Enhanced sensitivity of *Streptomyces seoulensis* to menadione by superfluous lipoamide dehydrogenase

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Abstract Lipoamide dehydrogenase from *Streptomyces seoulensis* could facilitate menadione-mediated cytochrome *c* reduction, which was mostly inhibited by superoxide dismutase, indicating the obvious involvement of superoxide radical anion. In this reaction, the production of superoxide radical anion occurred via a menadione semiquinone radical anion. When exposed to menadione, lipoamide dehydrogenase-overexpressing cells showed a much lower survival rate with a concomitant decrease of intracellular protein thiol than the wild-type strain. These results suggest that lipoamide dehydrogenase is a facilitating agent in the redox cycling of quinone compounds in vivo as well as in vitro and could inevitably increase the potential toxicity of the compounds.

Key words: Menadione; Lipoamide dehydrogenase; Redox cycling; Superoxide radical anion; *Streptomyces seoulensis*

1. Introduction

Lipoamide dehydrogenase (LPD) is a member of the pyridine nucleotide disulfide oxidoreductases [1], which are homodimeric proteins, each subunit of which contains a FAD cofactor and a redox-active disulfide. As a common component of the three 2-oxo acid dehydrogenase complexes, which catalyze the oxidative decarboxylation of pyruvate, 2-oxoglutarate, and the branched-chain 2-oxo acids, LPD fulfills the final reaction by catalyzing the NAD^+-dependent oxidation of dihydrolipoamide in these complexes [2]. LPD is also known to catalyze the reduction of various quinones using NADH as an electron donor [3]. LPD reduces quinones according to a 'ping-pong' mechanism with comparable rates to lipoamide, a physiological substrate [3]. More surprisingly, the quinone-reducing reaction of LPD may occur via a one-electron pathway producing semiquinone radicals [3]. The pig heart LPD is known to mediate a mixed mechanism involving one- and two-electron reduction of quinone compounds in the presence of NADH [4]. In aerobic conditions, the semiquinone radicals rapidly reduce molecular oxygen to generate superoxide radical anion with a concomitant regeneration of parental quinones, a process known as redox cycling. This process leads to oxidative stress and toxicity due to the formation of superoxide radical anion and subsequently other reactive oxygen species including hydrogen peroxide and hydroxyl radical.

In mammalian cells, several quinone-reducing flavoproteins have been identified and widely studied because many clinically important antitumor drugs contain the quinone nucleus [5–7]. Enzymes such as NADPH-cytochrome P-450 oxidoreductase, NADH-cytochrome b_5 oxidoreductase, and NADH-ubiquinone oxidoreductase [8–11] metabolize quinones to semiquinone radical intermediates via a one-electron pathway, resulting in antitumor and cytotoxic effects. In contrast, DT-diaphorase catalyzes the two-electron reduction of quinones to hydroquinones without the accumulation of semiquinone radicals [12]. Thus, DT-diaphorase has been proposed to protect cells against carcinogenicity, mutagenicity, and other toxic effects caused by quinone compounds [8,13,14].

Relatively little is known, however, about the metabolism of quinones in prokaryotic cells. As commercially interesting bacteria which produce many kinds of naphthoquinone family of antibiotics [15] and whose natural environments are abundant in quinones, streptomycetes are ideal materials for studying quinone metabolism.

In the present study, we show that the quinone reduction mediated by *Streptomyces seoulensis* LPD is accompanied by redox cycling in vivo as well as in vitro. For the purpose of monitoring this harmful reaction inside cells, we have overexpressed LPD in *S. seoulensis*, and then investigated the effect of menadione on the LPD-overexpressing cell.

2. Materials and methods

2.1. Materials

Menadione, NADH, NADPH, bovine erythrocytes, Cu,Zn-containing superoxide dismutase (Cu,Zn-SOD), nitroblue tetrazolium, horse heart cytochrome *c*, 5,5′-dithio-bis-(2-nitrobenzoic acid), *N*-ethylmaleimide, 5-bromo-4-chloro-3-indolyl phosphate, xanthine, xanthine oxidase, and *o*-dianisidine were purchased from Sigma. Hydrogen peroxide was purchased from Junsei (Japan).

2.2. Microorganisms and culture conditions

The plate culture of *S. seoulensis* was carried out in modified Bennett medium [16]. Modified YEME was used for the liquid culture of *S. seoulensis* [16]. HY303, an LPD-overexpressing *S. seoulensis*, was obtained by the transformation of pHY303 into *S. seoulensis* and HY702, a control strain, was obtained by the transformation of pIJ702 into *S. seoulensis*.

2.3. Preparation of cell-free extract

S. seoulensis mycelia were harvested by centrifugation at $12\,000\times g$ for 15 min and then washed with 50 mM sodium phosphate buffer (pH 7.4). After resuspension in the same buffer, mycelia were disrupted through sonication. After centrifugation at $12\,000\times g$ for 20 min, the supernatant was used as cell-free extract.

2.4. Purification of LPD

LPD was purified from *S. seoulensis* as described elsewhere [3].

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2.5. Enzyme assay and activity staining

The activity of LPD was measured by the reduction of NAD^+ in the presence of dihydrolipoamide as described by Snoep et al. [17]. One unit of LPD activity was defined as the amount of enzyme that reduced 1 μmol NAD^+ per minute. The activity of SOD was assayed by the indirect method based on the ability of SOD to inhibit the reduction of cytochrome *c* by scavenging superoxide radical anion produced by the xanthine/xanthine oxidase system [18]. One unit of SOD activity was defined as the amount of enzyme that gave 50% inhibition of cytochrome *c* reduction. Catalase activity was determined by measuring the decrease of the absorbance of H_2O_2 at 240 nm [19]. One unit of catalase activity was defined as the amount of enzyme that broke down 1 μmol of H_2O_2 per minute. Peroxidase activity was assayed by measuring the increase of absorbance at 460 nm using *o*-dianisidine as a substrate in the presence of H_2O_2 [20]. One unit of peroxidase activity was defined as the amount of enzyme that converted 1 μmol of *o*-dianisidine per minute. For activity staining, cell-free extract was subjected into 10% native gel electrophoresis. The quinone reducing activity staining was carried out as described elsewhere [21].

2.6. Construction of LPD-overexpression vector

The LPD-overexpression vector, pHY303, was constructed as follows: a 1.1-kb DNA fragment containing the promoter and N-terminal part of the open reading frame (ORF) of LPD was obtained by polymerase chain reaction (PCR). The nucleotide sequences of the two primers for PCR were 5′-ACCTCGGCTGAGCTCGCGGC-3′ (primer 1) and 5′-TGTACTCGTCGACCAGGACG-3′ (primer 2). The PCR product was cloned into pGEM-T easy vector and, at this stage, the nucleotide sequence of the PCR product was confirmed by DNA sequence analysis. And then the plasmid was *Sac*I-digested and self-ligated, giving pHY111. Into this plasmid, a *Sal*I–*Nco*I fragment of pHY102 [3] containing the C-terminal part of the LPD ORF was inserted subsequently, generating pHY112. A 1.7-kb *Sac*I–*Sph*I fragment of pHY112 was ligated into the *Sac*I–*Sph*I fragment of pIJ702, and the ligation mixture was used to transform protoplast of *Streptomyces lividans* under the screen of thiostrepton resistance. Then, the final expression plasmid pHY303 was prepared from the true transformant of *S. lividans*, since *S. seoulensis* is not transformable directly with the ligated DNA originating from *Escherichia coli*. Plasmid pHY303 was finally introduced into *S. seoulensis*. Protoplast preparation and transformation of *S. seoulensis* were carried out according to the method described by Hopwood et al. [22] with the following modification: a R2YE plate containing 5% rather than 10.3% sucrose was used for the *S. seoulensis* transformation. For unknown reasons, *S. seoulensis* protoplast was best regenerated on a R2YE plate containing 5% sucrose. Some of the LPD promoter sequence not published previously is as follows: 5′-ACCTCGGCTGACCTCGCGGCACACCCTGGGGGGCGCGGACCGACCCGGTCCGCGCCCCCGGCGTGAGGCGTCCGGAGCGGACGTGGGGCGTCTCACACGCCGGCCCGGCGTCCGTCGCACCCCCGACAGGTGCAAAGATGGAGCCCGGCAGGACAGGGCCC-3′ (underlined bases represent primer 1).

2.7. Measurement of protein thiol

Protein thiols were measured as described previously [23]. 1 ml of cell-free extract (about 1 mg of proteins) was precipitated with 5% trichloroacetic acid and washed twice. The protein pellet was then solubilized in 2.5 ml of 0.5 M Tris–HCl buffer (pH 8.8) containing 5 mM EDTA and 1% SDS and divided into two aliquots. One of the aliquots was treated with 25 mM *N*-ethylmaleimide for 10 min. 5,5′-Dithio-bis-(2-nitrobenzoic acid) was then added to the two aliquots with a final concentration of 0.25 mM. The aliquot treated with *N*-ethylmaleimide was used as blank. The amount of sulfhydryl groups was quantitated by measuring the absorbance increase at 412 nm using the molar absorption coefficient of 13 600 M^{-1} cm^{-1}.

2.8. Electron paramagnetic resonance (EPR) measurement of menadione semiquinone radical anion

EPR spectra were recorded at room temperature with a Bruker EMX EPR spectrometer operating at X-band with a TM_{110} cavity under the following conditions: 9.788 GHz of microwave frequency, 100 kHz of modulation frequency, 0.2 G of modulation amplitude, and 1 mW of microwave power. Spectral simulations were performed on a personal computer and matched directly with experimental data to extract the spectral parameters.

3. Results and discussion

3.1. Menadione-mediated production of superoxide radical anion by LPD

The addition of LPD to a reaction mixture containing NADH, menadione, and cytochrome *c* resulted in an increase of the reduction rate of cytochrome *c* by approximately 50-fold as shown in Table 1. When Cu,Zn-SOD was added to the reaction mixture, the rate of cytochrome *c* reduction was decreased by more than 80%, indicating that the major contributor to cytochrome *c* reduction was superoxide radical anion. Other minor contributors, such as menadione semiquinone radical anion or menadiol anion, to the cytochrome *c* reduction in the reaction should also exist since 20% of cytochrome *c* reduction was not inhibited by a sufficient amount of Cu,Zn-SOD, as shown by a plateau at a concentration of Cu,Zn-SOD above 10 μg/ml (Table 1). In the absence of menadione, LPD can also produce superoxide radical anion, consistent with the result of Massey et al. [24], in which the production of superoxide radical anion by pig heart enzyme was first reported. However, in our system the rate in the absence of menadione was very low compared to the rate in the presence of menadione (Table 1).

Superoxide radical anion can be produced from the relevant compounds supposed to be menadione semiquinone radical anion or menadiol anion in the following reactions:

$$M^{\bullet -} + O_2 \leftrightarrow M + O_2^{\bullet -} \quad (1)$$

or

$$MH^- + O_2 \leftrightarrow M^{\bullet -} + H^+ + O_2^{\bullet -} \quad (2)$$

where M, MH^-, $M^{\bullet -}$, and $O_2^{\bullet -}$ mean menadione, menadiol anion, menadione semiquinone radical anion, and superoxide radical anion, respectively. Both reactions contain a common menadione semiquinone radical anion as a key component. Accordingly, we tested whether LPD can produce menadione semiquinone radical anion in the quinone-reducing reaction. When purified LPD was added into a reaction mixture containing 5 mM NADH and 20 mM menadione in 50 mM sodium phosphate buffer (pH 7.4), an EPR spectrum assigned to menadione semiquinone radical anion was detected (Fig. 1a), in which the proton hyperfine coupling constants were $a_2 = 0.301$ mT, $a_3 = 0.234$ mT, $a_5 = a_7 = 0.066$ mT, $a_6 = 0.059$

Table 1
Menadione-dependent potentiation of the reduction of cytochrome *c* by purified LPD from *S. seoulensis*

Reaction mixture	Cu,Zn-SOD (μg/ml)	Cytochrome *c* reduction (μmol/min/mg protein)
	0	11.2
	2.5	7.2
	5	4.8
	10	2.4
	20	1.9
	100	2.1
With no LPD	0	0.21
With no menadione	0	0.52

The reaction mixture contained 0.2 mM NADH, 0.3 mM menadione, 50 μM horse heart cytochrome *c* with various concentrations of Cu,Zn-SOD in the presence of 2.5 μg LPD in 50 mM sodium phosphate buffer (pH 7.4). The reduction of cytochrome *c* was measured by the increase of absorbance at 550 nm using an extinction coefficient of 21 000 M^{-1} cm^{-1}.

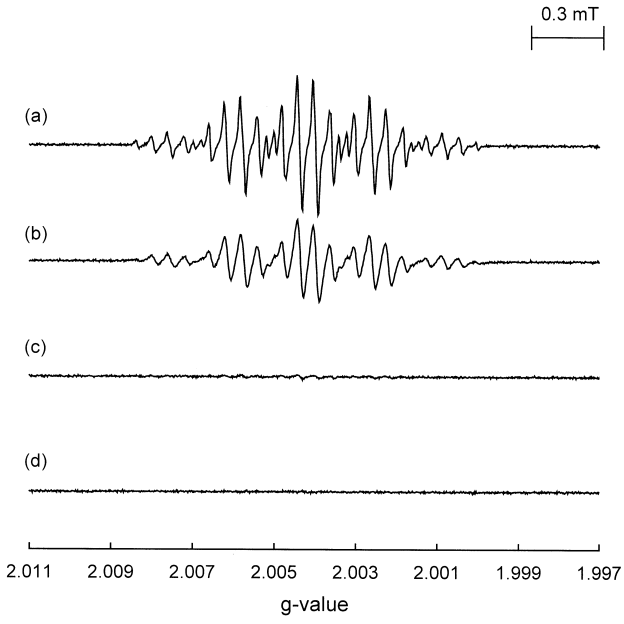

Fig. 1. EPR spectra of menadione semiquinone radical anion. The reaction mixture contained 5 mM NADH, 20 mM menadione in 50 mM sodium phosphate buffer (pH 7.4) with 2.5 μg of purified LPD (a), 100 μg of the cell-free extract of HY303 (b), HY702 (c), and with none (d).

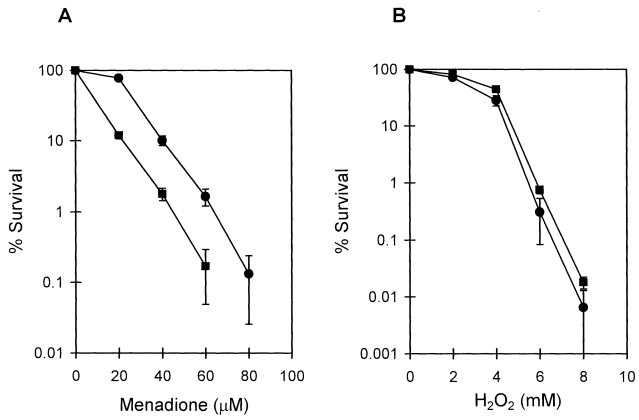

Fig. 2. Effect of LPD overproduction on the cell survival against oxidative stress. A: Survival curve of HY702 (circles) and HY303 (rectangles) against menadione. B: Survival curve against H_2O_2. Each spore was treated with individual oxidant at various concentrations for 15 min, and then plated in modified YEME. The measured values are given as means of three independent experiments.

mT, and $a_8 = 0.073$ mT having a center at a g-value of 2.00417. The production of menadione semiquinone radical anion is followed by reaction 1, in which the ratio of $[O_2^{\bullet-}]/[O_2]$ to $[M^{\bullet-}]/[M]$ was 7.03×10^{-3}, based on the facts that the reduction potentials of the $M/M^{\bullet-}$ couple and the $O_2/O_2^{\bullet-}$ couple are −203 mV and −330 mV, respectively [25]. The ratio of $[O_2^{\bullet-}]$ to $[M^{\bullet-}]$ will be 5.23×10^{-3} in the case of 0.3 mM menadione under aerobic conditions (Table 1), in which $[O_2]$ is estimated to be 0.223 mM [26]. Thus, per molecule, the cytochrome c reduction activity of $O_2^{\bullet-}$ is approximately 764-fold greater than that of menadione semiquinone radical anion ($M^{\bullet-}$), if the minor contributor to cytochrome c reduction is only menadione semiquinone radical anion ($M^{\bullet-}$). In this case, this postulation is reasonable because the menadiol anion is redox-stable [27], so reaction 2 is negligible.

Up to now, the catalytic mechanism of the formation of menadione semiquinone radical anion by LPD is not fully understood. However, it is generally accepted that the quinone reduction by LPD can be mediated by innate FAD [3,4], which is consistent with the fact that the enzymes containing quinone reductase activity are confined to flavoproteins.

3.2. Overexpression of LPD in S. seoulensis

LPD-overexpressing *S. seoulensis* (HY303) showed a bright yellow color corresponding to FAD, a prosthetic group of LPD, implying the overproduction of active LPD. As expected, the specific activity of LPD in the cell-free extract of HY303 was about 12-fold greater than that of the control strain (HY702) (Table 2). The level of LPD overexpression was also confirmed by Western blotting analysis (data not shown). Alternatively, we have measured the menadione-reducing activity of the cell-free extract of HY303 by checking the production of menadione semiquinone radical anion. As shown in Fig. 1, the signal intensity of the cell-free extract of HY303 (Fig. 1b) was about 20-fold higher than that of HY702 (Fig. 1c).

3.3. Potentiation of the toxic effect of menadione by the overexpression of LPD

We tested whether the increased level of LPD contributes to the survival under treatment with menadione. For this purpose, the spores of HY303 and HY702 were exposed to various concentrations of menadione for 15 min and the remaining viable cells were counted on modified Bennett plates. As shown in Fig. 2A, the increased level of LPD conferred more sensitivity on *S. seoulensis* toward menadione. Other quinones including 1,4-naphthoquinone showed almost the same results as menadione (data not shown), reflecting the broad range of substrate specificity of LPD [3]. We also measured the protein thiol content of the two cells grown in modified YEME liquid media at a 3-h interval after the cells were challenged with 40 μM menadione at the growth stage of $OD_{600} = 0.5$. In HY702, the level of protein thiol was decreased at first, but the level was gradually restored reaching 80% of the untreated level after 12 h (Fig. 3). However, in HY303, the level of protein thiol was not restored even after 12 h. Under this condition, HY303 showed no growth while HY702 showed a good growth comparable to menadione-untreated cells. Under treatment with menadione, HY303 confronted more harsh

Table 2
Levels of LPD and antioxidant enzymes in the cell-free extract of HY303, an LPD-overexpressing *S. seoulensis*

Strain	Lipoamide dehydrogenase (U)	Superoxide dismutase[a] (U)	Catalase (U)	Peroxidase (mU)
HY702	1.45	8.6	8.09	10.61
HY303	15.22	11.6	62.21	22.10

[a]The activities of superoxide dismutase were calculated from the amount of protein detected by Western analysis with the antibody against nickel-containing superoxide dismutase [28].

oxidative stress than HY702, which implies the role of LPD as facilitating the redox cycling reaction of quinone in vivo as well as in vitro. The H_2O_2 sensitivity of the HY303 was also examined along with HY702 (Fig. 2B); there was no marked difference between the two, indicating that the higher sensitivity of HY303 to various quinone compounds does not result from the increased susceptibility of the cell to general oxidative stress. On the contrary, HY303 is slightly more resistant to H_2O_2 than HY702, which is partly explained by the increased level of catalase (see below). Then, we investigated the effect of LPD overexpression on the expression level of antioxidant enzymes such as superoxide dismutase, catalase, and peroxidase. Interestingly, HY303 showed an elevated level of antioxidant enzymes (Table 2), particularly catalase (7.7-fold), without any treatment of oxidative stress, indicating that the H_2O_2 concentration increased in HY303. It is supposed to result from the redox cycling of an endogenous compound by LPD. The most probable one will be a naphthoquinone-family compound, since streptomycetes produce many kinds of naphthoquinone compounds including kalafungin, nanaomycins, granaticin, and actinorhodins [15]. However, this does not exclude the possibility of direct formation of superoxide radical anion because LPD can produce superoxide radical anion with NADH in the absence of menadione, as shown in Table 1.

3.4. *Cytosolic enzymes of S. seoulensis catalyzing the reduction of menadione*

The activity staining of cell-free extract of *S. seoulensis* has visualized at least four protein members (NQO1–4) apart from LPD mediating the reduction of menadione using NADH or NADPH as electron donor (Fig. 4). In the reaction, NQO2–4 were specific to NADPH, and NQO1 could use both NADH and NADPH as electron donor. The quinone reductase activities of LPD and NQO1–4 were not changed in activity staining upon treatment with menadione (data not shown), indicating no direct relation to quinone metabolism. However, like LPD, NQO1–4 are also supposed to facilitate the redox cycling of quinone compounds and thereby increase quinone-induced cellular toxicity in *S. seoulensis*.

In conclusion, an LPD-overexpressing *S. seoulensis* was produced which exhibited significantly increased sensitivity to menadione compared with the wild-type strain. This is the first report addressing the in vivo participation of LPD in facilitation of quinone redox cycling. However, this study does not address that the three 2-oxo acid dehydrogenase complexes, where LPD is an integral component, mediate quinone reduction, which remains to be studied further.

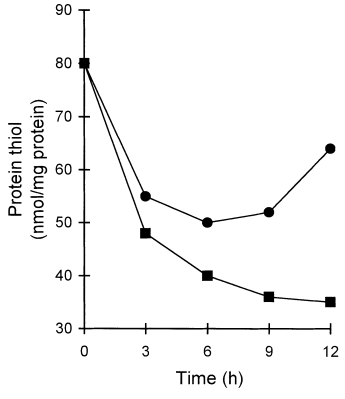

Fig. 3. Effect of menadione on protein thiol content of HY702 (circles) and HY303 (rectangles). Samples were taken at a 3-h interval after treatment with 40 μM menadione at the growth stage of $OD_{600} = 0.5$.

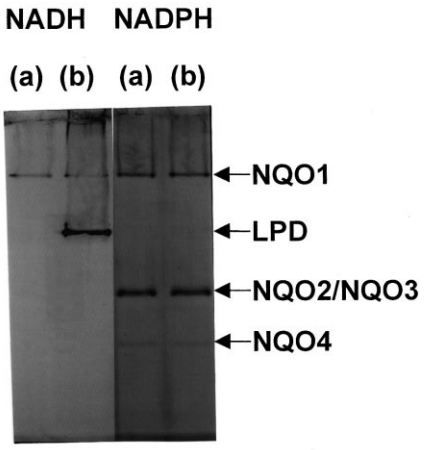

Fig. 4. Cytosolic enzymes containing menadione-reducing activity. The cell-free extracts of HY702 (a) and HY303 (b) were loaded onto 10% native gel and visualized using the reaction mixture containing 1 mM menadione, 0.1 mM nitroblue tetrazolium, and 50 μM NADH (or NADPH) in 0.2 M Tris–HCl buffer (pH 7.0).

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