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Biochemical Properties of NAD(P)H-Quinone Oxidoreductase from *Saccharomyces cerevisiae*

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The NAD(P)H-quinone oxidoreductase (EC 1. 6. 99. 2) was purified from *S. cerevisiae*. The native molecular weight of the enzyme is approximately 111 kDa and is composed of five identical subunits with molecular weights of 22 kDa each. The optimum pH of the enzyme is pH 6.0 with 1,4-benzoquinone as a substrate. The apparent K_m for 1,4-benzoquinone and 1,4-naphthoquinone are 1.3 mM and 14.3 μM, respectively. Its activity is greatly inhibited by Cu^{2+} and Hg^{2+} ions, nitrofurantoin, dicumarol, and Cibacron blue 3GA.

The purified NAD(P)H-quinone oxidoreductase was found capable of reducing aromatic nitroso compounds as well as a variety of quinones, and can utilize either NADH or NADPH as a source of reducing equivalents. The nitroso reductase activity of the purified NAD(P)H-quinone oxidoreductase is strongly inhibited by dicumarol.

Keywords: NAD(P)H-quinone oxidoreductase, *S. cerevisiae*.

Introduction

NAD(P)H-quinone oxidoreductase (EC 1. 6. 99. 2) catalyzes obligatory two-electron reductions of quinones to hydroquinones (Hojeberg *et al.*, 1981; Jaiswal *et al.*, 1988; Rao and Zigler, 1990; MacDonald, 1991; Arieli *et al.*, 1994; Favreau and Pickett, 1995). Quinones are derived from the fungal degradation of a variety of aromatic pollutants and also occur widely in nature (Valli *et al.*, 1991; 1992). Quinone oxidoreductase has a protective effect against carcinogenicity, mutagenicity, and other toxicities caused by quinones and their metabolic precursors (Bayney *et al.*, 1989; Gordon *et al.*, 1991; Shaw *et al.*, 1991; Prestera *et al.*, 1992; Zhang *et al.*, 1992; Chung *et al.*, 1994). It is highly inducible by a wide variety of compounds including polycyclic aromatic hydrocarbons and other planar aromatic compounds (Delong *et al.*, 1986; Prestera *et al.*, 1993; Ramchandani *et al.*, 1994). Quinone oxidoreductase has been purified from rat liver, mouse liver, and *Phanerochaete chrysosporium* by ion exchange, and hydrophobic and affinity chromatographies (Prochaska and Talalay, 1986; Prochaska, 1988; Constam *et al.*, 1991; Brock *et al.*, 1995). However, until now it had not been purified from *Saccharomyces cerevisiae*.

We have previously reported that *Saccharomyces cerevisiae* catalyzed the reduction of aromatic nitroso compound to the corresponding amine (Baik *et al.*, 1995; Kim *et al.*, 1995; 1996). In that study, aromatic nitroso compounds were selectively and rapidly reduced to their corresponding amino derivatives in good yields by *Saccharomyces cerevisiae* under neutral conditions.

It has been suggested 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 the purified nitroreductase can reduce quinones as well as several nitroaromatic compounds (Bryant and DeLuca, 1991). However, the nitroreductase is a minor component of the total cytosolic protein under basal conditions. Among the enzymes 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).

The experiments described herein were undertaken to identify the enzyme responsible for the reduction of aromatic nitroso compound using *S. cerevisiae*. In an attempt to examine whether quinone oxidoreductase could catalyze the reduction of the nitroso functional group or not, quinone oxidoreductase was purified from *S. cerevisiae* and its nitroso reductase activity was examined. We report here the characterization of NAD(P)H-dependent quinone oxidoreductase from *S. cerevisiae* and its nitroso reductase activity.

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Materials and Methods

Chemicals 2,6-Dichlorophenolindophenol, nitrosobenzene, 2-hydroxy-1,4-naphthoquinone, 1,4-benzoquinone, methyl-1, 4-benzoquinone, nitrofurantoin, 5-hydroxy-1,4-naphthoquinone, 4-nitrosophenol, 2-methyl-1,4-naphthoquinone, phenanthrenequinone, N,N-dimethyl-4-nitrosoaniline, 2-methyl-1,4-naphthoquinone, 2,6-dimethylbenzoquinone, dicumarol, 1,4-naphthoquinone, 1-aminoanthraquinone, 5-hydroxy-2-methyl-1,4-naphthoquinone, anthraquinone, 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from Aldrich (Milwaukee, USA). Coomassie brilliant blue G-250 was from Bio-rad (Richmond, USA). NADH, NADPH, NAD^+, glycine, Sephacryl, ethylenediamine tetraacetic acid (EDTA), N,N,N′,N′-tetramethylethylenediamine (TEMED), acrylamide, DEAE-Sephacel, sucrose, molecular weight standard, N,N′-methylenebisacrylamide, bromophenol blue, Coomassie brilliant blue R-250, bovine serum albumin, ammonium persulfate, lauryl sulfate were obtained from Sigma (St. Louis, USA). TLC sheets (silica gel 60 F-254) were obtained from Merck (Frankfurt, Germany). All other chemicals were of the highest purity grade commercially available.

Enzyme assay Standard reaction mixtures consisted of 25 mM Tris·HCl buffer (pH 8.0), 250 μM 1,4-benzoquinone, 200 μM NADH, and enzyme. Reactions were initiated by the addition of the enzyme. The decrease in absorbance at 340 nm due to NADH oxidation was monitored spectrophotometrically (Constam *et al.*, 1991). 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 (1976), using bovine serum albumin as standard. The protein content in fractions collected during each chromatographic procedure was determined by absorbance at 280 nm.

Enzyme purification All subsequent steps were carried out at 4°C.

Saccharomyces cerevisiae was homogenized in the extraction buffer consisting of 100 mM potassium phosphate (pH 7.5) and 1 mM EDTA. The homogenate was centrifuged at 10,000 × *g* for 15 min. The supernatant was subjected to fractional precipitation using ammonium sulfate. The crude extract was brought to 40% saturation by slowly adding solid ammonium sulfate. The mixture was allowed to incubate for an additional 1 h while stirring on ice. The resulting slurry was centrifuged at 20,000 × *g* for 15 min at 4°C. The supernatant was decanted and adjusted to 80% saturation by adding ammonium sulfate, and centrifuged as before. The pellet was then resuspended in a minimum volume of 10 mM Tris·HCl buffer (pH 8.0, containing 1 mM EDTA) and dialyzed against the same buffer. After centrifugation, the sample was applied to a column of DEAE-Sephacel anion exchange resin which had been previously equilibrated with 10 mM Tris·HCl buffer (pH 8.0, containing 1 mM EDTA). The column was washed with the same buffer and then eluted with a linear gradient of 0 to 0.5 M NaCl. The active fractions were pooled and concentrated, and then applied to a Sephacryl S-200 column equilibrated with 10 mM Tris·HCl, pH 8.0, containing 1 mM EDTA. The column was 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.

Results and Discussion

The NAD(P)H-quinone oxidoreductase was purified to apparent homogeneity by a combination of ammonium sulfate fractionation, ion-exchange and gel permeation chromatographies (Table 1). On polyacrylamide gel electrophoresis, the purified NAD(P)H-quinone oxidoreductase revealed a single band. To determine its native molecular mass by gel filtration, the purified NAD(P)H-quinone oxidoreductase was applied to a Sephacryl S-200 column. NAD(P)H-quinone oxidoreductase eluted from this column as a peak corresponding to 111 kDa. The subunit molecular weight was estimated by SDS-PAGE to be 22 kDa. Thus, it is concluded the enzyme is composed of five similar 22 kDa subunits as demonstrated by SDS-PAGE. In contrast, Brock *et al.* (1995) have isolated a *Phanerochaete chrysosporium* 1,4-benzoquinone reductase that is apparently composed of two subunits. The protein has a molecular mass of 44 kDa and a subunit molecular mass of 22 kDa.

The substrate specificity was tested with various substituted quinones (Table 2). In addition to the substituted benzo- and naphthoquinones, the enzyme readily reduced the 2,6-dichlorophenolindophenol, a quinoid redox dye. Substituted anthraquinones and phenanthrenequinone were also tested as substrates of quinone oxidoreductase. As is true for mouse liver quinone reductase (Prochaska and Talalay, 1986) only a slight NADH oxidation was observed. The purified enzyme utilizes either NADH or NADPH as the electron donor,

Table 1. Purification of the quinone reductase from *S. cerevisiae*.

Purification step	Total protein (mg)	Total activity (U)[a]	Specific activity (mU/mg)	Purification (fold)	Yield (%)
Crude extract	3563	7.200	2.02	1	100
$(NH_4)_2SO_4$ fractionation (40 ~ 80%)	832.4	3.204	3.85	1.9	44.5
DEAE-Sephacel chromatography	34.86	1.712	49.1	24.3	23.8
Sephacryl S-200 chromatography	3.195	0.799	250	124	11.1

[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 the quinone oxidoreductase purified from *S. cerevisiae*.

Substrate (100 μM)	Relative enzyme activity (% of control)	
	NADH (200 μM)	NADPH (200 μM)
1,4-Benzoquinone	100	149.3
1,4-Naphthoquinone	312.4	96.8
2,6-Dichlorophenolindophenol	425.5	57.7
5-Hydroxy-2-methyl-1,4-naphthoquinone	308.6	122.4
2,6-Dimethylbenzoquinone	357.5	84.7
Methyl-1,4-benzoquinone	176.2	109.0
Anthraquinone	9.5	13.3
1-Aminoanthraquinone	6.9	10.2
Phenanthrenequinone	13.7	12.8

Rates are expressed relative to the rate with 1,4-benzoquinone (100 μM) and NADH (200 μM) as equal to 100. One hundred mM sodium phosphate buffer (pH 7.0) was used. All quinone substrates were assayed at a final concentration of 100 μM.

whereas the purified *P. chrysosporium* quinone oxidoreductase (Constam *et al.*, 1991) utilized only NADH. The purified enzyme used not only substituted benzoquinone but also naphthoquinone as an electron acceptor. The broad substrate specificity of this enzyme, as well as that of the previously isolated quinone oxidoreductase (Brock *et al.*, 1995), suggests that this organism does not produce multiple specific quinone oxidoreductases. The apparent K_m for 1,4-benzoquinone and 1,4-naphthoquinone are 1.3 mM and 14.3 μM, respectively (Figs. 1, 2; Table 3). The comparison of kinetic constants for 1,4-benzoquinone and 1,4-naphthoquinone indicated that quinone oxidoreductase has somewhat higher affinity for the latter than the former (Table 3). The pH dependence of NADH-dependent quinone oxidoreductase activity is shown in Fig. 3. The pH profile of purified NAD(P)H-quinone oxidoreductase from *S. cerevisiae* with 1,4-benzoquinone as the substrate displays maximum activity at pH 6.0. This is in contrast to the pH profile obtained with quinone oxidoreductase purified from *Phanerochaete chrysosporium*, which displayed a broad pH optimum between pHs 5.0 and 6.5 (Brock *et al.*, 1995).

The inhibition of the quinone oxidoreductase by several metal ions was examined. The enzyme was not inhibited by 1 mM concentrations of $MgCl_2$, $BaCl_2$, or NaCl, whereas $NiCl_2$ (1 mM) and $ZnCl_2$ (1 mM) were slightly inhibitory (Table 4). *Solanum melongena* tyrosinase

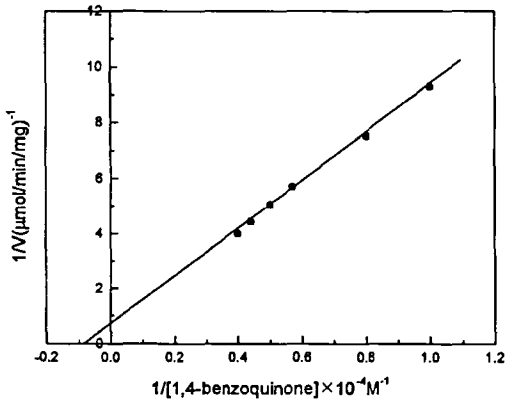

Fig. 1. The Lineweaver-Burk plot showing the quinone oxidoreductase activity as a function of 1,4-benzoquinone concentration. The reaction mixture contained 25 mM Tris·HCl buffer, pH 8.0, 200 μM NADH, purified quinone reductase, and varied concentrations of 1,4-benzoquinone.

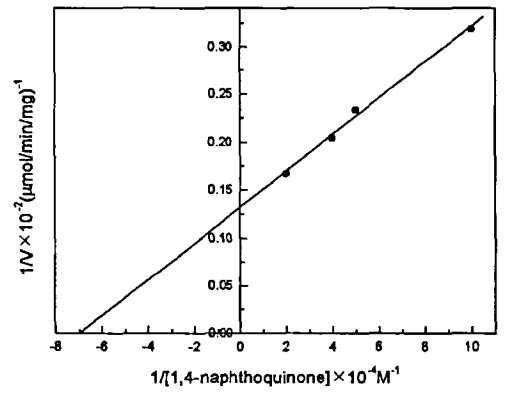

Fig. 2. The Lineweaver-Burk plot showing the quinone oxidoreductase activity as a function of 1,4-naphthoquinone concentration. The reaction mixture contained 25 mM Tris·HCl buffer, pH 8.0, 200 μM NADH, purified quinone reductase, and varied concentrations of 1,4-naphthoquinone.

Table 3. Kinetic constants for the purified *S. cerevisiae* quinone oxidoreductase toward 1,4-benzoquinone and 1,4-naphthoquinone.[a]

Substrate	V_{max} (μmol/min/mg)	K_m (μM)	V_{max}/K_m
1,4-Benzoquinone	1.56	1333.3	0.00117
1,4-Naphthoquinone	0.0758	14.3	0.00530

[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 25 mM Tris·HCl buffer, pH 8.0, 200 μM NADH, purified quinone reductase, and varied concentrations of 1,4-benzoquinone or 1,4-naphthoquinone.

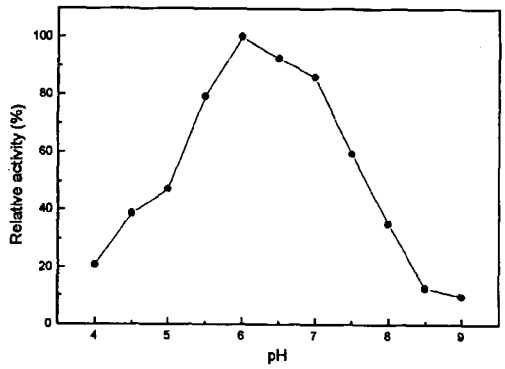

Fig. 3. The effect of pH on the quinone oxidoreductase activity. The reaction mixture contained 250 μM 1,4-benzoquinone, 200 μM NADH, 10 mM Glycine-HCl buffer at pH 4 to 4.5, 10 mM potassium phosphate buffer at pH 5 to 7.5, 10 mM Tris·HCl buffer at pH ≥8, and purified quinone reductase.

activity and rice isoperoxidase activity were also decreased in the presence of Zn^{2+} ion (Lee, 1997; Lee *et al.*, 1997). $CuCl_2$ and $SnCl_2$ inhibited the enzyme significantly (Table 4). 1-Aminocyclopropane-1-carboxylate oxidase activity was also strongly inhibited by metal ions such as Cu^{2+} and Mn^{2+} (Jin *et al.*, 1998). In contrast, the 1,4-benzoquinone reductase purified from *P. chrysosporium* was not inhibited by 1 mM concentrations of Cu^{2+} or Zn^{2+}. As is true for mammalian quinone oxidoreductase (Prochaska and Talalay, 1986; Prochaska, 1988), cibacron blue 3GA and dicumarol are efficient inhibitors, suggesting that the mechanism of this NAD(P)H-quinone oxidoreductase may be similar to that of mammalian quinone reductase (Tables 5, 6). In addition, nitrofurantoin strongly inhibited the

Table 4. The effect of various cations on the quinone oxidoreductase activity purified from *S. cerevisiae*.

Compound[a]	Residual activity (%)
None	100.0
$CuCl_2$	28.3
$HgCl_2$	9.1
$SnCl_2$	0.0
$MnCl_2$	71.2
$CaCl_2$	88.8
$NiCl_2$	90.6
$ZnCl_2$	90.1
$MgCl_2$	98.4
$BaCl_2$	102.9
NaCl	106.6
KCl	115.8

[a] Concentration of the compound was 1 mM.
The reaction mixture contained 25 mM Tris·HCl buffer, pH 8.0, 200 μM NADH, 250 μM 1,4-benzoquinone, and purified quinone reductase.

Table 5. Effects of some inhibitors on the quinone oxidoreductase activity purified from *S. cerevisiae*.

Compound	Concentration	Inhibition (%)
Cibacron blue 3GA	2 μM	77.4
Nitrofurantoin	50 μM	100.0
Dicumarol	10 μM	20.8
p-Chloromercurisulfonate	0.2 mM	7.9
Iodoacetamide	1 mM	22.5

The reaction mixture contained 25 mM Tris·HCl buffer, pH 8.0, 200 μM NADH, 250 μM 1,4-benzoquinone, and purified quinone reductase.

Table 6. Inhibition of the quinone oxidoreductase purified from *S. cerevisiae*.

Inhibitor (20 μM)	Substrate (100 μM)	Relative enzyme activity (% of control)	
		NADH (200 μM)	NADPH (200 μM)
Dicumarol	1,4-Benzoquinone	58.6	68.4
	1,4-Naphthoquinone	77.9	93.0
	2,6-Dichlorophenolindophenol	92.3	70.3
	2-Methyl-1,4-naphthoquinone	79.3	32.8
	5-Hydroxy-1,4-naphthoquinone	70.3	66.8
	5-Hydroxy-2-methyl-1,4-naphthoquinone	66.1	33.5
Nitrofurantoin	1,4-Benzoquinone	72.7	112.7
	1,4-Naphthoquinone	86.3	87.6
	2,6-Dichlorophenolindophenol	100.1	82.3
	2-Methyl-1,4-naphthoquinone	81.3	87.9
	5-Hydroxy-1,4-naphthoquinone	88.4	45.9
	5-Hydroxy-2-methyl-1,4-naphthoquinone	3.8	78.7

Inhibition depends on inhibitor, substrate, and whether NADH or NADPH is the coenzyme. Results are expressed as a percentage of the activity without inhibitor, with 100 representing no inhibition and 0 representing complete inhibition for each substrate. 100 mM sodium phosphate buffer (pH 7.0) was used.

Table 7. Activity of the *S. cerevisiae* quinone oxidoreductase as a NAD(P)H-dependent nitroso reductase.

Nitroso compound	Electron donor	Activity (μmol/min/ml)
4-Nitrosophenol	NADH	0.0665
	NADPH	0.08258
4-Nitroso-N,N-dimethylaniline	NADH	0.3266
	NADPH	0.3931
Nitrosobenzene	NADH	0.0056
	NADPH	0.01129

The reaction mixture contained 0.1 M sodium phosphate buffer (pH 7.0), 160 μM nitroso compound, 150 μM NAD(P)H, and purified quinone oxidoreductase.

Table 8. Inhibition of the nitroso reductase activity of the purified *S. cerevisiae* quinone oxidoreductase.

Inhibitor (20 μM)	Nitroso compound (160 μM)	Relative enzyme activity (% of control)	
		NADH (150 μM)	NADPH (150 μM)
Dicumarol	4-Nitrosophenol	75.9	60.4
	4-Nitroso-N, N-dimethylaniline	62.7	74.9
	Nitrosobenzene	34.6	83.6

The reaction mixture contained 0.1 M sodium phosphate buffer (pH 7.0), 160 μM nitroso compound, 150 μM NAD(P)H, 20 μM dicumarol, and purified quinone oxidoreductase.
Nitroso reductase activities were shown as percentage activity relative to the nitroso reductase activity of quinone reductase in the absence of dicumarol.

enzyme (Tables 5, 6). These results are in good agreement with those found for guinea pig quinone reductase. The quinone oxidoreductase purified from guinea pig was also inhibited by nitrofurantoin and dicumarol (Rao *et al.*, 1992).

Nitroreductase can reduce not only arylnitro compounds but also quinones (Bryant and DeLuca, 1991), and arylnitroso compounds are common intermediates in the metabolism of aromatic amines and arylnitro compounds. To elucidate the possibility of quinone oxidoreductase catalyzing nitroso reduction, the nitroso reductase activity of purified NAD(P)H-quinone oxidoreductase was also examined. The results of these experiments are presented in Table 7, showing the high activity of NAD(P)H-quinone oxidoreductase as a nitroso reductase. Purified quinone oxidoreductase catalyzed the reduction of arylnitroso compounds. The relative activity of NAD(P)H-dependent nitroso reduction followed the order 4-nitroso-N,N-dimethylaniline 〉 4-nitrosophenol 〉 nitrosobenzene. The purified quinone oxidoreductase utilizes either NADH or NADPH as the electron donor for the nitroso reduction. The effect of pH on the quinone oxidoreductase-dependent 4-nitrosophenol reduction by NADH was examined over the pH range of 5.0 – 8.0. The pH versus activity curve of quinone oxidoreductase is seen to decrease with increasing pH (data not shown). The activity of quinone oxidoreductase over the pH range studied might be proportional to the amount of 4-nitrosophenol in the non-ionized form. The effect of dicumarol on the nitroso reduction ability of NAD(P)H-quinone oxidoreductase is shown in Table 8. It is noted that dicumarol inhibited the NAD(P)H-dependent nitroso reductase activity of quinone oxidoreductase.

Aromatic nitroso-group reduction catalyzed by quinone oxidoreductase is presently under investigation in our laboratory.

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