin has been shown to be distantly related to the alcohol dehydrogenases, although it lacks alcohol dehydrogenase activity. Analysis of the sequence suggests that the molecule's three-dimensional structure is similar to that of alcohol dehydrogenase (9). In contrast to alcohol dehydrogenase, which uses NAD^+/NADH as cofactor, we have demonstrated the specific binding of NADPH by ζ-crystallin, and have shown a correlation between ζ-crystallin content and very high levels of NADPH/$NADP^+$ in guinea pig lenses (10). Tests of catalytic activity of ζ-crystallin using artificial electron acceptors have shown that ζ-crystallin has an NADPH:2,6-dicholorophenolindophenol (DCIP)[1] oxidoreductase activity (11). This paper reports the identification of potential physiological substrates for ζ-crystallin and the characterization of its catalytic activity. These findings have relevance to the following questions. First, since a mutation in the gene for ζ-crystallin is associated with an autosomal dominant hereditary cataract in a line of strain 13/N guinea pigs (7), elucidation of the protein's enzymatic function will allow us to address directly the possibility that loss of activity is a factor in the development of this cataract. Additionally, the function (or functions) of the enzyme/crystallins in the lens is currently an area of great interest. The guinea pig system provides the first opportunity for investigation of the effects on the lens of a mutation in an enzyme/crystallin. Understanding the enzymatic function of the protein will hopefully provide a means of evaluating the relative importance of its catalytic and putative structural roles in the lens.

EXPERIMENTAL PROCEDURES

Materials—All nucleotides, potential substrates, and inhibitors and other reagents were obtained from Sigma or Aldrich unless otherwise

* This work was supported in part by an Alcon Research Institute Award (to J. S. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: DCIP, 2,6-dichlorophenolindophenol; DMPO, 5,5-dimethylpyroline-1-oxide; TEMPOL, 4-hydroxy-2,2,6,6,-tetramethylpiperidine-1-oxyl; TEMPOL-H, hydroxylamine derivative of TEMPOL; HPLC, high performance liquid chromatography.

indicated. 5,5-Dimethylpyroline-1-oxide (DMPO) and 4-hydroxy-2,2,6,6,-tetramethylpiperidine-1-oxyl (TEMPOL) were also from Aldrich. DMPO was purified further by vacuum distillation before use. The hydroxylamine derivative (TEMPOL-H) of TEMPOL was prepared by hydrogenation of TEMPOL in the presence of platinum catalyst and retained less than 0.5% TEMPOL as impurity (12). Blue Sepharose CL-6B, PD-10 columns, Omega 30K ultrafiltration membranes, and Superose 12 HPLC column were from Pharmacia LKB Biotechnology Inc. The Synchropak CM-300 cation exchange HPLC column was from Synchrom, Inc. (Lafayette, IN).

Purification of ζ-Crystallin from Guinea Pig Lens—ζ-Crystallin was purified as described previously (11) from adult guinea pig lenses obtained frozen from Pel-Freez Biologicals (Rogers, AR). Its purity was assessed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13) and gel filtration (6). The preparation appeared to be homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as shown in Ref. 11. Protein determinations were by the method of Bradford (14).

Enzyme Assays—Enzyme activity was determined aerobically in a standard assay mixture containing the following in a total volume of 1.0 ml: 0.1 M Tris-HCl buffer, pH 7.8, 0.2 mM EDTA, 0.1 mM NADPH, purified ζ-crystallin and substrates at various concentrations as indicated in the tables and figures. Reactions were initiated by the addition of substrate, and the decrease in absorbance at 340 nm was monitored with a Beckman DU 50 recording spectrophotometer at 25 °C. Blanks, lacking either substrate or ζ-crystallin, were run routinely. Quinone substrates were dissolved in absolute alcohol (final concentration of alcohol in assay was 1%). For assessing the effect of inhibitors, assays were started after an initial preincubation of ζ-crystallin with the inhibitor for 5 min. For kinetic studies, reactions were started with the addition of ζ-crystallin.

Anaerobic Assays—Assays were carried out under nitrogen saturation using a two-compartment anaerobic cuvette (11).

Polarography—O_2 consumption was monitored (at 25 °C) using an oxygraph model 18172 fitted with a Clark electrode (Yellow Springs Instrument Co., Yellow Springs, OH). The reaction mixture in a final volume of 3.0 ml contained 0.1 M Tris buffer, pH 7.8, 0.2 mM EDTA, 0.1 mM NADPH, ζ-crystallin (24 μg), and 50 μM juglone. When 9,10-phenanthrenequinone (25 μM) was used as substrate 6.0 μg of protein was used. To measure oxygen consumption the electrode was calibrated to 100% air saturation after addition of NADPH and quinone to buffer, and O_2 consumption was monitored for the next 5 min with constant stirring to obtain a steady base line. Then, after the addition of ζ-crystallin, recording was continued until O_2 consumption ceased.

H_2O_2 Determination—H_2O_2 generation was monitored spectrophotometrically by the procedure of Allen *et al.* (15). 0.2-ml aliquots were withdrawn from standard reaction mixtures at different time intervals for estimation of H_2O_2 levels.

Electron Paramagnetic Resonance—EPR spectral measurements were carried out in a final volume of 0.5 ml, and concentrations of reactants were the same as above except that substrate concentrations were doubled. On starting the reaction with the addition of quinone, an aliquot of the reaction mixture (0.100–0.125 ml) was immediately withdrawn into a gas-permeable Teflon capillary tube (Zeus Industries, Raritan, NJ) of 0.81-mm inner diameter, 0.38-mm wall thickness, and 15-cm length. Each capillary was folded twice, inserted into a narrow quartz tube which was open at both ends (2.5-mm inner diameter), and then placed in the EPR cavity. During the experiment, gases of desired composition were blown around the sample without having to disturb the alignment of the tube within the spectrometer cavity. EPR spectra were recorded on a Varian E9 X-band spectrometer operating at 9.4 GHz and a modulation frequency of 100 Khz. A nonsaturating microwave power was used throughout the experiment. Spectra were simulated and matched with the experimental data.

RESULTS

Our earlier studies demonstrated that ζ-crystallin could catalytically reduce DCIP and that this reaction was inhibited by dicumarol (11). Although dicumarol, an anticoagulant, is known to be a potent inhibitor of several quinone reductases (16–18), we initially concentrated on other potential substrates such as aldoses and ketoses since the quinone menadione was found not to be a substrate for ζ-crystallin (11). When none of these compounds proved to be substrates, we decided to investigate a broad range of quinones and found certain of them to act as substrates for ζ-crystallin enzyme activity.

Table I lists the quinones found to be substrates of ζ-crystallin enzyme activity. Among the various quinones tested, the orthoquinones, 1,2-naphthoquinone and 9,10-phenanthrenequinone, were the most active, followed by methyl-1,4-benzoquinone, 1,4-benzoquinone, and juglone. Menadione, 9,10-anthraquinone, coenzyme Q_o, daunorubicin, vitamin K_1, and vitamin K_2 were inactive as substrates when tested at concentrations of 0.1–0.5 mM. The non-quinoidal compounds, 4-nitroacetophenone, naloxone, nitrofurans, paraquat dichloride and dehydroascorbic acid, were also not utilized as substrates over these concentrations. From these studies and our earlier data with substrates of aldoketo reductases (11), quinones are the only biological compounds so far identified as substrates of ζ-crystallin.

Characteristics of ζ-Crystallin Enzyme Activity—To characterize the NADPH:quinone oxidoreductase activity of ζ-crystallin, two of the better substrates, juglone and 9,10-phenanthrenequinone, were used since the background NADPH oxidation was very low with these compounds. Enzyme activity increased linearly with enzyme concentration (0–7 μg/assay) when juglone was used as substrate and from 0–1.6 μg/assay with 9,10-phenanthrenequinone as substrate. Activity was linear with time for more than 3 min with both substrates. Near maximal activity was obtained over a broad pH range (pH 6–9) with maximal activity at pH 7.8. Phosphate buffers (sodium or potassium salts) gave slightly lower activity than did Tris or triethanolamine buffers whereas activity in glycine buffer was only 40% of the activity observed with Tris. Increasing the Tris concentration from 25 to 100 mM increased enzyme activity by 26%; further increase in ionic strength did not influence the activity. Thus 100 mM Tris buffer was used for all standard assays. ζ-Crystallin exhibited maximal activity when the reaction was initiated by the addition of either substrate (quinone) or enzyme whereas activity was significantly reduced (>50%) when ζ-crystallin was preincubated with quinone and the reaction started with NADPH. The basis for this phenomenon is unclear, but it is consistent with previous results using DCIP as substrate (11). The enzyme was very stable when stored at −20 °C. More than 90% of the activity was retained over a 4–6-week storage period. Activity was sensitive to heating, decreasing to 35% of control after 10 min at 45 °C, and being completely abolished at 51 °C. The presence of NADPH (0.1 mM) during the heat treatment had a stabilizing effect on the enzyme activity.

Cofactor Specificity—The enzyme activity of ζ-crystallin was highly specific for NADPH; replacement of NADPH by NADH resulted in a >95% decrease in activity. This finding corroborates the specific binding of NADPH to ζ-crystallin reported earlier (10) from fluorescence quenching and blue Sepharose binding studies. Based on substrate and cofactor specificity we have chosen the name NADPH:quinone oxidoreductase for the enzymatic activity of ζ-crystallin.

Kinetic Properties—The velocity (v) *versus* substrate (S) plots showed typical Michaelis-Menten kinetics for both NADPH and quinone substrates. The kinetic constants were calculated from Lineweaver-Burk plots (Table II). The K_m for quinone substrates was in the μM range: 13.0 μM for 9,10-phenanthrenequinone, 27 μM for juglone, and 143.0 μM for 1,4-benzoquinone. In the presence of 9,10-phenanthrenequinone, ζ-crystallin exhibited a detectable activity with less than 1 μg of protein, and the V_{max} (16.6 μmol/min/mg protein) and turnover number (10/s) indicate that ζ-crystallin is an active enzyme. The estimated K_m was calculated to be 5.0 μM

TABLE I

Substrate specificity of ζ-crystallin enzyme activity

Assays were carried out in the presence of 1–6 μg of ζ-crystallin in 1.0-ml assay volumes by following the standard assay conditions as given under "Experimental Procedures." Activity obtained with juglone was set to 100%.

Substrate	Concentration required for maximal activity	Activity	Relative velocity
	μM	μmol/mg protein/min	%
1,2-Naphthoquinone	20	16.66	800
9,10-Phenanthrenequinone	25	8.25	400
Methyl-1,4-benzoquinone	500	3.42	165
1,4-Benzoquinone	250	2.53	122
5-Hydroxy-1,4-naphthoquinone (juglone)	50	2.10	100
2,6-Dichlorophenolindophenol[a]	100	0.63	30
1,4-Naphthoquinone	250	0.44	21
5-Hydryoxy-2-methyl-1,4-naphthoquinone (plumbagin)	250	0.16	7

[a] Activity was determined by following the DCIP reduction at 600 nM.

TABLE II

Kinetic constants of NADPH:quinone oxidoreductase activity of ζ-crystallin

Kinetic constants were calculated from Lineweaver-Burk plots.

Substrate	K_m	V_{max}	V_{max}/K_m	Turnover
	10^{-5} M	10^{-3} moles·liter^{-1}·min^{-1}	× 10^2	no./s
9,10-Phenanthrenequinone	1.3	16.6	12.7	10.0
Juglone	2.7	4.5	1.6	2.6
1,4-Benzoquinone	14.3	5.7	0.4	3.4
NADPH	0.5			

TABLE III

Inhibition of the NADPH:quinone oxidoreductase activity of ζ-crystallin

For all the inhibition studies the enzyme was preincubated with test compounds for 5 min before the addition of NADPH (0.1 mM) and quinone substrate. Activity was determined as described under "Experimental Procedures" using either 50 μM juglone or 25 μM 9,10-phenanthrenequinone substrates in the presence of 7.5 μg and 1.8 μg ζ-crystallin, respectively. Similar effects were obtained with either juglone or 9,10-phenanthrenequinone as substrate. Values shown were determined using juglone.

Compound	Concentration	Inhibition
		%
Inhibitors		
Dicumarol	0.1 mM	71
Nitrofurantoin	0.1 mM	70
Cibacron blue 3GA	10 μM	93
$NADP^+$	0.2 mM	11
Inactivators		
N-Ethylmaleimide	0.2 mM	98
5,5′-Dithiobis(2-nitrobenzoic acid)	0.2 mM	94
p-Chloromercurisulfonate	0.2 mM	53
Iodoacetamide	0.5 mM	0
H_2O_2	0.5 mM	77

for NADPH, indicating the high affinity of ζ-crystallin for NADPH, and is consistent with the K_m value of 5.2 μM reported earlier (11) for the catalytic reduction of DCIP.

Inhibition—Table III lists a number of compounds that were tested for inhibition of ζ-crystallin activity. As reported earlier (11) with the DCIP reduction, sulfhydryl reagents were strong inactivators, with *N*-ethylmaleimide producing 98% inhibition at 0.2 mM whereas the same concentration of 5,5′-dithiobis(2-nitrobenzoic acid) and *p*-chloromercurisulfonate inhibited enzyme activity by 93 and 52%, respectively. Iodoacetamide did not affect the activity. When the protein was preincubated with NADPH (0.1 mM), enzyme activity was

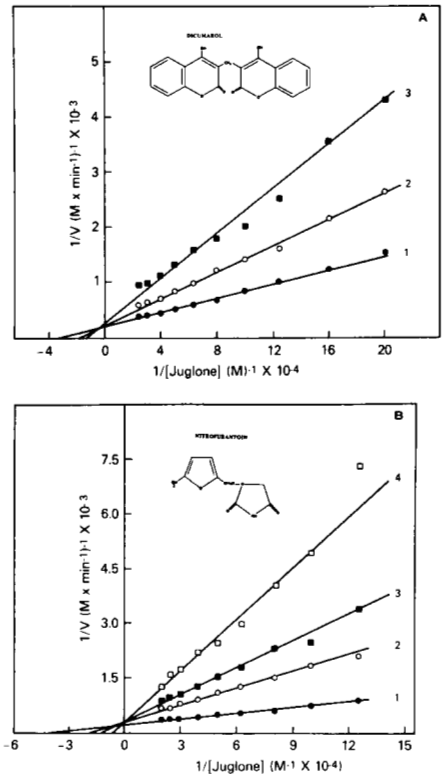


FIG. 1. **The effect of dicumarol (*A*) and nitrofurantoin (*B*) on the quinone reductase activity of ζ-crystallin.** In *panel A*, the concentrations of dicumarol are 0 (***1***), **10 μM (*2*)**, and 25 μM (*3*). In *panel B*, the concentrations of nitrofurantoin are 0 (*1*), 25 μM (*2*), 50 μM (*3*), and 100 μM (*4*). Enzyme assays were carried out in a final volume of 1.0 ml, containing 0.1 M Tris-HCl buffer, pH 7.8, 0.2 mM EDTA, 0.1 mM NADPH, juglone (0–50 μM), and ζ-crystallin (7.6 μg in the case of assays run with dicumarol, 7.0 μg in the case of assays with nitrofurantoin). Reactions were initiated with the addition of ζ-crystallin, and activity was measured at 25 °C by following the NADPH oxidation at 340 nM.

completely protected from the inhibition caused by *N*-ethylmaleimide. These reagents showed similar effects when either juglone or 9,10-phenanthrenequinone was used as substrate.

Dicumarol at 0.1 mM inhibited enzyme activity 70–85% with various substrates. Fig. 1 illustrates the inhibition kinetics of ζ-crystallin activity with dicumarol and nitrofurantoin. Dicumarol at various concentrations caused an increase in K_m for quinone with no effect on V_{max} (Fig. 1*A*). These changes are indicative of competitive inhibition. The K_i value was

calculated from the slopes of the plots to be 13 μM. Nitrofurantoin (0.1 mM) inhibited activity by 70% whereas 2-nitrofuran at the same concentration was not inhibitory. The inhibition kinetics with nitrofurantoin also demonstrated a competitive inhibition with respect to quinone substrate (Fig. 1*B*). The estimated K_i value was 14 μM. Dicumarol and nitrofurantoin both showed an uncompetitive inhibition with respect to NADPH.

$NADP^+$, at a concentration of 0.2 mM, caused 11 and 38% inhibition with juglone (50 μM) and 9,10-phenanthrenequinone (25 μM) as substrates, respectively. Incubation with 0.5 mM H_2O_2 for 5 min reduced enzyme activity by 77%; this effect was completely prevented by NADPH (0.1 mM). Superoxide dismutase (200 units/assay), catalase (250 units), or superoxide dismutase plus catalase did not affect activity. Neither EDTA nor diethylenetriaminepentaacetic acid at 0.5 mM affected the activity. Zinc or copper at 1 mM caused 60% inhibition with juglone as substrate; with 9,10-phenanthrenequinone less inhibition was noted. Calcium, magnesium, and manganese had no affect.

Mechanistic Aspects of the Reaction—It is well known that the enzyme-mediated reduction of quinones occurs either by one- or two-electron transfer (19, 20). The two-electron transfer results in the formation of hydroquinones which can be disposed of via conjugation reactions (17, 21, 22). The one-electron mechanism produces the semiquinone free radical which may be further nonenzymatically reduced to the hydroquinone or oxidized back to quinone in the presence of O_2. Under aerobic conditions, the one-electron reduction of quinone is generally associated with O_2 consumption and O_2^- and H_2O_2 generation (20–22).

In the ζ-crystallin/quinone reductase reaction semiquinone radical formation (under anaerobic conditions) was observed by EPR spectrometry; the EPR signal intensity of the semiquinone radical (Fig. 2) obtained from the complete reaction mixture with juglone as substrate was found to increase with time, reaching a steady-state level by 20 min. The EPR spectrum corresponded to that obtained when juglone was chemically reduced to its semiquinone form, and the hyperfine coupling constants are in close agreement with those reported previously (23). When the flow of argon was replaced with oxygen the EPR spectrum was rapidly abolished, indicating that the semiquinone radical is transferring an electron to molecular oxygen presumably generating O_2^-.

As seen in Fig. 3, O_2 consumption was also associated with the NADPH:quinone oxidoreductase activity of ζ-crystallin.

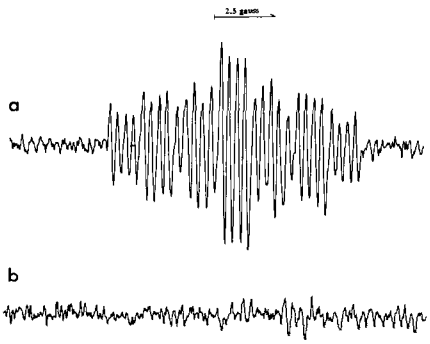


FIG. 2. **EPR spectroscopy of reaction products of the ζ-crystallin/NADPH:quinone oxidoreductase reaction with juglone as substrate.** Assays were carried out in a volume of 0.5 ml consisting of 100 mM Tris buffer, pH 7.8, 0.1 mM NADPH, 0.2 mM EDTA, 3.2 μg ζ-crystallin, and 100 μM juglone. *a* indicates the spectrum of juglone semiquinone formed in the complete reaction system at 30 min, and *b* shows the corresponding spectrum from control reaction lacking ζ-crystallin.

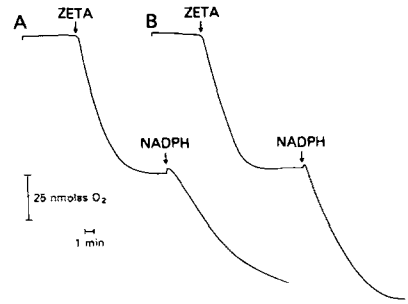


FIG. 3. **Measurement of O_2 consumption associated with NADPH:quinone oxidoreductase activity of ζ-crystallin.** In *A*, juglone (50 μM) was the substrate and was present from time 0; oxygen was consumed only after addition of enzyme at 5 min. Note that the reaction stops upon consumption of all NADPH as indicated by the resumption of O_2 consumption when more NADPH is added. *B* shows similar data with 9,10-phenanthrenequinone (25 μM) as substrate. Assay conditions are given under "Experimental Procedures."

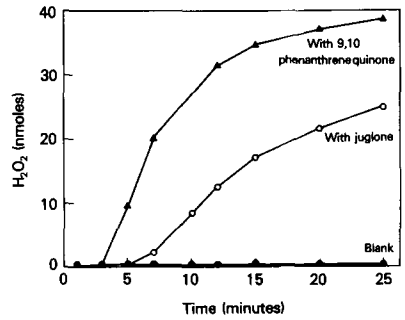


FIG. 4. **Formation of H_2O_2 in the NADPH:quinone oxidoreductase reaction catalyzed by ζ-crystallin.** The reaction mixture (2.0 ml) contained 50 μM juglone and 19.0 μg enzyme or 25 μM 9,10-phenanthrenequinone and 5.0 μg ζ-crystallin. 0.2-ml aliquots were withdrawn from the reaction mixture and assayed for H_2O_2 content at the indicated times. The *blank* lacked ζ-crystallin.

A reaction mixture including juglone but lacking ζ-crystallin did not consume O_2 whereas addition of ζ-crystallin led to O_2 consumption at an initial rate of 3.31 μmol/min/mg of protein. Furthermore, assays carried out under anaerobic conditions exhibited only one-third the activity of similar assays run aerobically (data not shown). Under aerobic conditions, ζ-crystallin (NADPH:quinone oxidoreductase) activity resulted in production of H_2O_2 (Fig. 4). Detectable levels of H_2O_2 were present in the reaction mixture with juglone as substrate after 7 min and increased with time. With 9,10-phenanthrenequinone, a better substrate, H_2O_2 was produced more efficiently (Fig. 4). Under aerobic conditions the NADPH:quinone oxidoreductase reaction of ζ-crystallin consumed an amount of NADPH in excess of the amount of quinone substrate in the reaction mixture indicating a nonstoichiometric oxidoreduction between NADPH and quinone.

Ferricytochrome *c* was not reduced in the presence of ζ-crystallin and NADPH; however, the addition of quinone substrates to the reaction mixture led to a linear reduction of ferricytochrome c (Fig. 5). In the presence of juglone, the rate of cytochrome reduction was 3.8 μmol/min/mg of protein. Superoxide dismutase (200 units/ml) inhibited the reduction of ferricytochrome c by 30%. Typically in O_2^--generating systems, superoxide dismutase can inhibit 80% or more of the ferricytochrome c reduction (24). Further, anaerobic conditions yielded the same rate of reduction of ferricytochrome *c* as that obtained under aerobic conditions, indicating that O_2^- is not obligatory for ferricytochrome *c* reduction in this system. This suggested that the semiquinone products were

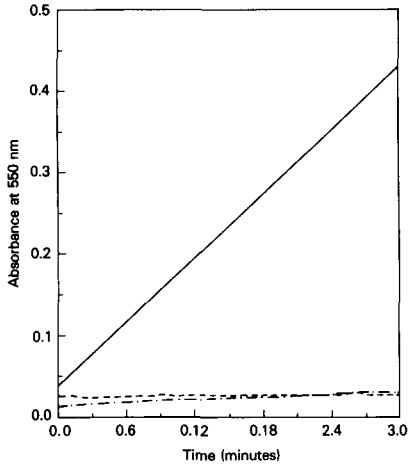
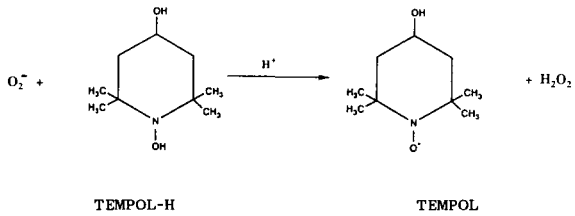


FIG. 5. **Juglone-mediated reduction of ferricytochrome *c* in the ζ-crystallin/NADPH:quinone oxidoreductase system.** Assays, carried out in a 1.0-ml final volume, contained 100 mM Tris buffer, pH 7.8, 0.1 mM NADPH, 0.2 mM EDTA, 50 μM cytochrome *c*, 1.78 μg of enzyme protein; reactions were started by the addition of 50 μM juglone. Activity was followed by recording the cytochrome *c* reduction at 550 nM. ——, complete system; –·–, without enzyme; -----, without juglone.



SCHEME 1

capable of reducing ferricytochrome *c*. The limited inhibition by superoxide dismutase probably reflects an "indirect inhibition" by superoxide dismutase of semiquinone-mediated reduction of ferricytochrome *c* as has been described by Winterbourn (25).

Since evidence of O_2^- generation was not clear from the ferricytochrome *c* studies, spin trapping experiments with DMPO (75 mM) were carried out in the complete reaction mixture with juglone as substrate. Despite the evidence obtained for the formation of the semiquinone of juglone and its instability to dissolved oxygen, neither the $OH^\cdot$ nor O_2^- spin adducts were detected. Similar results were obtained when 9,10-phenanthrenequinone was used as the substrate. These results indicate that either O_2^- is not formed in the system or that the spin adducts are not sufficiently stable to allow detection, perhaps because of reaction with the semiquinone. To test the latter possibility, 50 μM TEMPOL (a stable nitroxide radical) was added to the reaction system under anaerobic conditions with juglone as the substrate. The EPR signal intensity of TEMPOL was found to decrease with time, and no signal of the semiquinone radical was detected. These results indicated that the semiquinone was capable of reacting directly with stable nitroxides. Since spin adducts of DMPO are less stable than TEMPOL they may react with the semiquinone radicals more efficiently thereby preventing accumulation of the DMPO adduct to detectable levels.

It has been demonstrated (26) that TEMPOL is produced from its hydroxylamine via oxidation by O_2^- (Scheme 1). We have utilized this reaction to obtain clear evidence for generation of O_2^- by the ζ-crystallin reaction. To the reaction mixture under aerobic conditions, TEMPOL-H (1 mM) was added, and the formation of the stable nitroxide free radical (TEMPOL) was followed by EPR spectroscopy (Fig. 6). In the absence of ζ-crystallin, a small background production of TEMPOL was observed. Upon addition of ζ-crystallin, TEMPOL was formed at appreciable rates. TEMPOL formation was completely inhibited by superoxide dismutase but not by the protein control bovine serum albumin. These results support the conclusion that O_2^- is generated by the ζ-crystallin/NADPH/quinone system.

DISCUSSION

The present study demonstrates that ζ-crystallin, a major lens protein of the guinea pig, possesses an NADPH:quinone oxidoreductase activity. Among the various quinones tested, the orthoquinones (1,2-naphthoquinone and 9,10-phenanthrenequinone) were the best substrates. This enzyme appears to have a more limited substrate specificity than other quinone reductases such as DT-diaphorase (16) and carbonyl reductase (17, 27).

NADPH, one of the substrates of ζ-crystallin, protects against loss of activity on incubation with either sulfhydryl reagents or H_2O_2, suggesting the existence of a critical sulfhydryl group at the coenzyme binding site. Many other oxidoreductases have been shown to share this feature (16, 17). The protection of ζ-crystallin activity by NADPH against heat inactivation also indicates a protein stabilization effect of the NADPH. Dicumarol, a potent competitive inhibitor of DT-diaphorase (EC 1.6.99.2) (K_i 10^{-9} M), also inhibited ζ-crystallin/quinone oxidoreductase (K_i 1.3×10^{-5}). Interestingly, the inhibition was competitive with respect to quinone substrate, whereas with DT-diaphorase, dicumarol has been

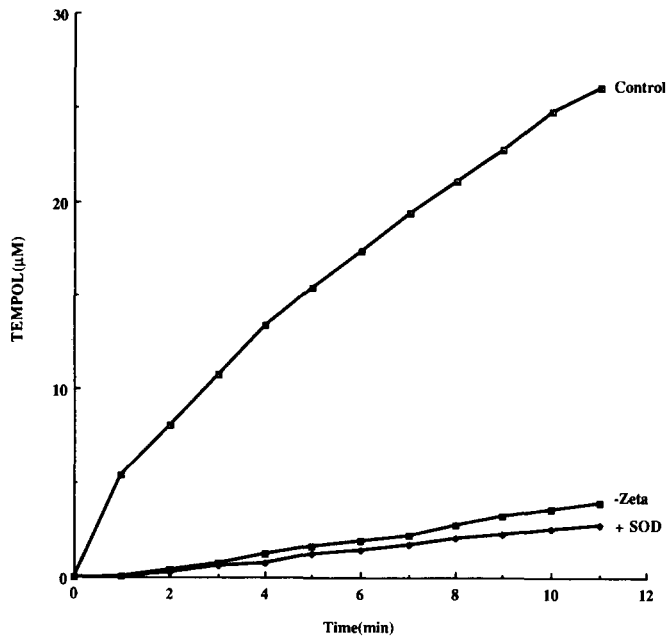
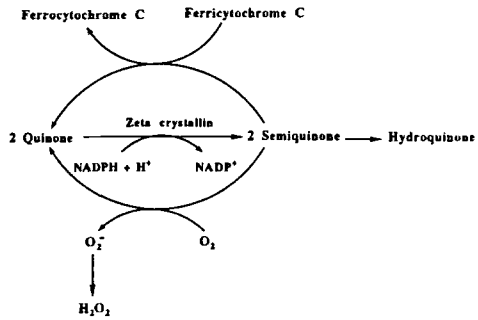


FIG. 6. **TEMPOL-H to TEMPOL oxidation during the NADPH:quinone oxidoreductase reaction of ζ-crystallin.** Assays were carried out aerobically in a final volume of 1.0 ml containing 50 mM phosphate buffer, pH 7.8, 0.1 mM NADPH, 0.1 mM diethylenetriaminepentaacetic acid, 1 mM TEMPOL-H, 5.5 μg of ζ-crystallin, and 50 μM juglone. Assays run in the presence of superoxide dismutase contained 250 units. TEMPOL formation was monitored by measuring the *middle line* of the *three-line* EPR spectrum of TEMPOL as a function of time. In the complete system (*control curve*) the TEMPOL radical is formed, whereas in the same system lacking ζ-crystallin (–ζ) only small background formation was observed. Addition of superoxide dismutase to the complete system prevents TEMPOL formation indicating the presence of O_2^-.



SCHEME 2

demonstrated to be a competitive inhibitor with respect to NADPH (16). Nitrofurans are an important group of physiologically active substances. A characteristic feature of these nitroheterocyclic compounds is their ability to be reduced to the corresponding anion-radical which is readily reoxidizable by oxygen. Many known dehydrogenases and reductases also exhibit a nitroreductase activity by reducing these heterocyclic compounds (28–30). When we tested nitrofurantoin and nitrofuran as electron acceptors for ζ-crystallin activity, there was no activity. On the other hand, nitrofurantoin inhibited the quinone reductase activity of ζ-crystallin competitively with respect to quinone substrate. Nitrofurantoin has previously been shown to be an inhibitor of glutathione and trypanothione reductases (31, 32).

The data are consistent with the concept that ζ-crystallin/NADPH:quinone oxidoreductase functions through a one-electron transfer mechanism. The strongest evidence for this conclusion is the demonstration by EPR spectrometry of semiquinone formation. The detection of oxygen consumption under aerobic conditions, H_2O_2 production, and the reduction of ferricytochrome *c* all support the one-electron transfer mechanism.

Based on these data the following hypothetical reaction scheme is proposed (Scheme 2). This scheme explains our observation that under aerobic conditions there is a nonstoichiometric relationship between the initial concentration of quinone in the reaction mixture and the amount of NADPH oxidized. Because of the O_2-mediated recycling of the semiquinone radical, the reaction continues until all NADPH is utilized. Further addition of NADPH reactivated the process (Fig. 3). Under anaerobic conditions, as predicted from Scheme 2, excess NADPH was not oxidized in the reaction; furthermore, when DCIP was utilized as substrate no such nonstoichiometry was observed. This is consistent with the fact that the product formed is redox inactive. With DCIP the reaction rate was not reduced in the absence of oxygen (11).

Quinone reductases are known to have several metabolic functions: (i) detoxification of quinones (DT-diaphorase and carbonyl reductase, EC 1.1.1.184) (16, 17, 21, 22, 27); (ii) electron transport (NADH:ubiquinone oxidoreductase, EC 1.6.99.3; NADH:cytochrome b_5 reductase, EC 1.6.2.2; and NADPH: cytochrome P-450 reductase, EC 1.6.1.4) (33–35) and (iii) vitamin K metabolism (18). Most well characterized quinone reductases are flavoproteins, the exception being carbonyl reductase (17). Aside from DT-diaphorase and carbonyl reductase these enzymes are one-electron transfer reductases and are known to be membrane bound. Interestingly ζ-crystallin is a soluble protein which does not contain a flavin moiety. We have also ruled out the possibility of ζ-crystallin being a quinoprotein by using the redox cycling staining procedure (36). Based on these properties, its substrate and cofactor specificities, and its primary sequence it is clear that ζ-crystallin is an enzyme distinct from previously reported quinone reductases. The physiological significance of this one-electron reduction of quinones is not known at present, although the enzyme might be involved in regulation of NADPH oxidation (10) or have a role in detoxification of quinones. The presence of this protein in other tissues including liver and kidney (7) at levels expected for an enzyme further supports the contention that ζ-crystallin has a discrete metabolic function in these tissues. That ζ-crystallin has an important function, catalytically or otherwise, in the physiology of the guinea pig lens is indicated by the association of a mutation in its gene with an autosomal dominant cataract (7). The mutant ζ-crystallin from cataractous lenses failed to bind coenzyme, and lenses from animals homozygous for this mutation lack this quinone reductase activity.[2] In this regard, the newly described quinone reductase activity of ζ-crystallin and further investigations into its physiological function may be helpful in elucidating the basis of enzyme/crystallin recruitment, as well as the molecular mechanism whereby this particular enzyme/crystallin is involved in the development of a hereditary cataract.

Acknowledgments—We thank Drs. D. Garland, P. Russell, and A. Russo for critically reading the manuscript and V. Blow for expert preparation of the manuscript.

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