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CHARACTERIZATION OF THE GLYCINE-DEPENDENT REDOX-CYCLING ACTIVITY IN ANIMAL FLUIDS AND TISSUES USING SPECIFIC INHIBITORS AND ACTIVATORS: EVIDENCE FOR PRESENCE OF PQQ

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SUMMARY: Pyrroloquinoline quinone, a redox cofactor first isolated from bacteria, efficiently catalyzes the nonenzymatic oxidation of glycine in the presence of nitroblue tetrazolium. We report that certain metalic cations and heterocyclic aromatic cations, like the N-methyl phenazonium cation and aryl-iodonium compounds, strongly and specifically inhibit this redox-cycling activity. The inhibition by metal cations is reversed by Tiron and that of the aromatic cations by Tiron and thyroxine. These inhibitors and activators affect authentic PQQ and the redox-activity of putative PQQ isolated from biological sources in a similar manner. This indicates that pyrroloquinoline quinone occurs naturally in animal tissues and fluids.

Pyrroloquinoline quinone (PQQ) is the cofactor of several bacterial dehydrogenases (1) and an essential nutrient for young mice (2). PQQ has also been identified in mammalian tissues and fluids (2-4) based on its ability to catalyze the oxidation of glycine with superoxide formation (3). There, PQQ appears to function as the dissociable redox-cofactor of the mitochondrial NADH-CoQ reductase (complex 1) (5, 6), the NADPH oxidase of phagocytic cells (7) and of the cytosolic flavin reductase (8).

The occurrence of PQQ in biological tissues and fluids has recently been confirmed by GC/MS where putative PQQ was oxidized and permethylated by reaction in air with phenyltrimethylammonium hydroxide (9, 10). However, PQQ

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ABBREVIATIONS: PQQ, pyrroloquinoline quinone; NBT, nitroblue tetrazolium; RP-HPLC, reverse phase high pressure liquid chromatography; PMS, phenazine methosulfate; DPI: diphenyleneiodonium; BPI: biphenyliodonium; VS: vanadyl sulfate.

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levels are significantly underestimated with this methodology, because it does not detect adducts between PQQ and amino acids (11). On the contrary, the redox-cycling method detects both free PQQ and PQQ adducts.

In this communication we describe the effect of specific inhibitors and activators on the redox-cycling activity on authentic and putative PQQ. The latter was isolated from biological samples by reverse phase HPLC. The results of the redox-cycling assay and the similarity of response to these inhibitors and activators confirms the presence of PQQ in nmolar concentrations in animal tissues and fluids.

MATERIALS AND METHODS

Materials: Unless otherwise stated the chemicals were obtained from Aldrich; PQQ was obtained from Fluka; Chelex 100, analytical grade (100-200 mesh) from Biorad; diphenyleneiodonium chloride (DPI) from Cookson Chemicals, Southampton, England; the bis-iodonium compound ($R\text{-}I^{+}\text{-}\phi\text{-}I^{+}\text{-}R$; R: p-$IC_6H_4$-) was a gift from Prof. PJ Stang, University of Utah, Salt Lake City. The oxazole of PQQ was prepared by exposing PQQ to excess glycine (12, 13).

Redox-cycling assay: The redox-cycling activity in NBT-glycinate was determined as previously described (14): To a 300 µl sample, containing the quinones/hydroquinones as well as inhibitors and or activators, were added 100 µl borate buffer (0.1M borate in 2M potassium glycinate, pH 10) and 100 µl of a borohydride-reduced, dialyzed albumin solution (10mg/ml). The reaction was started by addition of 1 ml NBT (0.24 mM NBT in 2M potassium glycinate, pH 10). After incubation in the dark for 20 minutes at 37°C, formazan formation was measured spectrophotometrically at 530nm. Albumin was included to keep the formazan in solution and borate to suppress interference from ascorbate, which directly reduces PQQ (15). To test for other compounds that may also directly reduce NBT, a control was run in valine (0.5 M potassium valinate, pH 10) which is not oxidized by PQQ.

Sample preparation: Protein-free dialysates were used for analysis of most materials. Sera, cerebrospinal fluid and acid whey were assayed directly. Skim milk, egg white and yolk, tissue homogenates, and sonicates of rat liver mitochondria (16) and guinea pig neutrophils (17) were dialyzed against 1.5 volumes cold water for 12 hours at 4°C, the protein free dialysis solution lyophilized and dissolved in cold water for analysis.

RP-HPLC: Separations were performed on an LC-18 Supelcosil column (10 mm x 25 cm), with a flow rate of 2ml/min. The column was equilibrated and washed with 0.1M formic acid adjusted to pH 3.3 with ammonia. After sample injection, the column was washed for 20 min and eluted with a linear gradient to 100% methanol. Elution was monitored at 280 and 340 nm. One minute fractions were collected and analyzed for redox-cycling activity in NBT/glycinate. Authentic PQQ elutes at 38% methanol as does putative PQQ. The fraction containing the putative PQQ precedes the yellow fluorescent fraction containing the riboflavins, which are most prominent with milk samples. The redox-inactive PQQ oxazole is not resolved from PQQ in this system. A blank run was always performed before biological materials were analyzed to exclude contaminating redox-cycling activity from authentic PQQ.

RESULTS

Inhibition and reversal of the redox-cycling activity of PQQ. The redox-cycling activity of PQQ in NBT/glycinate is inhibited by certain metal and organic cations as well as by carbonyl reagents (3). **Table 1** shows the IC_{50} of the more potent inhibitory cations. The IC_{50} of all other cations tested, including Zn, Sr, Fe(II/III), Co, Cd, Cr, Mg, Li, Ag, Be was >150 μM. The inhibition by metal cations is in part reversed by Tiron. The metal inhibitors do not affect the redox-cycling activity of quinones other than PQQ.

Organic cations like phenazine methosulfate (PMS), and iodonium compounds also selectively inhibit the redox-cycling of PQQ. PMS (80nM) inhibits the redox-activity of PQQ by 75% and that of neutral ortho-quinones by only 20%. This inhibition is released by thyroxine (T4), T3 and Tiron (**Table 2**). T3/T4 and Tiron have no effect on the inhibition of covalent inhibitors of PQQ.

Redox-cycling activity in biological fluids and tissues. Dialysates of biological fluids and tissue extracts test positive in the NBT/glycine redox-cycling assay (**Table 3**). The redox-cycling activity in these unfractionated samples is affected in a complex manner by inhibitory compounds. For instance, while the redox-cycling activity of authentic PQQ added to milk

Table 1: Cationic inhibitors of PQQ-catalyzed redox-cycling*

Inhibitor	IC_{50}	Reversal
Metals:		
In (III)	0.5μM	T
Mn (II)	3μM	T
Sn (II)	5μM	T
V=O, vanadyl	10μM	T
Pb (II)	11μM	T
Cu (II)	67μM	T
Organic Cations:		
Phenazine methosulfate (PMS)	67nM	T/T4
Bis-iodonium compound	33nM	T/T4
Diphenyleniodonium (DPI)	3μM	T/T4
Biphenyliodonium (BPI)	12μM	T/T4
Berberine	3μM	T/T4
Sanguinarine	33μM	T/T4

T: reversed by Tiron; T4: reversed by thyroxine.
* tested using 13.3 nM PQQ.

Table 2: Effect of inhibitors and activators on PQQ catalyzed redox-cycling

Inhibitor/activator	% redox cycling activity
PQQ	100
PQQ + PMS	24
PQQ + bis-iodonium compound	24
PQQ + T4	140
PQQ + PMS + T4	136
PQQ + bis-iodonium + T4	134

PQQ (13nM); PMS (80 nM); bis-iodonium (33nM); T4 (67 µM).

dialysates is recovered quantitatively after removal of inhibitory metal cations by Chelex treatment, no increase in basal values is seen.

HPLC-resolution of redox-active material. The effect of inhibitors and activators was further studied on the redox-active material separated by RP-HPLC. Two redox-active fractions were resolved, early eluting material and a later fraction containing the putative PQQ which elutes in the position of authentic PQQ (**Figure 1**). The relative proportion of the redox-activity in these two fractions varied greatly. Putative PQQ was obtained in higher yields if the column was exhaustively washed with acetonitrile between runs. Repeated sample injection without thorough column washes resulted in diminishing yields of putative PQQ. Sample preparation also affects the distribution of redox-cycling activity: Prolonged dialysis causes more redox-cycling activity to elute early from the column. This change in elution position was also observed with PQQ-spiked lyophilisates: 40-50% of the redox-cycling activity eluted early from the column.

Use of inhibitors for the characterization of redox activities. Inhibitors and activators of PQQ catalyzed redox-cycling similarly affect the redox-

Table 3: Redox-cycling activity in fluids and tissues

source	PQQ-equivalents, range
Serum	100-200 pmoles/ml
Erythrocyte	150 pmoles/ml RBC
Neutrophil	30 nmoles/ml cells
Cerebrospinal fluid	10-50 pmoles/ml
Liver mitochondria, rat	20 nmoles/g wet weight
Bovine skim milk	25- 50 pmoles/ml
Acid whey	150-200 pmoles/ml
Egg, chicken	350-400 pmoles/egg

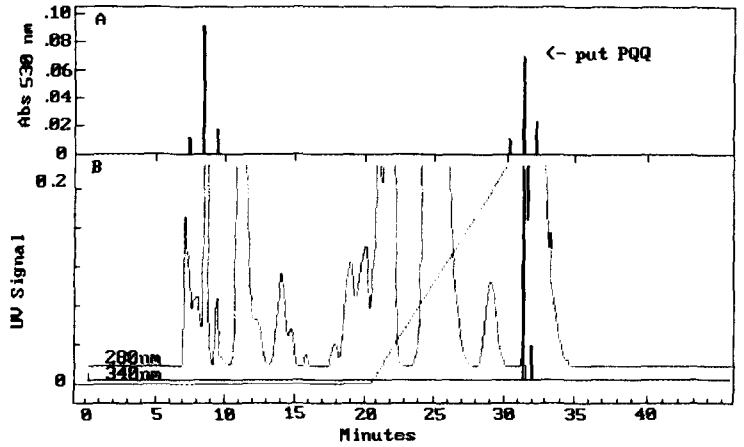

FIG 1. HPLC-resolution of the dialyzable redox-cycling activity from egg yolk: The redox-cycling in NBT/glycinate (panel A) elutes in two fractions, an early eluting one, and a later one (putative PQQ) which elutes in the position of authentic PQQ. Panel (B) shows the elution profile monitored at 280 and 340 nm.

cycling activity of putative PQQ and authentic PQQ (Figure 2). The percentage inhibition with PMS, $InCl_3$ and partial reversal of the PMS inhibition by Tiron is identical for authentic and putative PQQ. However, the extent of response of authentic and putative PQQ to vanadyl sulfate was somewhat different which may reflect the uncertain redox-state of this inhibitor.

The early eluting redox-cycling activity is not inhibited by PMS, and T4 increases it by 10-15%.

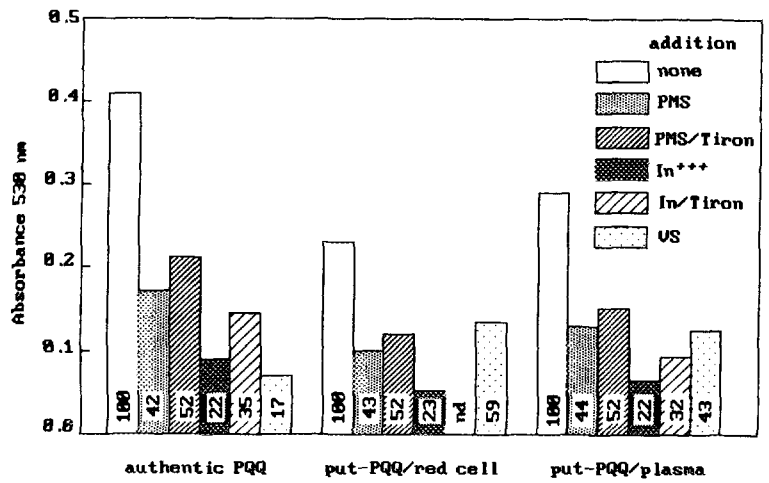

FIG 2. Effect of the inhibitors PMS, $InCl_3$ and vanadyl sulfate (VS) on the redox-cycling activity of authentic PQQ and putative PQQ isolated from red cells and plasma. Inserted is the percent activity. The concentrations were: PMS (70nM), $InCl_3$(590nM), VS (20 μM) and Tiron (84μM).

DISCUSSION

The inhibitors of PQQ-catalyzed redox-cycling recognize basic structural features of PQQ. Potent inhibition is caused by metal cations of a narrow size range (ionic radius 0.8-0.93 Å) and released by the quinoid transchelator Tiron, 4,5-dihydroxy-1,3-benzene disodium sulfonate (18). Metal cations therefore probe for the nature of the metal binding site, which in PQQ consists of the Schiff's base and quinoline nitrogens and the 7-carboxyl group.

The organic cations probe for an extended aromatic anionic ring system. They interact with PQQ not only by charge but also by π-π interactions. The involvement of a charge transfer complex is evidenced by the fact that the planar diphenyleneiodonium cation is more potent than the non-planar biphenyliodonium. Also this inhibition is reversed by water-soluble aromatic compounds like Tiron or T3/T4. These compounds are oxidyzed by PQQ to their quinoid forms (19) increasing the basal formazan production by PQQ.

The almost identical response to inhibitors of both putative and authentic PQQ indicates that these substances have in common a metal binding site and an extended anionic aromatic ring system.

The chromatographic and electrochemical properties of putative PQQ from milk also support the view that this material represents PQQ (3). Most of the redox-activity in milk, upon pH-gradient elution from a C-18 column, elutes in the position of authentic PQQ. Furthermore, this material in a multichannel electrochemical detector yielded a response identical to that of PQQ. This shows that putative PQQ undergoes reversible oxidation/reduction with the same electrocoulometric behavior as authentic PQQ.

The early-eluting redox-cycling activity also appears to be derived from PQQ since authentic PQQ added to biological materials also converts to an early eluting form. This material may represent adducts of PQQ with amino acids (12, 13). The initial condensation product, before decarboxylation, carries an additional negative charge and should elute earlier.

Such a redox-active quinoneimine can form an oxazole, which upon decarboxylation will elute in the position of PQQ, is redox-inactive and eludes detection. Such adduct formation can explain the variable yields of putative PQQ isolated in our studies and the lower concentrations reported by others (9,10). In fresh biological materials, ascorbate keeps PQQ in its reduced, less reactive form. When this reducing environment is lost during sample preparation, PQQ reacts with nucleophilic compounds and escapes detection.

The evidence presented here lends further support to the view that dialyzable PQQ occurs in animal fluids and tissues and explains why its concentration is easily underestimated.

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REFERENCES

1. Salisbury, S.A., Forrest, H.S., Cruse, W.B.T., and Kennard, O. (1979) Nature 280, 843-844.
2. Killgore, J., Smidt, C., Duich, L., Romero-Chapman, N., Tinker, D., Reiser, K., Melko, M., Hyde, D., and Rucker, R.B. (1989) Science 245, 850-852.
3. Flückiger, R., Paz, M.A., Bergethon, P.R., Henson, E., and Gallop, P.M. (1992) In: Principles and Applications of Quinoproteins (V.L. Davidson, Ed.), pp. 331-341. Marcel Dekker, Inc., New York.
4. Flückiger, R., Paz, M.A., Bergethon, P.R., Goodman, S., Henson, E., and Gallop, P.M. (1991) FASEB J. 5(6), A1510.
5. Mah, J., Paz, M.A., Flückiger, R., and Gallop, P.M. (1993) FASEB J. 7, A53.
6. Paz, M.A., Mah, J., Flückiger, R., Stang, P.J., and Gallop, P.M. (1993) ASBMB/DBC-ACS JOINT MEETING, Addendum LB80.
7. Bishop, A. (1993) Dissertation, Harvard University.
8. Xu, F., Mack, C.P., Quandt, K.S., Shlafer, M., Massey, V., and Hultquist, D.E. (1993) Biochem. Biophys. Res. Communun. 193, 434-439.
9. Kumazawa, T., Seno, H., Urakami, T., Matsumoto, T., and Suzuki, O. (1992) Biochim. Biophys. Acta 1156, 62-66.
10. Kumazawa, T., Seno, H., and Suzuki, O. (1993) Biochem. Biophys. Res. Communun. 193, 1-5.
11. Naito, Y., Kumazawa, T., Kino, I., and Suzuki, O. (1993) Life Sci.52, 909-1915.
12. vanKleef, M.A.G., Jongejan, J.A., and Duine, J.A. (1989) Eur. J. Biochem. 183, 41-47.

13. Adachi, O., Okamoto, K., Shinagawa, E., Matsushita, K., and Ameyama, M. (1988) Biofactors 1, 251-254.
14. Paz, M.A., Flückiger, R., Boak, A., Kagan, H.M., and Gallop, P.M. (1991) J. Biol. Chem. 266, 689-692.
15. Paz, M.A., Flückiger, R., and Gallop, P.M. (1990) FEBS Lett 264, 283-284.
16. Fleischer, S., McIntyre, J.O., and Vidal, J.C. (1979) Methods Enzymol. LV, 32-39.
17. Badwey, J.A. and Karnovsky, M.L. (1979) J. Biol. Chem. 254, 11530-11537.
18. Yoe, J.H. and Jones, A.L. (1944) Ind. & Eng. Chem. 16, 111-116.
19. Niemann, C. and Mead, J.F. (1941) J. Am. Chem. Soc. 63, 2685-2687.