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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND pH-DEPENDENT ELECTROCHEMICAL PROPERTIES OF PYRROLOQUINOLINE QUINONE AND THREE CLOSELY RELATED ISOMERIC ANALOGUES

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Pyrroloquinoline quinone **1** (PQQ, methoxatin, 4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid) is a novel coenzyme. Closely related isomeric analogues of PQQ may be formed in nature and may possess PQQ-like functions. Here, the electrochemical pH-dependent properties and the high performance liquid chromatographic (HPLC) separation protocols are reported for three major isomeric PQQ analogues likely to be formed in nature. The electrochemical data (cyclic voltammetry) provided the pK_as of the five ionizable groups in each isomeric PQQ. The HPLC protocols provide the means of investigating the formation of PQQ and the PQQ isomers reported here during the normal turnover of eukaryotic amine oxidases containing integral topaquinone residues, as well as from direct enzyme-mediated reactions which remain to be characterized. © 1995 Academic Press, Inc.

In 1979, pyrroloquinoline quinone **1** (PQQ, methoxatin, 4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid) was identified as a novel coenzyme for methanol dehydrogenase from the methylotrophic soil bacterium *Pseudomonas sp.*(1-2). An important role has been suggested for PQQ and its closely related analogues as growth and nutritional factors in eukaryotic development (3). In addition, PQQs may act as tissue-protective agents through highly specific mechanisms operating on enzyme surfaces as well as through electron transfer reactions which are not enzymatically mediated (4).

Complicating the elucidation of a specific role for PQQs in eukaryotic biochemistry is the facile formation of both PQQ and potentially its isomeric analogues, in particular the five membered ring isoanalogue **2** (4,5-dihydro-4,5-dioxo-3H-pyrrolo[3,2-f]quinoline-2,7,9-tricarboxylic acid), which may occur during the attempted isolation and derivatization of the PQQs from putative PQQ-containing enzymes (5-6). It is possible that the PQQs (**1-4,** Figure 1) may be formed nonenzymatically during the normal turnover of some amine oxidases, the redox-enabling cofactors of which have now been identified unambiguously as integral topaquinone residues (7). Recently, we synthesized **2**, **3** and **4**, all of which are isomeric analogues of PQQ (8). These,

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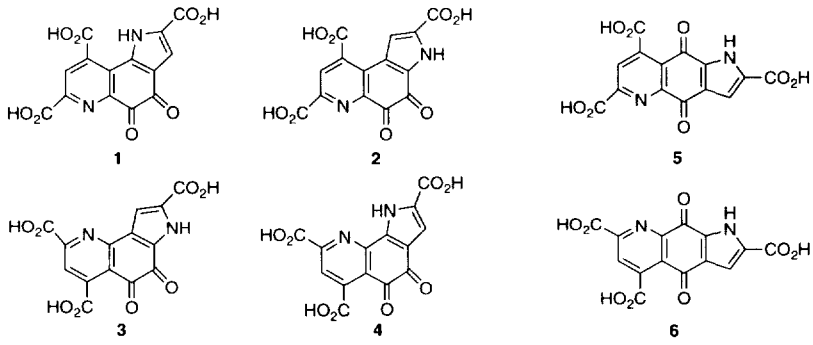

Figure 1. The structures of PQQ **1** and PQQ isomers **2-6**. The chemical names for compounds **1-6** are : **1,** 4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid; **2,** 4,5-dihydro-4,5-dioxo-3H-pyrrolo[3,2-f]quinoline-2,7,9-tricarboxylic acid; **3,** 5,6-dihydro-5,6-dioxo-7H-pyrrolo[2,3-h]quinoline-2,4,8-tricarboxylic acid; **4,** 4,5-dihydro-4,5-dioxo-1H-pyrrolo-[3,2-h]quinoline-2,6,8-tricarboxylic acid; **5,** 4,9-dihydro-4,9-dioxo-1H-pyrrolo[2,3-g]quinoline-2,6,8-tricarboxylic acid; **6,** 4,9-dihydro-4,9-dioxo-1H-pyrrolo[3,2-g]quinoline-2,5,7-tricarboxylic acid.

we anticipate to be the major isomeric PQQs formed. These compounds serve as authentic samples to define their possible formation in nature, and also serve as isosteric probes to assess the molecular basis of productive PQQ binding at active sites in PQQ-requiring enzymes. Here, we report their effective separation and analysis by high performance liquid chromatography, thus providing the basis for their analysis in biological fluids. In addition, we report the pH-dependence of the electrochemical properties of these new PQQ isomers determined by cyclic voltammetry. These measurements illustrate the expected similar redox behavior in comparison to PQQ and also provide a means of determining the pK_as of the five ionizable groups associated with each isomeric PQQ.

MATERIALS AND METHODS

Materials: PQQ was purchased from Fluka (purity >99%). Isomeric analogues of PQQ (**2, 3,** and **4**) were synthesized in this laboratory and will be reported elsewhere (8). The aqueous stock solutions (4×10^{-3} M) of PQQ and PQQ isomers were prepared by addition of aqueous K_2CO_3 (0.05 M) into a suspension of each of the compounds in water, followed by addition of H_2O to the desired volume.

Cyclic voltammetry was conducted with a Bioanalytical Systems Inc. (BAS) 100 B electrochemical analyzer, which was interfaced to a Gateway 386 PC and a Houston Instruments DMP-40 digital plotter. An aqueous Ag/AgCl (3 M NaCl) electrode (BAS) and a platinum wire were used as reference and auxiliary electrodes, respectively. The working electrode was a glassy carbon disk electrode (GCE, BAS), which was polished with 0.05 μm alumina polishing powder before each measurement. Each measurement at a given pH value was conducted three times. The standard deviation for each measurement was calculated and was too small to be visible as a deviation in each of the points plotted in Figure 3. All solutions were prepared from distilled and deionized water, which was purified to a resistivity of at least 18 MΩ-cm by a Barnstead Organic pure water system. The buffer solutions in cyclic voltammetry were as follows: HCl for pH 0-1, HCl /KCl for pH 1-2, citric acid/Na_2HPO_4 for pH 2.2-8.0, Na_2CO_3/$NaHCO_3$ for pH 9.2-10.7, Na_3PO_4/NaOH for pH 12, NaOH for pH > 12. The test solutions (2.5×10^{-4} M) for cyclic voltammetry were prepared from stock solution (0.5 ml) added to buffer (7.5 ml) just prior to each determination. The ionic strengths of the test solutions were adjusted to 0.5 with KNO_3. The

pH values of the test solutions were measured to ± 0.01 after each measurement. The pK_a values were determined by linear regression analysis of each set of data points.

High performance liquid chromatography (HPLC) was performed with a Varian Vista 5500 system. Compounds were chromatographed on a 10 cm Vydac C_{18} reversed phase column (218TP104) with water/methanol/85% H_3PO_4 (80:20:0.1) at the flow rate of 2 ml/min. The absorbance of the eluate was detected at 280 nm. HPLC samples (2.0×10^{-4} M) were prepared with 1 ml of stock solution of each compound being added to KH_2PO_4 buffer solution (19 ml, pH 7.0).

UV/Vis spectra were recorded on a Varian DMS 200 UV-Visible spectrophotometer at a scan rate of 200 nm/min. Samples (5.0×10^{-5} M) for the absorption spectrum measurement were prepared with 2 ml of HPLC solution (2.0×10^{-4} M) of each compound being added to KH_2PO_4 buffer solution (6 ml, pH 7.0).

RESULTS

The reduction of quinone to quinol is accompanied by protonation. The half-wave potentials ($E_{1/2}$) determined from the cyclic voltammograms are pH-dependent. As the pH increases, $E_{1/2}$ shifts negatively due to the decreasing availability of protons. In the acidic region, the reversible cyclic voltammograms of the PQQ isomers were observed. Figure 2 shows the typical cyclic voltammograms of PQQ isomer **3** at different pH values. As the pH values increased, quasi-reversible cyclic voltammograms were obtained. Plots of pH-dependence of half-wave potentials for PQQ isomers **2-4** are shown in Figure 3. The different pK_a values for PQQ isomers were obtained from computational analysis of the points of intersection in the above figures and are summarized in Table 1. The R factor in each case is larger than 0.99. The pK_a values for PQQ in Table 1 were those cited from Kano et al. (9-10). Their pK_as for PQQ **1** and our pK_as for the PQQ isomers studied here are similar for each corresponding microscopic pK_a.

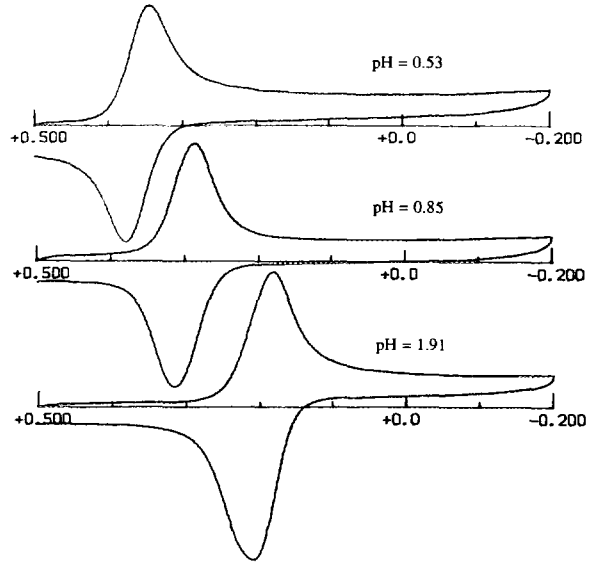

Figure 2. Cyclic voltammograms of PQQ isomer **3** at pH 0.53, 0.85 and 1.91.

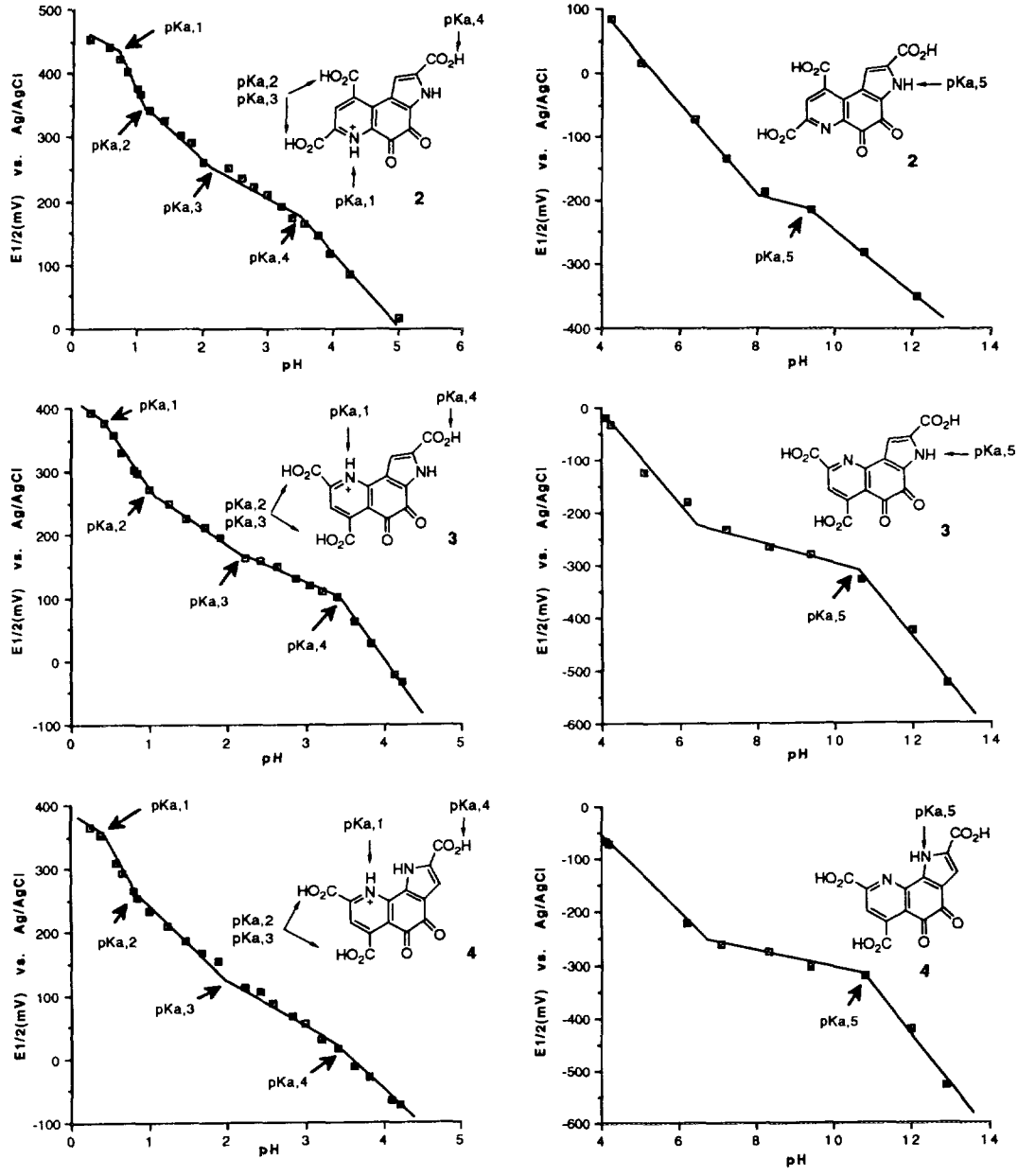

Figure 3. pH dependence of the half-wave potentials ($E_{1/2}$) of PQQ isomers **2-4**.

HPLC separations were carried out on a reversed phase Vydac C_{18} column and provided facile separations of isomers **2**, **3** and PQQ **1**. However, isomer **4** was difficult to separate from PQQ **1**. Figure 4 shows a representative separation of the isomeric PQQs and PQQ **1**. While we used λ_{280} nm to detect these PQQs, other wavelengths may be employed. The overlapping absorption spectra of the four PQQs are illustrated in Figure 5.

Table 1. The pKa values of PQQ **1**[a] and PQQ isomers **2-4**[b]

Compound	$pK_{a,1}$	$pK_{a,2}$	$pK_{a,3}$	$pK_{a,4}$	$pK_{a,5}$
PQQ **1**	0.30	1.60	2.20	3.30	10.30
2	0.62	1.22	2.32	3.35	9.85
3	0.40	1.01	2.18	3.37	10.71
4	0.27	0.97	1.96	3.34	10.88

a: Kano, K., Mori, K., Uno, B. and Kubota, T. (1990) Bioelectrochem. Bioenerg. 23, 227-138 and 24, 193-201.
b: Determined by linear regression analysis of each set of data points.

DISCUSSION

Compounds **2-4** may be regarded as the only isomeric analogues of PQQ likely to be formed from indiscriminate cyclization reactions involving oxidized tyrosine and glutamic acid precursors whether the process is enzymatic or nonenzymatic. PQQ **1** and compounds **2-4** represent the only four possible tricyclic isomers in which there is a fused pyridino- and a pyrrolo- ring attached to a central six membered ring containing an ortho quinone structure, and where the nitrogen within each respective 5- or 6- membered fused ring is directly attached to the central ortho quinone ring system. Two additional isomeric analogues, "linear PQQs" **5-6** (Figure 1), could be of interest, but would be expected to have major differences in redox supporting behavior due to their para quinone structure within the central ring. They have not been prepared and are, thus, not part of the present study. The formation of **5** and **6** from tyrosine and glutamic acid precursors is regarded as unlikely (11).

The assignment of pK_as for PQQ made previously by Kano et al. (9-10), and compared with those observed in the isoanalogues examined here requires brief comment. The pK_a values for the

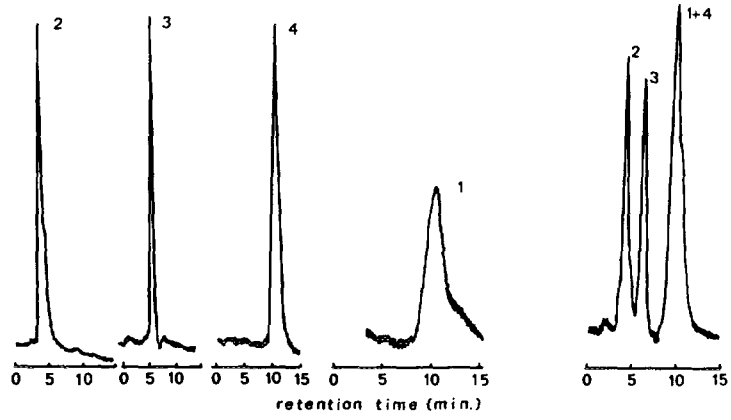

Figure 4. High-performance liquid chromatography of PQQ **1** and PQQ isomers **2-4**. Compounds were dissolved in 0.05 M K_2HPO_4 buffer solution (pH 7.0) and chromatographed on a Vydac C_{18} column in water/methanol/85% H_3PO_4 (80:20:0.1) at a flow rate of 2.0 ml/min. The absorbance was detected at 280 nm. 10 µl each of the compounds **1-4** (2×10^{-4} M) and 25 µl of a mixture of compounds **1-4** (5×10^{-5} M each) were loaded separately to the column.

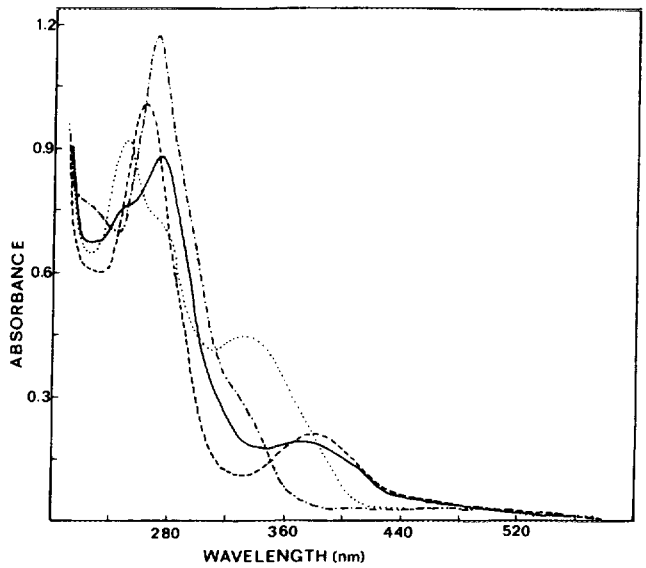

Figure 5. Absorption spectra of PQQ **1** and PQQ isomers **2-4** at concentrations of 5×10^{-5} M (pH 7.0). ····· PQQ **1**; —— **2**; — — — **3**; — · — · — **4**.

protonated pyridyl nitrogen of all of the isomers are all below 1.0. This observation is consistent with the prediction equation of Charton (12) ($pK_a = 5.39\text{-}5.70\Sigma\sigma$ for substituted pyridinium ions). Similarly, the pK_a values for the pyrrole nitrogens of all of the isomers are near 10.0, which is close to prediction based on the method of Barlin and Perrine (13), where $pK_a = 17.00\text{-}4.28\Sigma\sigma$ for substituted pyrroles. Because of the relative ease of hydrolysis of methoxycarbonyl group in the pyrrole ring of 2,7,9-trimethyl ester of PQQ, it is reasonable to assign the pK_a values between 3.3-3.4 to the carboxyl groups in pyrrole ring of PQQ and its isomers. The pK_a values for the other two carboxyl groups in pyridine rings are between 0.9-2.4. It appears reasonable to assign the lower pK_a to the carboxyl group attached ortho to the pyridine ring nitrogen.

HPLC separation of the four PQQ isomers provides the means to detect the presence of at least two PQQ analogues in some biological fluids. Obviously, all of these compounds are electroactive. This feature provides for their possible electrochemical detection in fluids where this may be appropriate, and should provide for lower limits of detectability. We are attempting the separation and analysis of these PQQs by capillary electrophoresis with specially crafted microelectrodes. Analysis by capillary electrophoresis (14) would be expected to provide femtomolar (10^{-15} M) to attomolar (10^{-18} M) detection levels for these substances in biological fluids in which similar electroactive interfering substances are not present. This strategy should also provide a more complete separation of PQQ **1** and isomer **4**.

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REFERENCES

1. Salisbury, S. A., Forrest, H. S., Cruse, W.B.T., and Kennard, O. (1979) Nature (London) 280, 843-844.
2. Duine, J. A., Frank, J. Jr., Van Zeeland, J. K. (1979) FEBS Lett. 108, 443-446.
3. Steinberg, F. M., Gershwin, M. E., Rucker, R. B. (1994) J. Nutri. 124, 744-753.
4. Quandt, K. S., Hultquist, D. E. (1994) Proc. Natl. Acad. Sci. 91, 9322-9326.
5. Janes, S. M., Mu, D., Wemmer, D., Smith A. J. Kaur, S., Maltby, D., Burlingame, A. L. and Klinman, J. P. (1990) Science 248, 981-987.
6. McIntire, W. S. (1992) Essays Biochem. 27, 119-134.
7. Klinman, J. P. and Mu. D. (1994) Annu. Rev. Biochem. 63, 299-344.
8. Zhang, Z. P., Tillekeratne, L.M.V., Hudson, R. A. (1995) submitted for publication.
9. Kano, K., Mori, K., Uno, B. and Kubota, T. (1990) Bioelectrochem. Bioenerg. 23, 227-138.
10. Kano, K., Mori, K., Uno, B., and Kubota, T. (1990) Bioelectrochem. Bioenerg. 24, 193-201.
11. Mackenzie, A.R. Moody, and Rees, C.W. (1986) Tetrahedron 42, 3259-3268.
12. Charton, M. (1964) J. Am. Chem. Soc. 86, 2033-2037.
13. Perrin, D.D., Dempsey, B. and Serjeant, E.P. (1981) pK_a Prediction for Organic Acids and Bases. p131. Chapman and Hall Ltd. New York.
14. Ewing, A.G., Mesaros, J.M. and Gavin, P.F. (1994) Anal. Chem. 66, 527A-537A.