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Enhancing survival of *Escherichia coli* by expression of azoreductase AZR possessing quinone reductase activity

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Abstract Quinone reductase activity of azoreductase AZR from *Rhodobacter sphaeroides* was reported. High homologies were found in the cofactor/substrate-binding regions of quinone reductases from different domains. 3D structure comparison revealed that AZR shared a common overall topology with mammal NAD(P)H/quinone oxidoreductase NQO1. With menadione as substrate, the optimal pH value and temperature were pH 8–9 and 50°C, respectively. Following the ping-pong kinetics, AZR transferred two electrons from NADPH to quinone substrate. It could reduce naphthoquinones and anthraquinones, such as menadione, lawsone, anthraquinone-2-sulfonate, and anthraquinone-2,6-disulfonate. However, no activity was detected with 1,4-benzoquinone. Dicoumarol competitively inhibited AZR's quinone reductase activity with respect to NADPH, with an obtained K_i value of 87.6 μM. Significantly higher survival rates were obtained in *Escherichia coli* YB overexpressing AZR than in the control strain when treated by heat shock and oxidative stressors such as H_2O_2 and menadione.

Keywords Quinone reductase · Azoreductase · Oxidative stress · Heat shock · Survival

Introduction

Compared to traditional physicochemical methods, microbial treatment of xenobiotic compounds, such as azo dyes and nitroaromatics, is a promising strategy. A wide range of bacteria have been reported capable of reducing azo dyes (Stolz 2001; Dos Santos et al. 2007). Enzymes that catalyze the reduction of azo groups are termed azoreductase. In the last decade, many azoreductases have been purified and characterized from different bacterial species (Chen 2006). Utilizing NAD(P)H as electron donor, they catalyze the successive two-electron-reduction of azo groups in vitro. Previously, we have cloned and characterized a gene *azr* (537 bp, GenBank accession number AY150311) coding for azoreductase AZR from *Rhodobacter sphaeroides*. AZR overexpressed in *Escherichia coli* catalyzes the reduction of various azo compounds with different structures (Yan et al. 2004). Further studies demonstrated that AZR adopts a flavodoxin-like fold with a three-layer α/β/α structure. AZR can also function as nitroreductase and flavin mononucleotide (FMN) reductase in vitro. It was shown that 2,4,6-trinitrotoluene was the most efficient nitro substrate and was reduced to hydroxylamino-dinitrotoluene (Liu et al. 2007). However, it has been argued that, as introduction of azo and nitro compounds into the environment is a recent anthropogenic event, enzymatic reduction of azo dyes and nitroaromatics may be secondary activities of reductases with different primary roles.

In addition to azo and nitro compounds, quinone is another kind of electrophilic compound. Quinones, characteristic of two carbonyl groups in an unsaturated six-member carbon ring, constitute an important class of ubiquitous and naturally occurring compounds, including several biologically important coenzymes, acceptors, or vitamins (Deller et al. 2008). Cellular oxidoreductases involved in the reduction of quinone compounds are termed quinone reductase. Quinones can be reduced by enzymes such as cytochrome P450 reductases via one-electron reduction to potentially harmful semiquinones. Semiqui-

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none intermediates in turn are highly prone to react with molecular oxygen to generate superoxide radicals, which lead to oxidative stress and cell damage. On the other hand, quinones can also be competitively reduced through two-electron pathway by NAD(P)H/quinone reductases to quinols, which is believed to help minimize the oxidative stress (Deller et al. 2008; Sollner et al. 2007; Gonzalez et al. 2005).

In this paper, the quinone reductase activity of AZR was studied in detail. It was also found that the survival of *E. coli* strain bearing the *azr* gene is significantly higher than that of the control strain when treated by heat shock and oxidative stressors.

Materials and methods

Chemicals

1,4-Benzoquinone, 2-methyl-1,4-naphthoquinone (menadione), 2-hydroxy-1,4-naphthoquinone (lawsone), anthraquinone-2-sulfonate (AQS), anthraquinone-2,6-disulfonate (AQDS), paraquat, and thrombin were obtained from Sigma. Ampicillin, bovine serum albumin (BSA), and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from TaKaRa Dalian. All other chemicals were of highest analytical grade available and used without further purification.

Bacterial strains and culture conditions

E. coli JM109 was obtained from TaKaRa Dalian. The gene *azr* encoding azoreductase from *R. sphaeroides* AS1.1737 was cloned and inserted into plasmid pGEX 4T-1 under the control of the *lac* promoter (Yan et al. 2004). The recombinant plasmid was transformed into $CaCl_2$-treated *E. coli* JM109 competent cell. The recombinant strain was named *E. coli* YB. All the strains were cultivated in Luria–Bertani (LB) media. The recombinant cells were induced by addition of 1 mM IPTG.

Sequence and structure analysis

Alignment of amino acid sequences was performed using GeneDoc (Nicholas et al. 1997). Structure comparison was conducted and viewed with Pymol (DeLano Scientific, San Carlos, CA, USA).

Enzyme assays

The purification of AZR was performed as described previously (Liu et al. 2007). Protein concentration was measured, according to the Bradford (1976) procedure, using BSA as a standard.

Quinone reductase activity was measured spectrophotometrically by monitoring NADPH disappearance at 340 nm (ε=6.22 mM^{-1} cm^{-1}). One-milliliter typical reaction mixtures contained 20 mM phosphate buffer (pH 7.0), 40 μM menadione, and a suitable amount of enzyme. The reaction was initiated by the addition of 100 μM NADPH. Menadione reduction was also monitored in a coupled assay (Wang and Maier 2004). Reduced menadione, menadiol, in turn reduces cytochrome *c*. The reaction mixtures contained 20 mM phosphate buffer (pH 7.0), 100 μM NADPH, 40 μM menadione, and 50 μM cytochrome *c*. Reduction of cytochrome *c* was monitored by the increase in absorbance at 550 nm (ε=29.5 mM^{-1} cm^{-1}). Optimal pH and temperature for the quinone reductase activity were determined with menadione. The optimal pH was determined with 20 mM acetate/Na-acetate buffer (pH 3–6), 20 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 5–7), 20 mM Tris–HCl buffer (pH 7–10), and 20 mM glycine/NaOH buffer (pH 10–11), respectively. The optimal temperature was determined by measuring the rate of reaction at temperatures ranging from 20 to 70°C under standard assay conditions. Thermal stability of the quinone reductase activity was also studied. The enzyme was incubated in a water bath with a temperature range of 20–90°C. After 15 min incubation, the enzyme was added to an assay mixture at 30°C to study its residual activity.

For the steady-state kinetic analysis, the reaction mixtures contained 20 mM phosphate buffer (pH 7.0), a suitable amount of enzyme, and 10–50 μM menadione. The reactions were initiated by the addition of 25, 50, 75 or 100 μM NADPH.

To study the inhibition of dicoumarol on AZR's quinone reductase activity, the initial NADPH oxidation rate was determined in the presence of various concentrations (0–100 μM) of dicoumarol. One-milliliter reaction mixtures contained 20 mM phosphate buffer (pH 7.0), 40 μM menadione, 30–100 μM NADPH, and a suitable amount of enzyme. The reaction was initiated by addition of NADPH. The dicoumarol stock solution (10 mM) was prepared in 0.1 M NaOH.

Survival of *E. coli* strains when treated by oxidative stressors and heat shock

Strains of *E. coli* JM109 and *E. coli* YB were grown overnight and then inoculated into fresh LB media, respectively, with an initial OD_{660} of 0.2. Strains were then cultivated aerobically until they reached an OD_{660} around 1.0. This represents around 3×10^7 cells ml^{-1}, which is the 100% survival rate. Appropriate amounts of stressors (H_2O_2, 10 mM; paraquat, 0.4 mM; and menadione, 0.6 mM) were then added to cultures. Heat shock was induced by incubating cultures at 48°C. Cell survival was

monitored by sampling at intervals, diluting in 20 mM phosphate buffer (pH 7.0), and plating aliquots onto LB plates to obtain viable cell counts.

All the experiments were carried out at least three times.

Results

Sequence and structure analysis

Amino acid sequence of AZR was aligned with those of other proteins possessing quinone reductase activity from *Bacillus subtilis*, *Saccharomyces cerevisiae*, *E. coli*, *Arabidopsis thaliana*, *Archaeoglobus fulgidus*, and *Homo sapiens* (Deller et al. 2006; Sollner et al. 2007; Patridge and Ferry 2006; Laskowski et al. 2002; Ross and Siegel 2004). The sequence length varies, and the overall sequence homology is not obvious. However, high similarities were found in the middle parts of their amino acid sequences. Regions of Pro72-Ser79 and Gly106-Gly111, corresponding to the two loops around the FMN group of AZR, respectively, are highly conserved (Fig. 1a). The two loops were reported to be involved in the binding of flavin cofactor and NAD(P)H, respectively (Liger et al. 2004; Liu et al. 2007).

As shown in Fig. 1b, although the overall sequences of AZR and NQO1 demonstrate low homology, monomers of the two proteins share a common α/β topology (rmsd of 9.5 Å over 467 atoms). They both adopt a typical flavodoxin-like fold, consisting of a central five-stranded parallel β sheet surrounded by α helices on both sides. In addition, different flavin cofactors (FMN for AZR while flavin adenine dinucleotide for NQO1) are bound in a similar manner, with the *si*-face partly exposed to the solvent and the *re*-face buried.

Characterization of quinone reductase

The quinone reductase had maximal activity at pH 8–9. Maximum quinone reductase activity was observed at 50°C. Thermal stability studies demonstrated that, although the enzyme was relatively stable up to 50°C, it lost activity rapidly at temperatures above 50°C.

Using NADPH as electron donors, several naturally occurring and artificial quinone substrates were studied for AZR's quinone reductase activity. The enzyme could use

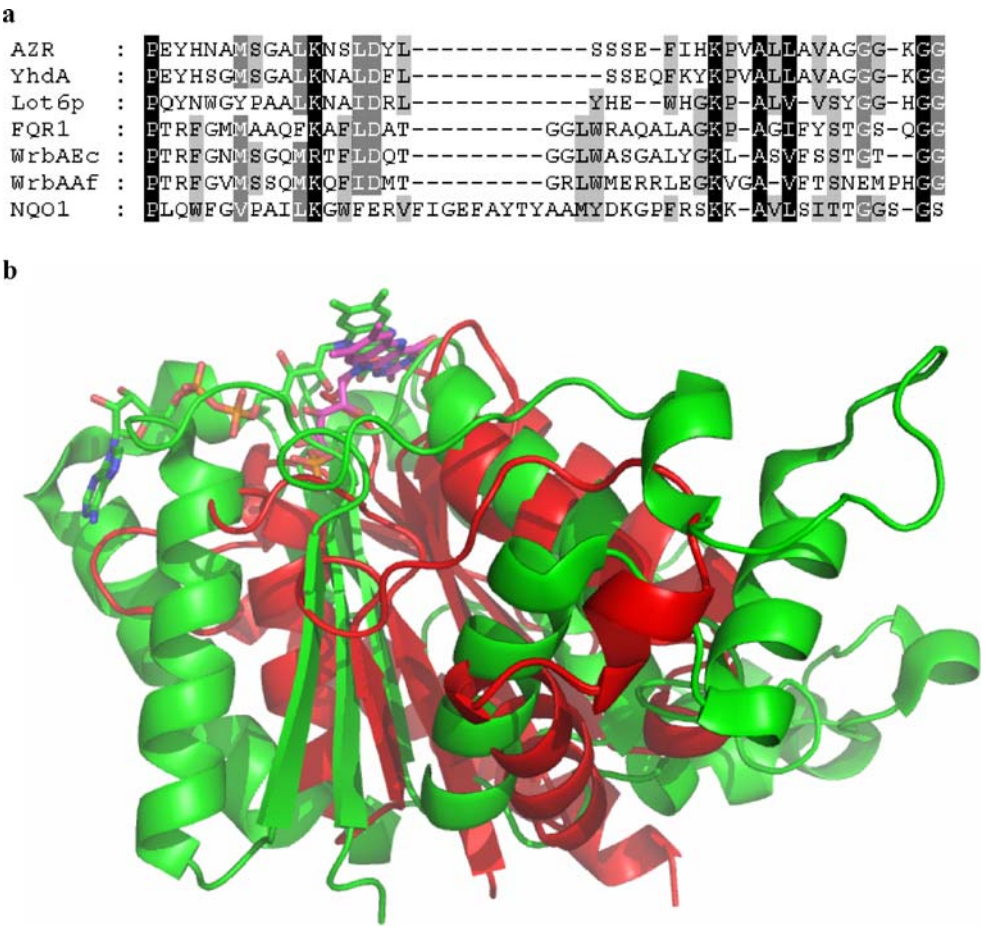

Fig. 1 Sequence and structure analysis of AZR. **a** Multiple sequence alignment of amino acid sequences of quinone reductases from *Rhodobacter sphaeroides* (AZR, GenBank accession number AAN17400), *Bacillus subtilis* (YhdA, GenBank accession number CAB12762), *Saccharomyces cerevisiae* (Lot6p, GenBank accession number 1T0IA), *Arabidopsis thaliana* (FQR1, GenBank accession number NP200261), *Escherichia coli* (WrbAEc, GeneBank accession number AAA24759), *Archaeoglobus fulgidus* (WrbAAf, GenBank accession number NP069179), and *Homo sapiens* (NQO1, GenBank accession number P15559). **b** Alignment of 3D structures of AZR and NQO1. Ribbon representation of AZR and NQO1 are shown in *red* and *green*, respectively. The flavin cofactors are shown as *stick models*

Table 1 Quinone reductase activities of AZR

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
1,4-Benzoquinone	ND	ND	ND
Lawsone	34.6±1.3	345±11.2	9.92±0.4
Menadione	16.2±0.4	3277.8±30.4	205.2±1.2
AQS	13.3±0.6	218.4±8.3	15.9±0.7
AQDS	16.1±0.2	211.2±6.5	13.1±0.3

All assays were done in triplicates. Values are mean ± standard deviation.
ND Not detectable.

menadione, lawsone, AQS, and AQDS as electron acceptors. No activity was detected with 1,4-benzoquione. According to the k_{cat}/K_m value, menadione was the best substrate for AZR among the quinones examined (Table 1).

The quinone reductase activity of AZR was determined using a coupled menadione-cytochrome *c* reduction assay that measures an increase in absorbance at 550 nm due to cytochrome *c* reduction. Using this assay, AZR showed an activity of 10.9±1.3 μmol of cytochrome *c* reduced per minute per milligram of protein.

Using menadione as electron acceptor at various fixed concentrations of NADPH, the reaction mechanism of AZR quinone reductase was investigated by steady-state kinetic analysis. The obtained parallel lines in double reciprocal plots (Fig. 2) indicated that AZR reduces quinones with bi–bi ping-pong kinetics.

The effects of dicoumarol on AZR's quinone reductase activity were studied. As shown in Fig. 3, dicoumarol was a competitive inhibitor of NADPH. The inhibition constant (K_i) for NADPH was 87.6 μM.

Enhancing survival of *E. coli* by expression of AZR

It was reported that quinone reductase might be involved in combating oxidative stress (Wang and Maier 2004; Gonzalez et al. 2005). Using stressors such as H_2O_2, menadione, and paraquat, the viabilities of *E. coli* JM109 and the recombinant *E. coli* YB overexpressing AZR were compared. The effect of heat shock, which may increase oxidative stress (Kim et al. 2005), was also studied.

When *E. coli* strains were treated with 10 mM H_2O_2, a protective effect of AZR was observed from the beginning. The survival rate of *E. coli* YB was over 30% higher than that of the control strain. When treated with 0.4 mM paraquat, similar enhanced resistance was obtained. The recombinant strain showed around 20–30% higher survival rates. When menadione was used as stressors, in contrast to the decreasing survival rate of *E. coli* JM109, the survival rate of *E. coli* YB increased in the first 3 h. While the survival rate of *E. coli* YB was almost four times higher than that at the beginning, nearly no viable count of the

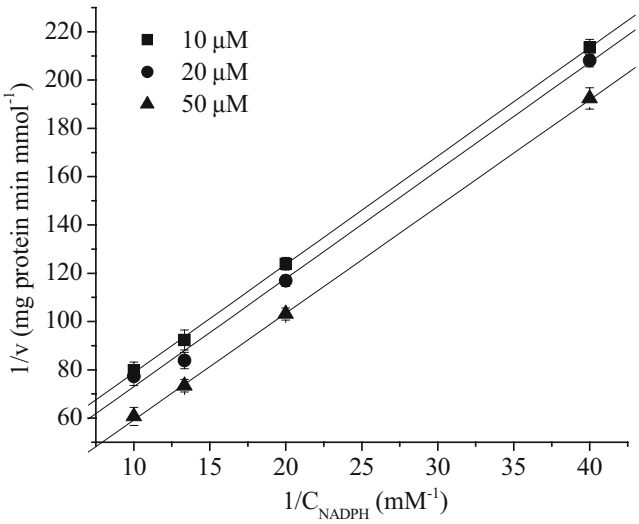

Fig. 2 Kinetics of quinone reductase activity of AZR. Double-reciprocal plot of initial rate (*v*) vs the concentration of NADPH (25–100 μM). The NADPH oxidation activity was assayed at varying concentrations of menadione

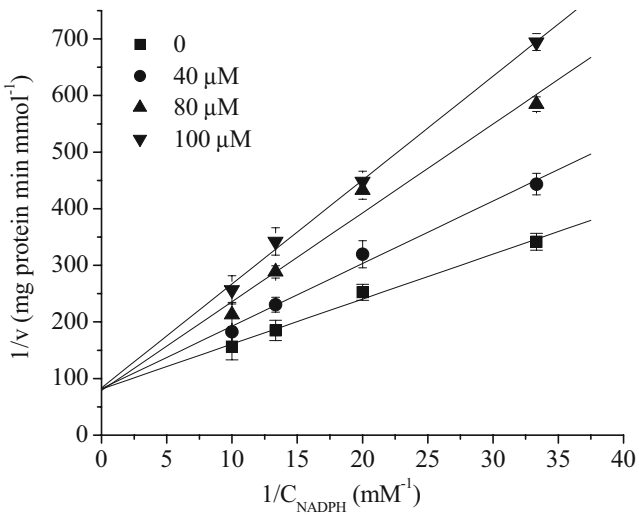

Fig. 3 Effects of dicoumarol on quinone reductase activity of AZR. Double-reciprocal plot of initial rate (*v*) vs the concentration of NADPH (30–100 μM) at a fixed concentration of 40 μM menadione and a varying concentration of dicoumarol

control strain was obtained at 4 h. A protection against heat stress by AZR was also observed from the beginning. The recombinant strain showed over 20% higher survival rate than the control one in 1 h (Fig. 4).

Discussion

Bacterial decolorization of azo dyes has been widely studied in recent years. Many NAD(P)H-dependent cytoplasmic azoreductases catalyzing the reduction of azo bond have been characterized and classified into two families (Chen 2006). However, these enzymes were shown to be unimportant in vivo, as their reductase activities were only significant when using cell extracts and not using intact cells. It was speculated that intracellular azoreductase might not be involved in bacterial decolorization (Russ et al. 2000; Blümel et al. 2002). In addition, actually, there exist very few naturally occurring azo compounds. Most azo compounds are introduced into the environment anthropogenically. The primary role of azoreductase protein remains unknown and controversial.

AZR was first identified as an NADH-dependent azoreductase (Yan et al. 2004). Its amino acid sequence shows 97% identity to that of azoreductase of *Bacillus* sp. OY1-2, which is representative of a flavin-dependent azoreductase family. Further studies showed the nitroreductase activity of AZR for the reduction of nitroaromatics such as nitrofurazone and trinitrotoluene (Liu et al. 2007). However, these nitro compounds are also xenobiotics produced by human activity and may not be physiological substrate of AZR.

It was revealed that AZR's amino acid sequences that involved in the binding of substrates and cofactors, namely, the signature sequence and the glycine-rich region, are relatively conserved among those of other quinone reductases from domains of *Bacteria*, *Archaea*, and *Eucarya*. Structure alignment further demonstrated that AZR and NQO1, a well-studied mammal quinone reductase, showed similar typical flavodoxin-like fold. Lot6p of *S. cerevisiae*

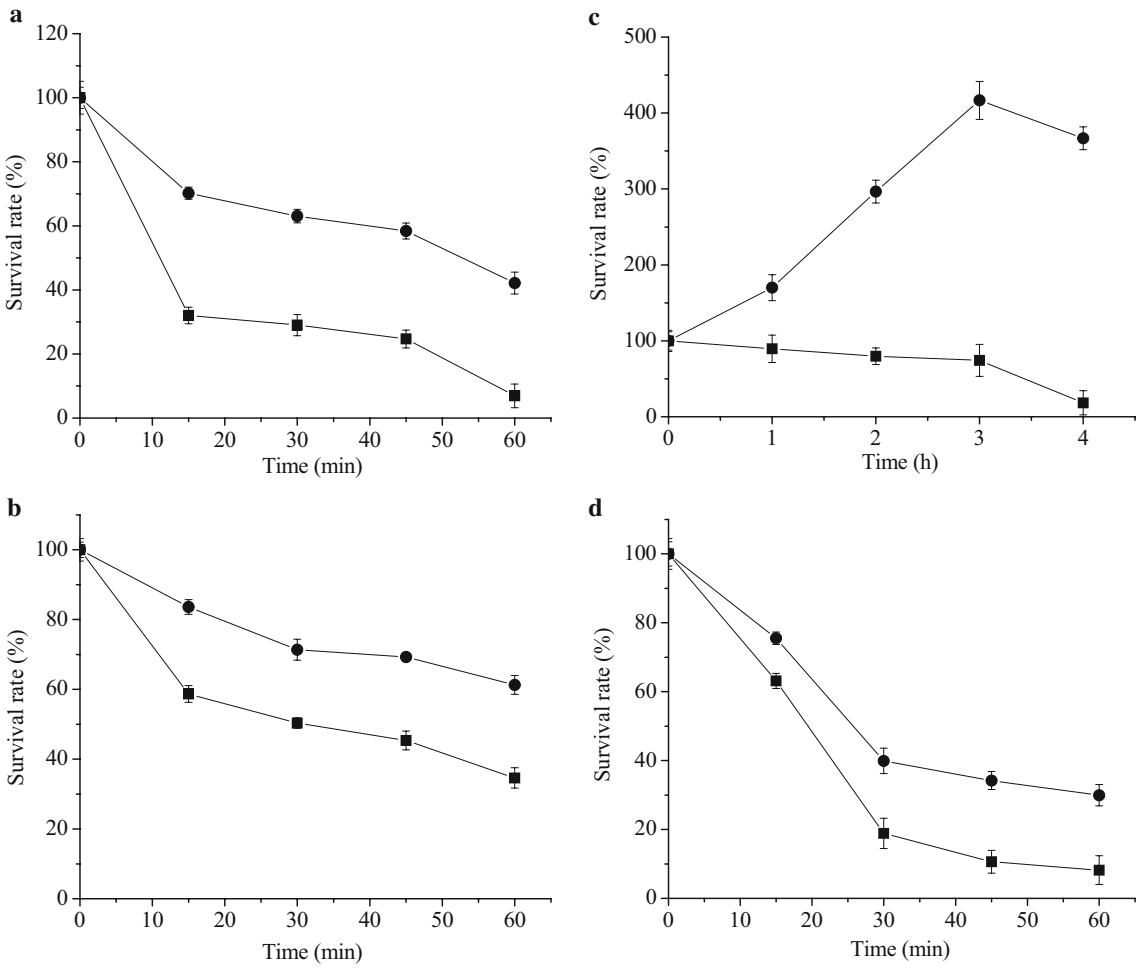

Fig. 4 Survival of *E. coli* treated with oxidative stressors or heat shock. Cells were treated with 10 mM H_2O_2 (**a**), 0.4 mM paraquat (**b**), 0.6 mM menadione (**c**), and heat shock (**d**). Samples were taken at intervals, diluted and plated on LB solid media to monitor cell viability. *Filled circles* and *squares* represent cells of recombinant and control *E. coli* strains, respectively

was firstly reported to be a ferric reductase. Recent study showed its quinone reductase activity. It displays a flavodoxin-like five-stranded α/β structure, which is similar with that of AZR (Liu et al. 2007). WrbA of *E. coli* has similar sandwich structure. However, it has a conserved and unique insertion forming an additional α/β unit after strand β4 (Patridge and Ferry 2006; Andrade et al. 2007). Thus, all these quinone reductases have a common flavodoxin-like α/β core.

The structure similarities of AZR with other quinone reductases led us to investigate its ability to catalyze the reduction of quinone substrates. AZR could reduce several naphthoquinone and anthraquinone compounds, among which menadione was shown to be the best substrate. No activity was detected when 1,4-benzoquinone was used as substrate. However, the study on Lot6p showed that 1,4-benzoquinone was a good substrate with the highest turnover number, and 1,4-anthraquinone was not reduced at all (Sollner et al. 2007). Thus, there are some differences in substrate recognition of the two quinone reductases possessing similar overall structures. Further studies are needed to elucidate these differences. It should also be noted that, by comparison of k_{cat}/K_m, quinones are much better substrates of AZR than azo and nitro compounds studied before (Liu et al. 2007). It was shown that AZR could catalyze the reduction of menadione via two-electron mechanism. NQO1 was shown to reduce quinone to quinol by a compulsory two-electron transfer (Iyanagi 1987), which is different from the deleterious one-electron pathway.

Similar to the results of its azoreductase and nitroreductase activities (Yan et al. 2004; Liu et al. 2007), AZR reduces quinones with bi–bi ping-pong kinetics. The FMN cofactor of AZR is firstly reduced to $FMNH_2$ by NADPH, and then, two electrons are transferred from $FMNH_2$ to the quinone substrates. This is also in agreement with previous observations for some other quinone reductases, such as NQO1, Lot6p, and ChrR (Bianchet et al. 2004; Sollner et al. 2007; Gonzalez et al. 2005).

Dicoumarol, a vitamin K antagonist, was shown to be an efficient inhibitor of NQO1 and Lot6p (K_i=2 and 410 nM, respectively). The inhibition is competitive with respect to NAD(P)H (Li et al. 1995; Prestera et al. 1992; Chen et al. 1999; Sollner et al. 2007). Our results demonstrated that the inhibition of dicoumarol for AZR is competitive with respect to NADPH. However, with a K_i value of 87.6 μM, dicoumarol is not an efficient inhibitor of AZR. The cofactors of AZR and NQO1 are bound in a similar manner. Recently, the crystal structure of NQO1 in complex with dicoumarol has been reported. It was shown that dicoumarol stacks parallel to the isoalloxazine ring of the flavin cofactor and forms a wall of the catalytic pocket (Asher et al. 2006).

Quinone reductase is suggested to be involved in quinone detoxification and oxidative stress resistance. It exercises its antioxidant effects directly by quinol-mediated quenching of reactive oxygen species and indirectly by diverting quinones away from one-electron reducers (Gonzalez et al. 2005). It was proposed that NQO1 serve as a cellular control device against quinone toxicity (Lind et al. 1982). Further studies indicated that NQO1 also plays a role in directly supporting the overall antioxidant functions of the cell (Beyer 1994). Lot6p was also shown to play a role in quinone detoxification and important for managing oxidative stress caused by quinones (Sollner et al. 2007). Similar protective roles were also reported for prokaryotic quinone reductases. The physiological role of WrbA of *E. coli* can be seen in the oxidative stress response. It is upregulated by various stressors, such as acids, salts, H_2O_2, and diauxie (Patridge and Ferry 2006). ChrR of *Pseudomonas putida* and MdaB of *Helicobacter pylori* and *Helicobacter hepaticus* were also reported to be involved in oxidative stress resistance (Gonzalez et al. 2005; Wang and Maier 2004; Hong et al. 2008).

To evaluate whether the expression of AZR could afford protection from oxidative species participating in bacterial killing, the control and the recombinant *E. coli* strains were treated with heat shock or several stressors, such as H_2O_2, paraquat, and menadione, respectively. The results clearly showed that the survival rates of the recombinant strain were higher than those of the control one. Quinone metabolism within a cell has a direct effect on the cell's ability to deal with oxidative stress (Soballe and Poole 1999). Quinols have been shown to lower the levels of superoxide ions in *E. coli* cell membrane (Soballe and Poole 2000). When using 0.6 mM menadione as stressor, the survival rate of the recombinant strain even increased in the first 3 h. This is in accordance with the fact that menadione is an effective substrate of AZR. However, when more than 3 mM menadione was used, no increase of the survival rate of *E. coli* YB was observed (data not shown). Our results indicated that the expressed AZR catalyzes the two-electron reduction of quinones to quinols, a mechanism protecting the recombinant strain from stress mediated damage. The survival rate seemed to show in two-step decay kinetics. This may be due to the transcription of other *E. coli* stress–response proteins after exposure to oxidative stress. For instance, NfsA and WrbA, both of which possess quinone reductase activities and are upregulated in response to various oxidative stressors, may help cells deal with oxidative stress (Zenno et al. 1996; Paterson et al. 2002; Patridge and Ferry 2006).

In addition to H_2O_2, which is generated as a by-product of aerobic respiration, there are various oxidative burdens from external sources including some pollutants. An immediate burst of H_2O_2 is released by plant cells coming

into contact with bacteria (Baker et al. 1991). Heavy metals are suggested to induce oxidative stress in cells (Ercal et al. 2001). Bioremediation of chromate through enzymatic reduction, which is a promising process, is unavoidably associated with H_2O_2 generation (Gonzalez et al. 2005). Nitroaromatics such as 2,4,6-trinitrotoluene are thought to induce oxidative stress in cell by enhancing superoxide and hydrogen peroxide production (Cenas et al. 2001). It was suggested that, due to its higher ability of overexpressing specific enzyme, gene-engineered microorganism may be more suited to environments where the pollutant is found at a high concentration (Garbisu and Alkorta 1999; Cases and de Lorenze, 2005). Our results demonstrated that enhancing activities of AZR azo/nitro/quinone reductase may not merely increase the bioremediation effects; it may also promote the ability of remediating cells to cope with oxidative stress. Further studies on modification of the recombinant strain with suicide system and its bio-safety assessment are underway for possible applications.

In summary, we have shown the quinone reductase activity of AZR, which was previously reported as azoreductase and nitroreductase in vitro. The expression of AZR in *E. coli* enhances its survival under oxidative stress conditions. It was demonstrated that quinones are better substrates compared to azo and nitro compounds. As synthetic azo and nitro compounds are not cognate substrates in an original physiological context, the finding of this new activity of AZR may shed some light on its primary role. Further biochemical and physiological studies of AZR are in process and will be reported later.

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