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Note

Pro-Oxidant Action of Pyrroloquinoline Quinone: Characterization of Protein Oxidative Modifications

Takeshi Ishii,[1,†] Mitsugu Akagawa,[2] Yuji Naito,[3] Osamu Handa,[3] Tomohisa Takagi,[3] Taiki Mori,[1] Shigenori Kumazawa,[1] Toshikazu Yoshikawa,[3] and Tsutomu Nakayama[1]

[1]*Department of Food and Nutritional Sciences, and Global COE Program, University of Shizuoka, Shizuoka 422-8526, Japan*
[2]*Department of Biological Chemistry, Division of Applied Life Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai 599-8531, Japan*
[3]*Department of Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan*

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Pyrroloquinoline quinone (PQQ), a putative essential nutrient, is a redox modulator in cell and animal models. Here we characterized PQQ-induced protein oxidative modifications in a model peptide and protein, and we propose that the mechanism of protein modification by PQQ is redox cycling-mediated oxidation. PQQ may contribute to the regulation of intracellular protein functions through its prooxidant action.

Key words: pyrroloquinoline quinone; prooxidant action; protein oxidative modification

Initially identified as a novel cofactor of various bacterial dehydrogenases,[1)] pyrroloquinoline quinone (PQQ) is indicated by various studies to be an essential animal nutrient. PQQ-Deficient animals display a variety of illnesses.[2)] Mice fed a PQQ-deficient diet grow slowly, have fragile skin and a reduced immune response, and do not reproduce well.[3)] Although PQQ is not synthesized in mammals, in young mice it is rapidly absorbed by the lower intestine after oral administration and is subsequently excreted by the kidneys.[4)] PQQ has also been found in a variety of animal cells and cell products, including milk, serum, and synovial fluid.[5)] Moreover, it has been reported that it acts as a mammalian redox cofactor for 2-aminoadipic acid dehydrogenase.[6)] These studies indicate that PQQ is a biologically active micronutrient in mammals. Although the potential physiological role of PQQ in mammals remains to be fully elucidated, it can acts as an antioxidant or a pro-oxidant. Previous studies have indicated that PQQ can protect against several types of oxidative damage.[7)] In contrast, it has also been found to cause extensive cell death among cells *in vitro*.[7)] This effect is inhibited by catalase and has been indicated to be due to the generation of H_2O_2 during the autoxidation of PQQ in the culture medium. Reactive oxygen species (ROS) can cause oxidative modifications to sensitive proteins that can lead to changes in protein function.[8)] Protein oxidation has emerged as a potential mechanism of dynamic, post-translational regulation of a variety of regulatory, structural, and metabolic proteins,[9)] but the regulation mechanism of the PQQ-mediated modulation of proteins remains unclear. In the present study, we used mass spectrometry techniques to characterize the protein oxidative modifications resulting from PQQ treatment in a model peptide and protein.

PQQ was purchased from Sigma-Aldrich (St. Louis, MO). In order to characterize PQQ-induced protein oxidation, we examined the oxidation behavior of PQQ with a model peptide of sequence MHRQETVDC obtained from Wako Pure Chemical Industries (Osaka, Japan), which contains several oxidation-sensitive amino acid residues: one methionine residue, one histidine residue, one arginine residue, and one cysteine residue. The peptide (0.1 mg/ml) was incubated with 0–1.0 mM PQQ in 50 mM sodium phosphate buffer (pH 7.4) at 37 °C for 24 h in the presence and the absence of 0.1 mM Cu^{2+}. To improve the ionization efficiency of MS, samples were purified with Zip Tip μ-C18 (Millipore, Bedford, MA) before MS and tandem MS (MS/MS) analysis. Peptides were mixed with 2.5 mg/ml of α-cyano-4-hydroxycinnamic acid from Bruker Daltonics (Bremen, Germany) containing 50% acetonitrile and 0.1% trifluoroacetic acid, and were dried on stainless steel targets at room temperature and pressure. The analyses were performed by an UltraFLEX matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF) MS (Bruker Daltonics). All analyses were carried out in the positive ion mode, and the instrument was calibrated immediately prior to each series of studies. As shown in Fig. 1A (first panel), MALDI-TOF MS analysis of the native peptide revealed a peak at m/z 1,118.37, in agreement with the theoretical molecular mass from the sequence. When the model peptide was incubated with 0.5 mM PQQ for 24 h, two peaks, corresponding to the addition of two or three molecules of oxygen (+32.0 or +48.0 Da) per peptide, were detected (Fig. 1A, second panel). Some oxidized peptides corresponding to the addition of one to four

† To whom correspondence should be addressed. Fax: +81-54-264-5551; E-mail: ishii_t@u-shizuoka-ken.ac.jp

Abbreviations: GSTP1, glutathione-*S*-transferase subunit-P1-1; HRP, horseradish peroxidase; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; MS, mass spectrometry; MS/MS, tandem MS; PAGE, polyacrylamide gel electrophoresis; PQQ, pyrroloquinoline quinone; $PQQH_2$, dihydroquinone PQQ; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate

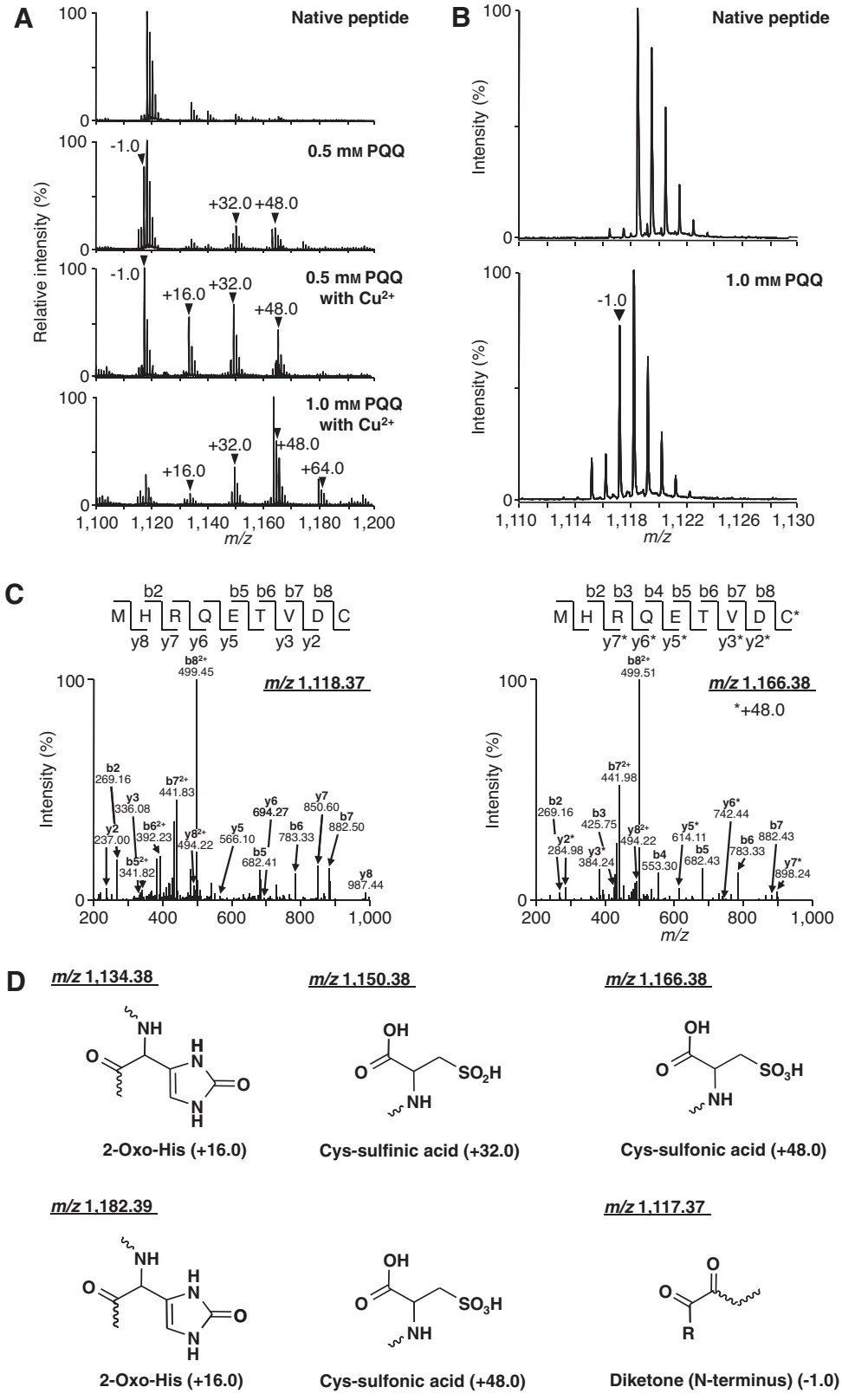

Fig. 1. PQQ-Induced Oxidative Modification of Amino Acid Residues in the Model Peptide.

A, MS spectra of PQQ-treated peptides. The model peptide was incubated with PQQ (0–1.0 mM) for 24 h in the presence and the absence of Cu^{2+}. B, Detection of the carbonylated peptide induced by PQQ. The model peptide was incubated with 1.0 mM PQQ. C, Identification of the oxidation site in the PQQ-treated peptide. MS/MS spectra of the native (m/z = 1,118.37, left panel) and oxidized (m/z = 1,166.38, right panel) peptides. D, Characterization of oxidative modification in the peptide.

molecules of oxygen per peptide (+16.0, +32.0, +48.0, and +64.0 Da) were observed in the presence of Cu^{2+} (Fig. 1A, third and fourth bottom panels). In addition, the PQQ-treatments induced 1.0 Da decreases as compared to the native peptide (Fig. 1A and B). In MS/MS analysis of the native peptide at m/z 1,118.37, N-terminal product ions (b2, b5, b6, b7, and b8) and C-terminal product ions (y2, y3, y5, y6, y7, and y8) were observed (Fig. 1C, left panel). MS/MS analysis of the oxidized peptide at m/z 1,166.38 revealed the N-terminal

product ions (b2, b3, b4, b5, b6, b7, and b8) (Fig. 1C, right panel). Compared to the native peptide, C-terminal fragment ions (y2, y3, y5, y6, and y7) showed a 48.0-Da increase. These results indicate that Cys-sulfonic acid is generated at the C-terminal cysteine residue in the peptide. Furthermore, MS/MS analysis confirmed the formation of Cys-sulfinic acid, 2-oxo-histidine, and diketone (N-terminus) in the peptide (data not shown). Fig. 1D summarizes the results of MS/MS analysis of the peptides by PQQ.

To detect the protein oxidative modifications by PQQ directly, we evaluated protein thiol oxidation and carbonyl formation due to PQQ-treatment using human recombinant glutathione-*S*-transferase subunit-P1-1 (GSTP1) as a convenient model protein. It contains two reactive thiol groups per subunit and is known to be highly sensitive to oxidation by pro-oxidant agents.[10,11] GSTP1 (1.0-mg/ml) (Sigma-Aldrich) was incubated with 0–1.0 mM PQQ in 50 mM sodium phosphate buffer (pH 7.4) at 37 °C for 2 h. The reaction was terminated by centrifugal filtration (Microcon 10, molecular weight cut-off of 10,000; Millipore) to remove the PQQ. The native and PQQ-treated GSTP1 were incubated with sodium dodecyl sulfate (SDS) sample buffer with and without a dithiothreitol. The samples ware separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 4–12% gel (NuPAGE-Bis-Tris Gel System, Life Technologies, Invitrogen, Carlsbad, CA). The gel was transblotted onto a nitrocellulose or PVDF membrane, incubated with EzBlock (ATTO, Tokyo) for blocking, washed, and incubated with anti-GSTP1 rabbit polyclonal antibody. This procedure was followed by the addition of HRP-linked anti-rabbit IgG and enhanced chemiluminescence reagents. The bands were visualized by Cool Saver AE-6955 (ATTO). When subjected to non-reducing SDS–PAGE, the native (unoxidized) form of GSTP1 migrated as a main protein band of 23-kDa, and the other immunoreactive bands, being higher than 23-kDa, were observed (Fig. 2A, left panel). However, upon incubation with PQQ, GSTP1 was converted to a higher molecular weight protein species, probably corresponding to the intermolecular cross-linking reactions. No formation of these modified proteins was detected during reducing SDS–PAGE (Fig. 2A, right panel), suggesting that PQQ induced reversible polymerization of GSTP1 with disulfide linkage through thiol oxidation. PQQ-treated GSTP1 has also been incubated with 5.0 mM biotin-LC-hydrazide at room temperature for 1 h in the dark.[11] The biotin-labeled proteins were subjected to SDS–PAGE using 4–12% gel and Western blotting with HRP-avidin. As shown in Fig. 2B, protein carbonyl increased remarkably due to the oxidation of GSTP1. These results strongly suggest that protein thiol oxidation and carbonyl formation are major events caused by PQQ-induced protein oxidation.

It has been reported that several reducing agents reduce PQQ to dihydroquinone PQQ ($PQQH_2$) *via* semiquinone PQQ ($PQQ^{\bullet}H$), and that quinone catalyzes the generation of O_2^- and H_2O_2 through its reduction and oxidation cycle.[12] In addition, *O*-quinone acts as a catalyst for the thiol-mediated reduction of O_2, which leads to the generation of ROS and thiol oxidation.[13] The present study indicate that PQQ oxidized Cys to

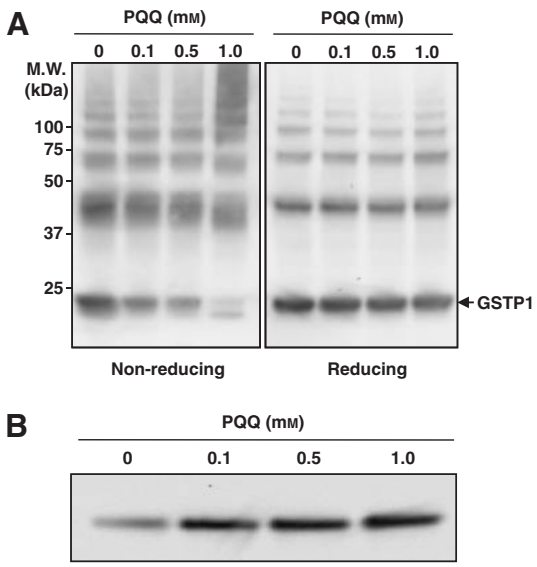

Fig. 2. Oxidative Modification of GSTP1 by PQQ.
GSTP1 was incubated with PQQ (0–1.0 mM) in sodium phosphate buffer (pH 7.4) at 37 °C for 2 h. A, Detection of protein thiol oxidation by non-reducing SDS–PAGE (left panel) and reducing SDS–PAGE (right panel) followed by Western blotting using the anti-GSTP1 antibody. B, Detection of protein carbonyl formation. PQQ-treated GSTP1 was incubated with 5.0 mM biotin-LC-hydrazid, and the biotin-labeled proteins were subjected to SDS–PAGE, followed by Western blotting with HRP-avidin.

Cys-sulfinic acid and Cys-sulfonic acid (Fig. 1). The thiol groups of proteins are particularly susceptible to oxidation by H_2O_2, and thiol can form sulfoxidation products as a result of these oxidation reactions. This suggests that PQQ generates H_2O_2 through its reduction and oxidation cycle under these experimental conditions. Oxidation of His to 2-oxo-His was also observed in the presence of Cu^{2+} (Fig. 1A and D). It is known that His residues are targets for metal-catalyzed protein oxidation.[14] In its simplest form, the mechanism of 2-oxo-His formation requires the addition of $HO^{\bullet}$ at the C-2 position of the imidazole ring by a Fenton-type reaction of Cu^+ with H_2O_2. These results and observations strongly suggest that PQQ oxidizes the amino acid residues in proteins through the generation of H_2O_2 and/or metal-catalyzed generation of $HO^{\bullet}$. PQQ catalyzes the continuous oxidation of primary amines. Shah have reported that PQQ catalyzes the nonspecific oxidation of peptidyl amine in elastin and collagen substrates.[15] Elastin oxidation under aerobic conditions results in the formation of aldehydes. Moreover, various quinone compounds are known to undergo the oxidative deamination reaction.[16] In the present study, we observed oxidative deamination of the N-terminal amino group to aldehyde in the PQQ-treated peptide (Fig. 1B and D). Amine oxidases carry out reactions in two steps in a ping-pong reaction.[15] The first part of the reaction initiates reduction of the cofactor and conversion of the amine substrate to an aldehyde product. In the second part of the reaction, ammonia and hydrogen peroxide are released with re-oxidation of the cofactor. Our results suggest that this action is attributable to the amine oxidase-like activity of PQQ.

On this basis, the proposed mechanism of protein oxidative modification by PQQ is redox cycling-medi-

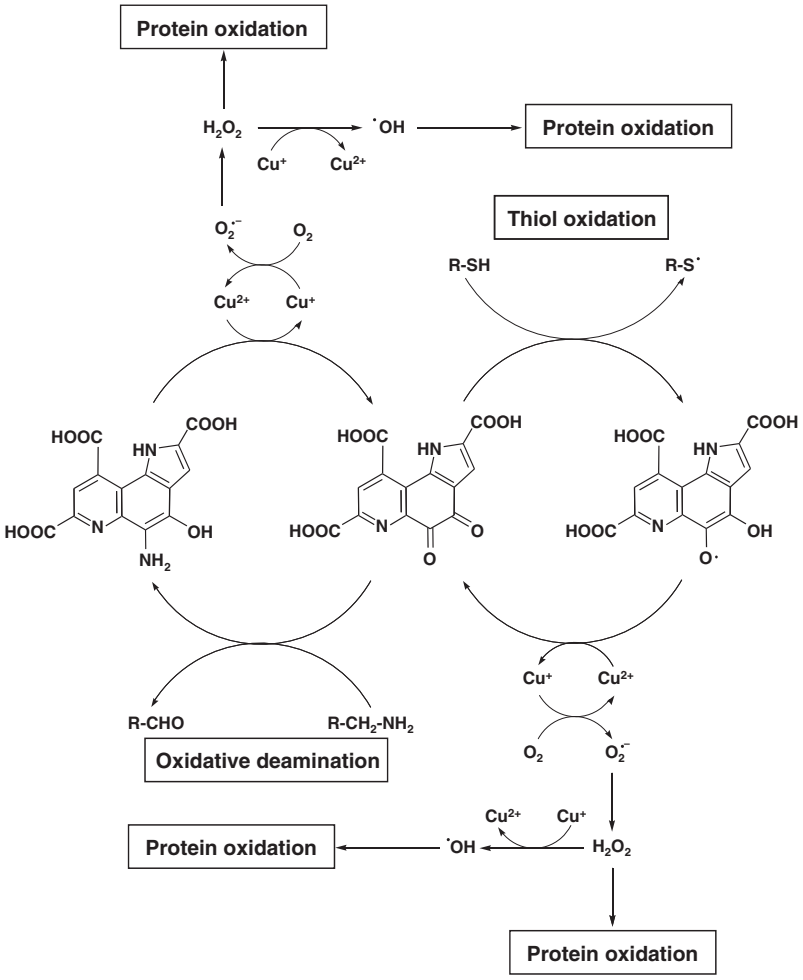

Fig. 3. Proposed Mechanism of Protein Oxidative Modification Induced by PQQ in the Presence of Cu^{2+}.

ated oxidation, as shown in Fig. 3. ROS play a central role as second messengers in many signal transduction pathways, where they can post-translationally modify proteins *via* the oxidation of redox-sensitive amino acid residues. In this study, using the model peptide and protein, we identified details of PQQ-induced protein oxidation under these experimental conditions, but oxidative modification in the other oxidation-sensitive amino acid residues, such as Tyr and Trp has not been completely characterized. Further research on protein oxidative modification by PQQ is needed to characterize fully the oxidation behavior of PQQ with protein.

In previous studies, PQQ protected against cell injury associated with oxidative stress.[7,17] Moreover, a recent study indicates that PQQ is easily reduced by GSH and Cys in buffer solution at neutral pH and results in $PQQH_2$, a powerful antioxidant.[18] Under these conditions, GSH and Cys are oxidized to GSSG and cystine by pro-oxidant action of PQQ through its redox cycle. Based on the present findings and observations, we conclude that PQQ contributes to the regulation of intracellular protein functions by pro-oxidant and anti-oxidant behaviors. Future studies are required to confirm this and to address the question whether PQQ is able to modulate the functions of intracellular protein through protein oxidative modification.

References

1) Anthony C and Zatman LJ, *Biochem. J.*, **104**, 960–969 (1967).
2) Killgore J, Smidt C, Duich L, Romero-Chapman N, Tinker D, Reiser K, Melko M, Hyde D, and Rucker RB, *Science*, **245**, 850–852 (1989).
3) Bishop A, Gallop PM, and Karnovsky ML, *Nutr. Rev.*, **56**, 287–293 (1998).
4) Smidt CR, Unkefer CJ, Houck DR, and Rucker RB, *Proc. Soc. Exp. Biol. Med.*, **197**, 27–31 (1991).
5) Gallop PM, Paz MA, Fluckiger R, and Kagan HM, *Connect. Tissue Res.*, **29**, 153–161 (1993).
6) Kasahara T and Kato T, *Nature*, **422**, 832 (2004).
7) He K, Nukada H, Urakami T, and Murphy MP, *Biochem. Pharmacol.*, **65**, 67–74 (2003).
8) Finkel T and Holbrook NJ, *Nature*, **408**, 239–247 (2000).
9) Rhee SG, *Science*, **312**, 1882–1883 (2006).
10) Baty JW, Hampton MB, and Winterbourn CC, *Biochem. J.*, **389**, 785–795 (2005).
11) Ishii T, Sakurai T, Usami H, and Uchida K, *Biochemistry*, **44**, 13893–13901 (2005).
12) Hiraku Y and Kawanishi S, *FEBS Lett.*, **393**, 317–320 (1996).
13) Kumagai Y, Koide S, Taguchi K, Endo A, Nakai Y, Yoshikawa T, and Shimojo N, *Chem. Res. Toxicol.*, **15**, 483–489 (2002).
14) Uchida K, *Amino Acids*, **25**, 249–257 (2003).
15) Shah MA, Scaman CH, Palcic MM, and Kagan HM, *J. Biol. Chem.*, **268**, 11573–11579 (1993).
16) Stites TE, Mitchell AE, and Rucker RB, *J. Nutr.*, **130**, 719–727 (2000).
17) Nishigori H, Yasunaga M, Mizumura M, Lee JW, and Iwatsuru M, *Life Sci.*, **45**, 593–598 (1989).
18) Ouchi A, Nakano M, Nagaoka S, and Mukai K, *J. Agric. Food Chem.*, **57**, 450–456 (2009).