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Bioreduction of N,N-dimethyl-*p*-nitrosoaniline

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Besides a variety of quinones, purified bovine liver quinone reductase catalyzed the reduction of N,N-dimethyl-*p*-nitrosoaniline to N,N-dimethyl-*p*-phenylenediamine. The formation of N,N-dimethyl-*p*-phenylenediamine was identified by TLC, GC, GC-MS and NMR. Quinone reductase can utilize either NADH or NADPH as a source of reducing equivalents. The apparent Km for 1,4-benzoquinone and N,N-dimethyl-*p*-nitrosoaniline was 1.64 mM and 0.22 mM, respectively. The reduction of N,N-dimethyl-*p*-nitrosoaniline was almost entirely hampered by dicumarol or Cibacron blue 3GA, potent inhibitors of mammalian quinone reductase. During the bovine liver quinone reductase-catalyzed reduction of N,N-dimethyl-*p*-nitrosoaniline, benzoquinonediiminium ion was produced.

Keywords: Bioreduction, N,N-Dimethyl-*p*-nitrosoaniline, N,N-Dimethyl-*p*-phenylenediamine

Introduction

Quinone reductase, also referred to as DT-diaphorase, catalyzes the two-electron reduction of quinones to hydroquinones (Constam *et al.*, 1991; Tang and Johansson, 1995; Trost *et al.*, 1995; Brock and Gold, 1996). Quinones and related compounds have received considerable attention recently due to their widespread environmental occurrence and toxicological potential. Quinone reductase is known to be a phase II detoxification enzyme, and is protective against quinone and quinone imine toxicity by virtue of a two-electron reduction to the hydroquinone, thereby facilitating conjugation with glucuronide, partitioning into the aqueous phase, and excretion (Gordon *et al.*, 1991; MacDonald, 1991; Shaw *et al.*, 1991; Prestera *et al.*, 1993; Brock *et al.*, 1995). Quinone reductase are ubiquitous throughout eukaryotes. In mammals, they are present in many organs, but are most abundant in the liver (Prochaska and Talalay, 1986; Chung *et al.*, 1994).

Recently, the use of *Saccharomyces cerevisiae* was developed in organic synthesis (Fronza *et al.*, 1994; Fogliato *et al.*, 1995). We reported the selective reduction of aromatic nitroso compounds using *S. cerevisiae* as a reductant (Baik *et al.*, 1995; Kim *et al.*, 1995). *S. cerevisiae* selectively and rapidly reduced nitroso compounds with high yields under neutral conditions. We then suggested that *S. cerevisiae* quinone reductase is responsible for the reduction of nitroso compounds using *S. cerevisiae* (Kim and Suk, 1999).

Quinone reductase is known to be a xenobiotic metabolizing enzyme, and is highly inducible in animals following pretreatment with various xenobiotic chemicals, including polycyclic aromatic hydrocarbons and other planar aromatic compounds (Hojeberg *et al.*, 1981; DeLong *et al.*, 1986; Bayney *et al.*, 1989; Zhang *et al.*, 1992; Ramchandani *et al.*, 1994; Faveau and Pickett, 1995). Nitroaromatics are used extensively in the synthesis of industrial chemicals. Some of the nitroaromatic compounds have been found to be mutagenic and carcinogenic in animal studies (Valli *et al.*, 1992). Nitroso aromatic compounds are also used extensively in textile and dyestuff industries. We also intake aromatic nitroso compounds as food additives. C. Bryant and M. Deluca (1991) insist that the reduction of the nitro functional group of the nitroaromatic compounds proceeds through the nitroso and hydroxylamine intermediates to the fully reduced amino adduct, and report that the purified nitro reductase can reduce quinones, as well as several nitroaromatic compounds. However, the nitroreductase is a minor component of the total cytosolic protein under basal conditions. Among the enzyme thus far isolated from mammalian tissues, only quinone reductase has been characterized as catalyzing two-electron reduction with respect to its ability to reduce nitroaromatic compounds (Bryant and DeLuca, 1991; Wu *et al.*, 1997). In an attempt to examine whether mammalian quinone reductase could catalyze the reduction of the nitroso functional group or not, quinone reductase was purified from bovine liver and its nitroso reductase activity was examined. N,N-dimethyl-*p*-nitrosoaniline, which has good spectral properties, was chosen as a chromophoric substrate.

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Materials and Methods

Chemicals Dicumarol, Cibacron blue 3GA, 2,6-dichlorophenolindophenol, N,N-dimethyl-*p*-nitrosoaniline, nitrosobenzene, 2,6-dimethylbenzoquinone, 2-hydroxymethyl-6-methoxy-1,4-benzoquinone, 2-methyl-1,4-naphthoquinone, 1,4-benzoquinone, 4-nitroacetophenone and 4-nitrobenzaldehyde were obtained from the Aldrich Chemical Co. (Milwaukee, USA). Coomassie brilliant blue G-250 was from the Bio-rad Chemical Co. (Richmond, USA). CM-Sepharose, bovine serum albumin, NADH, NADPH, glycine, Sephacryl S-200-HR, ethylenediamine tetraacetic acid (EDTA), ammonium persulfate, N,N,N',N'-tetramethylethylenediamine (TEMED), acrylamide, CM-Sepharose, sucrose, N,N'-methylenebisacrylamide, bromophenolblue, Coomassie brilliant blue R-250 and lauryl sulfate were purchased from the Sigma Chemical Co. (St. Louis, USA). TLC sheets (silica gel 60 F-254) were obtained from Merck (Darmstadt, Germany). All other chemicals were of the highest purity grade commercially available.

Enzyme assay Quinone reductase activity was measured in 25 mM Tris-HCl buffer (pH 8.0) containing 100 μM 1,4-benzoquinone and 200 μM NAD(P)H. Reactions were initiated by the addition of the enzyme. The decrease in absorbance at 340 nm, due to NAD(P)H oxidation, was monitored spectrophotometrically (Kim and Shin, 2000). 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 Protein concentration was determined according to the method of Bradford (Bradford, 1976), using bovine serum albumin as a standard. The protein content in fractions collected during each chromatographic procedure was determined by absorbance at 280 nm.

Enzyme purification Quinone reductase was purified to electrophoretic homogeneity from bovine liver by the method of Kim and Shin (2000). All subsequent steps were carried out at 4°C. Bovine liver was homogenized in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA. The homogenate was centrifuged and the supernatant was subjected to fractional precipitation using solid ammonium sulfate. The crude extract was brought to 50% saturation and the suspension was stirred for 1 h and then centrifuged. The supernatant was decanted and adjusted to 75% saturation by adding ammonium sulfate, and was centrifuged again. The supernatant was discarded and the pellet was resuspended in 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, and then dialyzed against the same buffer. The dialysate was applied to a CM-Sepharose column equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. After extensive washing with the same buffer, proteins were eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer. 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.

SDS-polyacrylamide gel electrophoresis SDS-polyacrylamide gel electrophoresis with a 9% running gel was performed according to the procedure described by Laemmli (1970). The gels were stained with Coomassie brilliant blue R-250.

Thin layer chromatography Substrates and products were analyzed by silica gel thin layer chromatography (TLC) using hexane/ethylacetate (6 : 4) as the solvent system.

Spectroscopy and kinetics UV-visible spectroscopy was performed using a Shimadzu Model 3100 UV-NIR spectrophotometer. Kinetic constants for quinone reductase-catalyzed reduction of N,N-dimethyl-*p*-nitrosoaniline 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 (GC) was performed using a Hewlett-Packard 6890 Plus gas chromatograph system equipped with a HP 5 column and a flame ionization detector (FID). The oven temperature was programmed to ramp from 100 to 300°C at 15°C/min. The injection port temperature was 200°C and the detector temperature was 300°C. Gas flow rates were 1 ml/min for nitrogen, 20 ml/min for hydrogen, and 200 ml/min for air.

GC-mass spectrometry (GC-MS) Gas chromatography-mass spectrometry (GC-MS) was performed at 70 eV on a HP 5973 mass spectrometer fitted with an HP 6890 Plus gas chromatograph. The oven temperature was programmed from 100 to 250°C at 15°C/min.

Nuclear magnetic resonance Spectrometry Proton NMR spectra were obtained in $CDCl_3$ at 300MHz on a Jeol-LA-300 NMR Spectrometer.

Results and Discussion

Quinone reductase was purified to electrophoretic homogeneity from bovine liver by the method of Kim and Shin (2000). Several different electron acceptors were tested as substrates for the purified enzyme. Table 1 shows the substrate specificities of quinone reductase. In addition to the substituted benzo-and naphthoquinones, the enzyme readily reduced the 2,6-dichlorophenolindophenol, a quinoid redox dye. The purified enzyme could accept electrons from either NADH or NADPH, whereas the *P.chrysosporium* quinone reductase utilized only NADH (Constam *et al.*, 1991). This enzyme exhibited a rather broad substrate specificity. The quinone reductase that was purified from *P.chrysosporium* also exerted a broad substrate specificity (Brock *et al.*, 1995).

To examine whether mammalian quinone reductase possesses nitrosoreductase activity, N,N-dimethyl-*p*-nitrosoaniline and nitrosobenzene were also used as substates. Purified bovine liver quinone reductase catalyzed the NADH- and NADPH-dependent reduction of nitrosobenzene or N,N-dimethyl-*p*-nitrosoaniline (Table 1). The kinetic constants

Table 1. Substrate specificity of bovine liver quinone reductase

Substrate (100 μM)	Relative enzyme activity (% of control)	
	NADH (200 μM)	NADPH (200 μM)
1,4-Benzoquinone	100	72.2
2,6-dimethylbenzoquinone	121.1	96.6
2-hydroxymethyl-6-methoxy-1,4-benzoquinone	28.6	
2-methyl-1,4-naphthoquinone	28.3	74.3
2,6-dichlorophenolindophenol	88.6	177
N,N-dimethyl-*p*-nitrosoaniline	25.4	82.0
Nitrosobenzene	19.6	10.3

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.
Reaction rates are expressed relative to the rate with 1,4-benzoquinone (100 μM) and NADH (200 μM) as equal to 100.

were calculated from Lineweaver-Burk plots and summarized in Table 2. The apparent K_m for 1,4-benzoquinone and N,N-dimethyl-*p*-nitrosoaniline are 1.64 mM and 0.22 mM, respectively. The reduction of N,N-dimethyl-*p*-nitrosoaniline by purified mammalian quinone reductase was followed by spectroscopic measurements. Spectra were recorded with a Shimadzu model 3100 Spectrophotometer, with a 1-cm path-length cell. N,N-dimethyl-*p*-nitrosoaniline exhibited a UV absorption with λ_{max} at 440 nm, and NADH exhibited a UV absorption with λ_{max} at 340 nm in aqueous sodium phosphate buffer (pH 7.0) . Tracings of the spectral transitions associated with the bovine liver quinone reductase-catalyzed reduction of N,N-dimethyl-*p*-nitrosoaniline are shown in Fig 1. After a quinone reductase treatment, the intensity of the local maximum absorbance (440 nm), corresponding to N,N-dimethyl-*p*-nitrosoaniline, was decreased. The intensity of the absorbance maximum at 340 nm, corresponding to NADH, was also decreased. This implies that the chemical structure of N,N-dimethyl-*p*-nitrosoaniline is changed by bovine liver quinone reductase. As indicated by the disappearance of the intense 340 and 440 nm absorption bands, mammalian quinone reductase was shown to catalyze the reaction of N,N-dimethyl-*p*-nitrosoaniline with NADH.

Cibacron blue 3GA and dicumarol, known inhibitors of mammalian quinone reductase (Prochaska and Talalay, 1986; MacDonald, 1991; Brock *et al.*, 1995; Brock and Gold, 1996), inhibited the reduction of N,N-dimethyl-*p*-nitrosoaniline by bovine liver quinone reductase (Table 3). The 5 μM Cibacron blue 3GA inhibited the NADH-dependent reduction of N,N-dimethyl-*p*-nitrosoaniline by quinone reductase to 44%. Dicumarol also inhibited the nitrosoreductase activity of quinone reductase. The effect of increasing concentrations of dicumarol on the reduction of N,N-dimethyl-*p*-nitrosoaniline by bovine liver quinone reductase is shown in Table 3.

Table 2. Kinetic constants for the purified bovine liver quinone reductase toward 1,4-benzoquinone and N,N-dimethyl-*p*-nitrosoaniline[a].

Substrate	V_{max} (μmol/min/mg)	K_m (mM)	V_{max}/K_m
1,4-Benzoquinone	9.692	1.643	5.899
N,N-dimethyl-*p*-nitrosoaniline	0.1498	0.2245	0.6673

[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. The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 200 μM NADH, purified quinone reductase and varied concentrations of 1,4-benzoquinone or N,N-dimethyl-*p*-nitrosoaniline.

Table 3. Inhibition of the nitrosoreductase activity of the purified bovine liver quinone reductase.

Inhibitor	Concentration (μM)	Residual activity (% of control)
Cibacron blue 3GA	0	100
	2.5	67.3
	5	44.0
	10	28.9
Dicumarol	0	100
	10	96.3
	70	52.9
	200	14.0

The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 100 μM N,N-dimethyl-*p*-nitrosoaniline, 200 μM NADH, inhibitor, and purified quinone reductase. Results are expressed as a percentage of the activity without an inhibitor, with 100 representing no inhibition and 0 representing complete inhibition.

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. Demonstration of quinone reductase induction after the administration of aryl nitroso compound to animals will support the fact that mammalian quinone reductase is responsible for the aryl nitroso reduction *in vivo*.

The production of N,N-dimethyl-*p*-phenylenediamine from N,N-dimethyl-*p*-nitrosoaniline by purified mammalian quinone reductase was confirmed by TLC, GC (Fig. 2), GC-MS (Fig. 3) and NMR analysis. The reaction in a preparative scale was performed by mixing purified enzyme and 110 μmol of N,N-dimethyl-*p*-nitrosoaniline with 110 μmol of NADH in 10 mM potassium phosphate buffer (pH 7.0), then extracting the mixture with methylene chloride. N,N-dimethyl-*p*-phenylenediamine in the methylene chloride extract was identified by comparing its retention time to that of an authentic reference compound (Fig. 2). It was also identified by comparing its R_f value to that of the authentic reference compound on the TLC plate. The methylene

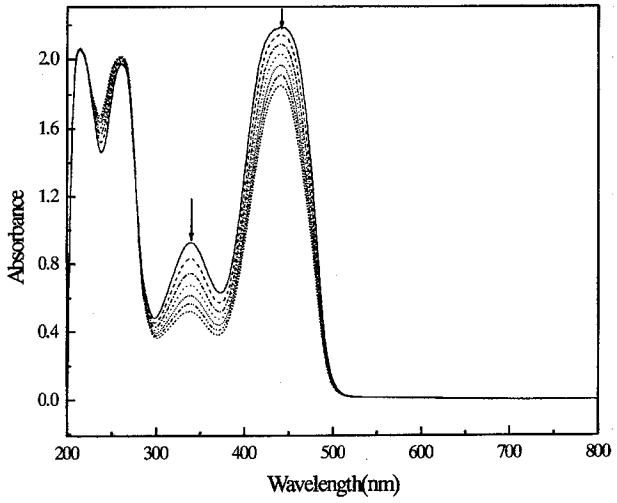

Fig. 1. Changes in absorption spectra during reduction of N,N-dimethyl-*p*-nitrosoaniline by quinone reductase. Reaction conditions were as follows: 100 μM N,N-dimethyl-*p*-nitrosoaniline, 200 μM NADH, 100 mM sodium phosphate buffer (pH 7.0) and purified bovine liver quinone reductase. Spectra were recorded at 5-min intervals. The arrows indicate decrease of the absorption band of N,N-dimethyl-*p*-nitrosoaniline and NADH.

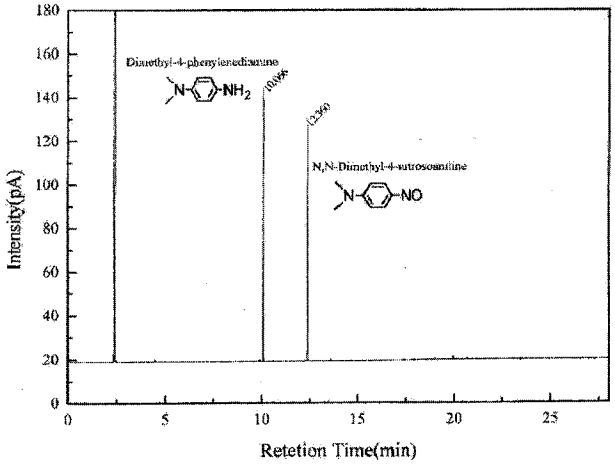

Fig. 2. GC chromatogram of the reaction mixture of N,N-dimethyl-*p*-nitrosoaniline, NADH and the purified quinone reductase.

chloride extract was subjected to TLC on a silica gel 60 F-254 plate and developed in hexane/ethylacetate (6 : 4). The major band migrated with a $R_f = 0.11$ as did an authentic sample of N,N-dimethyl-*p*-phenylenediamine. Its mass spectrum exhibits intense peaks at m/z values of 136, 121, 93 and 65. Proton NMR (300 MHz, $CDCl_3$) data was as follows: δ2.83 (6H, S), 6.66 (2H, dd, 2.6, 6Hz), 6.72 (2H, dd, 2.6, 6Hz). Consequently, the reduction product is identified as N,N-dimethyl-*p*-phenylenediamine on the basis of TLC,GC,GC-MS and NMR spectral comparisons with an authentic sample.

To identify the intermediate during the reduction of N,N-dimethyl-*p*-nitrosoaniline, the chemical trapping technique using 4-chloro-1-naphthol was employed. 4-chloro-1-naphthol has been shown to undergo oxidative condensation with benzoquinonediiminium cation, resulting in the formation of a characteristic dye with a broad, intense absorption maximum at ~600 nm (Tong and Glesmann, 1957). At pH 8.9, 4-chloro-*1*-naphthol existed as 4-chloro-1-naphthoxide ion, which reacted rapidly with arylnitrenium ion to form a characteristic blue dye. During the reduction of N,N-dimethyl-*p*-nitrosoaniline catalyzed by mammalian quinone reductase, the broad spectrum of the blue dye was seen with λ_{max} at 600 nm (data not shown). These results suggested that benzoquinonediiminium ion was produced during the mammalian quinone reductase-catalyzed reduction of N,N-dimethyl-*p*-nitrosoaniline. This may be due to the fact that the dimethylamino group is a good electron-donating group. Aromatic nitroso compounds are used extensively in textile and dyestuff industries, and we intake nitroso aromatic compounds as food additives. It could be suggested that mammalian quinone reductase might metabolize the nitroso compounds.

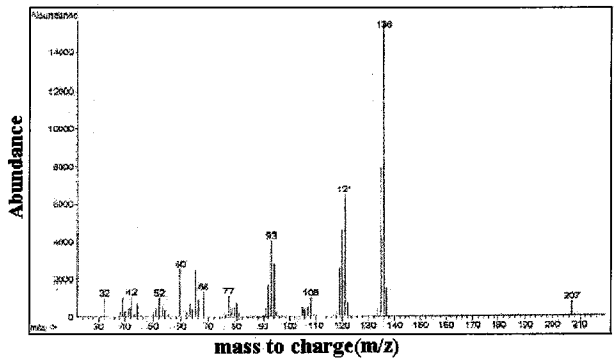

Fig. 3. GC-MS spectrum of the reaction product of N,N-dimethyl-*p*-nitosoaniline, NADH and the purified quinone reductase. Peak abundances are reported versus mass to charge (m/z).

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