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Reduction of Azobenzene by Purified Bovine Liver Quinone Reductase

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Quinone reductase was purified to homogeneity from bovine liver by using ammonium sulfate fractionation, ion-exchange chromatography, and gel filtration chromatography. The enzyme utilized either NADH or NADPH as the electron donor. The enzyme catalyzed the reduction of several quinones and other artificial electron acceptors. Furthermore, the enzyme catalyzed NAD(P)H-dependent reduction of azobenzene. The apparent Km for 1,4-benzoquinone and azobenzene was 1.64 mM and 0.524 mM, respectively. The reduction of azobenzene by quinone reductase was almost entirely inhibited by dicumarol or Cibacron blue 3GA, potent inhibitors of the mammalian quinone reductase. In the presence of 1.0 μM Cibacron blue 3GA, azoreductase activity was lowered by 45%, and almost complete inhibition was seen above 2.0 μM Cibacron blue 3GA.

Keywords: Azobenzene, Quinone reductase, Reduction.

Introduction

Quinone reductase, formerly known as DT diaphorase, promotes the obligatory two-electron reduction of various quinones to hydroquinones (Hojeberg *et al.*, 1981; Prochaska and Talalay, 1986; Constam *et al.*, 1991; MacDonald, 1991; Tang and Johansson, 1995). The enzyme can utilize either NADH or NADPH as the cofactor, and is highly inducible in animals following pretreatment with various xenobiotic chemicals. Quinone reductase is known to be a major enzyme of xenobiotic metabolism that carries out two-eletron reductions and thereby protects cells against the toxicities of quinones and their metabolic precursors (Prestera *et al.*, 1993; Brock *et al.*, 1995). In contrast, NADPH: cytochrome P-450 oxidoreductase, which produces toxic and mutagenic free radicals, mediates one-electron reduction (Brock *et al.*, 1995).

Quinone reductase is classified as a phase II enzyme since it is often induced coordinately with other phase II enzymes, and exerts protective functions (Zhang *et al.*, 1992; Prestera *et al.*,1993). Phase I enzymes, also designated as mixed-function oxidases, monooxygenases, or cytochromes P-450, functionalize xenobiotics (Gordon *et al.*, 1991). Phase II enzymes (e.g. NAD(P)H: quinone reductase, glutathione-S-transferase and UDP-glucuronosyl transferase) convert the reactive electrophiles to less toxic products. Phase II enzymes are widely distributed in mammalian cells and tissues, and inductions of phase II (detoxication) enzymes are major mechanisms for protecting animals against carcinogens and other toxic electrophiles (Zhang *et al.*, 1992; Prestera *et al.*,1993). Both classes of enzymes are induced by a wide variety of compounds. Monofunctional inducers elevate phase II enzymes selectively and bifunctional inducers raise the levels of both phase I and phase II enzymes (Bayney *et al.*, 1989; Zhang *et al.*,1992). It was reported that several compounds, including polycyclic aromatic hydrocarbons, act as bifunctional inducers causing induction of phase I and phase II detoxication enzymes (Ramchandani *et al.*,1994).

Quinone reductase is present at relatively high levels in liver and known to be induced by many xenobiotics in animal tissues. Such inductions are associated with an enhanced protection against electrophiles (DeLong *et al.*, 1986; Shaw *et al.*, 1991; Zhang *et al.*, 1992; Prestera *et al.*, 1993; Chung *et al.*, 1994; Brock *et al.*, 1995). In the rat hepatoma cell line, quinone reductase mRNA is induced by polycyclic aromatic hydrocarbons and other planar aromatic compounds (Favreau and Pickett, 1995).

Azo compounds, which are characterized by one or more R-N = N-R' (azo) bonds, are used widely in the textile, food and cosmetic industries (Heinfling *et al.*, 1997; Keck *et al.*, 1997; Hu, 1998). Some azo compounds have been shown to be toxic and to cause testicular damage or bladder cancer in human (Rafii *et al.*, 1997). Degradation of azo compounds consists of two steps. The first step is the cleavage of the azo bond. The compound is reduced and the azo bridge is cleaved by azoreductase to produce aromatic amines (Chung and Stevens, 1992; Keck *et al.*, 1997; Rafii *et al.*, 1997). Some azo polymers, which are useful for drug delivery, are also broken down by azoreductase (Samyn *et al.*, 1995; Shantha *et al.*, 1995; Kalala *et al.*, 1996).

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Bayney *et al.* (1989) demonstrated that Sudan III, an azo compound, induced the quinone reductase mRNA in the rat hepatoma cell line. They also showed that the induction of quinone reductase mRNA can be blocked by cycloheximide, suggesting a requirement for ongoing protein synthesis in the induction process (Bayney *et al.*, 1989). In addition, Ramchandani *et al.* (1994) reported that the administration of Metanil yellow and Orange II, both azo compounds, to rats caused a significant induction of quinone reductase activity. In rats, Metanil yellow and Orange II brought about a substantial increase in cytosolic detoxication enzyme activities, quinone reductase and glutathione S-transferase. They also reported that following oral administration, Metanil yellow undergoes azo reduction to metanilic acid and *p*-aminodiphenylamine (Ramchandani *et al.*, 1994). However, the enzyme that is responsible for the azo reduction has not yet been elucidated.

In the present study, we purified quinone reductase from bovine liver and demonstrated that the enzyme possesses azoreductase activity. Also in this study, quinone reductase was characterized as to substrate specificity, kinetic constants, susceptibility to inhibitor, and the ability to serve as an azoreductase.

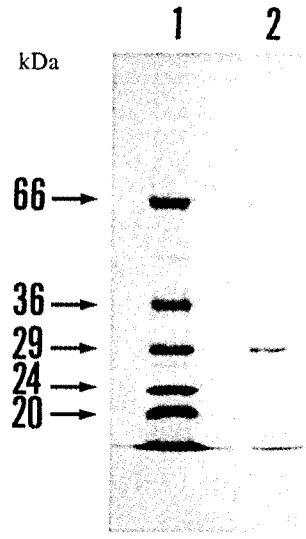

Fig. 1. SDS-PAGE of purified quinone reductase. Lane1: molecular weight marker proteins containing bovine abumin (66 kDa), glyceraldehyde-3-P-dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa) and soybean trypsin inhibitor (20 kDa). Lane 2: purified quinone reductase.

Materials and Methods

Chemicals Cibacron blue 3GA, dicumarol, 2,6-dichlorophenolindophenol, 2-methyl-1,4-naphthoquinone, azobenzene, 4,4'-azoxyanisole, benzotriazole, 1,4-benzoquinone and methyl viologen were purchased from Aldrich Chemical Co. (Milwaukee, USA). Coomassie brilliant blue G-250 was from Bio-rad Chemical Co. (Richmond, USA). CM-Sepharose, Sephacryl S-200-HR, NADH, NADPH, glycine, ethylenediaminetetraacetic acid (EDTA), N,N,N',N'-tetramethylethylenediamine (TEMED), acrylamide, sucrose, N,N'-methylenebisacrylamide, bromophenol blue, Coomassie brilliant blue R-250, bovine serum albumin, ammonium persulfate and lauryl sulfate were obtained from Sigma Chemical Co. (St. Louis, USA). All other chemicals were of the highest purity grade commercially available.

Activity measurement The 1,4-benzoquinone reductase enzyme activity was measured in 25 mM Tris · HCl buffer, pH 8.0, 200 μM NAD(P)H and 250 μM 1,4-benzoquinone. The reaction was initiated by the addition of enzyme. The reaction was followed by recording the oxidation of NAD(P)H at 340 nm (Kim and Suk, 1999). NADH was quantified from the extinction coefficient, 6.22 $mM^{-1}cm^{-1}$. Conditions for the specific reactions are presented in the related figure or table legends. One unit of enzyme was defined as the amount catalyzing the oxidation of 1 μmol of NADH per min.

Protein determination and electrophoresis Protein concentration was determined according to the method of Bradford (1976), using bovine serum albumin as a standard. The protein content in fractions collected during each chromatographic procedure was determined by measuring absorbance at 280 nm.

Polyacrylamide gel electrophoresis was carried out according to Laemmli (1970) and gels were stained with Coomassie brilliant blue R-250.

Enzyme purification The 1,4-benzoquinone reductase was purified to electrophoretic homogeneity from bovine liver. All procedures were carried out at 4°C unless otherwise indicated. Bovine liver was homogenized in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA. The homogenate was

Table 1. Purification of quinone reductase from bovine liver

Purification step	Total protein (mg)	Total activity (U)[a]	Specific activity (mU/mg)	Purification (fold)	Recovery (%)
Crude extract	2885	0.8355	0.2896	1	100
Ammonium sulfate fractionation	518.9	0.4077	0.7857	2.71	48.8
CM-Sepharose	30.64	0.4007	13.08	45.17	47.9
Sephacryl S-200-HR	3.406	0.3144	92.31	318.8	37.6

[a]1U = 1 μmol of NADH oxidized min^{-1}.
Enzyme activities and protein content were assayed as described under Materials and Methods.

Table 2. Substrate specificity of quinone reductase purified from bovine liver

Substrate (100 μM)	Relative enzyme activity (% of control)	
	NADH (200 μM)	NADPH (200 μM)
1,4-Benzoquinone	100	72.2
5-Hydoxy-2-methyl-1,4-naphthoquinone	29.6	58.6
Azobenzene	35	10.2
4,4'-Azoxyanisole	28.5	8.1
2-Methyl-1,4-naphthoquinone (menadione)	28.3	74.3
2,6-Dichlorophenolindophenol	88.6	177
Benzotriazole	3.2	
Methyl viologen	0.3	0

Reaction rates are expressed relative to the rate with 1,4-benzoquinone(100 μM) and NADH(200 μM) as equal to 100. The reaction mixture consisted of 100 μM of the indicated substrate, 200 μM NAD(P)H, 100 mM sodium phosphate buffer, pH 7.0, and the enzyme.

centrifuged at 10,000 × g for 15 min. Solid ammonium sulfate was added to the supernatant to make 50% saturation. The suspension was stirred for 1 h and then centrifuged at 20,000 × g for 15 min at 4°C. Then the supernatant was made to 75% saturation with ammonium sulfate and centrifuged as before. The precipitate was suspended in 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, and dialyzed against the same buffer. The dialysate was applied to a CM-Sepharose column, which was previously equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. The column was washed with the same buffer and then 1,4-benzoquinone reductase was eluted from the column with a linear gradient of 0-0.5 M NaCl in the same buffer. Fractions were assayed for protein concentration and reductase activities as indicated. The active fractions were pooled, concentrated, and then applied to a Sephacryl S-200 column equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. Proteins were eluted with equilibration buffer, and the active fractions were pooled and used for further study.

Spectroscopy and kinetics UV-visible spectroscopy was performed using a Shimadzu Model 3100 UV-NIR spectrophotometer. Kinetic constants for 1,4-benzoquinone reductase-catalyzed reduction of azobenzene were determined by monitoring the disappearance of NADH at 340 nm. Initial velocities were estimated and corrected for the nonenzymatic reaction. The extent of nonenzymatic reduction was determined under the same conditions and subtracted from the values obtained for the enzymatic reaction.

Gas chromatography Gas chromatography was performed using a Hewlett-Packard 6890 Plus gas chromatograph system equipped with an HP 5 column and a flame ionization detector.

Table 3. Kinetic constants for the purified bovine liver quinone reductase toward 1,4-benzoquinone and azobenzene[a]

Substrate	V_{max} (μmol/min/mg)	K_m (mM)	V_{max}/K_m
1,4-Benzoquinone	9.692	1.643	5.899
Azobenzene	0.218	0.524	0.416

[a]Assays were carried out as described in the text. A fixed NADH concentration of 200 μM was used in determining the K_m for the electron acceptors.

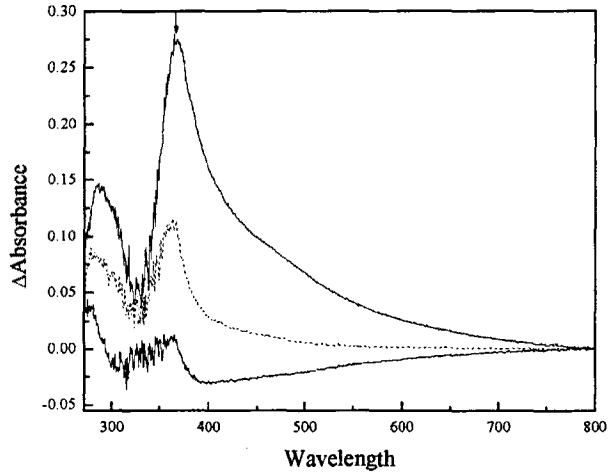

Fig. 2. Changes in absorption spectra during reduction of azobenzene by quinone reductase. Reaction conditions were as follows: 150 μM azobenzene, 200 μM NADH, 25 mM Tris · HCl buffer, pH 8.0, and purified bovine liver quinone reductase. Spectra were recorded at 5-min intervals. The decrease of the absorption band of azobenzene is indicated by the arrow.

The oven temperature was programmed to ramp from 100 to 250°C at 15°C/min. The injection port temperature was 250°C and the detector temperature was 280°C. Gas flow rates were 1 ml/min for nitrogen, 30 ml/min for hydrogen, and 300 ml/min for air.

Results and Discussion

Quinone reductase was purified to electrophoretic homogeneity from bovine liver by a combination of ammonium sulfate fractionation, ion-exchange chromatography and gel filtration chromatography. Details of the purification procedure have been described under Materials and Methods. Table 1 shows a typical result of purification. The enzyme was purified approximately 312-fold and the total recovery of the enzyme activity from this purification procedure was 37.6%.

Several different electron acceptors were tested as substrates for the purified enzyme (Table 2). Quinone reductase can utilize either NADH or NADPH as the cofactor, whereas the purified *P. chrysosporium* quinone reductase utilized only NADH (Constam *et al*, 1991). In addition to the

Table 4. Inhibition of the azoreductase activity of the purified bovine liver quinone reductase.

Inhibitor	Concentration (μM)	Residual activity (% of control)
Cibacron blue 3GA	0	100
	0.5	64.4
	1.0	55.1
	1.5	33.8
	2.0	0
Dicumarol	0	100
	10	76.5
	50	18.9

The reaction mixture contained 25 mM Tris · HCl buffer (pH 8.0), 100 μM azobenzene, 200 μM NADH, inhibitor, and purified quinone reductase. Results are expressed as a percentage of the activity without inhibitor, with 100 representing no inhibition and 0 representing complete inhibition.

benzo- and naphthoquinones, the enzyme also catalyzed the reduction of artificial electron acceptors, such as 2,6-dichlorophenolindophenol (DCPIP) and menadione (vitamin K_3). On the other hand, NADH oxidation was not detected when benzotriazole or methyl viologen were used as potential electron acceptors.

To examine whether or not quinone reductase possesses azoreductase activity, azobenzene and 4,4'- azoxyanisole were also used as substrates. Quinone reductase purified from bovine liver catalyzed the NADH- and NADPH-dependent reduction of azobenzene or 4,4' azoxyanisole (Table 2). The effect of increasing substrate concentrations on the NADH-dependent reduction of azobenzene catalyzed by quinone reductase was investigated. The rate of reduction of azobenzene was almost linearly proportional to the concentration of azobenzene until the maximum concentration of 100 μM. The kinetic constants were calculated from Lineweaver-Burk plots and summarized in Table 3. The apparent Km for 1,4-benzoquinone was 1.64 mM, and 0.524 mM for azobenzene. Fig. 2. shows the change of absorption spectra of azobenzene treated with bovine liver quinone reductase. After a quinone reductase treatment, a slight shift of local maximum absorbance (366 nm), corresponding to azobenzene with a shorter wavelength (361 nm), was observed and its intensity was almost completely decreased. The overall intensity in the visible range has also decreased. This implies that the chemical structure of azobenzene is broken down by quinone reductase. Using a GC analysis only, the product could not be identified (data not shown).

Dicumarol and Cibacron blue 3GA are known inhibitors of quinone reductase (Prochaska and Talalay, 1986; MacDonald, 1991; Brock *et al*, 1995; Brock and Gold, 1996). MacDonald (1991) reported that dicumarol inhibited the rat quinone reductase when NADPH was the substrate along with a quinone, but was slightly less effective when NADH, instead of NADPH, was the substrate. NADH-dependent reduction of azobenzene by quinone reductase was strongly inhibited by dicumarol (Table 4).

The 10 μM dicumarol inhibited the NADH-dependent reduction of azobenzene by quinone reductase to 76.5%. Cibacron blue 3GA also potently inhibited the azoreductase activity of quinone reductase. The effect of increasing concentrations of Cibacron blue 3GA on the reduction of azobenzene by quinone reductase is shown in Table 4. The 1.0 μM Cibacron blue 3GA decreased the azoreductase activity of the enzyme by 45%, and 2.0 μM Cibacron blue 3GA inhibited the reduction of azobenzene completely. These results combined with the absorption spectra supports the fact that the azo compound could be reduced by quinone reductase.

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