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Chemical structures critical for the induction of FMN-dependent NADH-quinone reductase in *Escherichia coli*

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An FMN-dependent NADH-quinone reductase is induced in *Escherichia coli* by growing the cells in the presence of menadione (2-methyl-1,4-naphthoquinone). Since the properties of induced enzyme are very similar to those of NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2), known as DT-diaphorase, from animal cells, structural requirements of quinone derivatives as an inducer of NADH-quinone reductase in *E. coli* were examined. Among quinone derivatives examined, it was found that 2-alkyl-1,4-quinone structure with C-3 unsubstituted or substituted with Br is critical as a common inductive signal. Michael reaction acceptors which have been reported to be strong inducers of DT-diaphorase in mouse hepatoma cells were not always effective inducers in *E. coli*. However, several compounds, such as 2-methylene-4-butyrolactone, methylacrylate and methyl vinyl ketone, showed a slight inductive activity. The efficient inducers of NADH-quinone reductase in *E. coli* contain 1,4-quinone structure as a part of the inductive signal. These compounds belong to Michael acceptors and are likely to conjugate with thiol compounds such as glutathione.

Introduction

Earlier studies in this laboratory [1] demonstrated that menadione (2-methyl-1,4-naphthoquinone) induces an FMN-dependent NADH-quinone reductase in *Escherichia coli*. The induced quinone reductase exclusively reacted with NADH and required FMN as a cofactor. Except for NADH-specificity and FMN-dependence, the properties of this enzyme were very similar to NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2), known as DT-diaphorase, from animal cells with respect to the electron acceptor specificity, reaction mechanism (ping-pong), dicoumarol sensitivity, and the two-electron transfer reduction of quinones [1].

The DT-diaphorase of animal cells is induced by a variety of xenobiotics [2]. Among these compounds, phenolic antioxidants elevated DT-diaphorase but not cytochrome *P*-450 levels [2]. By use of the mouse hepatoma cells (Hepa lclc7), Prochaska et al. [3] demonstrated that structurally dissimilar catechols (1,2-diphenols) and hydroquinones (1,4-diphenols) induce DT-diaphorase, but resorcine (1,3-diphenol) and its substituted analogues are inactive. Thus, the signal for the induction of DT-diaphorase was considered to be dependent on the redox-lability of these inducers [2,3]. After that, Talalay et al. [4] identified a common chemical signal for the induction of DT-diaphorase and glutathione transferases as Michael reaction acceptors characterized by olefinic bonds that are rendered electrophilic by conjugation with electron-withdrawing substituents. Furthermore, Spencer et al. [5] demonstrated that the potency of inducers of DT-diaphorase parallels their efficiency as substrates for glutathione transferases. At present, the induction of phase II enzymes such as DT-diaphorase, glutathione transferases and glucuronosyl-transferases, is considered to be a major mechanism whereby animals and their cells can be protected against the toxicity and carcinogenicity of many potentially harmful xenobiotics [2–5].

The NADH-quinone reductase of *E. coli* is induced by menadione and by 2-*tert*-butylhydroquinone [1], which are Michael reaction acceptors. Therefore, it is of interest to identify a chemical signal for the induction of NADH-quinone reductase in *E. coli* in comparison with that for the induction of DT-diaphorase in animal cells. In the work reported here, the inducible activities of various quinone derivatives and Michael reaction acceptors were examined.

Abbreviations: Menadione, 2-methyl-1,4-naphthoquinone; NQ, 1,4-naphthoquinone; BQ, 1,4-benzoquinone; duroquinone, 2,3,5,6-tetramethylbenzoquinone; NQR, NADH-quinone reductase; SOD, superoxide dismutase.

Materials and Methods

Materials. Bovine pancreas deoxyribonuclease I (Type IV), butter milk xanthine oxidase and horse heart cytochrome *c* (type III) were obtained from Sigma. 2,3-Dimethyl-, 2-chloro-, 2-methyl-3-chloromethyl-, 2-methyl-3-bromo-, and 2-hydroxy-1,4-naphthoquinones were kindly donated from Prof. Emeritus N. Ikeda of Chiba University. 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). The cell growth was monitored by the increase in absorbance at 600 nm with a Perkin-Elmer spectrophotometer, model 35.

Induction of NADH-quinone reductase. The cells were innoculated to a fresh medium at the cell density of 0.02 at 600 nm. When the cell density reached about 0.2, an appropriate amount of test compound was added to the culture. Most of test compounds were dissolved in ethanol as stock solutions and the concentration of ethanol in the growth medium was always maintained below 3%, where ethanol showed no inhibitory effect on the cell growth. By the addition of test compounds, the cell growth was suppressed or stopped for a while, and then the growth was resumed. At the cell density of 0.6–0.8, the cells were harvested and washed twice with 50 mM potassium phosphate (pH 7.5)/5 mM $MgSO_4$ by centrifugation. The washed cells were suspended in the same medium containing dithiothreitol (1 mM) and deoxyribonuclease I (10 μg/ml) at the cell density of about 2 at 600 nm. Then, the cells were disrupted by a pulse sonicator (Tomy UD-201) for 2.5 min at the output of 30 with 50% duty. The broken cells were centrifuged at 100000 × *g* for 1 h and the supernatant was used as a cytoplasmic fraction.

Enzyme assays. NADH-quinone reductase (NQR) was assayed at 30°C in the reaction mixture containing 20 mM Tris-HCl (pH 7.5), 0.2 mM NADH, 0.1 mM menadione, 5 μM FMN and enzyme in a total volume of 1.0 ml. The activity was measured from the decrease in absorbance at 340 nm. One unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADH per min.

Superoxide dismutase (SOD) was assayed and its unit is expressed as described by McCord and Fridovich [6] except that the reaction was carried out in a total volume of 1.0 ml.

Protein was determined by the method of Lowry et al. [7] with bovine serum albumin as a standard.

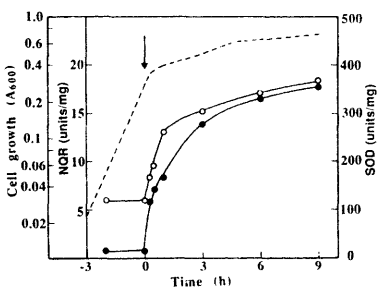

Fig. 1. Time-courses of the induction of NADH-quinone reductase (NQR) and superoxide dismutase (SOD). At a cell density of 0.25 at 600 nm (indicated by the arrow), 0.25 mM menadione was added to the culture. At the indicated time, the cells were harvested and then NOR (●) and SOD (○) activities in the cytoplasmic fraction were determined. The values are expressed as the mean of three separate experiments. The dotted line denotes the cell density at 600 nm.

Results and Discussion

Effect of menadione on the induction of NADH-quinone reductase (NQR) and superoxide dismutase (SOD)

Since FMN-dependent NADH-quinone reductase (NQR) and superoxide dismutase (SOD) of *E. coli* are markedly induced by menadione, the effect of menadione on the inductions of these enzymes was examined. As shown in Fig. 1, the cell growth was retarded after the addition of 0.25 mM menadione and then the cells continued to grow at a low rate. The induction of both NQR and SOD occurred immediately after the addition of menadione and reached about a half of the maximum induction in 1 h. Fig. 2 shows the effect of the concentration of menadione. The inducible ability of menadione for NQR and SOD increased with the increase in its concentrations and was saturated at about 0.35 mM. Thus, the time-courses and the concentration dependence of NQR induction were very similar to those of SOD induction.

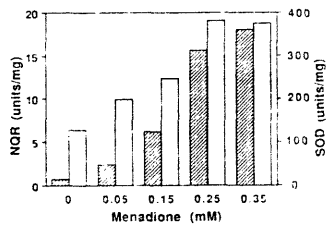

Fig. 2. Effect of the concentration of menadione on the inductions of NQR and SOD. After the addition of indicated amount of menadione to the culture at a cell density of 0.2, the cells were harvested at 0.8 and then NQR (▨) and SOD (□) activities in the cytoplasmic fraction were determined. The values are expressed as the mean of three separate experiments.

TABLE 1

Induction of NADH-quinone reductase (NQR) and superoxide dismutase (SOD)

Quinone derivative	Concentration (mM)	NQR (units/mg)	SOD (units/mg)
Without quinones		0.70 ± 0.09	127 ± 16
1,4-Naphthoquinone (NQ)	0.1	0.97 ± 0.20	122 ± 21
2-Methyl-NQ (menadione)	0.25	17.8 ± 1.2	381 ± 143
2,3-Dimethyl-NQ	0.3	0.79 ± 0.04	315 ± 31
2-Chloro-NQ	0.1	0.98 ± 0.18	148 ± 18
2-Methyl-3-chloromethyl-NQ	0.2	1.26 ± 0.2	285 ± 29
2-Methyl-3-bromo-NQ	0.4	21.7 ± 3.7	167 ± 8
2-Hydroxy-NQ (lawsone)	0.8	1.39 ± 0.27	275 ± 66
5-Hydroxy-NQ (juglone)	0.1	1.01 ± 0.42	205 ± 22
2-Methyl-5-hydroxy-NQ (plumbagin)	0.2	6.02 ± 1.36	376 ± 46
1,4-Benzoquinone (BQ)	0.3	7.79 ± 1.90	87 ± 5
2-Methyl-BQ	0.2	26.8 ± 6.1	109 ± 8
2,6-Dimethyl-BQ	0.2	8.74 ± 2.37	122 ± 24
2-Methyl-5,6-dimethoxy-BQ	0.3	3.51 ± 0.86	100 ± 9
2,3,5,6-Tetramethyl-BQ (duroquinone)	0.3	0.85 ± 0.06	266 ± 11
Thymoquinone	0.3	1.69 ± 0.03	113 ± 5
Dibromothymoquinone	0.4	22.4 ± 0.7	130 ± 53
2-*tert*-Butyl-4-hydroxyanisole (BHA)	1.0	0.49 ± 0.11	132 ± 12
3,5-Di-*tert*-butyl-4-hydroxytoluene (BHT)	0.2	0.67 ± 0.08	130 ± 22
2-*tert*-Butyl-1,4-hydroquinone (BHQ)	1.0	9.50 ± 0.34	122 ± 25

[a] The values are expressed as the mean of three separate experiments ± S.D.

As shown in our previous paper [1], the cytoplasm from non-induced cells contains several kinds of enzyme catalyzing NADH-menadione reductase activity. The total activity of NADH-menadione reductase in the cytoplasm increased from 0.32 to 0.80 units/mg protein by the addition of 5 μM FMN. In the presence of FMN, the activity was inhibited 59% by the addition of 3 μM dicoumarol. Thus, FMN-dependent and dicoumarol-sensitive NADH-menadione reductase was estimated to be 0.47 units/mg in the non-induced cells. With the cytoplasm from the induced cells, most of the NADH-menadione reductase was FMN-dependent and dicoumarol-sensitive. Since the total activity induced by 0.25 mM menadione was 17.8 units/mg, the FMN-dependent enzyme was calculated to be induced about 37-fold. On the other hand, SOD activity was high in the non-induced cells and it increased about 3-fold in the induced cells.

Induction of NQR and SOD by quinone derivatives

Table 1 shows the effect of naphthoquinone and benzoquinone derivatives on the induction of NQR and SOD. The amount of test compound was selected so as not significantly inhibit the cell growth. The concentration of each quinone used in Table 1 was nearly saturated for the induction of NQR. At higher concentrations, the induction of NQR was rather suppressed due to inhibitory effects on the cell growth. It was also confirmed that the NQR activity induced by these quinone derivatives required FMN as a cofactor and was inhibited more than 85% by 3 μM dicoumarol. These properties were very similar to those of the enzyme induced by menadione.

Among 1,4-naphthoquirone (NQ) derivatives, NQ and 2,3-dimethyl-NQ were ineffective as inducers. Inductive activity of menadione was lost by replacing the 2-methyl group with chloro or hydroxy group. The menadione derivative substituted at C-3 with chloromethyl (2-methyl-3-chloromethyl-NQ) was ineffective, but that substituted with bromo-group at C-3 (2-methyl-3-bromo-NQ) was a strong inducer of NQR. Although the 5-hydroxy derivative of menadione (plumbagin) still maintained an inductive activity with a significant decrease in efficiency, 5-hydroxy-NQ (juglone) was almost ineffective as an inducer of NQR.

With respect to 1,4-benzoquinone (BQ) derivatives, 2-methyl-BQ was a strong inducer, followed by 2,6-dimethyl-BQ. 2-Methyl-5,6-dimethoxy-BQ also showed a slight inductive activity. Duroquinone, however, showed no inductive activity. Dibromothymoquinone was found to be an efficient inducer. In this compound, the C-3 and C-6 positions adjacent to 2-methyl- and 5-isopropyl-groups are brominated. Although C-3 and C-6 positions are unsubstituted in thymoquinone, its inductive activity was very low as compared with dibromothymoquinone, possibly due to the effects of substituents in the quinone ring.

In contrast to NQ, unsubstituted BQ showed an apparent induction of NQR. In the presence of BQ, the growth medium turned to intensive red color due to the formation of quinhydrone. Furthermore, BQ is a highly reactive quinone, and Brunmark and Cadenas [8] reported that an electronically excited state is generated by the reaction with H_2O_2 in addition to the formation of 2,3-epoxy-BQ and 2-hydroxy-BQ. Since the induction of NQR did not occur immediately after the addition of BQ to the growth medium, BQ is likely to be converted to other reactive derivatives to function as an inducer of NQR.

As phenolic antioxidants, 2-*tert*-butyl-4-hydroxyanisole (BHA), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) and 2-*tert*-butyl-1,4-hydroquinone (BHQ) are known to act as inducers of DT-diaphorase in animal cells [2]. Among these compounds, only BHQ acted as an inducer of NQR in *E. coli*. The mild inductive activity of BHQ may be related to the presence of a bulky *tert*-butyl group at C-2 position. Inability of BHA and BHT as inducers of NQR may be due to the absence of

enzymes in *E. coli* which convert these compounds to a reactive form as the inducer.

From these results, it can be concluded that 2-alkyl-3-unsubstituted-1,4-quinone or 2-alkyl-3-bromo-1,4-quinone structure functions as a common inductive signal for NQR in *E. coli*. An apparent exception is the induction of NQR by BQ, which has already been mentioned above.

The induction of SOD reflects the generation of superoxide radicals during the metabolism of these quinone derivatives. As shown in Table I, the inductive potencies of SOD varied with naphthoquinone derivatives and the inducibilities of NQR and SOD were not correlated with each other. Since paraquat (methyl viologen), which is a typical redox-cycling agent, has no capacity to induce NQR [1], it is apparent that superoxide radicals and other reactive oxygen species do not function as an inductive signal for NQR. In *E. coli*, redox-cycling agents such as menadione and paraquat are known to induce at least 33 proteins in addition to those induced by hydrogen peroxide [9]. Furthermore, menadione, paraquat and hydrogen peroxide induced a few unidentified proteins unique to the respective agents, and five proteins induced by menadione only were detected in the two-dimensional gel analysis [9]. Although these proteins are not characterized yet, NQR apparently corresponds to one of these proteins.

Rossi et al. [10] demonstrated that BQ derivatives do not mediate oxidative stress in isolated rat hepatocytes and rapidly deplete cellular glutathione (GSH) by conjugation without oxidation to GSSG. Duroquinone is not able to conjugate with GSH, and its cytotoxicity was shown to be mediated by oxidative stress [10]. Therefore, it is interesting to note that only duroquinone significantly induced SOD among BQ derivatives (Table I). Duroquinone apparently mediates redox-cycling in *E. coli* also.

Induction of NQR by Michael reaction acceptors

Table II shows the effect of Michael reaction acceptors which have been reported to be efficient inducers of DT-diaphorase in mouse hepatoma cells [4]. To compare the inductive activities of these compounds, the concentrations required to double the basal specific activity of DT-diaphorase (designated CD) were cited from Ref. 4. From the examination of coumarine analogues, Talalay et al. [4] concluded that the structural feature critical for inductive activity is an electrophilic olefin conjugated with a carbonyl group. Since nitroolefins are highly efficient Michael acceptors, 1-nitro-1-cyclohexene was introduced and was found to be a strong inducer of DT-diaphorase (CD = 1–2 μM). This compound, however, was ineffective as the inducer of NQR in *E. coli* (Table II). Among the Michael acceptors examined, 2-methylene-4-butyrolactone, methylacrylate and methyl vinyl ketone showed slight

TABLE II

Induction of NQR of E. coli by Michael reaction acceptors

Compound	Concentration (mM)	NQR[a] (units/mg)	SOD[a] (units/mg)	CD[b] (μM)
1-Nitro-1-cyclohexene	0.06	0.90 ± 0.12	199 ± 14	1–2
2-Methylene-4-butyrolactone	3.3	3.05 ± 0.54	177 ± 41	22
Methylacrylate	3.3	2.27 ± 0.18	173 ± 53	20
Methyl vinyl ketone	1.5	2.48 ± 0.51	145 ± 22	40
Crotonaldehyde	1.25	0.89 ± 0.07	137 ± 10	9
Dimethyl fumarate	0.31	0.87 ± 0.02	121 ± 15	20
Dimethyl maleate	1.25	0.92 ± 0.06	125 ± 23	20

[a] The values are expressed as the mean of three separate experiments ± S.D.

[b] The data from Talalay et al. [4].

inductive activities for NQR, but unusually high concentrations were required as compared with the case of animal cells. Crotonaldehyde, dimethyl fumarate and dimethyl maleate were almost ineffective as inducers of NQR. All these compounds contain α,β-unsaturated carbonyl structure in their molecules. Dimethyl fumarate and dimethyl maleate have been shown to be efficient inducers of phase II enzymes such as DT-diaphorase and glutathione transferases in a variety of organs of mouse and rat [11]. These compounds are likely to be metabolized to other inactive compounds in *E. coli*. However, it is apparent that Michael reaction acceptors are not always effective inducers of NQR in *E. coli*. Many quinone derivatives examined belong to Michael acceptors, but only a few derivatives are active as the inducers of NQR (Table I). Since the Michael acceptors with quinone structure are far more efficient inducers than those without quinone structure, quinone structure seems to be an important inductive signal for NQR of *E. coli*.

Both BQ and NQ derivatives are able to undergo 1,4-reductive addition reaction with nucleophiles such as GSH to form the corresponding hydroquinone conjugates [10,12]. These hydroquinones are easily autoxidized by molecular oxygen. In vitro, the reaction rates of GSH with less substituted BQ derivatives are extremely rapid and decrease in the following order: BQ, 2-methyl-BQ, and 2,6-dimethyl-BQ [10]. The cytotoxicity of these compounds in isolated hepatocytes was considered to be due to the depletion of cellular GSH, leading to the oxidation or arylation of protein-thiols [10]. In *E. coli*, it is not known whether the inducers of NQR must be modified in the cells before functioning as true inducers. The chemical structure of efficient inducers, however, suggests that inducers are most able to conjugate with GSH or some other specific protein-thiols at the C-3 position. If these inducers interact with a putative repressor molecule in the cells, the

reactivity of inducers with the repressor to form an inactive conjugate seems to be critical for inductive activities. In this case, the formation of mono-substituted conjugate seems to be important, since the C-2 position is blocked by an alkyl group. At present, it is not known whether the quinone structure in the inducer molecules is active in the oxidized or reduced form. Further experiments are required to make clear these points.

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