Effect of N-Methyltetrazolethiol on Liver Microsomal Vitamin K Reductase

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Abstract—NADH-dependent vitamin K reductase activity in rat liver microsomes was measured by detecting the amount of the reduced form of vitamin K from the oxidized form of the vitamin. The enzyme activity was not detected when intact microsomes were employed as the enzyme source, but the solubilization of the microsomal enzyme with 1.5% Triton X-100 caused a development of the activity. Although the enzyme activity decreased gradually with time after the solubilization, the enzyme was stabilized by the addition of 20% glycerol and 2 mM vitamin C. Some optimal assay conditions for the vitamin K reductase were determined using the solubilized enzyme, and the standard assay method is described. Vitamin K reductase activity was not affected by warfarin and N-ethylmaleimide (NEM), but pyridoxal-5-phosphate (PAL-P) inhibited the activity, especially when microsomes were preincubated with PAL-P. The enzyme activity was not inhibited by N-methyltetrazolethiol (NMTT) and NMTT-containing antibiotics, suggesting that the hypoprothrombinemia caused by β-lactam antibiotics was not due to the inhibition of NADH-dependent vitamin K reductase.

Coagulopathy and bleeding diathesis are sometimes noted in aged, debilitated patients during treatment with cephalosporins. Antibiotic-induced hypoprothrombinemia is especially caused by several β-lactam antibiotics which contain an N-methyltetrazolethiol (NMTT) side chain (1-4). Lipsky (5) reported that the hypoprothrombinemic effect of these antibiotics is due to inhibition of the vitamin K-dependent γ-carboxylation of glutamyl residues by NMTT. However, Uchida et al. (6) and Suttie et al. (7) reported that NMTT or NMTT-containing antibiotics did not inhibit γ-glutamylcarboxylase when reduced vitamin K (vitamin KH₂) was used as a cofactor. γ-Glutamylcarboxylation activity is usually assayed using crude microsome preparations with either vitamin KH₂ or vitamin K plus NAD(P)H because vitamin K is reduced during the incubation in the presence of NAD(P)H (8, 9). Since Lipsky (5) employed systems of vitamin K plus NADH, the different conclusions of Lipsky (5) and Uchida et al. (6) or Suttie et al. (7) may be attributable to a problem with the vitamin K reductase activity. Various enzymes, such as vitamin K epoxide reductase, DT-diaphorase and microsomal NAD(P)H dehydrogenases, were reported to catalyze the conversion of vitamin K to vitamin KH₂, similar to the action of vitamin K reductase (10-12). However, we focused our attention on microsomal membrane-bound vitamin K reductase, because the vitamin K-dependent γ-glutamylcarboxylation system employed by Lipsky consisted of γ-glutamylcarboxylase and vitamin K reductase (5, 13). Here, we describe the enzymatic properties of vitamin K reductase and the effects of NMTT and NMTT-containing antibiotics on this enzyme activity.

Materials and Methods

Animals: Sprague-Dawley male rats were obtained from Japan Clea (Tokyo) and used for the experiments at the age of 8 weeks. The animals were kept in an air-conditioned room (25±1°C, 50-60% humidity) lighted 12 hr a day (8:00-20:00) and fed a vitamin K-
deficient diet containing about 50 ng of vitamin K₁/g diet ad libitum, which was prepared in our laboratory as described previously (14). During the period of the experiments, the animals were kept in cages which prevented coprophagy.

**Agents:** The following β-lactam antibiotics and reagents were used for the experiments: Latamoxef (LMOX) and flomoxef (FMOX) were obtained from Shionogi & Co., Ltd. (Osaka), cefazolin (CEZ) from Fujisawa Pharmaceutical Co. (Osaka), and ceftezole (CTZ) from Hoechst Aktiengesellschaft (Frankfort). N-Methyltetrazolethiol (NMTT), thiodiazolethiol (TDT), methylthiodiazolethiol (MTDT), and hydroxyethyltetrazolethiol (HTT) were synthesized in our laboratory. Vitamin K₁ was purchased from Wako Pure Chemical Industries, Ltd. (Osaka) and reduced vitamin K₁ was synthesized in our laboratory. Disulfiram was purchased from Wako Pure Chemical Industries, Ltd. (Osaka) and warfarin, from Warfinstitute, Inc. (Madison). Other commercially obtained chemicals were of the purest grade available.

**Preparation of microsomes and solubilization of enzyme:** Liver microsomes were prepared from one-day-fasted rats as described by Esmon et al. (8) and washed once by resuspending them in the homogenizing buffer followed by the centrifugation step. Microsomes were stored at -80°C for about one week without any loss of the enzyme activity. Next, microsomal pellets were solubilized with SIK buffer (25 mM imidazole buffer at pH 7.4 containing 0.25 M sucrose, 0.15 M KCl and 2 mM phenylmethylsulfonyl fluoride (PMSF)) containing 1.5% Triton X-100, 20% glycerol and 2 mM vitamin C. This preparation was centrifuged at 105,000×g for 60 min, and the supernatant fraction was used as an enzyme source.

**Determination of vitamin K reductase activity:** Incubation mixtures contained 200 μl of solubilized microsomal fraction (about 1.4 mg protein), 10 μl of 100 mM NADH in SIK buffer, 10 μl of the test compounds in SIK buffer and 10 μl of vitamin K₁ in ethanol (final concentration, 111 μM), and the final volume was brought to 500 μl with SIK buffer solution. The reaction was started by the addition of vitamin K₁. When the reaction was carried out under conditions in which the γ-glutamylcarboxylation reaction also proceeded, the substrates for the reaction, pentapeptide (0.3 mM) and sodium bicarbonate (0.36 mM), were added before the addition of vitamin K₁. The reaction mixtures were incubated at 17°C for 30 min, and an aliquot (50 μl) of incubation mixtures was taken into a vial containing 0.5 ml of methanol bubbled with O₂-free N₂ gas. The vials were shaken and centrifuged at 2000×g for 10 min, and a 5 μl portion of the supernatant fraction was directly subjected to vitamin K analysis. All procedures described above were carried out under dark conditions with orange light. Vitamin Ks were determined by high-performance liquid chromatography (HPLC) utilizing a Shimadzu HPLC Model LC3A (Shimadzu Co., Ltd., Kyoto) equipped with a recording integrator (Shimadzu Chromatopac C-R1A) and a fluorescence HPLC monitor (Shimadzu RF-535). A TSK gel ODS-120T column (250 mm×4.6 mm ID) was used and eluted isocratically with methanol-acetonitrile (6:4, v/v) at a flow rate of 1 ml/min at room temperature. Vitamin KH₂ was detected by fluorescence spectrophotometry at 340 nm emission and 430 nm excitation. Under these analytical conditions, vitamin KH₂ and vitamin K₁ were detected with tR values of 9.8 and 26.6 min, respectively. This assay method is reliable because we can separate and detect easily the reaction substrate (vitamin K₁) and its product (vitamin KH₂). The vitamin K reductase activity was expressed as reduced vitamin K₁ formed per mg microsomal protein per min. The protein concentrations were determined by the method of Bradford (15) using Bio-Rad reagents and bovine serum albumin as a standard.

**Results**

**Solubilization of vitamin K reductase**

Since the enzyme activity was not detected when intact microsomes were employed as the enzyme source, microsomes were solubilized under different conditions as shown in Fig. 1. Solubilization of microsomes with SIK buffer containing 1.5% Triton X-100 caused a development of the vitamin K reductase activity, but it decreased rapidly after preparation, and only 36% of the activity was...
Fig. 1. Effects of solubilization conditions of microsomes on the stability of vitamin K reductase. Microsomes were solubilized with SIK buffer containing 1.5% Triton X-100 (○); with SIK buffer containing 1.5% Triton X-100 and 20% glycerol (●); or with SIK buffer containing 1.5% Triton X-100, 20% glycerol and 2 mM vitamin C (□). The solubilized samples were kept at 0°C for 1–6 hr as shown in the figure, and their activities were determined as described in Materials and Methods. The control value (100%) was 0.157 nmole/mg protein/min.

Detected after 2.5 hr. Addition of 20% glycerol to the buffer markedly prevented enzyme inactivation, and further addition of vitamin C at 2 mM maintained considerable enzyme activity for about 6 hr. Solubilization of vitamin K reductase was also carried out using deoxycholate, Emulgen 913 and CHAPS. However, the enzyme activity detected in these preparations was 1/3 to 1/4 that of the Triton-solubilized preparation. The vitamin K reductase activity decreased rapidly in the samples solubilized by detergents other than Triton X-100 (data not shown). Triton X-100 was then chosen from these results as the detergent for enzyme solubilization.

Figure 2 shows the effect of Triton X-100 concentration on the vitamin K reductase activity in the reaction mixture containing 20% glycerol and 2 mM vitamin C. The enzyme activity increased almost linearly with increasing concentration of the detergent, and maximal activity was obtained at 1.5% of Triton X-100 or more. Therefore, we selected 1.5% as an appropriate concentration of the detergent.

Estimation of assay conditions
We examined enzymatic characteristics of vitamin K reduction. The enzyme activity depends on vitamin K concentration, and a Michaelis-Menten type curve was obtained as shown in Fig. 3 (inset). The $K_m$ value calculated from the Lineweaver-Burk plot of the data was 44.2 μM. The reduction of vitamin K also depended on the concen-
concentration of NAD(P)H. Figure 4 shows the relationship between NAD(P)H concentration and vitamin K reduction. The enzyme activity increased with increasing concentration of NADH and NADPH with apparent $K_m$ values of 0.32 and 0.5 mM, respectively. However, the efficacy of NADPH was about one-fourth that of NADH: $V_{max}$ values for NADH and NADPH under the experimental conditions shown in Fig. 4 were 0.164 and 0.042 nmole/mg protein/min, respectively. The optimum pH for the enzyme activity was not defined because the activity became higher with lower pH of reaction mixture, and we selected pH 7.4 for the reaction buffer. In the reaction system containing sufficient concentrations of substrate (vitamin K) and reductant NADH, the reaction proceeded linearly for about 30 min. When lower concentrations of microsomes were added to the reaction mixture (less than 5 mg protein/ml), a close correlation was obtained between the microsomal concentration and the formation of reduced vitamin K (data not shown). From these results, we finally established the assay system for vitamin K reductase as described in Methods.

Effects of $\beta$-lactam antibiotics and related compounds on vitamin K reductase: Figure 5 shows the time course changes in formation of vitamin KH$_2$ in the presence of NMTT and disulfiram. Disulfiram was used as a reference compound having an inhibitory action on the $\gamma$-glutamylcarboxylation reaction (16). In the control tube, reduction of vitamin K occurred almost steadily up to 1.5 hr. Although NMTT (2 mM) did not cause any inhibition of the reductase activity, disulfiram (0.3 mM) inhibited the reduction markedly. The effect of NMTT on the reductase activity was examined in the reaction mixture containing pentapeptide and sodium bicarbonate, which was the reaction mixture for the assay of $\gamma$-glutamylcarboxylation activity, but no inhibition was found (data not shown). Further examinations on the effect of some $\beta$-lactam antibiotics and their 3'-position side chains upon the vitamin K reductase activity were carried out in the presence of varying concentrations of compounds. As shown in Fig. 6, none of them inhibited the enzyme activity. The activity was not modified even by the addition of increasing concentrations of antibiotics or their side chains.

Effects of warfarin and chemical modification reagents: The effects of anticoagulant warfarin and the sulfhydryl-blocking reagent N-ethylmaleimide (NEM) on the vitamin K reductase activity were examined. Warfarin showed no inhibition of the NADH-dependent vitamin K reductase even at 0.5 mM.
Effect of NMTT on Vitamin K Reductase

Fig. 6. Effects of antibiotics and their side chains (heterocyclic thiol compounds) on vitamin K reductase activity. Antibiotics and heterocyclic thiol compounds were dissolved in SIK buffer and added to the reaction mixture at the concentrations of 5 mM and 1 mM, respectively. Other conditions are described in Materials and Methods. Each value represents the mean±S.E. of five determinations. LMOX, latamoxef; CTX, ceftezole; CEZ, cefazoline; FMOX, flomoxef; NMTT, N-methyltetrazolethiol; TDT, thiadiazolethiol; MTDT, methylthiadiazolethiol; HTT, hydroxyethyltetrazolethiol. The control value (100%) was 0.146 nmole/mg protein/min.

Fig. 7. Effects of warfarin and N-ethylmaleimide (NEM) on vitamin K reductase activity. Various concentrations of warfarin (●) and NEM (○) were added to the reaction mixture as shown in the figure. Other conditions are described in Materials and Methods. The control value (100%) was 0.149 nmole/mg protein/min.

Enhancement of microsomal γ-glutamylcarboxylation activity was reported by the addition of PAL-P (17), while vitamin K reductase activity was suppressed by 30 to 40% when the enzyme preparation was preincubated with PAL-P. However, the compound caused no inhibition without pre-

Fig. 8. Effect of pyridoxal-5-phosphate on vitamin K reductase activity. Solubilized microsomes were preincubated at 17°C in the presence (●) and absence (○) of 2 mM pyridoxal-5-phosphate. After addition of pyridoxal-5-phosphate, all procedures were carried out under a dark condition. Other conditions are described in Materials and Methods. The control value (100%) was 0.175 nmole/mg protein/min.

incubation (Fig. 8).

Discussion
Vitamin K reductase, one of the enzymes participating in the vitamin K cycle, is closely associated with the microsomal membrane, and its activity is not manifested unless it is
solubilized with detergents. In the present experiments, the enzyme was solubilized with Triton X-100, and its activity was maintained by the addition of glycerol and vitamin C. Although higher concentrations of Triton X-100 generally inactivate enzymes, as seen with dithiothreitol (DTT)-dependent vitamin K reductase, vitamin K epoxide reductase and \( \gamma \)-glutamylcarboxylation activities at the concentration of 1.5% Triton X-100 (17, 18), we found that the vitamin K reductase maintains its activity even in 1.5% Triton X-100 solution for at least 6 hr in the presence of glycerol and vitamin C (Fig. 1).

Although the microsomal \( \gamma \)-glutamylcarboxylase is not inhibited by NMTT in vitro (6, 7, 13), the carboxylation reaction is inhibited in a specific reaction mixture which contains vitamin K plus NADH (8, 13, 17), suggesting that the vitamin K reductase activity is impaired in the vitamin K plus NADH system. However, the vitamin K reductase activity was not decreased by NMTT and NMTT-containing antibiotics (Figs. 4 and 5). This observation agrees well with our previous findings that LMOX or NMTT inhibited neither \( \gamma \)-glutamylcarboxylase nor vitamin K reductase in vivo (13, 19). Suttie et al. (7) also reported that vitamin K reductase was not inhibited by NMTT even when the \( \gamma \)-glutamylcarboxylation was inhibited by NMTT in the presence of vitamin K and NADH. We also examined the effect of NMTT on vitamin K reduction in a system containing pentapeptide, sodium bicarbonate, vitamin K and NADH, which produced marked inhibition of \( \gamma \)-glutamylcarboxylation (13, 17), and found that vitamin K reduction was not decreased, confirming the result of Suttie et al. (7).

Vitamin K reductase requires reduced pyridine nucleotides as a cofactor. Usually NADH is used, but NADPH is also effective, although somewhat less efficacious. Uchida and Komeno (13) demonstrated that the \( \gamma \)-glutamylcarboxylation was inhibited by NMTT in the presence of vitamin K and NADH, but no inhibition occurred, when NADPH was used instead of NADH or together with NADH. The concentration of NADPH needed to overcome the inhibitory effect of NADH was one-fourth that of NADH (0.5 mM vs. 2 mM). Their observation also supports the present conclusion that NMTT or NMTT-containing antibiotics cause no inhibition of vitamin K reductase. However, it remains as a question why NADH and NMTT are inhibitory to \( \gamma \)-glutamylcarboxylation in such an in vitro system.

Warfarin is known to inhibit DTT-dependent vitamin K reductase (\( I_{50} \): 1 \( \mu \)M) and vitamin K epoxide reductase (\( I_{50} \): 2 \( \mu \)M) (10, 20), but our experiments demonstrated that warfarin did not inhibit the NADH-dependent vitamin K reductase at concentrations less than 0.5 mM. Hildebrandt and Suttie (21) and Fasco and Principe (11) also reported that warfarin did not inhibit DT-diaphorase. These results suggest that warfarin does not inhibit the NADH-dependent vitamin K reduction. Sulphhydryl-blocking reagents, which react with the free sulphydryl group of a cysteine residue, are reported to inhibit DTT-dependent vitamin K reductase (22), but NEM showed no inhibition of the NADH-dependent vitamin K reduction, even when the microsomes were primarily treated with NEM (Fig. 6).

Since the effect of pyridoxal-5-phosphate (PAL-P) on \( \gamma \)-glutamylcarboxylase activity was observed previously (17), the effect of PAL-P on vitamin K reductase activity was examined. PAL-P was found to inhibit the NADH-dependent vitamin K reductase, especially when the microsomes were preincubated with PAL-P. Since PAL-P reacts with an amino group of the Lys residue to form a Schiff's base, the inhibition may be caused by an area near the active site of vitamin K reductase.

In conclusion, we examined some characteristics of rat liver microsomal vitamin K reductase and the effects of some compounds on the reductase activity with special reference to that of antibiotics. The results indicated that disulfiram and pyridoxal-5-phosphate reduced the activity, but NMTT, TDT, MTDT, HTT, or antibiotics containing these side chains did not. The observation suggests that the hypoprothrombinemia caused by some \( \beta \)-lactam antibiotics is not due to the inhibition of NADH-dependent vitamin K reductase.

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