Journal of Biochemistry and Molecular Biology, Vol. 32, No. 4, July 1999, pp. 321–325

JOURNAL OF Biochemistry and Molecular Biology

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Reduction of Nitrosoarene by Purified NAD(P)H-Quinone Oxidoreductase

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Received 20 January 1999, Accepted 19 March 1999

NAD(P)H-quinone oxidoreductase (EC 1. 6. 99. 2) was purified from *S. cerevisiae*. The enzyme readily reduced 2,6-dichlorophenolindophenol, a quinonoid redox dye, as well as substituted benzo- and naphthoquinones, and could accept electrons from either NADH or NADPH. The purified NAD(P)H-quinone oxidoreductase turned out to be capable of reducing nitrosoarenes as well as a variety of quinones. A chemical-trapping technique using 4-chloro-1-naphthol was used to show that the N,N-dimethyl-*p*-benzoquinonediiminium cation was produced in the reduction of 4-nitroso-N,N-dimethylaniline catalyzed by NAD(P)H-quinone oxidoreductase.

Keywords: NAD(P)H-quinone oxidoreductase, Nitrosoarene.

Introduction

NAD(P)H-quinone oxidoreductase (EC 1. 6. 99. 2) catalyzes two-electron reductions of quinones to hydroquinones (Hojeberg *et al.*, 1981; Jaiswal *et al.*, 1988; Rao and Zigler, 1990; MacDonald, 1991; Prestera *et al.*, 1992; Arieli *et al.*, 1994; Favreau and Pickett, 1995). It is highly inducible by several polycyclic aromatic hydrocarbons and other planar aromatic compounds (Delong *et al.*, 1986; Prestera *et al.*, 1993; Ramchandani *et al.*, 1994).

We have previously reported that *Saccharomyces cerevisiae* catalyzes the reduction of arylnitroso compounds to their corresponding amino derivatives. (Baik *et al.*, 1995; Kim *et al.*, 1995; 1996). In that study, arylnitroso compounds were selectively and rapidly reduced to their corresponding amino derivatives in good yields by *Saccharomyces cerevisiae* under neutral conditions. However, the enzyme responsible for the reduction was not identified.

It was reported that the purified nitro reductase reduced quinones as well as several nitroaromatic compounds, and the reduction of the nitro group of nitroaromatic compounds proceeded through nitroso and hydroxylamine intermediates to the fully-reduced amino adduct (Bryant and DeLuca, 1991). In an attempt to examine whether quinone oxidoreductase could exert nitroso reductase activity or not, quinone oxidoreductase was purified from *S. cerevisiae* and its nitroso reductase activity was examined (Kim and Suk, 1999). The purified NAD(P)H-quinone oxidoreductase catalyzed the reduction of arylnitroso compounds to their corresponding amines, utilizing either NADH or NADPH as a source of reducing equivalents. The nitroso reductase activity of the quinone oxidoreductase was strongly inhibited by dicumarol, a potent inhibitor of NAD(P)H-quinone oxidoreductase.

The present work includes a detailed study on the quinone oxidoreductase-catalyzed reduction of nitrosoarene, including product identification, spectroscopic analysis, and reaction mechanism. 4-Chloro-1-naphthol has been shown to undergo oxidative condensation with the benzoquinonediiminium cation, resulting in the formation of a characteristic dye with a broad, intense absorption maximum at ~600 nm (Tong and Glesmann, 1957). To identify the intermediate during the reduction of 4-nitroso-N,N-dimethylaniline, a chemical-trapping technique using 4-chloro-1-naphthol was employed.

Materials and Methods

Chemicals 2-Hydroxymethyl-6-methoxy-1,4-benzoquinone, 1,4-benzoquinone, hydroquinone, 5-hydroxy-1,4-naphthoquinone, 4-nitrosophenol, 2-methyl-1,4-naphthoquinone, 2,6-dichlorophenolindophenol, nitrosobenzene, and N,N-dimethyl-4-

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nitrosoaniline were obtained from Aldrich (Milwaukee, USA). Coomassie brilliant blue G-250 was purchased from Bio-rad (Hercules, USA). NADH, NADPH, glycine, Sephacryl S-200-HR, ethylenediamine tetraacetic acid (EDTA), N,N,N′,N′-tetramethylethylenediamine (TEMED), acrylamide, DEAE-Sephacel, sucrose, N,N′-methylenebisacrylamide, bromophenol blue, Coomassie brilliant blue R-250, bovine serum albumin, ammonium persulfate and lauryl sulfate were obtained from Sigma (St. Louis, USA). Centriprep-10 concentrator was obtained from Amicon (Beverly, USA). TLC sheets (silica gel 60 F-254) was obtained from Merck (Darmstadt, Germany). All other chemicals were of the highest purity grade commercially available.

Enzyme assay Quinone reductase activity was determined in 25 mM Tris·HCl buffer (pH 8.0) containing 250 μM 1,4-benzoquinone, and 200 μM NADH. 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). 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.

SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250.

Enzyme purification Quinone oxidoreductase was purified to electrophoretic homogeneity from *S. cerevisiae* by the method of Kim and Suk (1999). All procedures were carried out at 4°C unless otherwise stated.

S. cerevisiae was homogenized in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA. The homogenate was centrifuged and enough solid ammonium sulfate was added to the supernatant to give 40% saturation. The suspension was stirred for 1 h and then centrifuged. The pellet was discarded, additional $(NH_4)_2SO_4$ was added to the resulting supernatant fraction to give 80% saturation, and the supernatant was centifuged again. The supernatant was discarded and the precipitate was dissolved in buffer A and then dialyzed against the same buffer. The dialysate was applied to a DEAE-Sephacel column equilibrated with buffer A. After extensive washing with the same buffer, proteins were eluted with a linear gradient of 0 to 0.5 M NaCl in buffer A. The active fractions were pooled, concentrated, and then applied to a Sephacryl S-200 column equilibrated with buffer A. Proteins were eluted with equilibration buffer, and the active fractions were pooled and used for further study.

Gas chromatography Gas chromatography was performed using a Hewlett-Packard 5890 series II gas chromatograph equipped with an HP 5 column and a flame ionization detector. 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.

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

UV-visible spectroscopy was performed using a Shimadzu Model 3100 UV-NIR spectrophotometer.

Results and Discussion

To elucidate the role of quinone oxidoreductase in the nitroso reduction by *S. cerevisiae*, NAD(P)H-quinone oxidoreductase was purified to apparent homogeneity by the method of Kim and Suk (1999). The native molecular weight of the enzyme was approximately 111 kDa and was composed of five identical subunits of 22 kDa each.

Several different electron acceptors were tested as substrates for the purified enzyme. Table 1 shows the substrate specificities of quinone oxidoreductase. The enzyme could accept electrons from either NADH or NADPH, and was capable of reducing both substituted benzo- and naphthoquinones. This rather broad substrate specificity suggests that this organism does not produce multiple specific quinone oxidoreductases. The quinone oxidoreductase purified from *P. chrysosporium* also exerts a broad substrate specificity (Brock *et al.*, 1995). As is true for mammalian quinone oxidoreductase (Prochaska and Talalay, 1986; Prochaska, 1988), dicumarol exerts an inhibitory effect on the *S. cerevisiae* quinone oxidoreductase activity (Kim and Suk, 1999). The dicumarol's inhibitory effect was affected by the identity of substrate and coenzyme (NADH or NADPH).

Table 1. Substrate specificity of quinone oxidoreductase.

Substrate (100 μM)	Relative enzyme activity (% of control)	
	NADH (200 μM)	NADPH (200 μM)
1,4-Benzoquinone	100	149.3
2-Hydroxymethyl-6-methoxy-1,4-benzoquinone	328.8	139.0
2,6-Dichlorophenolindophenol	425.5	57.7
2-Methyl-1,4-naphthoquinone	156.5	56.8
5-Hydroxy-1,4-naphthoquinone	452.8	377.5

Reaction rates are expressed relative to the rate with 1,4-benzoquinone (100 μM) and NADH (200 μM) set to 100. The reactions were performed in 100 mM sodium phosphate buffer (pH 7.0).

The production of hydroquinone from benzoquinone by purified quinone oxidoreductase was confirmed by GC analysis. The reaction in a preparative scale was performed by mixing purified enzyme and 88 μmol of 1,4-benzoquinone with 70 μmol of NADH in 10 mM Tris buffer (pH 8.0), then extracting the mixture with ethyl acetate. Hydroquinone in the ethyl acetate extract was identified by comparing its retention time to that of an authentic reference compound (Fig. 1). It was also identified by comparing its R_f value to that of the authentic reference compound on TLC plate (data not shown). The production of hydroquinone from benzoquinone by purified quinone oxidoreductase was also confirmed by spectroscopic measurements. Hydroquinone exhibited a UV absorption with λ_{max} at 284 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 reduction of 1,4-benzoquinone catalyzed by quinone oxidoreductase are shown in Fig. 2. Spectra were recorded with a Shimadzu model 3100 spectrophotometer, with a 1-cm path-length cell. These spectral transitions indicated that the decay of NADH at 340 nm is associated with the formation of product with an absorbance maximum at 284 nm. This absorbance maximum at 284 nm is consistent with that of hydroquinone obtained in 0.1 M sodium phosphate buffer at pH 7.0.

Bryant and DeLuca (1991) have argued that nitroreductase can reduce not only arylnitro compounds but also quinones, and arylnitroso compounds are common intermediates in the metabolism of aromatic amines and arylnitro compounds. In our laboratory, the possibility of quinone oxidoreductase catalyzing nitroso reduction was examined. Purified quinone oxidoreductase catalyzed the reduction of nitrosoarene (Kim and Suk, 1999). The enzyme utilized either NADH or NADPH as the electron donor for the nitroso reduction. Furthermore, dicumarol, an inhibitor of quinone oxidoreductase, inhibited NAD(P)H-dependent nitroso reductase activity of quinone oxidoreductase.

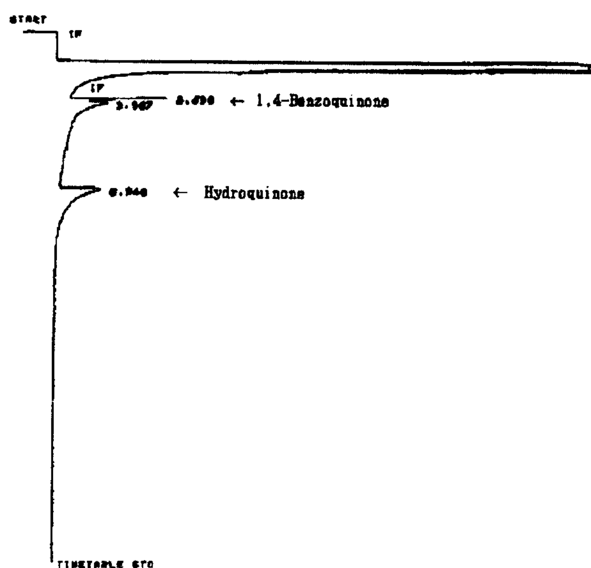

Fig. 1. GC chromatogram of the reaction mixture of 1,4-benzoquinone, NADH, and the purified NAD(P)H-quinone oxidoreductase.

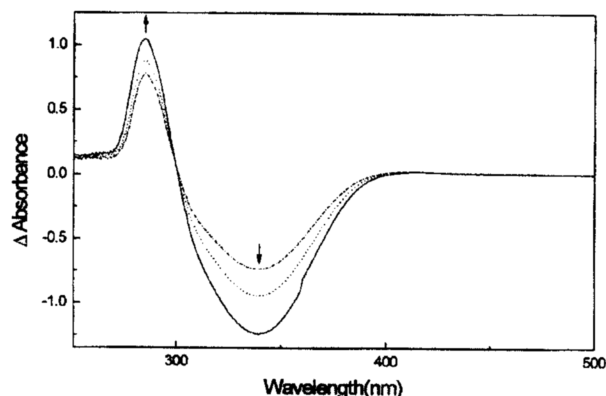

Fig. 2. Changes in absorption spectra during reduction of 1,4-benzoquinone by NAD(P)H-quinone oxidoreductase. Reaction conditions were as follows: 320 μM 1,4-benzoquinone, 300 μM NADH, 0.1 M phosphate buffer, pH 7.0, and purified NAD(P)H-quinone oxidoreductase. Spectra were recorded at 5-min intervals. The appearance of the 284 nm absorption band of hydroquinone and the disappearance of the 340 nm absorption band of NADH are indicated by arrows.

4-Nitrosophenol exhibited a UV absorption with λ_{max} at 400 nm in aqueous sodium phosphate buffer, pH 7.0, and NADH exhibited a UV absorption with λ_{max} at 340 nm. Tracings of the spectral transitions associated with the quinone oxidoreductase-catalyzed reduction of 4-nitrosophenol are shown in Fig. 3. As indicated by the disappearance of the intense 340 and 400 nm absorption bands, quinone oxidoreductase was shown to the reaction of NADH with 4-nitrosophenol.

To trap the enzymatic product of 4-nitroso-N,N-dimethylaniline reduction, 4-chloro-1-naphthoxide ion was employed. The result of quinone oxidoreductase-catalyzed reduction of 4-nitroso-N,N-dimethylaniline conducted in the presence of the chemical-trapping agent 4-chloro-1-naphthol is shown in Fig. 4. At pH 8.9, 4-chloro-1-naphthol exists as the 4-chloro-1-naphthoxide ion, which reacts rapidly with the arylnitrenium ion to form a characteristic blue dye (Tong and Glesmann, 1957). With quinone oxidoreductase, the broad spectrum of the blue dye is seen with λ_{max} at 600 nm. These results suggest that the benzoquinonediiminium ion was produced during the quinone oxidoreductase-catalyzed reduction of 4-nitroso-N,N-dimethylaniline. On the other hand, blue dye formation was not observed when 4-nitrosophenol was enzymatically reduced by quinone oxidoreductase in the

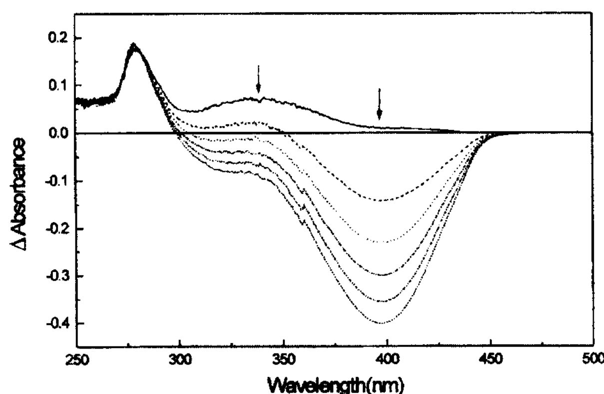

Fig. 3. Changes in absorption spectra during reduction of 4-nitrosophenol by NAD(P)H-quinone oxidoreductase. Reaction conditions were as follows: 320 μM 4-nitrosophenol, 300 μM NADH, 0.1 M phosphate buffer, pH 7.0, and purified NAD(P)H-quinone oxidoreductase. Spectra were recorded at 5-min intervals. The disappearance of the 340 and 400 nm absorption bands of NADH and 4-nitrosophenol are indicated by arrows.

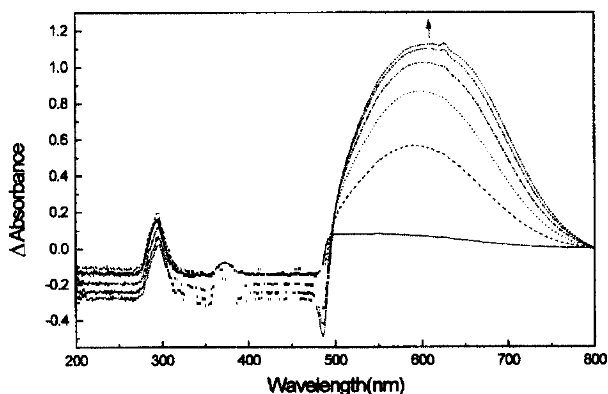

Fig. 4. Spectral transitions associated with quinone oxidoreductase-catalyzed reduction of 4-nitroso-N,N-dimethylaniline in the presence of 4-chloro-1-naphthol as a function of reaction time. The reduction of 4-nitroso-N,N-dimethylaniline catalyzed by quinone oxidoreductase was observed under the following conditions: 320 μM 4-nitroso-N,N-dimethylaniline, 300 μM NADH, 320 μM 4-chloro-1-naphthol, purified quinone oxidoreductase, and 0.1 M pyrophosphate buffer, pH 8.9. Spectra were recorded at 5-min intervals. The formation of a blue dye with a broad absorption maximum at 600 nm is indicated by the arrow.

presence of 4-chloro-1-naphthol (data not shown). In this case, only a loss of absorbance at 398 nm, i.e. the major absorbance band of 4-nitrosophenol at pH 8.9, was observed. This result indicates that the 4-hydroxy-benzylnitrenium ion is not generated during reduction of 4-nitrosophenol by quinone oxidoreductase. This may be due to the fact that the dimethylamino group is a better electron-donating group than the hydroxyl group. During the reduction of 4-nitroso-N,N-dimethylaniline, the N,N-dimethyl-*p*-benzoquinonediiminium ion could be readily produced by donation of an unshared electron pair from nitrogen, while 1,4-benzoquinoneimine was produced during the reduction of 4-nitrosophenol.

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