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Inhibitory Effects of 1,4-Naphthoquinone Derivatives on Rat Cytochrome P4501A1–Dependent Monooxygenase Activity in Recombinant Yeast Microsomes[1]

Kuniyo Inouye,*[,2] Akitoshi Saito,* Manabu Orita,* Ben'ichiro Tonomura,*[,3] Hiromasa Imaishi,† and Hideo Ohkawa†

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502; and †Department of Biological and Environmental Science, Faculty of Agriculture, Kobe University, Nada-ku, Kobe 657-0013

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We reported previously that various naphthoquinone derivatives inhibited cytochrome P450–dependent monooxygenase of liver and placenta microsomes [Muto, N. *et al.* (1987) *Biochem. Biophys. Res. Commun.* 146, 487-494]. To understand the complex inhibitory behaviors that were observed, it is desirable to study the relationship between structure and inhibitory activity of naphthoquinones in a simplified system containing a single P450 species. In the present study, the inhibitory effects of six derivatives of 1,4-naphthoquinone (hereafter referred to as NQ) on rat cytochrome P4501A1–dependent 7-ethoxycoumarin *O*-deethylation were examined using yeast microsomes containing overexpressed rat P4501A1. Of these, 2-methyl-5-hydroxy-NQ, 2-methyl-NQ, 2-hydroxy-NQ, and NQ showed competitive inhibition, whereas 5,8-dihydroxy-NQ and 5-hydroxy-NQ showed noncompetitive inhibition. Judging from the inhibitor constant (K_i), the binding affinity of the four competitive inhibitors for the substrate-binding pocket of P4501A1 is in the order: 2-CH_3-5-OH-NQ > 2-CH_3-NQ > NQ ≫ 2-OH-NQ. On binding with P4501A1, 2-CH_3-5-OH-NQ, 2-CH_3-NQ, and NQ induced distinct Type II, Type I, and reverse Type I spectra, respectively. These results indicate that methyl and hydroxyl groups introduced into NQ have unique effects on their binding mode and binding affinity.

Key words: cytochrome P450, inhibition, monooxygenase, NADPH-cytochrome P450 reductase, 1,4-naphthoquinone.

1,4-Naphthoquinone derivatives are found in various parts of higher plants, such as leaves, flowers, roots, bark, and wood, and have interesting activities in physiology and pharmacology (*1*). 2-Methyl-1,4-naphthoquinone (menadione or vitamin K_3), phylloquinone (vitamin K_1), and menaquinone (vitamin K_2) were reported to have antihemorrhagic activity in chicks (*2*). 5,8-Dihydroxy-2-(1-hydroxy-4-methyl-3-pentenyl)-1,4-naphtoquinone (shikonin) was found to have antimicrobial and antifungal activities, and antitumor activity against Sarcoma 180 (*3*). These activities of 1,4-naphthoquinones appear to be based on their reduction by NADPH-P450 reductase (*4–6*) or DT-diaphorase (*7*) to yield superoxide radicals.

We have reported that various naphthoquinone derivatives, including 1,2-naphthoquinone and 1,4-naphthoquinone, which are known ingredients of Chinese medicines, inhibit monooxygenase activities of rabbit liver microsomes and human placenta microsomes (*1*). The inhibitory behaviors appear to be complicated, indicating the desirability of studying the relationship between structure and inhibitory activity of naphthoquinones in a simplified system containing a single P450 species. Recently, we have found that vitamins K_1 and K_2, which contain a 2-methyl-1,4-naphthoquinone ring, inhibit P4501A1-dependent 7-ethoxycoumarin *O*-deethylation (*8*). In the present study, we investigated the inhibitory effects of various 1,4-naphthoquinone derivatives (NQs) on cytochrome P4501A1–dependent monooxygenase activity to elucidate the inhibition mechanism, to survey the active site geography, and to design a potent inhibitor of P4501A1. Evidence obtained in this study might be pharmacologically interesting, because P4501A1 is induced in liver microsomes by polycyclic aromatic hydrocarbons such as dioxins, and its arylhydrocarbon hydroxylase activity is closely related with mutation of cells and induction of tumors (*9–11*). The recombinant yeast microsomal membrane containing rat liver microsomal P4501A1 contains substantially only one P450 species, P4501A1, and yeast NADPH-P450 reductase (*12*). This reductase can efficiently transfer electrons to mammalian microsomal P450s (*13*). Accordingly, the recombinant yeast microsomal membrane seems to be a well-organized reconstituted system, and the interactions of P4501A1 with the

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[2] To whom correspondence should be addressed. Tel: +81-75-753-6266, Fax: +81-75-753-6265, E-mail: inouye@kais.kyoto-u.ac.jp

[3] Present address: Research Institute of Biotechnological Sciences, Kinki University.

Abbreviations: P450, cytochrome P450; NQ, 1,4-naphthoquinone.

reductase and substrate might be closer to those in the cells than those in a reconstituted system composed of purified proteins and artificial membranes.

In this study, we discuss the relationship between the structure of NQs and their inhibitory effect on P4501A1-dependent 7-ethoxycoumarin *O*-deethylation by using the recombinant yeast microsomal membrane. Unique spectral changes induced by NQs are also reported.

MATERIALS AND METHODS

Materials—1,4-Naphthoquinone-related compounds (NQs) were purchased from the following sources: 1,4-naphthoquinone (NQ), 5-hydroxy-1,4-naphthoquinone (5-OH-NQ or juglone), 5,8-dihydroxy-1,4-naphthoquinone [5,8-$(OH)_2$-NQ or naphthazarin] and 2-methyl-1,4-naphthoquinone (2-CH_3-NQ, menadione or vitamine K_3), 2-hydroxy-1,4-naphthoquinone (2-OH-NQ or lawson) from Tokyo Chemical Industry (Tokyo); 2-methyl-5-hydroxy-1,4-naphthoquinone (2-CH_3-5-OH-NQ or plumbagin) from Sigma (St. Louis, MO). The following chemicals were also used: 7-ethoxycoumarin from Aldrich (Milwaukee, WI); 7-hydroxycoumarin from Sigma (St. Louis, MO); and NADPH from Oriental Yeast (Tokyo). All other chemicals were of the best commercially available grade.

The expression plasmid pAMR2 for rat liver microsomal P450 1A1 and yeast NADPH-P450 reductase was constructed and expressed in *Saccharomyces cerevisiae* AH22 as reported previously (*13*). The content of P450 in microsomes of the recombinant *S. cerevisiae* was estimated to be 0.27 nmol/mg protein on the basis of reduced CO-difference spectra. The content of NADPH-P450 reductase in the microsomes was estimated to be 0.24 nmol/mg protein on the basis of the cytochrome *c* reductase activity of the purified yeast NADPH-P450 reductase sample (*14*). Microsomes prepared from *S. cerevisiae* AH22 did not show a reduced CO-difference spectrum or any activity of 7-ethoxycoumarin *O*-deethylation. The microsomes were judged not to contain a significant amount of mitochondrial fragments from assays of mitochondria-specific enzymes, monoamine oxidase and cytochrome *c* oxidase (*1, 8, 14*).

Measurement of 7-Ethoxycoumarin O-Deethylation Activity and Inhibition by 1,4-Naphthoquinones—*O*-Deethylation of 7-ethoxycoumarin in the recombinant yeast microsomal fractions was assayed by monitoring production of 7-hydroxycoumarin (*8, 14*). To estimate the initial rate of 7-hydroxycoumarin production, the fluorescence change was continuously monitored at 37°C with an excitation wavelength of 366 nm and an emission wavelength of 452 nm using a Hitachi 850 fluorescence spectrophotometer (Tokyo). The reaction mixture contained 0–60 μM 7-ethoxycoumarin, 1.0% methanol, 400 μM NADPH, and the microsomal fraction prepared from AH22/pAMR2 cells containing 2.6 nM P4501A1 and 1.6 nM NADPH-P450 reductase in 100 mM potassium phosphate buffer, pH 7.4, in a final volume of 2.0 ml. The concentrations of 1,4-naphthoquinones were as follows: 1,4-naphthoquinone (NQ), 4.0 and 8.0 μM; 2-methyl-1,4-naphthoquinone, 5.0 and 10.0 μM; 2-methyl-5-hydroxy-1,4-naphthoquinone, 2.0 and 4.0 μM; 2-hydroxy-1,4-naphthoquinone, 75 μM; 5-hydroxy-1,4-naphthoquinone, 1.25 and 2.5 μM; and 5,8-dihydroxy-1,4-naphthoquinone, 4.0 μM. The reaction was initiated by adding NADPH to the mixture of other components, and the fluorescence change was continuously monitored at 37°C for more than 10 min. At the end of the monitoring, a known amount of 7-hydroxycoumarin was added to the reaction mixture in order to calibrate the fluorescence change. Since fluorescence changes originating from the decrease of 7-ethoxycoumarin and NADPH oxidation are less than 1% of the fluorescence change caused by 7-hydroxycoumarin production, these effects can be neglected. The Michaelis constant, K_m, and the maximum velocity, V_{max}, were calculated by the nonlinear least-squares regression method (*15*), and the inhibitor constant, K_i, was calculated from the apparent K_m and V_{max} values obtained in the presence of 1,4-naphthoquinone derivatives (hereafter designated as K_p and V_p, respectively) by using the following equations.

Competitive inhibition:

$$K_p = K_m (1 + [I]/K_i)$$
$$V_p = V_{max}$$

Noncompetitive inhibition:

$$K_p = K_m$$
$$V_p = V_{max} (1 + [I]/K_i)$$

The type of inhibition was judged from Hanes-Woolf plots ($[S]/v$ *vs.* $[S]$) and Lineweaver-Burk plots ($1/v$ *vs.* $1/[S]$) (*16*), where $[S]$ and v are the substrate concentration and reaction rate, respectively.

NADPH Oxidation by Microsomes in the Presence of 1,4-Naphthoquinones—NADPH oxidation in the microsomal fraction was measured by following the decrease in absorbance at 339 nm with a Shimadzu UV-240 spectrophotometer (Kyoto) at 37°C. The reaction mixture contained 0–10 μM 1,4-naphthoquinone derivatives, 2.6 nM P4501A1, 1.6 nM NADPH-P450 reductase, 1% methanol, and 100 mM potassium phosphate, pH 7.4. The reaction was initiated by the addition of NADPH at a final concentration of 400 μM. The initial rate was calculated from the molar absorption change of NADPH oxidation, $\Delta\varepsilon$ = 6,200 M^{-1} cm^{-1}. The data were fitted to the Michaelis-Menten equation using a nonlinear least-squares method (*15*) to determine the kinetic parameters K_m and V_{max}.

Difference Spectra of P4501A1 with 1,4-Naphthoquinones—Difference spectra were measured using a Hitachi U-3200 spectrophotometer with a head-on photomultiplier in a double tandem cell system composed of one set of matching cells having two compartments with a 0.45-cm light path at 37°C. The double tandem cell system was used to avoid the effect of the absorbance of 1,4-naphthoquinone derivatives. The microsomal fraction was suspended in 100 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 1% methanol (buffer A) and transferred into both sample and reference cells, only one compartment proximal to the photomultiplier. An equivalent volume of buffer A was poured into the other compartments of both cells. After recording the base line from 350 to 500 nm, each of the 1,4-naphthoquinones in buffer A was added to the proximal compartment of the sample cell containing the microsomal fraction and the distal compartment of the reference cell containing buffer A.

Other Methods—The concentration of P4501A1 was determined from the reduced CO-difference spectrum using an absorption coefficient of 91 mM^{-1} cm^{-1} (*17*). The concentration of NADPH-P450 reductase in the microsomal fraction was estimated on the basis of cytochrome *c* reductase activity (*18*). Protein concentration was determined by the Lowry method using bovine serum albumin as a standard

(*19*). The concentrations of 7-ethoxycoumarin, 7-hydroxycoumarin, and NADPH were determined by using absorption coefficients of 13.56 mM^{-1} cm^{-1} at 320 nm, 14.5 mM^{-1} cm^{-1} at 324 nm, and 6.20 mM^{-1} cm^{-1} at 339 nm, respectively.

RESULTS

Inhibition of P4501A1-Dependent 7-Ethoxycoumarin O-Deethylation by 1,4-Naphthoquinone Derivatives—The chemical structures of 1,4-naphthoquinones used in this study are shown in Fig. 1. Figures 2 and 3 show Hanes-Woolf plots of inhibition by 1,4-naphthoquinone (NQ), 2-CH_3-NQ, 5-OH-NQ, 2-CH_3-5-OH-NQ, 2-OH-NQ, and 5,8-$(OH)_2$-NQ of P4501A1-dependent 7-ethoxycoumarin *O*-deethylation. With four 1,4-naphthoquinones, namely, NQ, 2-CH_3-NQ, 2-CH_3, 5-OH-NQ, and 2-OH-NQ, the apparent K_m (K_p) values increased with increasing concentrations of the respective inhibitors, while the apparent V_{max} (V_p) values showed no significant change (Fig. 2). Thus, the inhibition type of these 1,4-naphthoquinones was judged to be competitive. However, with 5-OH-NQ and 5,8-$(OH)_2$-NQ, the V_p values decreased with increasing inhibitor concentrations, while the K_p values showed no significant change (Fig. 3). Accordingly, the inhibition type of 5-OH-NQ and 5,8-$(OH)_2$-NQ was judged to be noncompetitive. Table I summarizes the inhibition types and the K_i values of the six 1,4-naphthoquinones. It should be noted that both noncompetitive inhibitors bear a 5-hydroxyl group on NQ. The K_i value of 5,8-$(OH)_2$-NQ was six times larger than that of 5-OH-NQ, indicating that an 8-hydroxyl group reduced the inhibitory effect. Judging from the K_i values, the binding affinity of the four competitive inhibitors for substrate-binding pocket of P4501A1 is in the order: 2-CH_3-5-OH-NQ > 2-CH_3-NQ > NQ ≫ 2-OH-NQ.

Effect of 1,4-Naphthoquinones on NADPH-Oxidation—Several 1,4-naphthoquinone derivatives are known to accelerate oxidization of NADPH in the presence of NADPH-P450 reductase (*4*). Electrons from NADPH are transferred to 1,4-naphthoquinones by the reductase, and the resultant semiquinone reduces dioxygen to produce a superoxide radical (*20*). Thus, it seems reasonable to assume that 1,4-naphthoquinones efficiently accept electrons from the reductase and inhibit electron transfer from the reductase to P4501A1. The NADPH-oxidation rate was markedly accelerated by 1,4-naphthoquinones except for 2-OH-1,4-naphthoquinone (Fig. 4). The kinetic parameters K_mNQ (K_m for 1,4-naphthoquinones) and V_{max} are summarized in Table II. Almost the same values of K_mNQ and V_{max} were obtained for 5,8-$(OH)_2$-NQ and 5-OH-NQ. The V_{max} values of 2-CH_3-5-OH-NQ, NQ, and 2-CH_3-NQ correspond with each other, whereas the K_mNQ values are significantly different. The two noncompetitive inhibitors of P4501A1-dependent 7-ethoxycoumarin *O*-deethylation, 5,8-$(OH)_2$-NQ and 5-OH-NQ, showed almost the same acceleration of NADPH oxidation, but the K_i values for 7-ethoxycoumarin *O*-deethyla-

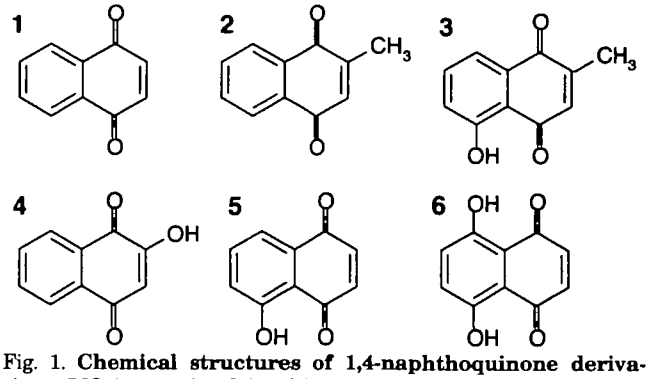

Fig. 1. **Chemical structures of 1,4-naphthoquinone derivatives (NQs) examined in this study.** 1, 1,4-naphthoquinone or NQ; 2, 2-methyl-1,4-naphthoquinone or 2-CH_3-NQ; 3, 2-methyl-5-hydroxy-1,4-naphthoquinone or 2-CH_3-5-OH-NQ; 4, 2-hydroxy-1,4-naphthoquinone or 2-OH-NQ; 5, 5-hydroxy-1,4-naphthoquinone or 5-OH-NQ; 6, 5,8-dihydroxy-1,4-naphthoquinone or 5,8-$(OH)_2$-NQ.

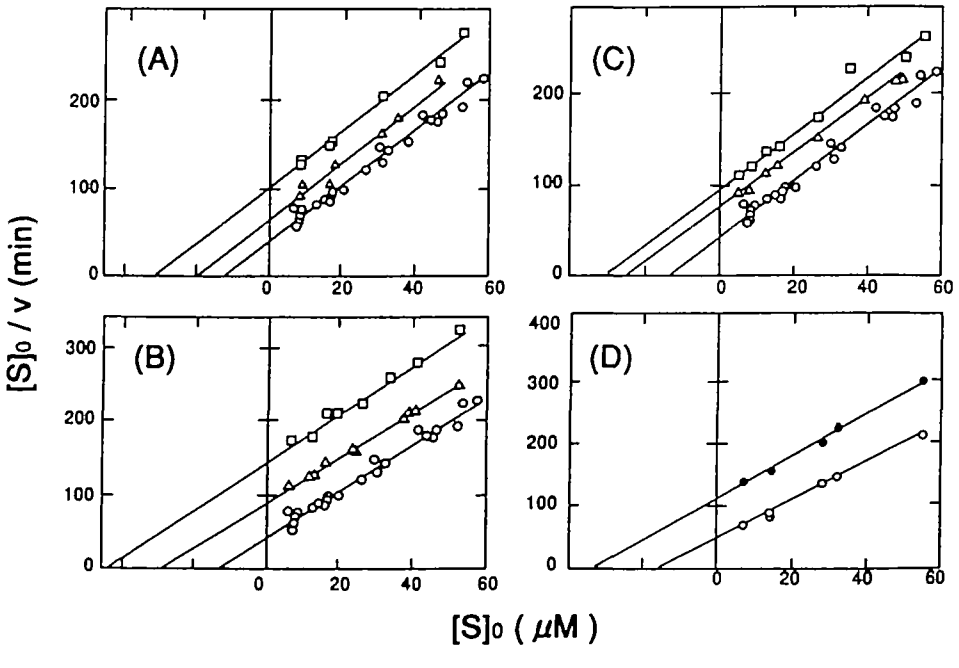

Fig. 2. **Hanes-Woolf plots of 7-ethoxycoumarin *O*-deethylation catalyzed by cytochrome P4501A1–dependent monooxygenase system in the presence of 1,4-naphthoquinone derivatives (NQs) that showed competitive inhibition.** The activity was measured in the reconstituted system containing the microsomal fractions prepared from AH22/pAMR2 cells at a final P450 concentration of 2.6 nM and that of NADPH-P450 reductase of 1.6 nM in the presence of the NQs. (A) NQ at concentrations of 0 μM (○), 4.0 μM (△), and 8.0 μM (□); (B) 2-CH_3-NQ at concentrations of 0 μM (○), 5.0 μM (△), and 10.0 μM (□); (C) 2-CH_3-5-OH-NQ at concentrations of 0 μM (○), 2.0 μM (△), and 4.0 μM (□); and (D) 2-OH-NQ at concentrations of 0 μM (○) and 75 μM (●).

tion were quite different (Table I).

Effect of NADPH and NADP⁺ Concentration on P4501A1-Dependent 7-Ethoxycoumarin O-Deethylation—The effect of NADPH was examined in a reaction mixture containing 4.1 nM P4501A1, 2.5 nM NADPH-P450 reductase, 8.3 μM 7-ethoxycoumarin, 1.0% methanol, and 12.5–400 μM NADPH in 100 mM potassium phosphate buffer, pH 7.4, at 37°C. The initial rate of 7-hydroxycoumarin formation was constant (0.19 μM/min) over the range of NADPH concentration examined. The *O*-deethylation was also performed at 4.1 nM P4501A1, 2.5 nM reductase, and 0–60 μM 7-ethoxycoumarin in the presence of 100 and 400 μM NADPH. The K_m and V_{max} values at both NADPH concentrations were determined to be (13 ± 1) μM and (0.32 ± 0.01) μM/min, respectively. To examine inhibition of 7-ethoxycoumarin *O*-deethylation by NADP⁺, the activity was measured in the presence 400 μM NADPH and various concentrations (0–800 μM) of NADP⁺. With increasing NADP⁺ concentration, the enzyme activity was reduced progressively to 85% of the activity in the absence of NADP⁺. The inhibitory effect of NADP⁺ on the *O*-deethylation was examined in the presence of 100 μM NADPH and in the absence or presence of 300 μM NADP⁺. In the presence of 300 μM NADP⁺, the K_p and V_p values were determined to be (13 ± 1) μM and (0.27 ± 0.01) μM/min, respectively. Thus, NADP⁺ was found to be a noncompetitive inhibitor of the reaction with the K_i value of 2.0 mM. Judging from this value, NADP⁺ produced by NADPH oxidation mediated by 1,4-naphthoquinones would not affect the initial rate of 7-ethoxycoumarin *O*-deethylation.

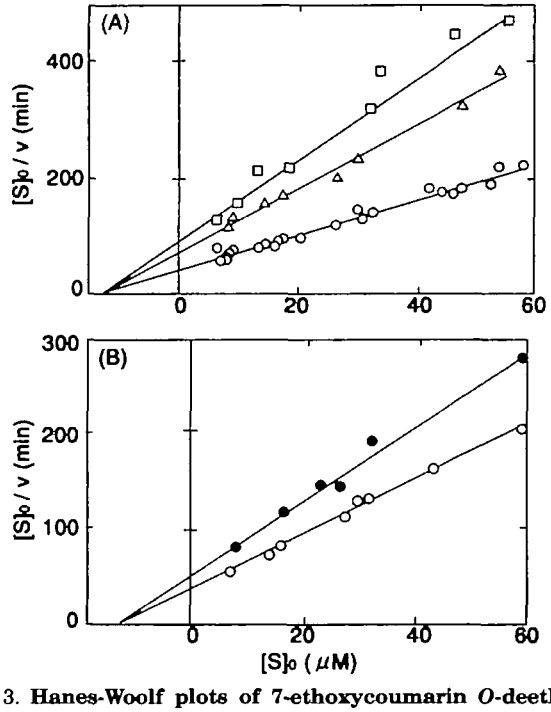

Fig. 3. **Hanes-Woolf plots of 7-ethoxycoumarin *O*-deethylation catalyzed by cytochrome P4501A1–dependent monooxygenase system in the presence of the NQs that showed noncompetitive inhibition.** The activity was measured in the reconstituted system containing the microsomal fractions prepared from AH22/pAMR2 cells at 2.6 nM P450 and 1.6 nM NADPH-P450 reductase in the presence of the NQs. (A) 5-OH-NQ at concentrations of 0 μM (○), 1.25 μM (△), and 2.5 μM (□); and (B) 5,8-$(OH)_2$-NQ at concentrations of 0 μM (○) and 4.0 μM (●), respectively.

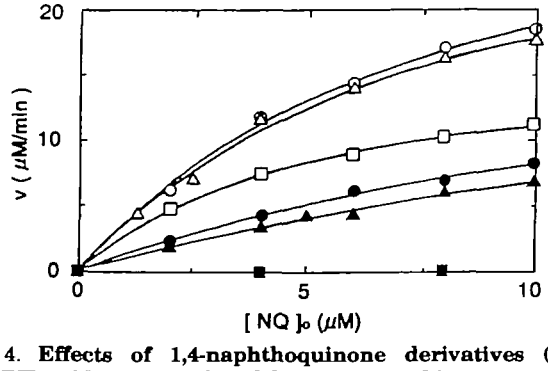

Fig. 4. **Effects of 1,4-naphthoquinone derivatives (NQs) NADPH oxidation catalyzed by the recombinant yeast microsomes.** The NADPH oxidation was performed in the reconstituted system containing the microsomal fractions prepared from AH22/pAMR2 cells at 2.6 nM P450 and 1.6 nM NADPH-P450 reductase in the presence of 5,8-$(OH)_2$-NQ (○), 5-OH-NQ (△), 2-CH_3-5-OH-NQ (□), NQ (●), 2-CH_3-NQ (▲), and 2-OH-NQ (■). The K_mNQ and V_{max} values obtained are listed in Table II.

Difference Spectra of P4501A1 with 1,4-Naphthoquinone—Addition of selected 1,4-naphthoquinone derivatives, 2-CH_3-NQ, 5-OH-NQ, NQ, and 2-CH_3-5-OH-NQ, to the microsomal fraction containing P4501A1 induced spectral changes due to the changes of the spin state of heme iron (Fig. 5). The binding difference spectrum of 2-CH_3-NQ showed a peak at 382 nm and a trough at 422 nm, designated as Type I spectrum (*21, 22*), suggesting conversion of the spin state of heme iron from a low spin state (O-coordination) to a higher one. The difference spectrum induced by 5-OH-NQ showed a peak at 424 nm and a trough at 380 nm, designated as Type II (N-coordination). The difference spectrum induced by 2-CH_3-5-OH-NQ was similar. These results with 5-OH-NQ and 2-CH_3-5-OH-NQ were similar to those obtained from untreated rabbit liver microsomes as described previously (*1*). On the other hand, the binding

TABLE I. **Inhibition of P4501A1-dependent 7-ethoxycoumarin *O*-deethylation by 1,4-naphthoquinone derivatives (NQs).**[a]

Competitive	K_i (μM)	Non-competitive	K_i (μM)
NQ	6.5 ± 1.0	5-OH-NQ	1.8 ± 0.2
2-CH_3-NQ	4.1 ± 0.1	5,8-$(OH)_2$-NQ	11
2-CH_3-5-OH-NQ	2.6 ± 0.5		
2-OH-NQ	62		

[a]Each value represents the mean ± SD of three or more separate experiments except for 2-OH-NQ and 5,8-$(OH)_2$-NQ, which were examined once.

TABLE II. **Kinetic parameters of NADPH oxidation in the presence of 1,4-naphthoquinone derivatives (NQs).**[a]

1,4-Naphthoquinones	K_mNQ (μM)	V_{max} (μM/min)
5,8-$(OH)_2$-NQ	8.1 ± 1.0	34 ± 3
5-OH-NQ	7.8 ± 1.0	32 ± 3
2-CH_3-5-OH-NQ	5.1 ± 0.4	17 ± 1
NQ	15 ± 7	21 ± 7
2-CH_3-NQ	26 ± 9	25 ± 9
2-OH-NQ	0	0

[a]The K_mNQ (K_m for 1,4-naphthoquinones) and V_{max} values were calculated by the method of Sakoda and Hiromi (*15*). Each value represents the mean ± SD of three or more separate experiments.

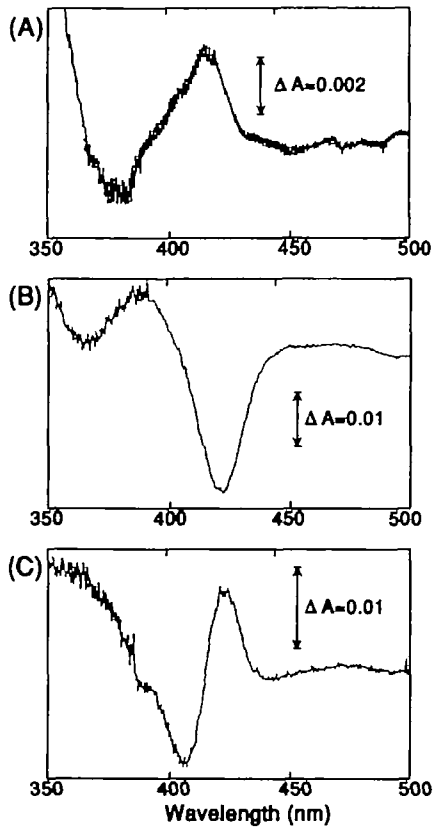

Fig. 5. **Difference spectra of P4501A1 induced by NQ (A), 2-CH3-NQ (B), and 5-OH-NQ (C).** The spectra were measured at 1.9 μM P450 and 6.8 μM NQ (A); 3.7 μM P450 and 5.9 μM $2CH_3$-NQ (B); and 3.7 μM P450 and 1.8 μM 5-OH-NQ (C).

difference spectrum of NQ showed a peak at 416 nm and a trough at 380 nm, designated as the reverse Type I spectrum (*22*), suggesting conversion of the spin state of the heme iron from a high spin state to a lower one (O-coordination). Because the oxidized form of the purified P4501A1 showed a Soret peak at 417 nm (*22, 23*), it is reasonable to assume that P4501A1 in the recombinant yeast microsomes mostly has the heme iron in a low spin state (O-coordination). The intensity of the reverse Type I difference spectrum induced by NQ was lower than that of the Type I spectrum induced by 2-CH_3-NQ. Accordingly, it is suggested that a small amount of P4501A1 contains the heme iron in a high spin state, which is converted to a low spin state by binding of 1,4-naphthoquinones. Neither 1% methanol nor 20% glycerol in the buffer caused spectral change of P4501-A1 (data not shown).

DISCUSSION

Our previous study suggested that inhibition of microsomal monooxygenation by 1,4-naphthoquinone derivatives selected from the components of Chinese medicines is not due to formation of superoxide radicals but due to their binding to cytochrome P450 (*1*). Judging from the IC_{50} values, the inhibition would be physiologically significant. However, the microsomal fraction prepared from rabbit liver and human placenta in the previous study contain multiple forms of P450. On the other hand, it has been reported that 1,4-naphthoquinones are reduced by NADPH-P450 reductase (*4–6*). In this study, we performed kinetic analysis using recombinant yeast microsomes containing a single P450 species, P4501A1. We also examined the possibility that 1,4-naphthoquinones act as substrates of the P4501A1-dependent monooxygenase system, but no metabolite of the enzyme system was detected (data not shown). Thus, the six 1,4-naphthoquinones examined in this study were regarded as inhibitors of the monooxygenase system. The inhibitory effects of 1,4-naphthoquinones can be classified into three types. One of them involves binding of 1,4-naphthoquinones to the P450 active site, which interferes with binding of the substrate to the active site. In this case, the 1,4-naphthoquinones may show competitive inhibition. The second type involves binding of 1,4-naphthoquinones to a secondary site other than the active site, and the 1,4-naphthoquinone may inhibit the enzyme in a noncompetitive manner. In the third type, 1,4-naphthoquinone interferes with electron-transfer from NADPH-P450 reductase to P4501A1, and the inhibition pattern might be noncompetitive. If these types of inhibition occurred simultaneously, a mixed-type inhibition would be observed. The inhibitory potency and pattern of 1,4-naphthoquinones seem to be dependent on their structure and the nature of their substituents (Table I). In this study, 7-ethoxycoumarin *O*-deethylation was performed at 2.6 nM P4501A1 and 1.6 nM NADPH-P450 reductase under the standard conditions. No clear change in the inhibitory potency and pattern was observed when the reductase concentration was varied in the range between 0.16 and 4.8 nM, where the P4501A1 concentration was in the range between 0.26 and 7.8 nM (not shown). Accordingly, it is considered that change in the reductase concentration does not influence the inhibitiory behaviors of 1,4-naphthoquinones.

Two noncompetitive inhibitors of 7-ethoxycoumarin *O*-deethylation, 5,8-$(OH)_2$-NQ and 5-OH-NQ, showed almost the same acceleration of NADPH oxidation (Table II). Thus, these inhibitors may have an equal ability to affect the electron transfer. If their inhibitory effect is mainly derived from their interference with electron-transfer from NADPH-P450 reductase to P4501A1, both inhibitors should show similar inhibition profiles with similar K_i values. However, the K_i values observed with 5,8-$(OH)_2$-NQ (11 μM) and 5-OH-NQ (1.8 ± 0.2 μM) differ significantly. This suggests that the major inhibitory effect of 5,8-$(OH)_2$-NQ and, particularly, 5-OH-NQ, which has a low K_i value, is not due to interference with the electron-transfer. The binding difference spectrum of 5-OH-NQ strongly suggests that it binds to the substrate-heme pocket of P4501A1, that is, 5-OH-NQ shows two modes of inhibition: binding to the P450 active site and interfering with the electron-transfer. It is still unclear why 5-OH-NQ and 5,8-$(OH)_2$-NQ show noncompetitive inhibition, especially if we accept that 5-OH-NQ binds to the substrate-heme pocket of P4501A1. The possibility remains that 5-OH-NQ binds to a secondary site other than the active site to result in a Type II spectral change of the heme. On the other hand, it has been demonstrated, according to the two-site model (*24*), that P4501A1 could accommodate two 7-ethoxycoumarin molecules in the substrate-binding pocket when the substrate concentration is higher than 60 μM under the conditions used in the present study (K. Inouye *et al.*, submitted). On the other hand, four 1,4-naphthoquinones (2-CH_3-, 5-OH-NQ, 2-CH_3-

NQ, NQ and 2-OH-NQ) showed typical competitive inhibition with binding affinity for P4501A1 in the order: 2-CH_3-5-OH-NQ > 2-CH_3-NQ > NQ ≫ 2-OH-NQ (Table I). However, the binding difference spectra of 2-CH_3-5-OH-NQ (Type II), 2-CH_3-NQ (Type I), and NQ (reverse Type I) were quite different. These observations on the inhibition patterns and spectral changes of the 1,4-naphthoquinones are too complicated to be explained unambiguously. The effects of substituents and the structure–function relationship of 1,4-naphthoquinone derivatives should be studied further.

The present study indicates that the 1,4-naphthoquinone derivatives are potent inhibitors of P4501A1 with K_i values of μM order. Inhibitory effects of the 1,4-naphthoquinones on P4501A1-dependent monooxygenase are derived from their binding to the substrate-heme pocket of P4501A1. The methyl and hydroxyl groups of the 1,4-naphthoquinones had unique effects on their binding affinity and modes of binding. Therefore, the 1,4-naphthoquinones appear to be useful probes to elucidate the structure of the substrate-heme pocket of P4501A1. In addition, the lines of evidence obtained in this study give us useful information to design potent inhibitors of the cytochrome P4501A1–dependent monooxygenase system, and the inhibitors could be used to suppress carcinogenesis derived from the activity of P4501A1.

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