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Pyrroloquinoline-quinone: a reactive oxygen species scavenger in bacteria

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Abstract Transgenic *Escherichia coli* expressing pyrroloquinoline-quinone (PQQ) synthase gene from *Deinococcus radiodurans* showed superior survival during Rose Bengal induced oxidative stress. Such cells showed significantly low levels of protein carbonylation as compared to non-transgenic control. In vitro, PQQ reacted with reactive oxygen species with rate constants comparable to other well known antioxidants, producing non-reactive molecular products. PQQ also protected plasmid DNA and proteins from the oxidative damage caused by γ-irradiation in solution. The data suggest that radioprotective/oxidative stress protective ability of PQQ in bacteria may be consequent to scavenging of reactive oxygen species per se and induction of other free radical scavenging mechanism.

Keywords: Pyrroloquinoline-quinone; Antioxidant; ROS scavenging; Oxidative stress; Radioprotection; *Deinococcus*

1. Introduction

Microorganisms that exhibit mineral phosphate solubilization (MPS) phenotype possess a glucose dehydrogenase that requires pyrroloquinoline-quinone (PQQ) as a redox co-factor [1]. Many bacteria either lack the gene for glucose dehydrogenase or the PQQ synthase system, and thus, do not express MPS phenotype. *Deinococcus radiodurans* lacks the gene for glucose dehydrogenase and other known dehydrogenases,which require PQQ as a cofactor. The role of PQQ in this microbe is, therefore, an interesting aspect to investigate. *D. radiodurans* shows resistance to several abiotic stresses, including extreme ionizing radiation [2,3]. Earlier we cloned the PQQ synthase gene, homologous to the *pqqE* genes reported from other mineral phosphate solubilizing bacteria, from *D. radiodurans* and demonstrated the activity of recombinant protein in *Escherichia coli* [4]. Such transgenic *E. coli* cells expressing PQQ synthase gene showed higher tolerance to oxidative stress and higher activities of catalase and superoxide dismutase, in addition to the MPS phenotype.

PQQ has been shown to protect the mitochondrial activity of rats from oxidative stress [5]. Further, the requirement of PQQ by several dehydrogenases and its role in the lysine metabolism in mammalian system has been demonstrated recently. Consequently, PQQ has been classified as a member of B-group of vitamins [6]. The structural analysis of PQQ with other antioxidants such as indole and pyrrole derivatives, which act as reactive oxygen species (ROS) scavenger, showed that PQQ exhibits comparatively higher reactive electron density making it a relatively strong antioxidant. This supports the idea that PQQ probably functions by directly neutralizing the ROS. Pulse radiolysis technique provides an excellent tool to demonstrate the reactivity of ROS with other compounds. Using this technique, the transients produced between the ROS and its neutralizing compound at nanosecond time intervals under in vitro conditions can be monitored [7].

In this paper, we report the experimental fevidence describing the mechanism by which PQQ acts as an antioxidant under oxidative stress conditions in vivo and in solution. Using nanosecond pulse radiolysis technique, PQQ was shown to react with radiolytically produced reactive oxygen species, such as super-oxide radicals and hydroxyl radicals in vitro. In solution, PQQ protected plasmid DNA from nicking and proteins from oxidative damage caused by γ-rays. Transgenic *E. coli* cells expressing PQQ showed decreased levels of protein oxidation during oxidative stress. These results suggest that PQQ acts as ROS scavenger by directly neutralizing the reactive species and protects the bacterial cells from oxidative stress.

2. Materials and methods

2.1. Bacterial strains, plasmids and reagents

The *E. coli* strain BL21 (DE3) pLysS harboring pETpqq, PQQ synthase gene from *D. radiodurans* cloned in pET28a+ [4], and plasmid vector pET28a+ (Novogen, USA) were used in this study. *E. coli* cells were grown as batch cultures in LB broth with shaking at 180 rpm at 37 °C. *E. coli* harboring pET28a+ and its derivatives were grown in the presence of kanamycin (Kan) (25 μg/ml). All the chemicals used were of molecular biology grade and were obtained from Sigma Chemical Company, USA, Bethesda Research Laboratory, USA and Sisco Research Laboratory, India.

2.2. Expression of recombinant protein in E. coli

The *E. coli* BL21 (DE3) pLysS harboring pETpqq used in this study was assessed for the expression of recombinant protein in every experiment. Inducible expression of recombinant protein was estimated as described earlier [4]. In brief, the mid logarithmic phase *E. coli* cells growing in the presence of kanamycin (25 μg/ml) were induced with 1 mM isopropyl-β-thiogalactopyranoside (IPTG) and allowed to grow at 37 °C for 3 h. These cells were harvested and used for further studies. The expression of recombinant protein was ascertained by SDS–polyacrylamide gel electrophoresis using the protocols described earlier [8].

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2.3. Mineral phosphate solubilizing activity assay

MPS activity of transgenic *E. coli* was measured in liquid cultures grown for 36 h in glucose minimal medium and supplemented with tricalcium phosphate (TCP) (TCP broth) as a sole source of phosphorous [4,9]. In brief, the IPTG induced cultures were used to inoculate TCP broth containing 1 mM IPTG. The cultures were grown under shaking at 180 rpm at 37 °C. Aliquots were drawn and culture supernatants were taken to estimate the release of phosphorous from insoluble phosphates as described earlier [10].

2.4. Estimation of protein carbonylation

E. coli cells harboring pET28a+ and pETpqq induced with IPTG were treated with Rose Bengal as described earlier [4]. Cells were broken by sonication and cell free extracts were used for measurement of protein carbonylation using the protocol described earlier [11]. In brief, the cell-free extracts containing approximately 2 mg soluble proteins in 50mM potassium phosphate buffer, pH 7.4, were taken and total proteins were precipitated with ice chilled 10% trichloroacetic acid (TCA). The pellet was suspended in 0.2% dinitrophenyl hydrazine (DNPH) in 2 N HCl and incubated at room temperature for 2 h. Proteins were reprecipitated with TCA and excess DNPH was removed with several washes of 50% ethyl acetate in ethanol. Decolorized protein pellet was dissolved in 6 N guanidine hydrochloride and the optical density was measured at 370 nm against a protein control that was processed in parallel by replacing DNPH with 2 N HCl. Protein concentration was determined by the method of Lowry et al. [12].

2.5. Pulse radiolysis experiments and optical measurements

Pulse radiolysis studies were carried out with high energy electron pulses (7 MeV, 50 ns) obtained from a linear electron accelerator whose details were described earlier [7]. Hydroxyl radicals were produced exclusively by radiolysis of water under N_2O-saturated condition [13], while super-oxide radicals were produced by pulse radiolysis of 0.1 M formate solution under oxygenated conditions [14]. Here, the radical species formed upon radiolysis react with formate ion to produce $CO_2^{\cdot-}$ radical, which subsequently react with oxygen to produce super-oxide. High purity gases and nanopure water were used for the experiment. All the samples were freshly prepared. Aerated aqueous solution of potassium thiocyanate (KSCN) (1×10^{-2} M) was used for determining the dose per pulse [15]. The dose per pulse was close to 12 Gy (1 Gy = 1 J kg^{-1}). The reactivity of free radicals with PQQ was monitored by a kinetic spectrometer using a 450 W pulsed xenon arc lamp and a Kratos model GM-252 monochromator. The photomultiplier output was digitized with a 100 MHz storage oscilloscope that was interfaced to a computer for kinetic analysis. The transient absorption spectrum and decay and formation kinetics were used to determine the nature, lifetime and reactivity of the radical species produced on reaction with different concentrations of PQQ (30–70 μM) with reactive oxygen species. The pulse radiolysis experiments were performed in suprasil cuvettes with a cross-sectional area of 1 cm^2 at 25 °C. Steady state γ-radiolysis was carried out to produce hydroxyl radicals in the presence of different effectors and absorption spectra were recorded on single beam spectrophotometer.

2.6. Radiation protection of biomolecules under in vitro conditions

The effect of PQQ in ROS scavenging and in protection of plasmid DNA and proteins was studied under in vitro conditions. The plasmid pET28a+, DNA was prepared from a *E. coli* culture growing in the presence of Kanamycin (25 μg/ml) and covalently closed circular (CC) molecular form of the plasmid (5.369 kb) was extracted from agarose gel using QIAquick Gel Extraction Kit (Qiagen GmbH, Germany). Approximately 200 ng plasmid DNA was taken in 20 μl of phosphate buffered saline to which different concentrations of PQQ were added. Control sample was prepared by substituting PQQ with buffer. All subsequent operations were carried out in dark. Samples were saturated with N_2O as described previously [13] and chilled on ice before they were irradiated with 5 Gy of γ radiation (Gamacell 220, Atomic Energy of Canada Limited) as described earlier [16]. DNA was mixed with sterile DNA loading dye and separated on 1% agarose gel containing 0.50 ng/ml ethidium bromide in dark. The DNA pattern in gel was documented on GeneGenius Bioimaging System and the intensities of fluorescent DNA bands were estimated using GeneTools software (Syngene, UK).

Protein oxidation was monitored as change in the levels of protein carbonylation using Bovine Serum Albumin (Sigma, Cat. No. A4503). Two mg protein was dissolved in 1 ml of 50 mM Na^+/K^+ phosphate buffer (pH 7.5). Three sets were prepared each with three replicates. One set was treated as control and other sets were treated with PQQ. Two different concentrations (5 μM and 10μM) of PQQ were added and tubes were shielded from light. All the tubes were saturated with N_2O and irradiated with 50 Gy γ radiation (the minimum dose that gives reproducible and scorable difference in protein carbonylation as compared to unirradiated samples). The levels of protein carbonylation were measured using standard protocol described earlier [11].

All data presented represent the average of three replicates, wherein the variation among replicates was less than 15%. All experiments were repeated at least two times and results of a typical experiment are shown.

3. Results and discussion

Earlier, we have reported the cloning and expression of PQQ synthase gene from *D. radiodurans* [4] into *E. coli.* The transgenic *E. coli* expressed an expected 42 kDa protein and exhibited functional complementation of mineral phosphate solubilizing (MPS) phenotype of *E. coli*, which is otherwise MPS minus. The functional complementation of MPS activity in transgenic *E. coli* is possible only when these cells make PQQ that is required for the activity of glucose dehydrogenase, the enzyme responsible for MPS phenotype in the microorganisms [9]. The transgenic *E. coli* cells expressing PQQ synthase gene possessed improved tolerance to oxidative stress induced by Rose Bengal and higher activities of catalase and super-oxide dismutase [4]. It was speculated that PQQ either directly interacts with and scavenges ROS or induces other ROS scavenging enzymes. The mechanism underlying in the protective effect of PQQ against oxidative stress has been investigated further.

3.1. Reactivity of PQQ with reactive oxygen species

The nanoseconds pulsed radiolysis technique was used to generate ROS under in vitro conditions and to monitor the reactivity of PQQ with ROS. The data in Fig. 1 shows the transient spectrum after the reaction of 3.63×10^{-5} M PQQ with hydroxyl radicals in the wavelength range from 420 nm to 550 nm (Fig. 1A, 40 μs after the pulse). The rate constant for the reaction of OH radicals with PQQ was determined by monitoring the growth of 440 nm peak (Inset L of Fig. 1A). The pseudo-first order rate (k_{obs}) increased linearly with PQQ concentration and from the slope of the linear plot, the bimolecular rate constant was determined to be $(3.63 \pm 0.07) \times 10^9$ M^{-1}s^{-1} (inset R of Fig. 1A). Time resolved studies (800 μs after the pulse) showed the formation of a new transient species having absorption maximum at 400 nm (Fig. 1A). This transient did not show any decay in maximum detectable time scale (5 s), indicating probability of formation of molecular products. This ·OH reaction product of PQQ was tested for its ability to oxidize compounds like $ABTS^{2-}$ (2,2′-azinobis (3-ethylbenzothiozoline-6-sulfonate)) and TMPD (*N*,*N*,*N*′,*N*′-*tetra*-methyl-*p*-phenylenediamine dihydrochloride) by monitoring the characteristic absorption due to the formation of $ABTS^{\cdot-}$ at 645 nm and $TMPD^{\cdot+}$ at 605 nm. Steady state γ-radiolysis of samples containing 50 μM ABTS-2 in the presence and in the absence of 200 μM PQQ was carried out under hydroxyl radicals producing conditions and the relative absorbance at 645 nm was monitored. Fig. 2 shows the levels of $ABTS^{\cdot-}$ produced in the presence or in the absence of PQQ under γ radiolysis. The results show that $ABTS^{2-}$ reacts with

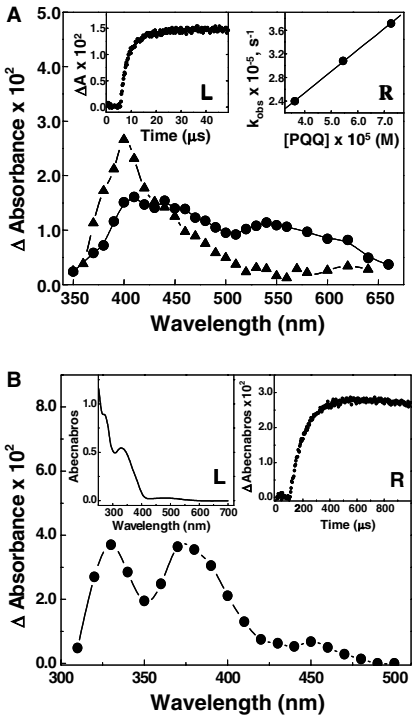


Fig. 1. (A) Absorption spectrum of the transient produced by the reaction of OH radical with PQQ (3.63×10^{-5} M) under N_2O saturated condition after 40 μs (●) and 800 μs (▲) of electron pulse. *Inset L* shows the reaction kinetics of the transient formed by OH radical reaction with PQQ monitored at 440 nm. *Inset R* shows the linear plot of k_{obs} values against different concentrations of PQQ. (B) Absorption spectrum of the transient produced by the reaction of superoxide radical with PQQ (5.08×10^{-5} M) under 0.1 M sodium formate and 1:1 (v/v) N_2O and O_2 saturated condition after 800 μs of electron pulse. *Inset L* shows the steady state absorption spectra of PQQ at pH 7.0. *Inset R* shows the reaction kinetics of transient formed by superoxide radical reaction with PQQ monitored at 390 nm.

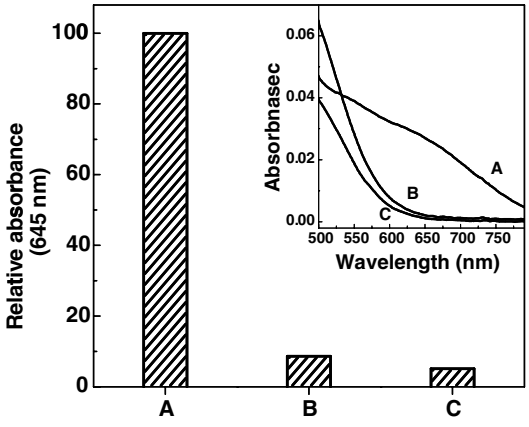
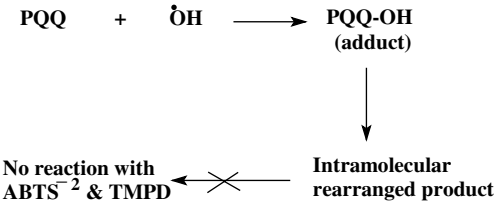


Fig. 2. Effect of PQQ on the change in the relative yield of $ABTS^{\cdot-}$ radicals. The absorbance of $ABTS^{\cdot-}$ was measured at 645 nm in samples containing 50 μM $ABTS^{2-}$ (A), 200 μM PQQ with 50 μM $ABTS^{2-}$ (B) and 200 μM PQQ (C) under γ-radiolysis. *Inset:* A portion of the typical spectra of $ABTS^{2-}$ (A), $ABTS^{2-}$ with PQQ (B) and PQQ (C) under hydroxyl radicals producing conditions showing the change in the absorbance at 645 nm.

hydroxyl radicals and gets converted to $ABTS^{\cdot-}$ radical, which absorbs at 645 nm, in agreement with earlier findings [17]. However, in the presence of PQQ the levels of $ABTS^{\cdot-}$ decreased to only 10%, while the PQQ reaction with hydroxyl radical alone and $ABTS^{2-}$ along with PQQ in the absence of hydroxyl radical (data not given) do not show significant levels of $ABTS^{\cdot-}$. The transient spectra of PQQ under hydroxyl radical producing conditions show the formation of an adduct that absorbs at 440 nm (Fig. 1A), which was absent in the absorption spectra of PQQ in the absence of hydroxyl radicals (Fig. 1B, inset, R). This result indicated that $ABTS^{\cdot-}$ radicals are not generated from $ABTS^{2-}$ in the presence of hydroxyl radical when PQQ was present, further suggesting the non-reactivity of adduct formed between PQQ and hydroxyl radical, with $ABTS^{2-}$. Similar results were obtained with TMPD (data not shown). PQQ did not react with other powerful and specific oxidizing species like azide radicals and $SO_4^{\cdot-}$ radical suggesting that PQQ itself is resistant to oxidation and the reaction with hydroxyl radicals proceeds only by adduct formation, in which hydroxyl radical possibly adds to one of the double bonds of the pyrrole or quinoline structure. In several cases, the hydroxyl radical adducts also exhibit redox nature, thereby possessing the ability to propogate the reaction with biomolecule. But unlike many other compounds, the hydroxyl radical adduct of PQQ undergoes unimolecular transformation to give new products, which do not cause further oxidation. This unique feature may be responsible for its ability to act as a powerful antioxidant. The proposed hydroxyl radical reaction is summarized below.



PQQ was also found to react with super-oxide radicals with a rate constant of 2.1×10^8 $M^{-1}s^{-1}$. Fig. 1B gives the transient spectrum obtained from reaction of 5.08×10^{-5} M PQQ with superoxide radicals. The formation trace at 390 nm is given as inset of Fig. 1B. The absorption spectrum differs from that of OH radical reaction but matches well with that produced by one-electron reduction mechanism (results not shown), suggesting that PQQ can scavenge super-oxide radicals by accepting one-electron which can be depicted as below.

$$PQQ + O_2^{\cdot-} \xrightarrow{H^+} \text{Semi quinone} + O_2$$

Comparison with the rate constants of other antioxidants such as ascorbate [13,14] and trolox [18] with hydroxyl and superoxide radicals (Table 1) clearly shows that PQQ is a

Table 1
Comparison of reaction rate constants (K) of different antioxidants with reactive oxygen species

Antioxidants	Hydroxyl radicals K (M^{-1} S^{-1})	Superoxide radicals K (M^{-1} S^{-1})
PQQ	3.63×10^9	2.10×10^8
Trolox C [18]	6.90×10^9	1.70×10^4
Ascorbate anion [14,21]	1.10×10^{10}	5.75×10^4

Data presented represent the average of three replicates of a typical experiment, where variations among the replicates were less than 10%. Values within parentheses indicate the reference describing the original work related to these antioxidants.

powerful scavenger of reactive oxygen species.The probable products produced from these reactions do not show any subsequent reactivity towards other reductants in the low concentration (nanomolar) range.

3.2. PQQ-based protection of biomolecules from oxidative damage under in vitro conditions

The effect of ROS produced through γ radiolysis under in vitro conditions, in causing the damage to DNA and proteins, was studied in the presence and absence of PQQ. The response of different doses of γ radiation on the formation of single strand breaks (ssbs) in plasmid DNA has been reported earlier [16]. It was seen that the formation of ssbs was optimum in between 4 and 6 Gy dose of γ radiation. Data given in Fig. 2 show the effect of 5 Gy dose of γ radiation on producing single strand breaks in plasmid DNA in the presence and absence of different concentrations of PQQ. The conversion of CC form to OC form decreased as a function of PQQ concentration up to 10 μM (Fig. 4A). Quantitative analysis of the proportion of CC and OC forms of the plasmid DNA showed a gradual decrease in OC form that was inversely proportional to the concentration of PQQ up to 5 μM beyond which the effect saturated (Fig. 4B).

The effect of different doses of γ radiation on protein oxidation was determined in solution. An absorbed dose of 50 Gy was the minimum dose required to detect the significant effect of γ irradiation on protein oxidation (data not given). Fig. 3A shows that at this dose of irradiation, levels of protein carbonylation decrease as the concentration of PQQ increases from 1 to 5 μM. The levels of protection at 5 and 10 μM concentration of PQQ become nearly equal with 45% and 55%,

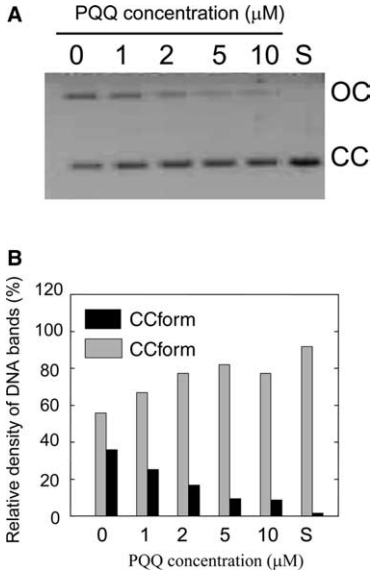


Fig. 3. Protection of plasmid DNA from oxidative damage by PQQ. (A) Agarose gel analysis of plasmid, pET28a+, DNA exposed to 5 Gy dose of γ radiation. Approximately 200 ng of covalently closed circular form of plasmid DNA (lane S) was irradiated with 5 Gy dose of γ radiation in the presence of different concentrations of PQQ (0–10 μM) in phosphate-buffered saline. Samples were electrophoresed on 1% agarose gel containing 0.5 ng/ml ethidium bromide, in TAE buffer (pH 8.5). (B) Quantitative analysis of the γ radiation-mediated conversion of covalent form of the plasmid DNA to open circular form in the presence and absence of PQQ. Quantitation of fluorescent intensity of each DNA band was carried out from the data as shown in panel A.

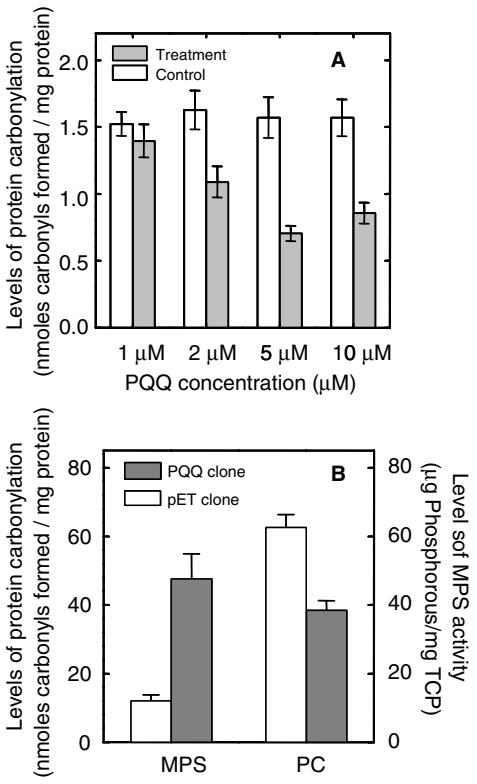


Fig. 4. (A) Effect of PQQ on protein carbonylation in vitro. About 30μM Bovine Serum Albumin in 50 mM sodium phosphate buffer, pH 7.0, was exposed to 50Gy dose of γ radiation and levels of protein carbonylation were measured. (B) Effect of PQQ on the Rose Bengal (5 μg/ml) induced protein carbonylation in vivo. The transgenic *E. coli* cells expressing PQQ synthase gene from *D. radiodurans* and complemented for MPS phenotype were used. The MPS activity (MPS) of the cells and protein carbonylation (PC) were determined in control (pET clone) and transgenic *E. coli* expressing PQQ synthase gene (PQQ clone).

respectively, as compared to the controls. This suggested that PQQ can, thus, protect DNA and proteins in solution from the oxidative damage triggered by ionizing radiation in a dose and concentration dependent manner.

3.3. PQQ synthase expression and antioxidant response of E. coli to oxidative stress

The oxidative damage protective effect of PQQ was also observed in vivo. Cell free extracts prepared from the Rose Bengal treated transgenic and control *E. coli* cells were compared for protein carbonylation, an indicator of protein oxidation. The levels of protein carbonylation in MPS positive *E. coli* cells were 1.7, folds less than the MPS negative cells (Fig. 3B). Thus, the cells synthesizing PQQ could protect the cellular proteins from oxidative damage and such protective effect of PQQ may be attributed to its antioxidant properties. An interesting question to resolve is whether the protection conferred by PQQ is due to ROS scavenging inside the cells or due to scavenging of the ROS generated by Rose Bengal in extracellular medium. We observed that exogenously added PQQ in the medium did not affect the intracellular levels of protein carbonylation in *E. coli* cells treated with Rose Bengal (data not given). This suggests that the PQQ effect in vivo might be due to the neutralization of ROS produced inside the cells.

Since the protein-bound PQQ reportedly cannot scavenge ROS, it is tempting to speculate that PQQ may exist in both free as well as enzyme bound form in transgenic *E. coli* cells.

Data presented in this paper clearly show that in vivo synthesis of PQQ in transgenic *E. coli* cells makes them resistant to oxidative stress. The reactivity of PQQ with radiolytically produced ROS elaborates the possible mechanism of action of this compound and consequent protection during oxidative stress. The reactions of PQQ with super-oxide and hydroxyl radicals have been studied earlier using electron spin resonance spectroscopy [19]. It was shown that superoxide and hydroxyl radicals react to PQQ with IC50 values $1–6 \times 10^{-8}$ and $4–6 \times 10^{-5}$ M, respectively, and protect the animals from the oxidative stress effects such as hydrocortisone-induced cataract, endotoxin shock and carbon tetrachloride induced liver injury. Subsequently, the role of PQQ as a neuroprotectant has been argued based on its ability to neutralize the super-oxide radicals and thereby suppressing the formation of peroxynitrite [20]. As has been demonstrated in this study, PQQ neutralizes the ROS by directly reacting with them through single electron transfer mechanism and the adducts, thus formed, are non-oxidant in nature. PQQ also functions in a concentration-dependent manner in protecting the proteins and DNA from the oxidative damage caused by γ radiation in solution, suggesting a role of PQQ as a radioprotector. Similar protection of cellular proteins against protein carbonylation is observed in vivo in transgenic *E. coli*. Although the levels of PQQ inside the transgenic *E. coli* cells are not known, the comparison of the data on protection of proteins from oxidation under in vivo and in vitro conditions suggests that approximately 5 μM PQQ may be present in these cells. In addition to such direct role in ROS scavenging, PQQ may also contribute to radioprotection by triggering other oxidative stress alleviation mechanisms, such as induction of catalase and super-oxide dismutase activities in bacteria, as shown earlier [4].

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