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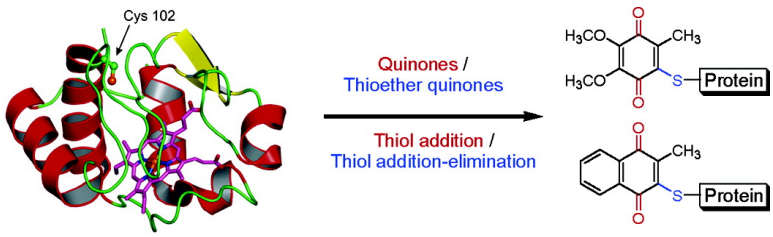
J. Am. Chem. Soc., **2005**, 127 (17), 6140-6141• DOI: 10.1021/ja050974x • Publication Date (Web): 09 April 2005



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Published on Web 04/09/2005

Site-Specific Binding of Quinones to Proteins through Thiol Addition and Addition−Elimination Reactions

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Enzymes and biological complexes often utilize noncovalently or covalently bound cofactors for their functions. Quinones are such molecules. Three classes of *p*-quinone: ubi-, mena-, and plastoquinone are noncovalently bound components in photosynthetic electron transfer (ET) and respiratory chains.[1] Nature has also crafted a variety of covalently bound quinone cofactors[2] including a recently found cysteine tryptophylquinone (CTQ).[3] CTQ consists of an *o*-quinone-modified tryptophan side chain cross-linked to a cysteine via a thioether bond. These natural constructs are in favor of generating new functions through chemical modification of proteins with quinones.[4] Recently, 3,4-dihydroxy-L-phenylalanine was incorporated into proteins by expanded genetic-code technique to generate *o*-quinone bound to proteins.[5] However, is it possible to simply modify side chains of amino acids with *p*-quinones to realize a similar goal? The sulfur addition and substitution reaction to quinones has been thoroughly investigated,[6] which together with their redox cycling is attributed to one of their toxic mechanisms.[7,1b] For instance, 2,3-dimethoxy-5-methyl-1,4-benzoquinone (UQ-0),[8] and 2-methyl-1,4-naphthoquinone (menadione, MQ-0)[9] were covalently bound to the β-93 cysteinyl residue of human oxyhemoglobin via 1,4-Michael-type of thiol addition to quinones (Scheme 1A). These quinones have also been shown to induce the dimerization of proteins such as ArB sensor kinase via an intermolecular disulfide bond[10] and even the polymerization of the phosphorylated tubulin binding region of tau protein.[11] To better understand the molecular action mechanisms of these quinones and to provide a general and simple method of binding quinones to proteins, we studied the interactions of UQ-0, MQ-0, and 2,3,5-trimethyl-1,4-benzoquinone (TMBQ) with proteins.

Cytochrome *c* (cyt *c*) as a common protein electron carrier is often used to prepare ET models by chemical modification.[12] Here yeast iso-1 cyt *c*[13] from *Saccharomyces cerevisiae* with free Cys-102 was used as a model protein. For the coupling reactions, UQ-0, MQ-0, and TMBQ (dissolved in acetonitrile 1.0 mg/mL, all in 20-fold molar excess) were incubated with cyt *c* at a concentration of 1.0 mg/mL (79 μM) in degassed 50 mM Tris-HCl buffer, 100 mM NaCl, pH 7.5. The solutions were then stirred for 2−6 h at RT under argon in the dark. Reverse phase HPLC of reaction products between UQ-0, MQ-0 (Figure 1A, solid line), and TMBQ and cyt *c* at pH 7.5 shows the formation of the respective quinone cyt *c* adducts. (Yields, ESI mass spectra, and ESI-MS data of all products in Supporting Information.) The chromatograms show also a disulfide dimer of cyt *c* and a mixture of unreacted cyt *c* and oxidized cyt *c* with sulfinic acid (SO_2H). At pH 4 all reactions occurred with an increase of quinone−cyt *c* adducts and a decrease of disulfide dimer as compared to pH 7.5. The difference masses between cyt *c* and UQ−cyt *c*, MQ−cyt *c*, TMBQ−cyt *c* were 180, 167, and 155 ± 1.2, respectively, which indicated that only one molecule of each quinone was attached. The quinone-modified proteins were isolated by HPLC.

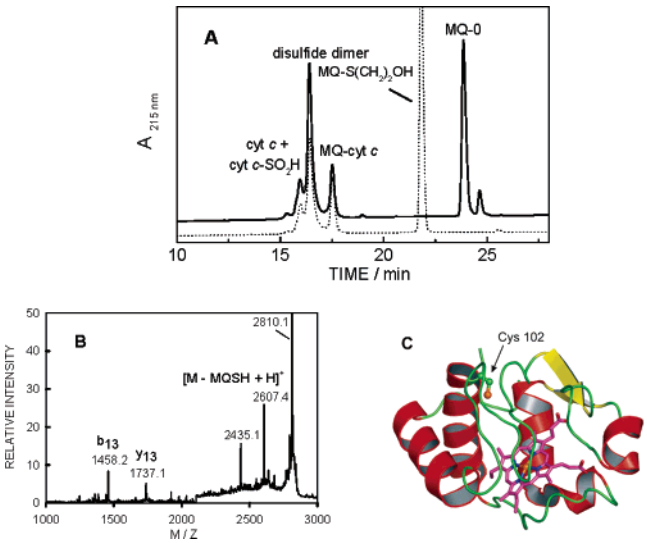
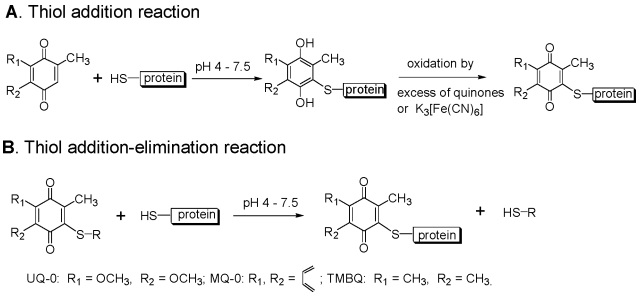


Figure 1. (A) HPLC chromatograms monitored at 215 nm of reactions between MQ-0 (solid line), MQ-S$(CH_2)_2$OH (dotted line) and cyt *c* at pH7.5 after 3 h. (B) PSD-MALDI-mass spectrum of MQ−peptide 81−103. (C) Cartoon structure of yeast iso-1 cyt *c* (1ycc PDB) drawn with PyMOL. Cys-102 is modified by quinones via thioether bond formation.

Scheme 1. Covalent Attachment of Quinones to Cysteine of Proteins via Thiol Addition (A) and Thiol Addition−Elimination Reaction (B)



To further investigate the properties of such thioether quinones, model compounds were synthesized by coupling of UQ-0, MQ-0, and TMBQ with 2-mercaptoethanol [HS$(CH_2)_2$OH] and/or *N*-acetyl L-cysteine [(N-Ac)Cys] using a reductive addition and oxidation reaction (Supporting Information). Unexpectedly, the oxidized thioether quinone adducts showed reactivity toward cyt *c* similar to that of their parent quinones through a thiol addition−elimination reaction (Scheme 1B). Figure 1A (dotted line) shows the formation of the same products as that of MQ-0 by reaction of MQ-S$(CH_2)_2$-OH (an anti-cancer substance)[14] with cyt *c*. This demonstrated the covalent binding of thioether quinone adduct to protein via a thiol-exchange reaction, which provided experimental evidence for the

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10.1021/ja050974x CCC: $30.25

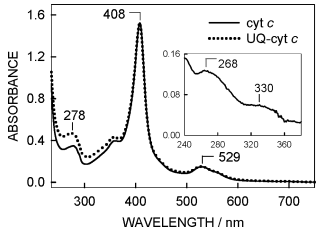

Figure 2. Normalized UV−vis spectra of UQ-cyt *c* (dotted line) and cyt *c* (solid line) in 50 mM Tris-HCl, 100 mM NaCl, pH 8.0. (Inset) Difference between spectra.

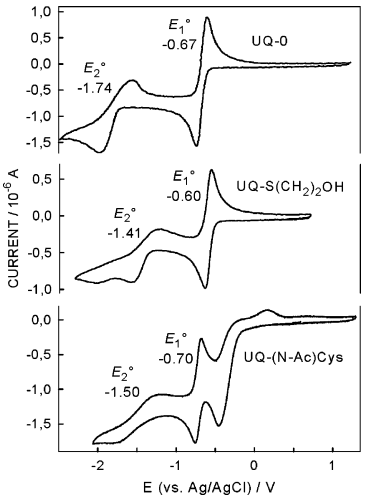

Figure 3. Cyclic voltammograms of UQ-0, UQ-S(CH$_2$)$_2$OH, and UQ-(N−Ac)Cys in CH$_3$CN/0.1 M tetrabutylammonium hexafluorophosphate.

mechanism of its inhibition of tyrosine phosphatase.[14] It also corrects the conclusion that thioether quinone conjugates are inactive toward other thiols.[15] For ET studies free thiols should be removed to avoid a possible loss of thioether quinone moiety in proteins.

The specific binding site of the three quinones in cyt *c* was investigated by bromide cyanogen cleavage. The peptide fragments cleaved at Met-80 [sequence 81−103: AFGGLKKEKDRNDLITYL-KKA**C**(102)E] containing a quinone moiety were isolated by HPLC. Post-source decay-matrix assisted laser desorption ionization mass spectrometry (PSD-MALDI-MS) was used to investigate their fragmentation. The mass spectrum of the MQ−peptide (*m*/*z* 2810) (Figure 1B) shows two significant fragment ions at *m*/*z* 1737 and 1458 which correspond to the y_{13} ion with a menaquinone moiety and b_{13} ion without such modification, respectively. Therefore, MQ-0 should be located within the last 10 amino acids. In addition, a significant fragment ion at *m*/*z* 2607 can be attributed to the peptide after loss of menaquinone moiety with a thiol group (MQSH) via β-elimination.[16] This confirmed that MQ-0 was covalently bound to Cys-102 (Figure 1C). A similar fragmentation pattern for UQ− and TMBQ−peptide 81−103 indicated the same binding site as that found with MQ-0.

The UV−vis spectrum of UQ−cyt *c* (Figure 2) showed in addition to the absorption of cyt *c* distinct features with maxima at 268 and 330 nm characteristic for the quinone with thioether linkage to the protein, as highlighted in the inset.

Changes of redox properties of the quinones by sulfur substitution were investigated by cyclic voltammetry in acetonitrile. The cyclic voltammograms (CV) of UQ-0 and UQ-S(CH$_2$)$_2$OH (Figure 3, *top* and *middle*) show two characteristic one-electron reduction steps with redox potentials $E_1°$ and $E_2°$ and a minor change of $E_1°$ by the thioether bond from −0.67 to −0.60 V. The CV of UQ-(N-Ac)Cys (Figure 2, *bottom*) shows, in addition to the two reduction steps, an extra one at a more positive potential due to the carboxylic acid group of Cys.[17] The CVs of MQ-0, MQ-(N-Ac)Cys, TMBQ, and TMBQ-(N-Ac)Cys were similar to those of the UQ derivatives except for more negative values (Supporting Information). The small redox shift of $E_1°$ is probably due to a modification of the electron density by sulfur addition. This negative shift of the redox potentials is consistent with previous data in aqueous solution found for MQ-(N-Ac)Cys and MQ-0 glutathione conjugates.[7,18]

Taken together, the electrophilicity of quinones enables their specific modification of the free Cys-102 of yeast iso-1 cyt *c* as that of oxyhemoglobin.[8,9] The oxidative property causes the intermolecular disulfide bond formation as also found for ArB sensor kinase[10] and even polymerization[11] of proteins. Through separation of quinone thioether protein adducts from dimers of protein the quinones bound to proteins were available. The generated quinones with a thioether bond possess common structural features of both noncovalently bound ubi-, mena-, and plastoquinone and covalently bound CTQ.[3] The method provides a general, simple, and fast route to attach different quinones to cysteine-containing proteins. Thioether quinone conjugates show addition−elimination reaction toward thiol groups of proteins, which suggests that these quinones may be transferred between proteins. These results should also contribute to the understanding of biological activities, toxicity, and the anti-cancer mechanism of quinones and thioether quinone adducts. De novo design and chemical synthesis of proteins with quinones, heme, and flavin are under investigation for ET studies.[19]

Acknowledgment. Support from the Volkswagen-Stiftung is gratefully acknowledged. We thank Bettina Knapp for the measurements of the ESI and PSD-MALDI mass spectra.

Supporting Information Available: Experimental materials and details for the synthesis of model compounds, UV−vis spectra of quinone−cyt *c*, ESI- and MALDI-PSD mass spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA050974X