Characterization of 1,4-Benzoquinone Reductase from Bovine Liver

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Abstract 1,4-Benzoquinone reductase was purified to electrophoretic homogeneity from bovine liver, and the purified enzyme found to have a molecular mass of 29 kDa, as determined by sodium dodecyl sulfate- polyacrylamide gel electrophoresis. The enzyme exhibited pH optimum between 8.0 and 8.5. The apparent K_m for 1,4-benzoquinone was 1.643 mM, and the apparent Km for NADH was 1.837 mM. Various divalent cations, such as Hg^{2+}, Cu^{2+} , and Zn^{2+}, exhibited strong inhibitory effects. The enzyme activity was also strongly inhibited by quercetin, dicumarol, and benzoic acid. Incubation of the enzyme with *N*-bromosuccinimide and pyridoxal 5'-phosphate led to inhibitions of 100% and 99%, respectively. Accordingly, these results suggest that tryptophan and lysine residues are involved at or near the active sites of the enzyme.

Keywords : biochemical property, 1,4-benzoquinone reductase

INTRODUCTION

Quinones and related compounds have received considerable attention recently, due to their widespread environmental prevalence and toxicological potential. Quinone reductase catalyzes the two-electron reduction of quinones and quinonoid compounds to hydroquinones [1-9]. Current evidence favors the hypothesis that quinone reductase is protective against quinone and quinone imine toxicity, by virtue of the two-electron reduction to hydroquinone, compared with the one -electron reduction mediated by cytochrome-P450 reductase, which produces toxic and mutagenic free radicals [10-14]. Quinone reductase is classified as a phase II enzyme since the enzyme can convert reactive electrophiles to less toxic products.

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 [15,16]. Quinone reductase activity has also been shown to increase several-fold in cultures of rat liver and human hepatoblastoma (Hep-G2) cells in response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) treatment. In the rat hepatoma cell line, quinone reductase mRNA is induced by polycyclic aromatic hydrocarbons and other planar aromatic compounds [17].

It is widely recognized that biotransformation enzymes are concentrated in the liver, although they are also found in most tissues. Broad substrate specificity is a characteristic property of the xenobiotic-metabolizing enzymes. Azocompounds are used widely in the textile food and cosmetic industries, yet certain azo compounds have been shown to be toxic [18-21]. The first step in the degradation of azo compounds is the cleavage of the azo bond by reductase [19,21,22].

The current author previously reported that bovine liver quinone reductase could catalyze the reduction of azo compound [23]. In that study, the reduction of an azo compound was almost entirely inhibited by dicumarol, a potent inhibitor of quinone reductase. Accordingly, the current study reports on the characterization of 1,4-benzoquinone reductase purified from bovine liver.

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MATERIALS AND METHODS

Chemicals

1,4-Benzoquinone, diethylmalonic acid, dicumarol, nitrofurantoin, 5,5'-dithiobis(2-nitrobenzoic acid), diphenic acid, N-ethylmaleimide, acetaldoxime, benzoic acid, *syn*-benzaldehyde oxime, quercetin and pyrazole were obtained from Aldrich. Coomassie brilliant blue G-250 was from Bio-Rad. Phenylmethylsulfonyl fluoride, *N*-bromosuccinimide, pyridoxal 5'-phosphate, NADH, glycine, iodoacetamide, acrylamide, 2,2'-dipyridyl, sephacryl, ethylenediamine tetraacetic acid (EDTA), *N*, *N*, *N'*, *N'*-tetramethylethylenediamine (TEMED), DEAE-Sephacel, sucrose, *N*, *N'*-methylene-bisacrylamide, bromophenol blue, molecular weight standard, Coomassie brilliant blue R-250, bovine serum albumin, lauryl sulfate, and ammonium persulfate were obtained from Sigma. All other chemicals were of the highest purity grade commercially available.

Activity Measurement

The standard reaction mixtures consisted of 25 mM Tris-HCl buffer (pH 8.0), 250 μM 1,4-benzoquinone, 200 μM NADH and the enzyme. The reactions were initiated by the addition of the enzyme. The decrease in absorbance at 340 nm was monitored spectrophotometrically [24]. The conditions for the specific reactions are presented in the related Figure or Table legends.

Protein Determination and Electrophoresis

The protein concentration was determined according to the method of Bradford [25], using bovine serum albumin as a standard. The protein content in the fractions collected during each chromatographic procedure was determined by measuring the absorbance at 280 nm.

SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli [26], and the gels were stained with Coomassie brilliant blue R-250.

Enzyme Purification

1,4-Benzoquinone reductase was purified to electrophoretic homogeneity from bovine liver by the method of Kim and Shin [23]. All procedures were carried out at 4°C unless otherwise stated. Bovine liver was homogenized in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA. The homogenate was then centrifuged at 10,000 g for 15 min. Solid ammonium sulfate was added to the supernatant to achieve 50% saturation, then the suspension was stirred for 1 h, and centrifuged at 20,000 g for 15 min. Additional amount of solid ammonium sulfate was added to the supernatant to make 75% saturation, then the mixture was centrifuged as before. The precipitate was suspended in 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA (buffer A), then the suspension was dialyzed against the same buffer. The dialysate was loaded to a CM-Sepharose column pre-equilibrated with buffer A. The column was washed with buffer A until the protein content of the effluent returned to the baseline level. Elution was carried out with a linear gradient of 0-0.5 M NaCl in buffer A. The active fractions were pooled, concentrated, and then applied to a Sephacryl S-200 column pre-equilibrated with buffer A. The fractions containing 1,4-benzoquinone reductase activity were pooled and the purified enzyme preparation was used for further study.

RESULTS AND DISCUSSION

SDS-PAGE of the purified enzyme revealed a single protein band. The molecular mass of the 1,4-benzoquinone reductase purified from bovine liver was calculated to be 29 kDa on the basis of its mobility relative to those of standard proteins (Fig. 1). This value is somewhat higher than that for *Phanerochaete chrysosporium* quinone reductase, where the molecular mass of the enzyme was estimated to be 22 kDa [27]. Activity of the 1,4-benzoquinone reductase was tested using different substrate concentrations. The data was plotted according to Lineweaver and Burk, and the Michaelis-Menten constant, K_m, was calculated from the intercept and the slope of the line (Figs. 2, 3). The Km values for 1,4-benzoquinone and NADH were estimated to be 1.643 mM and 1.837 mM, respectively (Table 1). In the case of *P.chrysosporium* quinone reductase, the K_m values of the enzyme for 2-methoxy-1,4-benzo-quinone and NADH were previously reported to be 2.4 μM and 55 μM, respectively [27].

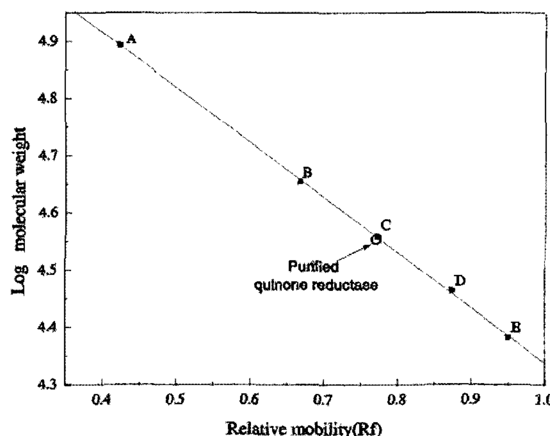

Fig. 1. Determination of molecular weight of 1,4-benzoquinone reductase by SDS-polyacrylamide gel electrophoresis. 9% acrylamide gel was used. A: bovine albumin (66 kDa), B: glyceraldehyde-3-P-dehydrogenase (36 kDa), C: bovine carbonic anhydrase (29 kDa), D: bovine pancreas trypsinogen (24 kDa) and E: soybean trypsin inhibitor (20 kDa).

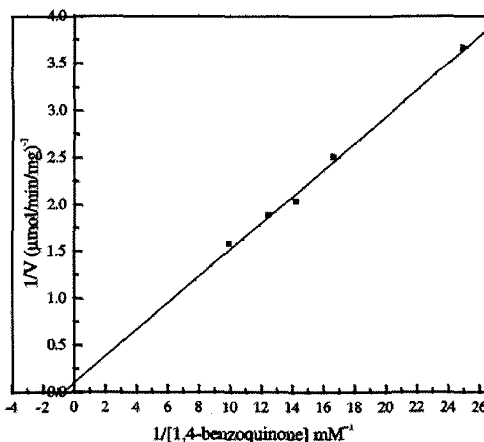

Fig. 2. The Lineweaver-Burk plot showing the 1,4-benzoquinone reductase activity as a function of 1,4-benzoquinone concentration. The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 200 μM NADH, purified enzyme and varied concentrations of 1,4-benzoquinone.

The effects of various metal ions on the 1,4-benzo-

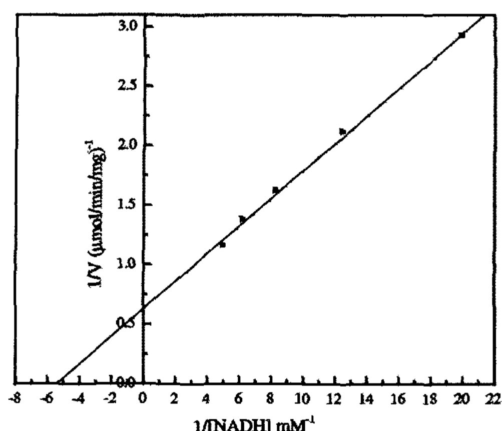

Fig. 3. The Lineweaver-Burk plot showing the 1,4-benzoquinone reductase activity as a function of NADH concentration. The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 100 μM 1,4-benzoquinone, purified enzyme and varied concentrations of NADH.

Table 1. Kinetic constants for the purified 1,4-benzoquinone reductase toward 1,4-benzoquinone and NADH[a]

Compound	K_m (mM)	V_{max} (μmol/min/mg)	V_{max}/K_m
1,4-Benzoquinone	1.643	9.692	5.899
NADH	1.837	1.587	0.864

[a] Assays were carried out as described in the experimental section. A fixed NADH concentration of 200 μM was used in determining the K_m for 1,4-benzoquinone. A fixed 1,4-benzoquinone concentration of 100 μM was used in determining the K_m for NADH.

Table 2. Effect of some metal ions on purified 1,4-benzoquinone reductase activity

Compound[a]	Relative activity (%)
None	100
$CuCl_2$	0.4
$HgCl_2$	0
$ZnCl_2$	26.7
$SnCl_2$	96.9
$BaCl_2$	111.1
$MgCl_2$	88.4

[a] Concentration of the compound was 1 mM.
The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 200 μM NADH, 100 μM 1,4-benzoquinone, 1 mM cation and the purified 1,4-benzoquinone reductase.

Table 3. Effect of various inhibitors on 1,4-benzoquinone reductase activity

Inhibitor	Residual activity (%)
None	100
KCN	100.8
NaN_3	104.1
Diethylmalonic acid	103.3
Pyrazole	93.7
2-Mercaptoethanol	0
Dicumarol	0
Quercetin	0
Nitrofurantoin	0
2,2'-Dipyridyl	84.7
Diphenic acid	91.4
N-ethylmaleimide	91.6
Iodoacetamide	96.6
Acetaldoxime	105.9
Benzoic acid	14.6
syn-Benzaldehyde oxime	28.5

The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 100 μM 1,4-benzoquinone, 200 μM NADH, 1 mM inhibitor and the purified enzyme. The results are expressed as a percentage of the activity without inhibitor, with 100 representing no inhibition and 0 representing complete inhibition.

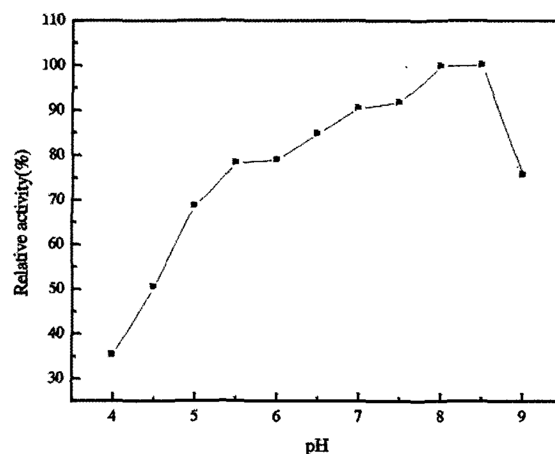

Fig. 4. Effect of pH on the 1,4-benzoquinone reductase activity. The reaction mixture consisted of 100 μM 1,4-benzoquinone, 200 μM NADH, 10 mM buffer, and the purified enzyme. Glycine · HCl buffer was used from pH 4.0 to 4.5, potassium phosphate buffer from pH 5.0 to 7.5 and Tris · HCl buffer from pH 8.0 to 9.0.

quinone reductase activity were examined. The concentrations of the metal ions tested were 1 mM in all cases. As shown in Table 2, almost complete inhibition of the 1,4-benzoquinone reductase activity was observed with Cu^{2+} and Hg^{2+}. Zn^{2+} had a significant inhibitory effect, and the enzyme activity remained unaffected by Sn^{2+}. In contrast, *P. chrysosporium* quinone reductase was not inhibited by 1 mM concentrations of Cu^{2+} or Zn^{2+}. Benzoic acid and syn-benzaldehyde oxime exerted a strong inhibitory effect along with dicumarol and quercetin (Table 3).

The pH dependence of the purified enzyme is shown in Fig. 4. The 1,4-benzoquinone reductase activity was measured in the pH range of 4.0-9.0. Three different buffers were used: glycine · HCl (pH 4.0-4.5), potassium phosphate (pH 5.0-7.5), and Tris · HCl (pH 8.0-9.0). The

Table 4. Effect of chemical modifiers on quinone reductase activity

Chemical modifier	Residual activity (%)
N-bromosuccinimide	0
Pyridoxal 5'-phosphate	0
Phenylmethylsulfonyl fluoride	48
N-ethylmaleimide	68
N-acetylimidazole	76

The enzyme was preincubated with various chemical modifiers at room temperature for 5 min. The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 1 mM modifier, 100 μM 1,4-benzoquinone, 200 μM NADH and purified enzyme.

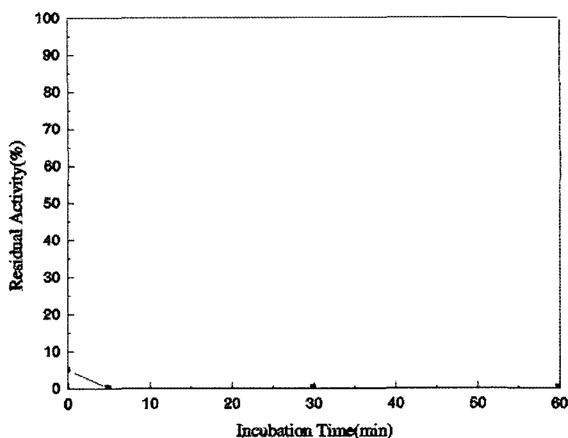

Fig. 5. Inactivation of 1,4-benzoquinone reductase by *N*-bromosuccinimide. The purified enzyme was incubated with 1 mM *N*-bromosuccinimide in 100 mM sodium phosphate buffer, pH 7.0, at room temperature. At time intervals, aliquots were removed for measurements of the residual enzyme activity.

enzyme exhibited a rather broad pH optimum between pH 8.0 and 8.5 in contrast to the *P. chrysosporium* quinone reductase, which displayed a broad pH optimum between pH 5.0 and 6.5 [5].

An investigation was conducted to examine the amino acid residues at or near the active sites of the enzyme, based on determining the residual activity after incubation with the group-specific potential inactivators. Incubation of the enzyme with 1 mM *N*-bromosuccinimide and pyridoxal 5'-phosphate led to inhibitions of 100% and 99%, respectively (Table 4, Fig. 5). However, incubation of the enzyme with 1 mM N-acetylimidazole, *N*-ethylmaleimide or phenylmethylsulfonyl fluoride only led to a partial loss of activity (Table 4). Even when the enzyme was incubated with *N*-ethylmaleimide or *N*-acetylimidazole for 1 h, over 40% of the enzyme activity still remained (Figs. 6, 7). Accordingly, these results suggest that tryptophan and lysine residues are involved at or near the active sites of the quinone reductase.

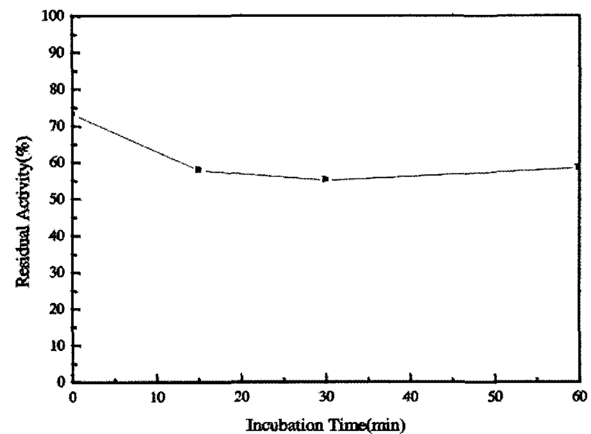

Fig. 6. Effect of *N*-ethylmaleimide on 1,4-benzoquinone reductase activity. The purified enzyme was incubated with 1 mM *N*-ethylmaleimide in 100 mM sodium phosphate buffer (pH 7.0) at room temperature. At time intervals, aliquots were removed for measurements of the residual enzyme activity.

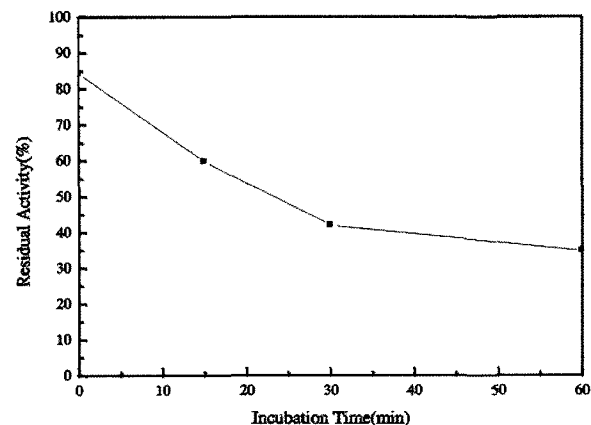

Fig. 7. Effect of *N*-acetylimidazole on 1,4-benzoquinone reductase activity. The purified enzyme was incubated with 1 mM *N*-acetylimidazole in 100 mM sodium phosphate buffer (pH 7.0) at room temperature. At time intervals, aliquots were removed for measurements of the residual enzyme activity.

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