ζ-Crystallin catalyzes the reductive activation of 2,4,6-trinitrotoluene to generate reactive oxygen species: a proposed mechanism for the induction of cataracts

Yoshito Kumagai[a,*], Toshihiko Wakayama[a], Song Li[b,c], Azusa Shinohara[d], Akihiro Iwamatsu[d], Guifan Sun[c], Nobuhiro Shimojo[a]

[a] *Department of Environmental Medicine, Institute of Community Medicine, Master's Program in Environmental Sciences, Tsukuba University, Tsukuba, Ibaraki 305-8575, Japan*
[b] *Doctoral Program in Medical Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*
[c] *Department of Labor Hygine and Occupational Health, School of Public Health, China Medical University, Shengyang 110001, PR China*
[d] *Central Laboratories for Key Technology, Kirin Brewery Co., Ltd., 1-13-5 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236, Japan*

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Abstract Exposure to 2,4,6-trinitrotoluene (TNT) has been shown to cause induction of cataract in which oxidative stress plays a critical role. From bovine lens we purified to homogeneity and identified an enzyme that catalyzes the reduction of TNT, resulting in the production of reactive oxygen species. The final preparation of TNT reductase showed a single band with a subunit molecular weight of 38 kDa on SDS–PAGE. Sequence data from peptides obtained by digestion with lysylendopeptidase *Achromobacter* protease I (API) revealed that TNT reductase is identical to ζ-crystallin. Superoxide anions were formed during reduction of TNT by ζ-crystallin, though negligible enzyme activity or protein content for superoxide dismutase, a superoxide scavenging enzyme, was found in the lens. Thus, the present results suggest that the induction of cataracts by TNT may be associated with increased oxidative stress, as a result of reductive activation of TNT generating superoxide anions, there being minimal antioxidant enzyme activity for defense against reactive oxygen species exogenously produced in the lens.

Key words: 2,4,6-Trinitrotoluene; ζ-Crystallin; Active oxygen; Cataract; Bovine; Lens

1. Introduction

2,4,6-Trinitrotoluene (TNT) is an industrial chemical used as an explosive; chronic human exposure to TNT can result in aplastic anemia, liver damage and cataracts [1–3]. Cataracts in workers exposed to TNT typically exhibit an annular, peripheral, symmetric dark area of turbidity [2–4] believed to be characteristic of lens changes produced by chemical agents [5]. Anshou [3] reported a relatively high frequency of cataract formation in TNT-exposed workers and details of the characteristics of TNT lens impairment. Although numerous factors can cause induction of cataracts, oxidative stress is an initiating factor in the development of cataracts [6,7]. For example, Spector et al. [8] reported that exposure of cultured lenses to hydrogen peroxide caused an apparent cataract formation whereas treatment of lenses with AL-3823A, a mimic of glutathione peroxidase to decompose hydrogen peroxide, resulted in prevention of photochemically induced cataracts. It has also been shown that patients with cataract exhibit elevated lipid peroxidation, a biomarker for oxidative stress [9].

It has generally been recognized that the one-electron reduction of nitroaromatic compounds by flavin enzyme in the presence of NAD(P)H yields not only its nitro radical but also superoxide anion [10,11]. The hydrogen peroxide and hydroxyl radical generated from the superoxide anion cause damage to proteins, lipids and nucleic acids [12]. With regard to the biotransformation of TNT, it has been reported that TNT is converted to 4-hydroxyamino-2,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene, but not a TNT nitro radical, by liver microsomes [13]. Zitting et al. [14] showed production of reactive oxygen species during incubation of enzyme preparations from brain, liver and kidney with TNT in the presence of NAD(P)H. They argued that TNT was a substrate for nitroreductase(s), leading to production of superoxide. However, identification of the enzyme producing the reactive oxygen species was not attempted. The lens appears to be vulnerable to peroxidation and thus any cumulative effect of reactive oxygen species may be important because the lifetime of lens constituents is extremely long. Although to our knowledge no evidence for the existence of an enzyme system responsible for the one-electron reduction of TNT in the lens has been reported, we postulate that TNT-induced cataract formation may result from the presence of protein(s) in the lens capable of reducing TNT that may produce reactive oxygen species. This communication describes for the first time the purification and identification of an enzyme from bovine lens that acts as a TNT reductase and generates superoxide anions.

2. Materials and methods

2.1. Materials

Column gels were obtained from the following suppliers: Affi-Gel Blue (100–200 mesh) from Bio-Rad Laboratories (Richmond, CA, USA); single-stranded DNA (ssDNA) cellulose from Sigma Chemical Co. (St. Louis, MO, USA) and Sephadex G-200 was from Pharmacia LKB Biotechnology (Uppsala, Sweden). All other chemicals used were of the highest grade available. TNT was synthesized by the method of McGookin et al. [15]. Cu,Zn-superoxide dismutase (SOD), Mn-SOD and antibodies against these SOD isozymes were prepared as described previously [16,17]. Acetylated cytochrome *c* was synthesized by the method of Kakimura and Minakami [18].

*Corresponding author. Fax: (81)-298-53 3039.
E-mail: yk-em-tu@md.tsukuba.ac.jp

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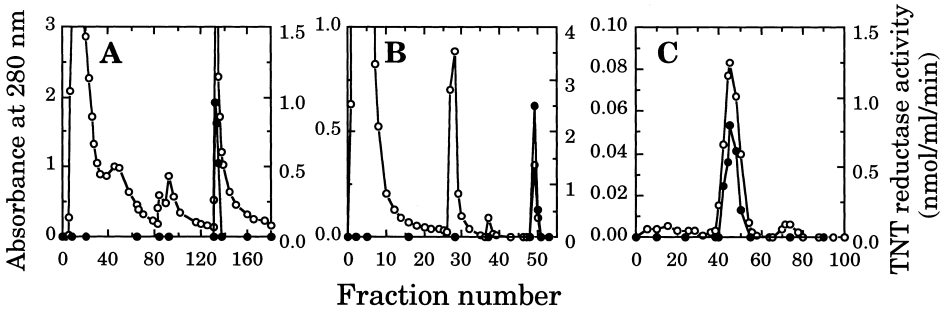


Fig. 1. Separation of TNT reductase from bovine lens cytosol by column chromatographies. A: Affi-Gel Blue column. B: ssDNA cellulose column. C: Sephadex G-200 column. Open and closed circles indicate absorbance at 280 nm and TNT reductase activity, respectively. Cytosol fraction (47 ml) was applied on an Affi-Gel Blue column (16×2.5 cm, i.d.), which had been equilibrated with 20 mM potassium phosphate buffer (pH 7.6)–0.5 mM EDTA–5 mM 2-mercaptoethanol (buffer A). The column was successively washed with buffer A at a flow rate of 30 ml/h, followed by buffer A–0.1 M NaCl (from fraction 76). TNT reductase was eluted using buffer A–2 M NaCl (from fraction 125). Affi-Gel Blue fractions (Nos. 132–135, 41 ml) containing TNT reductase activity were combined and then dialyzed against 20 mM potassium phosphate buffer (pH 7.6)–1 mM 2-mercaptoethanol (buffer B). The resulting dialyzed sample obtained was applied to a ssDNA cellulose column (4.7×1 cm, i.d.), which had been equilibrated with buffer B. The column was successively washed with buffer B at a flow rate of 30 ml/h, followed by buffer B–0.1 M NaCl (from fraction 27), and then buffer B–sodium dextran sulfate (0.5 mg/ml) (from fraction 37). TNT reductase was eluted with buffer B–2 M NaCl (from fraction 49). ssDNA-eluted fractions (Nos. 49–50, 19 ml) were combined and directly applied to a Sephadex G-200 column (71×2.5 cm, i.d.), which had been equilibrated with 20 mM potassium phosphate buffer (pH 7.6)–0.15 M NaCl at a flow rate of 20 ml/h. Fractions containing TNT reductase activity (Nos. 42–45, 45 ml) were combined and concentrated to 1.5 ml using an Amicon Ultrafiltration system with a PM10 membrane.

2.2. Enzyme assay

Bovine eyes were collected from the slaughterhouse (Shimodzuma, Ibaraki, Japan) on the day the animals were slaughtered. Lenses were extracted the same day and stored at −70°C. All operations were performed at 2–4°C. Lenses (8.3 g) were well chopped and then homogenized in 6 volumes of 20 mM potassium phosphate buffer (pH 7.6)–1 mM EDTA–5 mM dithiothreitol. The homogenate was centrifuged at 9000×*g* for 20 min. Supernatants obtained were further centrifuged at 105 000×*g* for 60 min. All reactions were performed at 25°C. Reduction of TNT was measured by the decrease in absorbance at 340 nm, based on the oxidation of NADPH to $NADP^+$, using an extinction coefficient of 6.22 mM^{-1} cm^{-1}, in a Shimadzu UV1600 double beam spectrometer (Kyoto, Japan). Incubation mixtures (1.5 ml) consisted of 0.5 mM TNT, enzyme preparation (10–30 μg), 0.1 mM NADPH and 0.1 M potassium phosphate buffer (pH 7.4). The reactions were initiated by addition of the NADPH. Superoxide anion generation was determined by measuring SOD-inhibitable reduction of acetylated cytochrome *c* as described previously [19]. The reaction mixture (1.5 ml) contained purified TNT reductase, 25 μM acetylated cytochrome *c*, 0.5 mM TNT, and 0.1 M potassium phosphate buffer (pH 7.4) in the absence and presence of human Cu,Zn-SOD (1.00 U). SOD activity and immunoblot analysis with antibody against mouse Cu,Zn-SOD and Mn-SOD was performed by our previously established method [17,20]. The protein concentration was determined by the Bradford method [21], using bovine serum albumin as the standard.

2.3. Identification of protein by peptide mass mapping

The final preparation of the TNT-reducing enzyme from the Sephadex-G 200 column was subjected to SDS–PAGE, on a 15% gel. After electrophoresis, the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane, and stained with Ponceau S. The 38 kDa band was reduced, *S*-carboxymethylated, and digested in situ with *Achromobacter* protease I (API, a Lys-C) as described previously [22]. Molecular mass analysis of the API fragments was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) using a PerSeptive Biosystems Voyager-DE/RP. Identification of the 38 kDa protein was carried out by comparison between the molecular weights determined by MALDI-TOF/MS and the theoretical peptide masses from the proteins registered in NCBInr (10.04.99) [23].

3. Results

The enzyme(s) responsible for the reduction of TNT was localized in the bovine lens cytosol, while no enzyme activity was found in the microsomes. The TNT reductase activity was highly specific for NADPH; substitution of NADH for NADPH resulted in a minimal enzyme activity. As pyridine nucleotide-dependent oxidoreductases such as α,β-ketoalkene double bond reductase [24], sorbitol dehydrogenase [25] and ζ-crystallin [26] in bovine lens cytosol have been found to show high affinity to Blue-Sepharose resin, we attempted first to separate the NADPH-dependent enzymes responsible for TNT reduction from bovine lens cytosol by Blue-Sepharose column chromatography. As shown in Fig. 1A, the enzyme(s) catalyzing the reduction of TNT was tightly bound to this dye and was eluted with buffer containing 2 M NaCl. Dialyzed Blue-Sepharose fractions containing TNT reductase activity exhibited a strong affinity to a ssDNA cellulose column (Fig. 1B). The ssDNA agarose fractions eluting with a high concentration of NaCl contained a 38 kDa protein as an abundant band and a few faint bands on SDS–PAGE as shown in Fig. 2. The TNT reductase was further purified by Sephadex G-200 column chromatography, to give a final preparation of a 38 kDa protein showing a single bond on SDS–PAGE with a specific activity of 8.6 nmol/min/mg (see Table 1 and Fig. 2). Interestingly, the purified enzyme cata-

Table 1
Purification of TNT reductase from bovine lens

Step	Volume (ml)	Protein (mg)	Total activity (U)	Specific activity (nmol/mg/min)	Purification (%)
Cytosol	47	2120	46	0.022	1
Affi-Gel Blue	51	163	62	0.38	17
ssDNA cellulose	19	8.3	31	3.7	168
Sephadex G-200	1.5	2.1	18	8.6	391

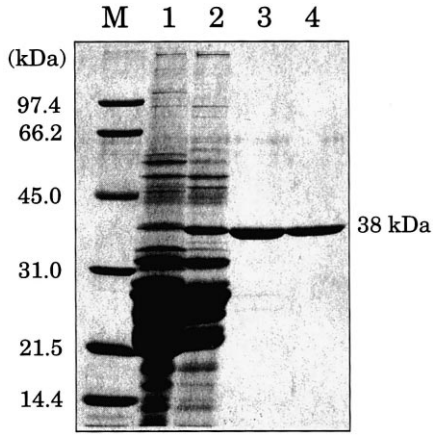


Fig. 2. SDS–polyacrylamide gel (12%) electrophoresis of enzyme preparations possessing TNT reductase activity. Lane M, markers; lane 1, cytosol fraction (68 μg); lane 2, Affi-Gel Blue fraction (27 μg); lane 3, ssDNA fraction (2.2 μg); lane 4, Sephadex G-200 fraction (1.9 μg).

lyzed the reduction of 9,10-phenanthraquinone, defined as a ζ-crystallin activity [27]. We next examined substrate specificity toward other nitroaromatic compounds for ζ-crystallin. Among the nitrotoluenes examined, 2,4-dinitrotoluene was readily reduced but its 2,6-isomer and 4-nitrotoluene were poorly reduced; 2-nitrotoluene was not reduced at all. The specific activity for 1,2- or 1,3-nitrobenzene was about 75% of that for TNT reduction whereas 1,4-dinitrobenzene was the best substrate for ζ-crystallin among the nitroaromatics tested (6.3 times that of TNT reduction).

The subunit molecular weights, specific affinity to ssDNA, and high substrate specificity for 9,10-phenanthraquinone suggested that the isolated protein catalyzing reduction of TNT was ζ-crystallin. Consistent with this, elution patterns of 9,10-phenanthraquinone reductase activity were identical to those of TNT reductase activity during column chromatography purification (data not shown). To further confirm this hypothesis, the observed molecular masses of the fragments of TNT reductase obtained by digestion with API were analyzed and compared with the theoretical masses of the corresponding peptides from ζ-crystallin, using the sequence in the protein data base (Table 2). All the masses determined were almost the same as the theoretical API-digested fragment masses of bovine ζ-crystallin [26]. Furthermore, the N-terminal amino acid sequences of AP-1 and AP-6 were identical with those of bovine ζ-crystallin (data not shown).

Table 2
Fragment sequences of bovine lens TNT reductase determined using molecular mass analysis

Fragment	Observed mass	Theoretical mass	Δ[a]	Sequences from database
AP-1	664.33	664.35	0.02	(196)VFNHK
AP-2	852.45	852.41	0.04	(201)EANYIDK
AP-4	1166.78	1166.68	0.10	(23)LQSDVAVPIPK
AP-5	1722.04	1721.86	0.18	(307)ATQAHENIIHSSG-ATGK
AP-6	1962.29	1962.10	0.19	(6)LMRAIRVFEFGG-PEVLK

The number in parentheses represents the position from N-terminal amino acid of bovine ζ-crystallin.
[a]The difference between the observed and theoretical mass.

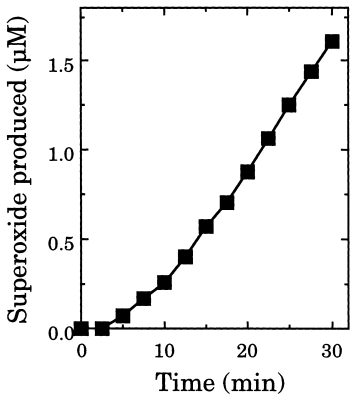


Fig. 3. Production of superoxide anions during reduction of TNT by ζ-crystallin. ζ-Crystallin (36 μg) was incubated with 0.5 mM TNT under the conditions described in Section 2. Each point is the average of duplicate determinations.

When bovine ζ-crystallin was incubated with TNT in the presence of NADPH, a time-dependent generation of superoxide anions was detected (Fig. 3). However, the enzyme activity of SOD which scavenges superoxide in bovine lenses was minimal; the specific activity in the lens was only 0.14% of that in the liver (lens, 0.067 ± 0.021 U/mg, $n = 4$; liver, 49.1 ± 2.7 U/mg, $n = 3$). As shown in Fig. 4, appreciable amounts of Cu,Zn-SOD protein and Mn-SOD protein of bovine were not detected.

4. Discussion

The present study demonstrates that an enzyme catalyzing the reduction of TNT is present in bovine lens and that the purified protein was ζ-crystallin. It has been shown that crystallins consists of two major classes; one is the ubiquitous crystallins, the α-, β- and γ-isoforms, present in all vertebrates, and the other is the 'taxon-specific crystallins', which are found in large amounts in lenses from phylogenetically restricted groups [28,29]. These taxon-specific crystallins are pyridine nucleotide-dependent oxidoreductases, present at enzymatic levels in non-lenticular tissues [28,29]. ζ-Crystallin was identified as a member of the taxon-specific crystallin family and has been shown to belong to the alcohol/polyol dehydrogenase superfamily even though it possesses no such enzymatic activity [30]. It was subsequently reported that ζ-crystallin acts as a novel NADPH-dependent quinone reduc-

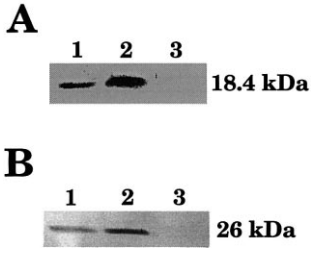
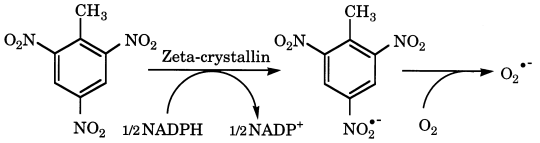


Fig. 4. Western blot analysis of bovine Cu,Zn-SOD and Mn-SOD. A: With anti-mouse Cu,Zn-SOD. B: With anti-mouse Mn-SOD. Lane 1, purified mouse SOD (A, 0.1 μg; B, 0.4 μg); lane 2, liver cytosol (40 μg); lane 3, lens cytosol (40 μg). Each cytosol fraction contains mitochondrial matrix where Mn-SOD is localized.



Scheme 1. ζ-Crystallin-catalyzed reduction of TNT and generation of superoxide anions.

tase (EC 1.6.5.5) [26,27,31]. Although ζ-crystallin exhibits species specificity [28], this protein is expressed in humans [32].

Using the purified enzyme activity, superoxide anions were produced during metabolism of TNT by ζ-crystallin, but the purified enzyme did not catalyze reductions of TNT to its four- and six-electron reduction products; 4-hydroxyamino-2,6-dinitrotoluene (four-electron reduction) and 4-amino-2,6-dinitrotoluene (six-electron reduction) were not seen under either aerobic or anaerobic conditions (data not shown). This suggests that one-electron reduction of TNT is mediated by ζ-crystallin (Scheme 1); NADPH was oxidized during the reaction, suggesting that the TNT nitro groups were reduced. However, no four- and six-electron reduction products were seen. If, however, a one-electron reduction of TNT was catalyzed by ζ-crystallin, the resulting nitro anion radical would readily react with molecular oxygen to form a superoxide anion, as we detected.

Although many multi-factors can cause cataract formation, oxidative stress is thought to be critically important in this condition [6,7]. SOD is extensively expressed in a variety of organs and protects tissues from oxidative stress by scavenging superoxide anion [33]. However, we have also shown that total SOD activity and protein contents for Cu,Zn-SOD and Mn-SOD in the lens were quite low. Markedly low levels of SOD in mammalian lenses have also been reported by others [34,35]. It has also been reported that exposure of animals to quinoid compounds such as naphthoquinone, plumbagin and juglone can also cause cataract formation [36–38]. Rao et al. [27] reported that ζ-crystallin obtained from guinea pig reduced various quinoid compounds effectively, and produced superoxide and hydrogen peroxide.

In conclusion, it seems likely that cataract formation by prolonged exposure to TNT may be associated with minimal expression of lens SOD isozymes, resulting in increased oxidative stress as a result of superoxide anions generated during the ζ-crystallin-catalyzed reduction of TNT. It also seems quite reasonable that reductive activation by ζ-crystallin may contribute to the reactive oxygen species-mediated induction of cataracts by quinoid compounds.

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