

Communications

Menadione-Mediated Phosphate Ester Hydrolysis

Yunjung Choi, Changwon Oh, and Seung Wook Ham*

Department of Chemistry, Chung-Ang University, Seoul 156-756, Korea. *E-mail: swham@cau.ac.kr
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Menadione (2-methyl-1,4-naphthoquinone, or vitamin K₃), a synthetic congener of naturally occurring vitamin K₁ and K₂, has increasingly been of interest given its broad range of antitumor activity in human cells.¹ Although oxidative stress was a common mechanism of tumor growth inhibition by menadione,² it was reported in 1993 by Juan and Wu that menadione induces alterations in the phosphorylation status of p34^{cdc2} kinase (cdk1) and the activity of protein tyrosine phosphatases (PTPases) during cell cycle progression.³ On the basis of this result, this lab demonstrated that menadione is an inactivator of cdc25 phosphatase because cdc25 phosphatase dephosphorylates cdk at the Thr-14 and Tyr-15 residues and thus stimulates cdk activity.⁴ Since then, another possible mechanism of menadione cytotoxicity has been ascribed to inactivation of cdc25 phosphatases.⁵

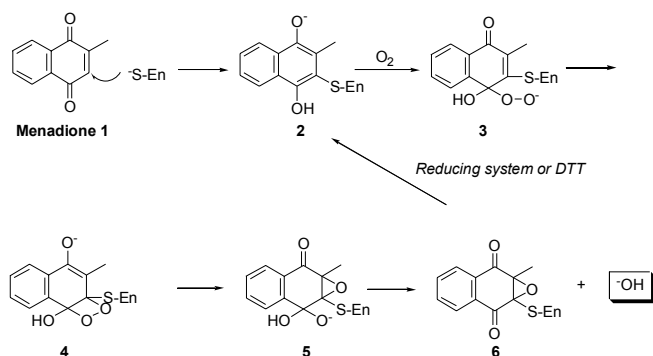
However, some time earlier, Statdman discovered that the phosphatase derived from *Clostridium Sticklandii* required the presence of menadione and of added thiol for its activity.⁶ The Wu group also pointed out that the p34^{cdc2} activity of menadione-treated cells was increased at early recovery times for unknown reasons.³ These observations are interesting as menadione acts as a phosphatase cofactor under certain conditions, differing from the mechanism of action of menadione as a phosphatase inactivator.

Meanwhile, the mechanistic investigations of vitamin K₁ have provided evidence for the production of a strong base as the outcome of oxygenation.⁷ It has been suggested that the gen-dialkoxide or hydroxide ion in a hydrophobic environment might be possible candidates for the active base. At this

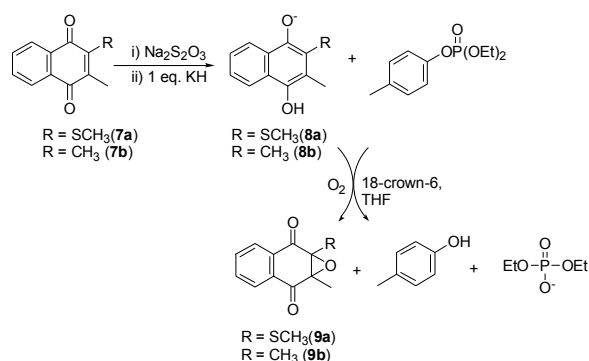
point, it is the contention of the authors that this capacity to produce the hydroxide ion can be applied to the intriguing observations of phosphatase action. As shown in Scheme 1, addition of the thiolate group in the enzyme active site would lead directly to hydroquinone anion **2**. If molecular oxygen were a constituent of the active site of the enzyme, trapping with oxygen would then lead to naphthoquinone oxide **6** formation and the production of a hydroxide ion. In the proper hydrophobic setting, the latter can be sufficient to hydrolyze an aromatic phosphate.

To examine the chemical behavior of the hydroquinone anion in the presence of oxygen, first, thiomethyl vitamin K derivative **7a** was prepared by treatment of menadione with methane thiol, which was then readily converted to the hydroquinone by reduction of the aqueous sodium hydrosulfite. Next, the resulting hydroquinone was treated with 1.0 eq. of potassium hydride and 18-crown-6 in THF, and the reaction mixture stirred under an atmosphere of oxygen in the presence of diethyl *p*-tolyl phosphate. We expected that hydrolysis of the phosphate ester would occur by the resulting hydroxide anion during oxygenation. However, treatment with oxygen did not lead to the formation of the expected hydrolysis product. The ¹H-NMR spectrum of the crude product showed small amounts of the corresponding epoxy quinone **9a**,⁸ no hydrolysis product was detected.

In another model experiment, a tetrahydrofuran solution containing potassium hydroquinone anion **8b**, from the reaction of potassium hydride and 2,3-dimethyl naphthohydroquinone, was stirred at room temperature with 18-crown-6. Syringe pump



Scheme 1



Scheme 2

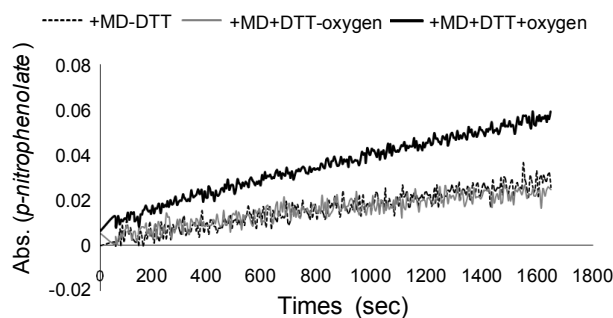


Figure 1. Production of *p*-nitrophenolate by preincubated Cdc25A phosphatase with a high concentration of menadione in the various conditions.

infusion of 2.0 eq of oxygen into the solution yielded the hydrolysis product in 20 ~ 30% yield, and the naphthoquinone epoxide **9b** in 40 ~ 50% isolated yield. The ^1H -NMR spectrum of the crude product, obtained by the ethereal extraction, showed epoxy quinone **9b** with a minor quantity of naphthoquinone **7b**, diethyl *p*-tolyl phosphate, and two products from phosphatase ester hydrolysis. As expected, no hydrolysis product was observed in a control reaction carried out under an oxygen-free atmosphere of nitrogen, showing that the monoanion of 2,3-dimethyl naphthohydroquinone **8b** was not a sufficient nucleophile to hydrolyze the phosphate ester under the reaction conditions. Less than 1% of tolyl alcohol would readily have been detected by the presence of the three-proton singlet of tolyl alcohol at 2.35 ppm in the 600 MHz ^1H -NMR spectra of the total crude reaction products. It was important to conduct the control reaction under scrupulously oxygen-free conditions; otherwise, trace amounts of hydrolysis product were observed.

On the basis of these results, the effects of menadione on enzymatic activity were further investigated. Thus, Cdc25A phosphatase was preincubated in the incubation buffer containing 20 mM Tris (pH 8.0), 1.0 mM EDTA, and 0.1% β -mercaptoethanol, with excess menadione (100 μM) that was 20-fold greater than the IC_{50} value of menadione against Cdc25A. After a 20-min preincubation, the enzyme activity was monitored at 410 nm, using 40 μM *p*-nitrophenyl phosphate as a chromogenic substrate under various conditions. As shown in Fig. 1, there was an increase in the production of *p*-nitrophenolate in the presence of DTT under atmospheric pressure of oxygen, when compared to those in the absence of DTT or without oxygen. These results supported the production of the hydroxide ion by trapping with oxygen as proposed in Scheme 1. It has been established that vitamin K oxide is easily transformed to vitamin K by the reductase or in the reducing system.⁹ Therefore, the resulting epoxide **6** could also be returned to the starting hydroquinone anion by the reducing agent, DTT, indicating that the irreversibly inactivated enzyme with menadione might dephosphorylate the substrate by the hydroxide ion through the proposed oxygenation cycle.

It is well established that PTPase completes its action in two major steps.¹⁰ In the first step, the phosphoryl group from the substrate is transferred to the nucleophilic cysteine, forming a phosphoenzyme intermediate. This intermediate is hydro-

lyzed, leading to the regeneration of the enzyme and release of an inorganic phosphate in the second step, which has been suggested as a rate-limiting step by the use of kinetic techniques. Therefore, if molecular oxygen and reducing agents were supplied, the hydroxide ion could be continuously produced as shown in Scheme 1 and readily hydrolyze the phosphate monoester of proteins without the rate-limiting hydrolysis of the phosphoenzyme intermediate. Finally, consistent with observations of phosphatase action by Wu and Stadtman, the model and enzymatic analyses presented herein, therefore, lead to a new interpretation of the role of menadione as a phosphatase cofactor.

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References and notes

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- ^1H NMR (300 MHz, CDCl_3); δ 1.71 (s, 6H), 7.68-7.71 (m, 2H), 7.92-7.95 (m, 2H). ^{13}C NMR (75 MHz, CDCl_3); δ 11.7 (q, J = 129 Hz), 65.1 (s), 127.1 (d, J = 164 Hz), 132.1 (s), 134.2 (dd, J = 163, 8 Hz), 193 (s). The IR spectrum (neat) showed at 1693 (s). The mass spectrum showed peaks at m/e (rel. intensity), 202 (M^+ , 23), 187 (13), 160 (47), 131 (22). Exact mass calculated for $\text{C}_{12}\text{H}_{10}\text{O}_3$: 202.0630, found: 202.0630.
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