

**PYRROLOQUINOLINE QUINONE ACTS WITH FLAVIN REDUCTASE
TO REDUCE FERRYL MYOGLOBIN *IN VITRO* AND
PROTECTS ISOLATED HEART FROM RE-OXYGENATION INJURY**

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SUMMARY: Pyrroloquinoline quinone has been isolated from bacteria and recently has been detected in mammalian tissues and fluids. We report *in vitro* studies which show that pyrroloquinoline quinone serves as a high-affinity substrate for an erythrocyte "flavin reductase" and that the pyrroloquinoline quinol generated by this catalysis reacts rapidly with ferryl myoglobin radical. Western blot analysis of rat and rabbit heart homogenates detects a cross-reactive protein which has a molecular weight identical to the erythrocyte reductase from the same species. Low concentrations of pyrroloquinoline quinone protect isolated rabbit heart from re-oxygenation injury, serving as an effective tissue-protective agent in this model for cellular oxidative damage. We propose that this tissue protection is due to a pyrroloquinoline quinol-mediated reduction of reactive oxygen species. © 1993 Academic Press, Inc.

Pyrroloquinoline quinone (PQQ) is synthesized by a variety of bacteria and has been identified as a component of several bacterial oxidoreductases (1). This ortho-quinone has also been reported to be an essential growth factor in mice (2, 3) and the presence of free PQQ in mammalian fluids and cells has been suggested on the basis of redox cycling and chromatographic studies (4, 5). Very recently, nanogram-quantities of PQQ have been isolated from human and rat tissues, derivatized, and identified by gas chromatography/mass spectrometry (6). In addition, this compound has been observed to mediate a number of non-enzymic redox reactions (7). These findings suggest possible physiological roles for PQQ as a coenzyme, a mediator of non-enzymic redox reactions, and an antioxidant and possible roles as a therapeutic agent (7-11).

PQQ has recently been observed to serve as a substrate for a bovine cytosolic flavin reductase which has been isolated from erythrocytes and detected in liver (12, 13). Riboflavin serves as a

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ABBREVIATIONS USED: PQQ, pyrroloquinoline quinone; PQQH₂, pyrroloquinoline quinol; metMb, metmyoglobin; Mb, myoglobin; cyt c, cytochrome c; BPSS, bicarbonate-buffered physiological saline solution; PMSF, phenylmethanesulfonyl fluoride; LDH, lactate dehydrogenase.

low-affinity substrate for erythrocyte flavin reductase and the product of this catalysis, dihydroriboflavin, rapidly reduces the higher oxidation states of heme proteins (14). These reactions have been proposed to constitute the mechanism by which riboflavin protects tissues from reoxygenation injury (13-15). In this paper we report (a) that PQQ is even more effective than riboflavin as a tissue-protective agent, (b) that the product of the reductase-catalyzed reduction of PQQ rapidly reduces the higher oxidation states of heme proteins, and (c) that heart contains a protein that immunochemically cross-reacts with erythrocyte flavin reductase. Some of these data have been presented in abstract form (15-17).

MATERIALS AND METHODS

Materials. Horse heart metmyoglobin (metHb) was obtained from ICN, horse heart ferricytochrome *c*-type III (cyt *c*), PQQ, horseradish peroxidase, and lactate dehydrogenase (LDH) standards from Sigma, and riboflavin from Eastman. H₂O₂ (30%) obtained from EM Science was quantitated by its absorbance at 240 nm ($\epsilon = 43.6 \text{ M}^{-1}\text{cm}^{-1}$). Bovine erythrocyte flavin reductase was prepared as described previously (18). Hearts were freshly isolated from New Zealand White rabbits (1.5 kg) and from Sprague Dawley rats (350 g).

Assay of reductase activity. Rates of PQQ reduction were measured under aerobic conditions (a) by monitoring NADPH oxidation at 341 nm, an isosbestic wavelength for PQQ/PQQH₂ ($\Delta\epsilon = 6.2 \text{ mM}^{-1}\text{cm}^{-1}$) using 50 μM NADPH and 0.5 μM reductase in 5 mM phosphate buffer, pH 7.5, or (b) by monitoring ferric cyt *c* reduction at 550 nm ($\Delta\epsilon = 21 \text{ mM}^{-1}\text{cm}^{-1}$ (19)) in a coupled reaction using 1 mM NADPH, 1 μM reductase, 35 μM ferric cyt *c*, and 1 mM EDTA in 50 mM Tris chloride buffer, pH 8.0. A Uvikon Kontron 810 spectrophotometer and 1-cm light-path quartz cuvettes were used.

PQQ-mediated reduction of ferryl Mb radical. Peroxidation of metMb under anaerobic conditions was initiated by mixing equimolar amounts of H₂O₂ and metMb (1.7 μM) in 10 mM phosphate buffer, pH 7.5. The formation of ferryl Mb radical was monitored at 432 nm and detected by its spectrum (20). The reduction of ferryl Mb radical to Mb was achieved by adding reductase, NADPH, and PQQ to give final concentrations of 0.5 μM , 50 μM , and 10 μM , respectively, and reduction was monitored at 432 nm. To test the effect of the reduced form of PQQ on metMb peroxidation, a preincubated mixture of reductase, NADPH, and PQQ was added at the time metMb and H₂O₂ were mixed. Effect of peroxidation on Mb function was assessed by spectral estimation of O₂ affinity. The MbO₂/Mb ratio was estimated following the addition of aliquots of aerated buffer to a Mb solution in an anaerobic cuvette. O₂ solubility in the buffer was assumed to be the same as that in water (1.4 mM/atm).

Immunodetection of cardiac flavin reductase. Rat and rabbit hearts were perfused for 5 min to remove residual blood, using BPSS (25 mM NaHCO₃, 118 mM NaCl, 4 mM KCl, 1.2 mM MgCl₂, 2.4 mM CaCl₂, and 11 mM D-glucose) that had been oxygenated by equilibration with 95% O₂ / 5% CO₂. Ventricles were excised and homogenized at 4°C with a Tekmar Tissuemizer using 10 mM Tris buffer, pH 7.4, containing 250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, and 0.02 mM leupeptin. A cytosolic fraction was obtained by differential centrifugation, the last spin occurring at 73,000 $\times g$ for 60 min. Aliquots (20 μg total protein) of this fraction and hemolysates of rat and rabbit erythrocytes were subjected to SDS-PAGE on a 15% gel and electrotransferred to nitrocellulose using a Trans-Blot cell. Immunoblotting and color development were performed using rabbit antisera to purified bovine erythrocyte flavin reductase as described previously (18).

Measurement of reoxygenation injury in isolated rabbit heart. Rabbit hearts were isolated, perfused, and subjected to hypoxia/reoxygenation injury as described previously (21). All hearts were immediately perfused via the aorta with oxygenated BPSS. The effluent was not recirculated. After heart rate had been stabilized in response to pacing, left ventricular end-diastolic pressure was adjusted to 5 mm Hg and hearts were equilibrated by perfusing with oxygenated BPSS for 30 min. The hearts were then made hypoxic by perfusing with BPSS that had been equilibrated with 95% N₂ / 5% CO₂ (PO₂ \leq 7.5 mm Hg). After 60 min of hypoxia, the hearts were reoxygenated by perfusing with oxygenated BPSS for 30 min. In PQQ-treated hearts, 20 μM PQQ was added to the BPSS after 55 min of hypoxia (5 min before reoxygenation) and remained in the perfusate throughout reoxygenation. In control hearts, no PQQ was added to

the BPSS. The combined pulmonary artery effluent plus myocardial lymph drainage was collected continuously. At the end of each experiment, a sample of left ventricular free wall was removed, weighed, and dried to constant weight.

LDH activity in the effluent was measured photometrically (22) and used as a measure of cardiac injury. Enzyme activity was normalized per gram of ventricle dry weight and expressed as LDH released/g/min or in total LDH units during the periods of equilibration, hypoxia, and reoxygenation. PQQ, at the concentration present in the samples from the treated hearts, was shown to have no effect on the LDH assay. Data were expressed as arithmetic means (± 1 S.E.M.) and were analyzed for statistically significant differences using analysis of variance.

RESULTS

Catalysis of PQQ reduction by flavin reductase. Bovine erythrocyte flavin reductase was shown to catalyze the reduction of PQQ with NADPH as reductant. The resulting reduced form of PQQ transferred electrons to O_2 and ferricytochrome *c* at rates sufficiently rapid so that PQQ reductase activity could be measured in coupled reaction. An apparent K_m for PQQ of $2 \mu M$ was obtained by measuring PQQ-dependent NADPH oxidase activity at a saturating concentration of NADPH and pH 7.5 in the presence of air. The observed V_{max} was the same as that obtained with FMN as electron acceptor (12). An apparent K_m for PQQ of $2 \mu M$ was also obtained by measuring PQQ-dependent catalysis of ferricytochrome *c* reduction at a saturating concentration of NADPH and pH 8.0 in the presence of air.

Detection of rabbit and rat cardiac proteins that immunochemically cross-react with erythrocyte flavin reductase. Probing the soluble fractions derived from erythrocytes and heart with antibody prepared against bovine erythrocyte flavin reductase detected cross-reactive proteins as shown in Fig. 1. Rat and rabbit erythrocyte hemolysates each showed one major cross-reactive band at 27 kD and 26 kD, respectively. The soluble fractions from rat and rabbit hearts each showed a cross-reactive band that migrated indistinguishably from the flavin reductase detected in the hemolysate from the same species. The rat heart showed an additional cross-reactive band and rabbit heart showed several additional bands.

Mediation by PQQ of the enzymic reduction of ferryl myoglobin radical. Mixing equimolar amounts of metMb and H_2O_2 under anaerobic conditions resulted in the slow appearance of spectral properties characteristic of ferryl Mb radical (Fig. 2). Addition of flavin reductase, NADPH, and PQQ (see arrow, inset of Fig. 2) resulted in the catalytic reduction of this ferryl Mb radical to a product showing spectral properties characteristic of ferrous Mb. In this experimental time frame, reduction was not observed in the absence of either reductase or PQQ. Myoglobin, after being subjected to the ferryl radical formation and subsequent PQQ-mediated catalytic reduction, is present in a modified form as evidenced by an observed decrease of its O_2 -affinity (K_d of $7 \mu M$ for the O_2 -complex, as compared to $1.2 \mu M$ for native oxymyoglobin).

The extent of ferryl myoglobin radical formation is greatly diminished if flavin reductase, NADPH, and PQQ are preincubated to generate PQQH₂ before the mixing of metMb and H_2O_2 . Such preincubation also diminishes the extent of protein modification, as evidenced by the values of $5 \mu M$ and $2.5 \mu M$ observed for the K_d of the O_2 -complexes for myoglobin that had been protected against peroxidative damage by preincubation for 33 min and 91 min, respectively.

PQQ protection of isolated rabbit hearts from reoxygenation injury. Treatment of hypoxic rabbit hearts with $20 \mu M$ PQQ provided significant protection against reoxygenation injury

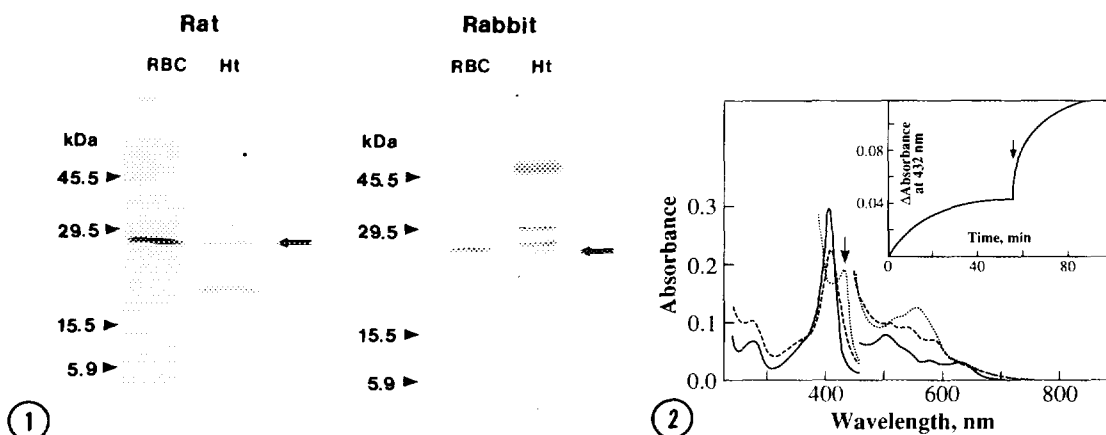


FIG. 1. Detection of rabbit and rat cardiac flavin reductase. Rat and rabbit hearts that had been perfused with oxygenated BPSS were homogenized and cytosolic fractions (Ht) were obtained using differential centrifugation. Proteins (20 μg per lane) were separated on a 15% SDS polyacrylamide gel, transferred to nitrocellulose, and blotted using rabbit antisera to purified bovine erythrocyte flavin reductase. For comparison, rat and rabbit hemolysates (RBC) were run alongside the heart cytosolic preparations. Arrow indicates a cross-reactive protein with molecular mass equal to that of flavin reductase.

FIG. 2. PQQ-mediated reduction of ferryl Mb radical. —, Spectrum of 1.7 μM metMb; - - -, spectrum of ferryl Mb radical generated by reacting 1.7 μM metMb and 1.7 μM H_2O_2 for 58 min at 25°C; ···, spectrum of product resulting from a 120-min incubation of ferryl Mb radical with 0.5 μM reductase, 1 mM NADPH, and 10 μM PQQ. Visible spectra are shown at five times the indicated absorbance. Arrow indicates the 432 nm wavelength at which the kinetic profile was obtained. *Inset:* Absorbance change at 432 nm corresponding to the kinetic profiles for the formation of ferryl Mb radical followed by its reductase-catalyzed, PQQ-mediated reduction to Mb. Arrow indicates the time at which reductase, PQQ, and NADPH were added. Experimental conditions are described in *Materials and Methods*.

measured as LDH activity released into heart effluent. In control hearts, the rate of LDH release was low during hypoxia but increased nearly 7-fold upon reoxygenation. PQQ added 5 min prior to reoxygenation attenuated this rise in LDH release (Fig. 3). PQQ had no effect ($p = 0.44$) on total LDH activity released during the last 5 min of hypoxia but significantly reduced total LDH activity released during reoxygenation ($p < 0.02$) (Fig. 4).

DISCUSSION

Kinetic studies demonstrate that flavin reductase catalyzes an NADPH-dependent reduction of PQQ. The observed apparent K_m of 2 μM for PQQ is much smaller than the values observed for riboflavin, FMN, or FAD (23). In light of the recent evidence that PQQ is present in mammalian tissues and fluids (6) and is an essential nutrient for the growth of mice (2, 3), it is conceivable that flavin reductase functions *in vivo* as a "PQQ reductase". The detection of soluble, cardiac proteins that immunochemically cross-react with the erythrocyte flavin reductase suggests that this enzyme is present in heart as well as in erythrocytes and liver.

Our results demonstrate that the reaction between PQQH_2 (formed in rxn 1) and ferryl myoglobin radical (formed in rxn 2) generates (by rxns 3 and 4) a product similar in spectral properties to ferrous Mb, but with an O_2 affinity weaker than that of Mb:

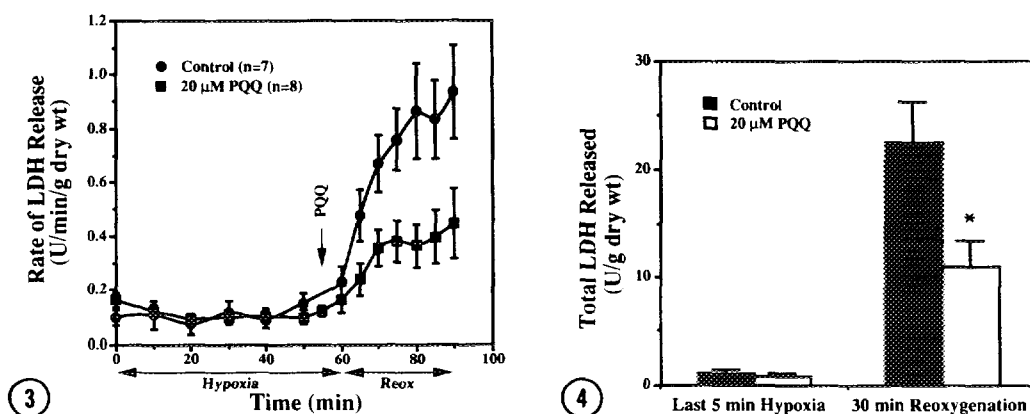
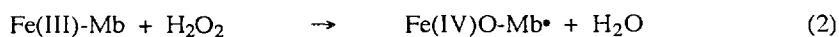


FIG. 3. Effect of PQQ on LDH release rate during heart perfusion. Isolated rabbit hearts were subjected to 60 min of hypoxia followed by 30 min of reoxygenation. In treated hearts, 20 μM PQQ was added to the perfusate 5 min prior to reoxygenation (as indicated by arrow) and was present throughout reoxygenation. Effluent was assayed for LDH. Values are means ± S.E.M.

FIG. 4. Effect of PQQ on total LDH released. The total amounts of LDH in the effluent from perfused rabbit hearts were measured during PQQ treatment. PQQ did not affect LDH release during the last 5 min of hypoxia, but significantly reduced LDH release during reoxygenation relative to controls (* $p < 0.02$).



Preincubation of NADPH, PQQ, and flavin reductase before the generation of ferryl Mb results in less formation of ferryl Mb radical and diminished modification of the protein. Preincubation leads to an accumulation of PQQH₂ which then presumably reacts with ferryl Mb radical before this unstable species can undergo self-destruction. We have not ruled out the possibility that a direct reaction between PQQH₂ and H₂O₂ might also occur.

Our observed protection of hypoxic rabbit heart from reperfusion injury by inclusion of 20 μM PQQ five min prior to reoxygenation is similar to the protection previously observed in this system with 150 μM riboflavin. PQQ and riboflavin have three properties in common which suggest a common mechanism of protection: (1) they both readily pass into cells; (2) they both serve as electron acceptors for flavin reductase; and (3) their reduced forms, the products of catalysis by this reductase, readily reduce reactive oxygen species that have been implicated in tissue damage. These similarities suggest that the catalysis of PQQ reduction by intracellular reductases and the subsequent reduction by PQQH₂ of reactive oxygen species may be responsible for the observed protection of isolated heart by administered PQQ.

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