Cataract Induction by 1,2-Naphthoquinone
I. Studies on the Redox Properties of Bovine Lens Proteins

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Conditions of oxidative stress may lead to cataract formation. Reaction of certain flavoproteins, the NADH : oxidoreductases, with different quinones is well known to form hydrogenperoxide. This reaction was investigated to get more information on cataract induction by naphthalene and its quinone metabolites. Protein extracts from bovine lens cortex exhibit "diaphorase" activity, indicated as dye reduction in the presence of NADH and dichlorophenol-indophenol (DCPIP) or ferricyanide. Different redox cycling compounds are shown to be active in this "diaphorase" reaction by lens protein extract (LCE): Oxygen consumption can be detected in the presence of pyrroloquinoline quinone and juglone whereas 1,4-naphthoquinone, menadione and paraquat are no redox cyclists in this flavoprotein catalyzed reaction.

Introduction

Cataract induction by naphthalene and its metabolite 1,2-naphthoquinone (1,2-NQ) is one of the most common models for the investigation of different forms of the so-called "senile" cataracts. Naphthoquinones can react with thiol groups in a nucleophilic Michael-type reaction and thus form protein-SH adducts [1, 2]. As a redox cycling quinone, 1,2-naphthoquinone can lead to a decreased glutathione level as well as to protein damage by forming higher protein aggregates and protein-glutathione adducts.

Naphthoquinone can be reduced chemically by glutathione, ascorbic acid or NADH, and also enzymatically by reduced flavoproteins [3]. Thus, we investigated "diaphorase"-reactions with NADH and 1,2-naphthoquinone, eventually responsible for oxygen radical formation and thus oxidative damage in lens epithelial cells. Flavoprotein-driven two electron transport from reduced nicotinamide to quinoid species and to oxygen results in the formation of hydrogenperoxide, which has been shown to be elevated in the aqueous humour of cataract patients and is thought to play an important role in cataract formation. Hydrogenperoxide has been shown to cause oxidative damage to both, lipids and proteins [4–7] and also leads to DNA modification [8].

Testing for diaphorase reactions in homogenates of isolated bovine lenses, we used well known electron acceptors of flavoprotein activity such as dichlorophenol-indophenol (DCPIP) and ferricyanide as well as different quinones which might act in electron cycling between NADH and oxygen; these quinones included 1,2-naphthoquinone and 1,4-naphthoquinone as metabolites of naphthalene in human metabolism known to induce cataracts [9, 10]. 1,2-Naphthoquinone has approximately the same redox potential as ubiquinone [11], and was therefore thought to be active in flavoprotein redox reactions.

Menadione (vitamin K) is a redox cycling *p*-quinone [12] known to be reduced by a diaphorase reaction in human liver [13]. It was found to decrease cellular glutathione and protein-SH-levels [14].

Juglone, another 1,4-naphthoquinone, couples to flavoproteins and can be used for enzyme catalyzed production of reactive oxygen species [15–17] and may also induce cataract *in vitro* [18].

Pyrroloquinoline-quinone, cofactor of the so-called "quinoproteins", as an *o*-quinone also can undergo redox cycling [19], detectable as formazan formation from nitrobluetetrazolium [20, 21]. The PQQ redox state depends on oxygen availability. PQQ is reduced by aminoacids as well as by amines and ascorbic acid.

The non-quinoid, but potently redox cycling compound paraquat, a bipyridylium salt, was also

Abbreviations: LCE, lens cortex extract; DCPIP, dichlorophenol-indophenol; DTNB, dithionitrobenzoic acid; PQQ, pyrroloquinoline quinone.

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used in our investigation. It has also been shown to induce cataract *in vitro*, leading to decreased levels in antioxidants such as ascorbic acid and glutathione [22].

In the following report, we investigated general quinone redox cycling properties of bovine lens homogenates in order to get more information on possible mechanisms of cataract formation by naphthalene.

Materials and Methods

Oxygen uptake has been determined potentiometrically in 2 ml vessels with an oxygen electrode (Rank Brothers, England) in combination with a two-channel recorder. The temperatures of the reaction mixtures were 22, 25 and 37 °C, corresponding to different oxygen saturations with 532, 506 and 414 nmoles O_2/2 ml, respectively.

Bleaching of dichlorophenol-indophenol (DCPIP) has been measured photometrically at room temperature. DCPIP can be detected at 601 nm, the molar extinction coefficient is 18,200. The extinction of DCPIP was set as 1,050 at the beginning.

Reduction of ferricyanide is detected at 420 nm. The extinction at the reaction start was 1,0.

All reactions contained 100 mM phosphate buffer, pH = 7.8.

Chemicals were from Sigma, Merck or Boehringer.

Lens proteins were prepared from bovine lenses by ammoniumsulphate fractionation (35–75% saturation) followed by 16 h dialysis against 50 mM phosphate buffer, pH = 7.8. The post-mortem age of the bulbi between slaughtering and lens protein precipitation was approximately 4 h. Precipitated and dialyzed protein extracts were stored in small portions at −16 °C. The protein content was determined with the Biorad-protein assay according to Bradford [23] yielding 2.54 mg/ml protein. Determination of SH-groups was done according to Ellman [24, 25] with 5,5′dithiobis-nitrobenzoic acid (DTNB) and spectrophotometric quantification at 412 nm. The protein extract with 2.54 mg/ml protein was 3.6 mM in SH-content, according to a SH-density of 1.42 µmol SH/mg protein.

Experiments were done at least twice or as often as indicated.

Results and Discussion

Not-autoxidizable electron acceptors of the diaphorase reaction

Ferricyanide is reduced by certain flavoproteins at the expense of NAD(P)H. The stable reduced form, ferrocyanide, cannot undergo redox reactions with oxygen. The amount of chemically bleached ferricyanide by NADH or NADH/diaphorase can be monitored at 420 nm. The extinction of yellow ferricyanide at the reaction start was 1.0.

Fig. 1 shows a significant stimulation of ferricyanide reduction by lens protein and NADH, indicating the presence of factors catalyzing the electron transfer from NADH to ferricyanide.

Dichlorophenol-indophenol (DCPIP) is a blue dye in the oxidized and is colourless in the reduced state. DCPIP reduction can be detected photometrically at 601 nm.

DCPIP is well known as an artificial electron acceptor in isolated chloroplasts and in several flavoprotein/NADH reactions.

Both, NADH and SH-groups in the protein homogenate can reduce DCPIP, where concentration-dependent bleaching is observed (data not shown).

In combination, NADH and LCD show a diaphorase-type activity (Table I).

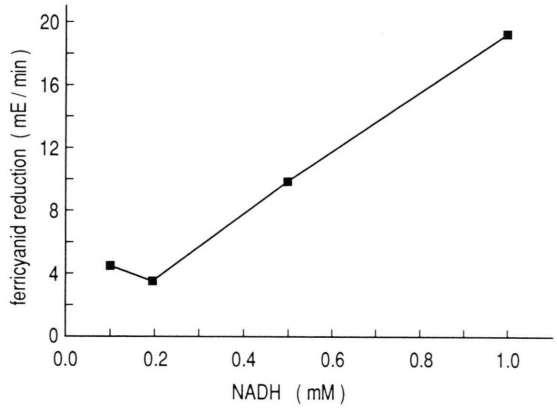

Fig. 1. Ferricyanide reducing activity of lens cortex extract in the presence of NADH.
Controls: Activities in the absence of either LCE or NADH are substracted from the corresponding values at different NADH concentrations. LCE activity (100 µl): 7.0 mE/min.

Table I. DCPIP bleaching by NADH and LCE.

Reaction conditions:
NADH: 500, 200, 100 μM
LCE: 50, 100 μl
DCPIP: E_{601} = 1.1 (bleaching in nmol/min).

NADH	0	100	200	500 μM
		0.72 (n = 2)	1.43 (n = 2)	3.85 (n = 3)
+ LCE 50 μl	8.55 (n = 4)	–	–	18.6 (n = 2)
+ LCE 100 μl	10.4 (n = 2)	15.3 (n = 2)	15.8 (n = 2)	17.5 (n = 2)

Comparison of redox cycling of different quinones

First, wie analyzed the oxygen uptake of various quinones as electron acceptors with NADH as reductant and tried to evaluate the reducing power of lens cortex extract (LCE) in order to get some information on a possible involvement of diaphorases in this redox process.

Menadione (2-methyl-naphthoquinone), with the physiological properties of vitamin K, was used in its water-soluble form (sulfonic acid). Neither as chemical electron acceptor with NADH alone nor in the presence of flavoproteins, oxygen-uptake was observed.

Paraquat (methylviologen), a low-potential (−440 mV) bipyridylium salt, is a commercially used contact herbicide producing reactive oxygen species such as superoxide [26]. Like with menadione there was no measurable oxygen uptake either with NADH or with LCE or both.

1,4-Naphthoquinone dissolved in hot water at a maximal concentration of 1 mM, catalyzes oxygen uptake in the presence of LCE (Table II).

Protein addition shows a significant increase of O_2 uptake by the *p*-quinone but there is no further stimulation after the addition of NADH. Furthermore, 1,4-naphthoquinone showed no effect as an electron acceptor with the commercially available diaphorase from *Cl. kluyveri* and NADH as electron donor.

1,2-Naphthoquinone-sulfonic acid as water soluble form of *o*-naphthoquinone like the *p*-quinone is not a cofactor of the lens cortex flavoproteins. Table III shows the oxygen uptake in the presence of 1,2-NQ and NADH, or in the presence of 1,2-NQ and LCE. A combination of protein and NADH leads to additive effects and does not yield an enhanced rate of oxygen reduction.

Methoxatin (pyrroloquinolinequinone, PQQ) is a tricarboxylated pyrrolo-quinoline-quinone with unique redox properties allowing efficient and reversible electron transfer. PQQ has been described as coenzyme in oxygenases, aminoxydases, dehydrogenases, hydratases and decarboxylases [27].

Since PQQ can operate as electron cycler between NADH and oxygen in the reaction with *Clostridium*-diaphorase, we tested this activity in lens homogenates (Table IV).

Table II. Oxygen consumption by 1,4-naphthoquinone [nmol O_2/min].

1,4-NQ	+ NADH	+ LCE	+ LCE/NADH
0	0	6.7	6.7

Reaction conditions:
NADH: 500 μM LCE: 100 μl
1,4-NQ: 50 μM T = 25 °C

Tab. III. Oxygen uptake by 1,2-naphthoquinone-sulfonic acid and NADH or lens proteins (nmol/min).

1,2-NQ 100 μM	+ NADH 100 μM	+ LCE		+ NADH + LCE	
0	25.5	100 μl	6.7	100 μl	33.4
	25.5	200 μl	11.8	200 μl	38.0

Reaction conditions:
NADH: 100 μM
LCE: 100, 200 μl
1,2-NQ: 100 μM
T = 25 °C.

Table IV. Influence of LCE on the oxygen consumption [nmol/min].

PQQ	+ NADH 500 µM	+ NADH 500 µM + LCE	+ NADH 1 mM	+ NADH 1 mM + LCE
1 µM	0	3.5	0	4.5
10	2.7	4.2	1.88	6.35
50	23.8	26.1		

Reaction conditions:

PQQ: 1, 10, 50 µM
NADH: 0.5, 1 mM
LCE: 100 µl
T = 25 °C

Table V. Oxygen consumption by juglon and NADH/LCE [nmol/min].

Juglone	25 µM	50 µM
+NADH	0	0
+ LCE	8.7	9.0
+ NADH + LCE	12.4	21.8

Reaction conditions:

Juglone: 25, 50 µM
NADH: 500 µM
LCE: 100 µl
T = 25 °C

PQQ can not be reduced by SH-groups of the protein extract, since no oxygen consumption by protein and PQQ can be detected (data not shown).

Table IV shows that no clear enzymatic effect of the protein can be seen at a "higher" concentration of PQQ (50 µM) while 10 and 1 µM PQQ show a small but significant stimulation of oxygen reduction by LCE.

Juglone, 5-hydroxy-1,4-naphthoquinone isolated from *Juglans regia* represents the aglycon of a secondary plant ingredient with germination-inhibiting and antibiotic effects coupling to certain diaphorases [17]. It is also active with LCE in the presence of NADH.

LCE causes a significant increase in oxygen consumption in the presence of juglone. The clear effect of the protein is again stimulated by NADH which in the absence of LCE is inactive (Table V).

Conclusions

1) Diaphorase activity in the lens cortex extract is detectable by ferricyanide and DCPIP reduction at the expense of NADH.

2) The diaphorase is also active with juglone and PQQ, whereas menadione, 1,4-naphthoquinone and paraquat cannot couple to this type of enzyme.

3) 1,2-Naphthoquinone reacts chemically with SH-groups, but there is no detectable diaphorase-catalysis. Thus, the cataractogenetic potential of 1,2-naphthoquinone can be explained merely by a chemical, and not an enzyme-catalyzed reaction.

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[1] M. G. Peter, Angew. Chem. Int. Ed. Engl. **28,** 555–570 (1989).
[2] E. Cadenas, Ann. Rev. Biochem. **58,** 79–110 (1989).
[3] R. Kröner *et al.*, Z. Naturforsch. **46c,** 285 (1991).
[4] R. C. Augusteyn, in: Mechanisms of Cataract Formation in the Human Lens (G. Duncan, ed.), p. 72–112, Academic Press, New York 1981.
[5] A. Spector, Invest. Ophthalmol. Vis. Sci. **25,** 130–145 (1984).
[6] K. C. Bhuyan and D. K. Bhuyan, Molecular Mechanism of Cataractogenesis. II. Evidence of Lipid Peroxidation and Membrane Damage, In: Oxygen Radicals and their Scavenger Systems. II. Cellular and Medical Aspects (R. Greenwald and G. Cohen, eds.), p. 349–356, Elsevier, New York 1983.
[7] K. R. Hightower, J. R. Reddan, and D. C. Dziedzic, Invest Ophthalmol.Vis. Sci. **30(3),** 569–574 (1989).
[8] A. Spector, N. J. Kleiman, R.-R. Huang, and R.-R. Wang, Exp. Eye Res. **49,** 685–698 (1989).
[9] B. M. Lubek, S. Kubow, P. K. Basu, and P. G. Wells, Lens and Lens Tox. Res. **6(1+2),** 203–209 (1989).
[10] J. Rees and A. Pirie, Biochem. J. **102,** 853–863 (1967).
[11] R. van Heyningen and A. Pirie, Biochem. J. **102,** 842–852 (1967).
[12] G. E. N. Kass, S. K. Duddy, and S. Orrenius, Biochem J. **260,** 499–507 (1989).
[13] R. Wallin, D. Patrick, and L. F. Martin, Biochem. J. **260,** 879–884 (1989).
[14] D. Di Monte, D. Ross, G. Bellomo, L. Eklöw, and S. Orrenius, Arch. Biochem. Biophys. **235,** 334–342 (1984).
[15] E. Paur, R. J. Youngman, E. Lengfelder, and E. F. Elstner, Z. Naturforsch. **39c,** 261–267 (1984).
[16] R. J. Youngman and E. F. Elstner, in: CRC Handbook of Methods for Oxygen Radical Research (Greenwood, ed.), 1987.
[17] E. F. Elstner, W. Schütz, and G. Vogl, Free Rad. Res. Comm. **1(6),** 355–358 (1986).
[18] K. C. Bhuyan, S. M. Podos, and D. K. Bhuyan, in: Fifth Conference on Superoxide and Superoxide Dismutase (Program + Abstracts), p. 142, Jerusalem, Israel 1989.
[19] P. M. Gallop, E. Henson, M. A. Paz, S. L. Greenspan, and R. Flückinger, Biochem. Biophys. Res. Comm. **163(2),** 755–763 (1989).
[20] P. M. Gallop, M. A. Paz, R. Flückinger, and H. M. Kagan, TIBS **14(8),** 343–346 (1989).
[21] M. A. Paz, P. M. Gallop, B. M. Torrelio, and R. Flückinger, Biochem. Biophys. Res. Comm. **154(3),** 1330 (1988).
[22] D. Bhuyan and K. Bhuyan, in: Fifth Conference on Superoxide and Superoxide Dismutase (Program + Abstracts), p. 141, Jerusalem, Israel 1989.
[23] M. M. Bradford, Anal. Biochem. **72,** 248–254 (1976).
[24] G. L. Ellman, Arch. Biochem. **74,** 443–450 (1958).
[25] G. L. Ellman, Arch. Biochem. **82,** 70–77 (1959).
[26] A. P. Autor, Biochemical Mechanisms of Paraquat Toxicity. Academic Press, New York 1977.
[27] J. A. Duine and J. A. Jongejan, Ann. Rev. Biochem. **58,** 403–426 (1989).