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NADPH-specific quinone reductase is induced by 2-methylene-4-butyrolactone in *Escherichia coli*

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Abstract

2-Methylene-4-butyrolactone (MBL), an inducer of NAD(P)H:(quinone acceptor) oxidoreductase (EC 1.6.99.2) in animal cells, was found to induce NADPH-specific quinone reductase about 25-fold in *Escherichia coli*. MBL induced NADPH-quinone reductases with relative mobilities (Rm) of 0.70, 0.76 and 0.91 on polyacrylamide gel electrophoresis (PAGE). These three enzymes were found to be charge isomers with the same molecular size of 42 kDa. Two NADPH-quinone reductases (A and B) were purified to single proteins both with an apparent mass of 21 kDa on SDS-PAGE. Enzyme A corresponded to the activity of the band at Rm 0.76 with a minor active band at Rm 0.70, and enzyme B to the activity of band Rm 0.91. Both enzymes reacted exclusively with NADPH and were most active toward quinone derivatives and ferricyanide with the optimum pH at 7.0. The reaction followed a ping-pong mechanism with K_m values for NADPH and menadione of 10.5 μM and 6 μM, respectively. The sequences of the 20 amino acids at the N-terminal of enzymes A and B were identical, and furthermore coincided with that of the *E. coli* modulator of drug activity (mda66) submitted under the accession number U18656.

Keywords: NADPH-quinone reductase; Induction; 2-Methylene-4-butyrolactone; Two-electron reduction; Drug activity modulator; (*E. coli*)

1. Introduction

The anticancer and cytotoxic effects of quinonoid drugs are considered to be mediated through their one-electron reduction by microsomal NADPH-cytochrome P450 reductase (EC 1.6.2.4) to semiquinone radicals, which are able to react with molecular oxygen to form superoxide radicals [1]. Hassan and Fridovich [2] demonstrated that several quinonoid compounds cause increased intracellular production of superoxide radicals and hydrogen peroxide in *Escherichia coli*. The toxicity of menadione (2-methyl-1,4-naphthoquinone) in hepatocytes has been shown to be due to the oxidative stress induced by redox cycling of menadione [3–7]. NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2), known as DT-diaphorase, catalyzes the two-electron reduction of quinones to quinols [8–10], and has been shown to protect against menadione toxicity by competing with the potentially toxic one-electron reduction pathway [3,11,12]. Thus, the toxicity of quinones is greatly influenced by the species and amounts of enzymes participating in their metabolism.

Earlier studies in this laboratory demonstrated that menadione induces an FMN-dependent NADH-quinone reductase in *E. coli* [13] and that a 2-alkyl-1,4-quinone structure with the C-3 unsubstituted or substituted with Br is critical as a common inductive signal for this enzyme [14]. Except for its FMN-dependence and NADH-specificity, the properties of the induced enzyme were very similar to those of the DT-diaphorase from animal cells [13]. Since several Michael reaction acceptors, characterized by olefinic bonds that are rendered electrophilic by conjugation with electron-withdrawing substituents, have been reported to be efficient inducers of DT-diaphorase in mouse hepatoma cells [12], the inductive activities of these compounds were examined in *E. coli*. Most of the Michael reaction acceptors were ineffective as inducers of NADH-quinone reductase in *E. coli*. However, 2-methylene-4-butyrolactone (MBL), methylacrylate and methyl vinyl ketone functioned as inducers of NADH-quinone reductase [13]. Further examination revealed that these compounds

Abbreviations: menadione, 2-methyl-1,4-naphthoquinone; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; MBL, 2-methylene-4-butyrolactone.

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induce NADPH-specific quinone reductases in addition to NADH-quinone reductase. Since the toxicity of quinone derivatives is influenced by the nature of enzymes participating in their metabolism, two of the NADPH-specific quinone reductases induced by MBL in *E. coli* were purified and their properties examined. From their N-terminal amino acid sequence, the induced enzymes were found to correspond to the gene product of the *E. coli* modulator of drug activity (*mda66*).

2. Materials and methods

Materials. Ubiquinone-1 (Q-1) was kindly supplied by Eizai (Tokyo, Japan). FMN, FAD, dicumarol and menadione were obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were of the highest commercial grade available.

Bacterial growth. E. coli K12, C600 was shake-cultured at 37°C in a complex medium containing 1% polypeptone, 0.5% yeast extract and 20 mM sodium phosphate (pH 7.2). Cell growth was monitored by the increase in absorbance at 600 nm with a Perkin-Elmer spectrophotometer, model 35.

Induction of quinone reductases. The cells were inoculated into fresh medium at a cell density of 0.02 at 600 nm. At a cell density of 0.2–0.3, the test compound was added to the culture, which partly suppressed cell growth. The cells were harvested by centrifugation at the cell density of 0.6–0.8, and washed twice with 50 mM potassium phosphate (pH 7.5)/5 mM $MgSO_4$. Soluble extracts (the cytosol fractions) were prepared as previously described [14].

Enzyme assays. Menadione reductase activity was assayed at 30°C. The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 0.1 mM menadione, 0.2 mM NADH or 0.1 mM NADPH and the enzyme in a total volume of 1.0 ml. FMN was used at 5 μM when specified. The reaction was started by the addition of enzyme and the activity was calculated from the decrease in absorbance at 340 nm using the absorption coefficient of 6.22 mM^{-1} cm^{-1}.

The formation of menadiol in the presence of NADPH was measured from the changes in the absorbance difference at the wavelength pair, 263–240 nm, using a Hitachi 557 dual-wavelength spectrophotometer. The reaction was carried out in the presence of 0.1 mM NADPH and 20 μM menadione. The absorption coefficient for menadiol at the above wavelength pair was estimated to be 27.2 mM^{-1} cm^{-1}.

For the assay of ferricyanide reductase, 1.0 mM ferricyanide was used as the electron acceptor and the activity was measured from the decrease in absorbance at 420 nm using the absorption coefficient of 1.0 mM^{-1} cm^{-1}.

One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADH or NADPH, or the reduction of 1 μmol quinone or 2 μmol ferricyanide per min.

Purification of enzymes. DEAE-Sephacel (Pharmacia) and pre-packed high-performance columns, TSK gel DEAE-5PW (2.15 × 15 cm, Toyo Soda, Tokyo) and Biofine HAC-5CP (0.75 × 10 cm, Nippon Bunko, Tokyo), were employed for the purification of the enzymes using the conditions described in our previous paper [13]. HiLoad Superdex 200 (1.6 × 60 cm, Pharmacia) and Sephacryl S-200 (1.7 × 26 cm, Pharmacia) were used for the gel permeation chromatography with a buffer system containing 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 0.1 mM EDTA and 5% (w/v) glycerol.

Polyacrylamide gel electrophoresis (PAGE). PAGE and SDS-PAGE were performed using the discontinuous buffer system of Laemmli [15]. For the electrophoresis of native enzymes, 7.5% acrylamide was used as the running gel, and enzyme activities were detected using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by incubating the gels at 30°C in a reaction mixture containing 0.2 mM NADPH, 0.5 mM MTT, 0.1 mM menadione and 50 mM Tris-HCl (pH 7.5). The electrophoretic mobility of the stained bands were expressed as the relative mobility to bromophenol blue dye (Rm).

The molecular mass of the native enzymes was determined according to the method of Hedrick and Smith [16], where the acrylamide gel concentrations were varied between 6 and 13.5%.

Amino acid sequence analysis. Proteins separated by SDS-PAGE were transferred to a Problot membrane (Applied Biosystems) and then each protein band was analyzed by an Applied Biosystems 477A protein sequencer.

Other methods. For kinetic analyses, the value of each experimental point was obtained as the mean of at least three determinations. The line of best fit for each set of double-reciprocal plots, kinetic constants, and their standard errors were calculated according to the method of Cleland [17] as described previously [18]. Flavins were analyzed as described previously [19], and FAD and FMN contents were determined by the method of Faeder and Siegel [20].

Protein was determined by the method of Bradford [21] with bovine serum albumin as a standard.

3. Results

3.1. Induction of quinone reductases by 2-methylene-4-butyrolactone

During our studies on the induction of FMN-dependent NADH-quinone reductase by various compounds, a few Michael reaction acceptors such as MBL, methyl vinyl ketone and methylacrylate were found to act as inducers of quinone reductase in *E. coli* [14]. Although the FMN-dependent NADH-quinone reductase induced by several quinone derivatives was confirmed to be identical to that induced by menadione, the reductase activities induced by

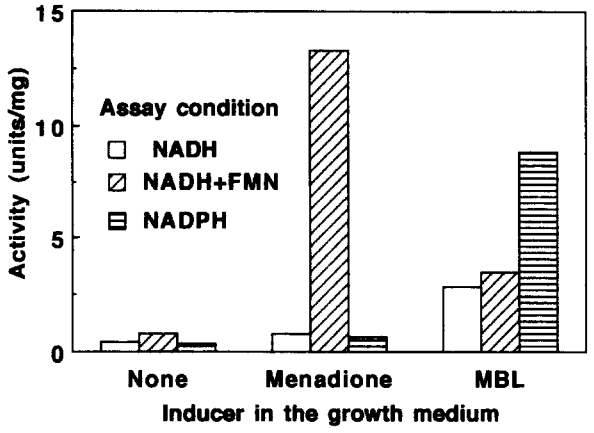

Fig. 1. Induction of NADH- and NADPH-menadione reductases in the cytosol of *E. coli*. Enzyme activities in the cytosol from *E. coli* cells grown in the absence or presence of 0.25 mM menadione or 2 mM MBL were assayed under the conditions shown in the figure, and the activity was expressed as the mean of triplicates in units/mg protein.

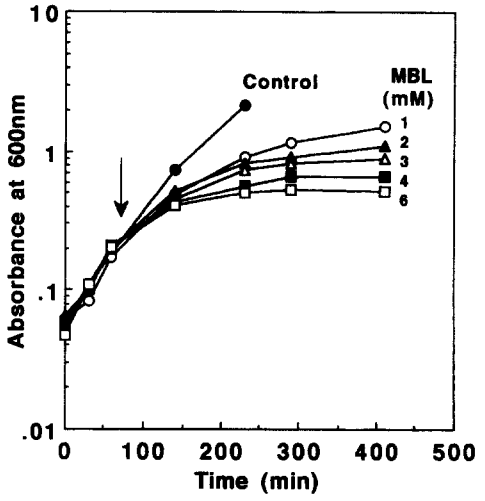

Fig. 2. Effect of MBL on the growth of *E. coli*. MBL was added at 80 min (arrow) to cultures at the concentration shown in the figure.

the above Michael reaction acceptors have not been analyzed in detail.

Fig. 1 shows the reductase activities in the soluble extracts (cytosol) of *E. coli* cells grown in the presence or absence of inducers. The concentrations of inducers were selected so as to partly inhibit the growth rate of *E. coli*. NADH-dependent menadione reductase activity was assayed in the presence and absence of 5 μM FMN to determine FMN dependence. Since NADPH-dependent menadione reductase activity was unaffected by the addition of flavins, this activity was assayed in the absence of flavins. As reported in our previous paper [13], menadione strongly induced the FMN-dependent NADH-menadione reductase activity. On the other hand, MBL induced more FMN-independent NADH-menadione reductase activity (Fig. 1). Moreover, NADPH-menadione reductase activity was strongly induced by MBL. Although not shown here, other compounds such as methyl vinyl ketone and methylacrylate behaved similarly to MBL but were less efficient as inducers.

When MBL was added to cultures of *E. coli* at the exponential phase of growth, the rate and extent of cell growth decreased with increasing MBL (Fig. 2). The effect of MBL, however, was not immediate and required a prolonged time to completely arrest cell growth. The concentration dependence of the induction of NADPH-menadione reductase was measured, and the addition of 2 mM MBL was found to induce more than 70% of the maximum induction observed at 5 mM.

Menadione reductase activity in the cytosol from non-induced *E. coli* cells was separated into four fractions by DEAE-Sephacel column chromatography (Fig. 3). Fractions I and III mainly contained NADH-specific and fraction II, NADPH-specific menadione reductases. In the cells induced by menadione, the activity in fraction I greatly increased [13]. When the cells were induced by MBL, the activity in fraction II greatly increased with a significant increase also in fraction I.

3.2. Purification of NADPH-quinone reductases induced by MBL

Since NADPH-specific quinone reductases increased about 25-fold by induction with MBL, these enzymes were purified from the cytosol of MBL-induced cells. The cytosol was first fractionated by DEAE-Sephacel column chromatography as described in Fig. 3, fraction II was rechromatographed on DEAE-Sephacel, and the active fraction was applied to DEAE-5PW. The latter column chromatography separated the NADPH-quinone reductase activity into two fractions, A and B. Fraction A was eluted

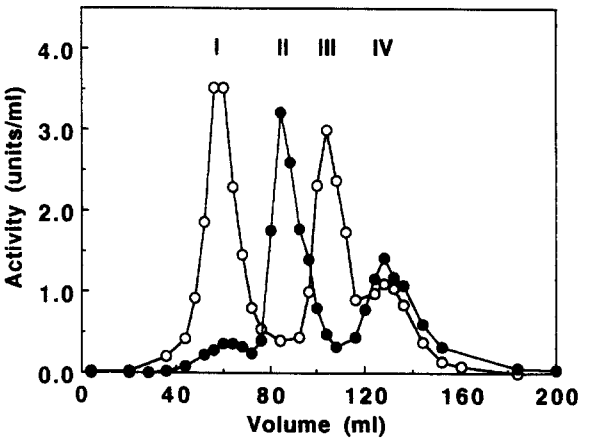

Fig. 3. Fractionation of menadione reductases by DEAE-Sephacel column chromatography. About 200 mg cytosolic protein from non-induced *E. coli* cells were fractionated by a DEAE-Sephacel column (1.5 × 17 cm) as described in Materials and Methods. The menadione reductase activity in the effluent was assayed in the presence of 5 μM FMN using NADH (○) or NADPH (●) as the electron donor.

Table 1
Purification of MBL-induced NADPH-quinone reductases A and B

Fraction	Protein (mg)	NADPH-menadione	
		units	units/mg
Cytosol	1210	11253	9.3
1st DEAE-Sephacel	264	8659	32.8
2nd DEAE-Sephacel	173	6868	39.7
DEAE-5PW			
Fraction A	20.8	5762	277
Fraction B	16.4	346	21.1
Fraction A			
DEAE-5PW	1.40	388	277
Superdex 200	0.40	306	765
Sephacryl S-200	0.22	157	715
Biofine HAC-5CP	0.08	85.4	1067
Fraction B			
DEAE-5PW	14.6	308	21.1
2nd DEAE-5PW	2.10	133	63.3
Superdex 200	0.28	73.6	263
Biofine HAC-5CP	0.17	44.4	261

Table 2
Electron acceptor specificity of NADPH-quinone reductases A and B

Electron acceptor	Concn. (μM)	Relative activity [a]	
		A	B
Menadione	10	0.48	0.52
	100	1.00	1.00
1,4-Naphthoquinone	10	0.78	0.80
	100	0.85	0.91
1,4-Benzoquinone	20	0.60	0.62
	100	0.81	0.90
Q-1	10	0.21	0.30
Ferricyanide	1000	0.89	1.06
DCIP [b]	40	0.15	0.18

[a] The activity with 100 μM menadione was taken as 1.00.
[b] Dichlorophenolindophenol.

at an NaCl concentration of 120 mM, and fraction B at 140 mM.

A portion of each fraction was further purified by ion-exchange and gel chromatographies. Table 1 shows a typical purification result. As calculated on the basis of the cytosol from the induced cells, NADPH-quinone reductase fraction A was purified about 115-fold and NADPH-quinone reductase fraction B about 28-fold.

3.3. Molecular masses and the correlation of three activity bands

As shown in Fig. 4, purified enzyme A contained a major activity band with relative mobility (Rm) of 0.76 and a minor activity band of Rm 0.70. Purified enzyme B contained a single activity band of Rm 0.91. To determine the molecular masses of these active bands, gel concentrations were varied from 6 to 13.5%, and the log of relative mobilities were plotted against gel concentrations (Ferguson plots). A linear plot was obtained for each band and

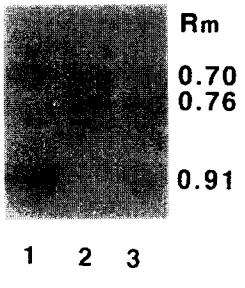

Fig. 4. Staining of the purified NADPH-quinone reductases A and B after polyacrylamide gel electrophoresis. Lane 1, enzyme B; lane 2, enzyme A; lane 3, cytosol from MBL-induced cells. Enzyme activity corresponding to 0.05 units was applied to each lane.

the slopes of all three were identical (data not shown). This result is evidence that these three proteins are identical in size but are different in charge. From the slope–molecular mass relationship of standard proteins, the molecular sizes of the three bands were estimated to be 41.7 kDa. On SDS-PAGE, enzymes A and B each gave a single protein band of 21 kDa. Thus, these three proteins appear to be charge isomers with the same molecular size of 42 kDa, composed of two identical subunits of 21 kDa.

The amino acid sequences of the N-termini of purified enzymes A and B were analyzed by an amino acid sequencer. The N-terminal amino acid sequences were identical: SNILIINGAKKFAXSNGQLN, where X is an unidentified amino acid residue. These results indicate that both enzymes were derived from the same structural gene. Furthermore, it was found that the N-terminal sequence is identical to that of the *E. coli* modulator of drug activity (mda66) submitted under accession number U18656.

3.4. Properties of NADPH-quinone reductases A and B

Substrate specificity. The purified NADPH-quinone reductases A and B exclusively reacted with NADPH, and NADH was ineffective as an electron donor. As shown in Table 2, the electron acceptor specificities of the enzymes A and B were essentially identical. Both enzymes were most active toward quinone derivatives and ferricyanide, but Q-1 and also DCIP reacted less efficiently.

Optimum pH. Using either menadione or ferricyanide as the electron acceptor, enzymes A and B exhibited the same pH-activity curve with the optimum at pH 7.0.

Inhibitor studies. The enzymes A and B were insensitive to *p*-chloromercuribenzoate (100 μM), heavy metals such as Cu^{2+}, Co^{2+}, Cd^{2+} and Mn^{2+} (10 μM each), and chelating agents such as EDTA, *o*-phenanthroline and diethyldithiocarbamate (100 μM each). Dicumarol was also ineffective; 50 μM was required to inhibit the activity

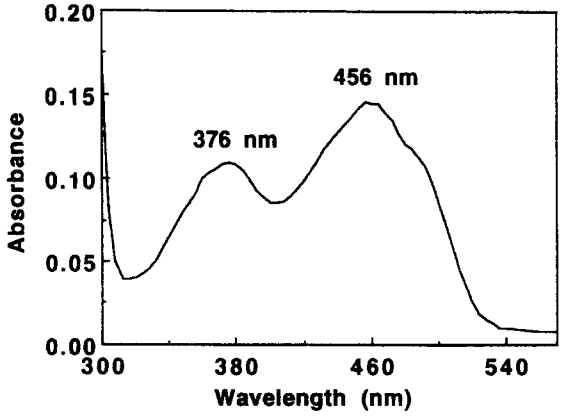

Fig. 5. Absorption spectrum of the purified NADPH-quinone reductase A. The spectrum was recorded at a concentration of 0.36 mg protein/ml.

about 15%. A specific inhibitor for these enzymes could not be found.

Kinetic studies. For both enzymes A and B, double-reciprocal plots of initial velocity versus NADPH at fixed levels of menadione (10, 15, 20, 25, 30 and 40 μM) gave parallel lines, indicating a ping-pong mechanism. Secondary plots of intercepts against the reciprocal of menadione concentration gave linear plots. From these data, the K_m values for NADPH and menadione were calculated to be 10.4 ± 1.7 and 6.4 ± 0.9 μM for enzyme A, and 10.9 ± 2.0 and 5.8 ± 1.1 μM for enzyme B. Thus, both enzymes have the same K_m values for NADPH and menadione.

Flavin contents. The purified NADPH-quinone reductase A showed a characteristic flavin absorption spectrum with peaks at 376 and 456 nm and a shoulder at around 484 nm (Fig. 5). Flavins were extracted from the enzymes by boiling for 5 min at neutral pH, and then FAD and FMN content were determined by the method of Faeder and Siegel [20]. Both enzymes contained FAD as the cofactor, and FMN was not detected in either. Assuming that the molecular mass of the subunits is 21 kDa, purified enzymes A (1067 units/mg protein) and B (261 units/mg protein) contained 0.74 and 0.28 mol FAD/subunit, respectively. Thus, the low specific activity of enzyme B may be related to its low FAD content; however, the addition of FAD to the assay medium showed no significant activation of either enzyme.

Mode of quinone reduction. The formation of menadiol from menadione was spectrophotometrically measured by the technique described in our previous paper [22,23]. In the presence of NADPH, absorbance changes using the wavelength pair 263–240 nm were measured and the amount of menadiol was calculated using the absorption coefficient of 27.2 mM^{-1} cm^{-1}. Both NADPH-quinone reductases A and B consumed NADPH with a stoichiometric formation of menadiol (data not shown). Apparently, both enzymes reduce menadione by a two-electron transfer pathway, similar to the DT-diaphorase from animal cells [10].

4. Discussion

NADPH-specific quinone reductases with relative mobilities of 0.70, 0.76 and 0.91 are induced by MBL, but not by menadione. The three active bands appear to be charge isomers of the same protein that has an apparent molecular size of 42 kDa, composed of identical subunits of 21 kDa. The purified NADPH-quinone reductases A and B have very similar enzymatic properties. Moreover, the sequence of the first 20 amino acids of enzymes A and B are identical, which makes it likely that both enzymes are derived from the same structural gene. Furthermore, this sequence is identical to that of N-terminal amino acid sequence of the modulator of drug activity (mda66). Although the amino acid sequence of the latter has been submitted by Chatterjee and Sternberg under the accession number of U18656, the properties of the mda66 protein are not yet published. Mda66 is composed of 193 amino acid residues with a mass of 21890 Da, which is very similar to the subunit size of the induced enzyme. Since the MBL-induced enzyme functions in the metabolism of quinone derivatives, it may also function as a modulator of some drug activities.

MBL belongs to Michael reaction acceptors, which have been reported to be efficient inducers of DT-diaphorase in mouse hepatoma cells [12]. Michael reaction acceptors, however, were not always effective as inducers of quinone reductases in *E. coli*. Among them, MBL and, to a lesser extent, methyl vinyl ketone and methylacrylate were found to act as inducers of NADPH-quinone reductases. Since these compounds have a methide group as a common structure, the methide group is likely to be an inductive signal for these enzymes.

The toxicity of MBL against *E. coli* is not so strong and requires millimolar concentrations of MBL and time to arrest cell growth. MBL is not reduced by the induced enzyme, nor does it inhibit its activity. Therefore, a direct interaction between MBL and the induced enzyme is unlikely. MBL seems to indirectly provide an inductive signal by affecting cell metabolism, but the mechanism by which this induction occurs is unknown.

It is worthy of note that the induced enzyme is insensitive to dicumarol and reduces quinone derivatives by a two-electron reduction pathway. In animal cells, DT-diaphorase is considered to protect against the toxicity of quinones by a two-electron reduction [3,11,12]. Hydroquinone products, however, are not always stable and are also capable of autoxidations, thereby generating superoxide anion and related active oxygen radicals [24]. Therefore, the induction of two-electron transfer quinone reductases may not necessarily be related to a protective response against oxidative stress. Recently, NADPH-fer-

redoxin oxidoreductase [25] and NADPH-sulfite reductase [26] of *E. coli* have been reported to act as a paraquat reductase, but the MBL-induced enzyme does not react with paraquat. Further studies are required to clarify the physiological role of this inducible NADPH-quinone reductase in *E. coli* and the correlation with mda66 protein reported to be a modulator of drug activity.

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