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EFFECT OF *N*-ETHYLMALEIMIDE ON BEEF AND RAT LIVER VITAMIN K_1 EPOXIDE REDUCTASE

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There is little difference in the extent of inactivation of beef liver microsomal vitamin K_1 epoxide reductase by *N*-ethylmaleimide (NEM) whether or not the microsomes are pre-treated with dithiothreitol (DTT). The rat liver microsomal enzyme, however, is inactivated by NEM to a much greater extent if the microsomes are pre-treated with DTT. The beef liver enzyme activity is protected from NEM inactivation by the substrate, vitamin K_1 epoxide. Ping-pong kinetics are exhibited by the beef liver enzyme. These results support a mechanism for vitamin K_1 epoxide reductase in which the function of the required dithiol is to reduce an active site disulfide bond; however, the geometry of the active sites of the enzyme from rat and beef may be different.

KEY WORDS: Active site disulfide, dithiothreitol, *N*-ethylmaleimide, ping-pong kinetics, vitamin K_1 epoxide reductase

INTRODUCTION

Vitamin K_1 epoxide reductase is a dithiol-dependent enzyme that catalyzes the last step in the vitamin K_1 cycle, namely reduction of vitamin K_1 epoxide to vitamin K_1. The dithiol commonly utilized in the assay of this enzyme is dithiothreitol (DTT), however, recent evidence by Silverman and Nandi[1] suggests that the *in vivo* reductant may be reduced thioredoxin. On the basis of chemical model studies, Silverman[2] suggested that the function of the required thiol is to reduce an active site disulfide bond which activates the enzyme for catalysis. This hypothesis has since been supported by studies with the rat liver microsomal enzyme and warfarin,[3] with *N*-ethylmaleimide (NEM),[4] and by kinetic analyses which indicated that a ping-pong mechanism was important.[5,6] Lee and Fasco[4] showed that only 10–18% of the enzyme activity was lost in the presence of the sulfhydryl reagent NEM (1.0 mM) but 80% of the activity was destroyed if the enzyme was pre-reduced with dithiothreitol (0.1 mM), then incubated with NEM. This supports the active-site disulfide hypothesis.

Recently, we presented results to suggest that DTT may be acting as a direct substrate for vitamin K_1 epoxide reductase rather than as a reducing agent.[7] However, these results were obtained with the beef liver microsomal enzyme not the rat liver enzyme that was used earlier.[4] We now show that the reactivity of the enzyme from beef liver toward NEM is different from the rat liver enzyme, but the kinetics of the reaction are the same. Consequently, the same catalytic mechanism appears to be relevant to the enzymes from both species.

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MATERIALS AND METHODS

Biochemicals

Dithiothreitol, dithioerythritol, iodoacetamide, CHAPS (3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), sodium cholate, Lubrol PX, *N*-ethylmaleimide, and vitamin K_1 were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Vitamin K_1 Epoxide

Vitamin K_1 2,3-epoxide was synthesized by the method of Tishler *et al.*[8] and purified by HPLC using a 10 μ C_{18} reversed-phase semi-preparative column (Alltech, 50 cm × 10 mm), eluting with methanol at 4 ml/min. After evaporation of the solvent it was dissolved either in ethanol or in water containing 10% (w/v) Emulgen 911. The final concentrations of ethanol or Emulgen in the assay system were 4% or 0.05%, respectively. An ethanolic solution of the substrate was used for the inhibition studies with sulfhydryl blocking agents, when different detergents were required. The nature of the vehicle used for vitamin K_1 epoxide did not alter the inhibition results.

Preparation and Solubilization of Microsomes

Beef liver microsomes were prepared by the homogenization of well minced liver with three times its volume of 50 mM potassium phosphate buffer, pH 7.4, containing 250 mM sucrose, 0.5 mM EDTA in a Waring blender (six 20 s pulses) at 4°C and made into a smooth suspension with 20 additional strokes of a Teflon cap hand homogenizer. The homogenate was centrifuged at 12,000 rpm (17,000 × g) for 30 min to remove the cell debris. The supernatant was centrifuged for 90 min at 105,000 × g. The pellets were washed by resuspension in 50 mM Tris-HCl buffer, pH 8.0 containing 0.5 mM EDTA, and recentrifugation at 105,000 × g for 90 min. The pellets were stored at −70°C until further used. Frozen microsomal pellets were thawed and resuspended in 50 mM Tris-HCl, pH 7.4, containing 250 mM sucrose and 0.5 mM EDTA by homogenization with 20 strokes of an all-glass homogenizer. The microsomal suspension usually contained 8–12 mg protein/ml. Partially purified CHAPS-solubilized beef liver microsomal vitamin K_1 epoxide reductase was prepared as previously described.[7] Solubilization of the microsomes with different detergents was carried out as described earlier.[7] Rat liver microsomes, prepared from female Wistar rats, were obtained as a gift from Prof. John W. Suttie (University of Wisconsin). The frozen microsomal pellet was thawed overnight and suspended as described for the beef liver microsomes in 50 mM Tris-HCl, pH 7.4, containing 250 mM sucrose and 0.5 mM EDTA. The rat microsomal suspension usually contained 10 mg of protein/ml.

Protein Determination

The protein content was determined either with the use of BCA or Coomassie blue reagent as developed by the Pierce Chemical Company.

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Enzyme Assay

Vitamin K_1 2,3-epoxide reductase activity was assayed by incubating the microsomal preparation at room temperature in a total volume of 0.5 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose, 5 mM DTT and 40 μM vitamin K_1 epoxide (added in ethanol or as an aqueous solution in Emulgen 911) for 20 min, during which time the reaction was linear. The reaction was quenched by the addition of 2 ml of 25 mM $HgCl_2$ which minimizes the non-enzymatic reaction products. The reaction mixture was then extracted with 3 ml of an isopropanol-*n*-hexane (1:1) mixture. The organic solvent was removed under vacuum in a Savant speed vacuum concentrator. The residue was dissolved in 140 μl of methanol and 100 μl of this solution was analyzed by HPLC (Beckman model 330 with a UV detector and a Hewlett-Packard integrator) using a 5 μ C_{18} reversed phase column and eluting with methanol at 2 ml/min. The vitamin K_1 peaks were calibrated, when necessary, against a standard curve.

RESULTS AND DISCUSSION

Experiments similar to those described by Lee and Fasco[4] for the unsolubilized rat liver microsomal enzyme were carried out on partially-purified CHAPS-solubilized beef liver microsomal vitamin K_1 epoxide reductase. Figure 1 shows the effect of

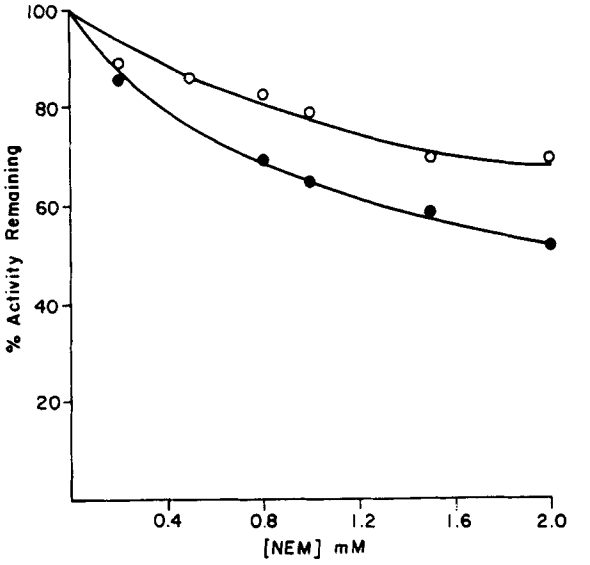

FIGURE 1 Effect of *N*-ethylmaleimide on partially purified beef liver vitamin K_1 epoxide reductase activity[7] in the presence (●) and absence (○) of 0.2 mM dithiothreitol. The experimental procedure was as follows: the partially purified enzyme (100 μg, 0.2 ml) was incubated for 1 min in a total volume of 0.5 ml of 50 mM Tris-acetate pH 8.0 containing 1.5% CHAPS, 10% glycerol, and 0.5 mM EDTA, and then incubated with or without 0.2 mM DTT for 1 min, followed by incubation with various concentrations of NEM for 1 min. DTT (final concentration 5 mM) and vitamin K_1 epoxide (final concentration 20 μM) were added, and after incubation for 30 min the enzyme activity remaining was assayed as described in Materials and Methods.

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pre-treatment of the enzyme with DTT on the loss of enzyme activity by increasing amounts of NEM. At a concentration of 1.0 mM NEM 23% of the enzyme activity was lost without DTT pre-treatment, but only 35% of enzyme activity was lost with DTT pre-treatment. This is in sharp contrast to the results on the rat liver enzyme reported by Lee and Fasco[4] in which case 10–18% and 80% of the enzyme activity was destroyed without or with DTT pre-treatment, respectively. The difference in these results could be caused by a difference arising from the use of solubilized versus unsolubilized microsomes in these two studies or a difference in the beef enzyme versus the rat enzyme, or both.

In order to test the former possibility, the experiment was repeated with resuspended unsolubilized beef liver microsomes. As shown in Table I, when compared with the rat liver results of Lee and Fasco,[4] there is not nearly as much difference in the beef liver results whether or not there is pre-reduction of the enzyme. In order to determine the generality of this observation, the experiment was repeated with two detergents other than CHAPS and with iodoacetamide, another sulfhydryl reagent (Table II). In all cases there was little difference in the results with the non pre-reduced and the pre-reduced enzyme. Vitamin K_1 epoxide, however, protected the enzyme from inactivation by NEM (Table I), indicating that the sulfhydryl reagent was acting at the active site of the enzyme.

When the experiment was repeated with unsolubilized rat liver microsomes, however, a result similar to that reported by Lee and Fasco[4] was obtained, namely, in the

TABLE I

Effect of 1.0 mM NEM on vitamin K_1 epoxide reductase activity in resuspended beef liver microsomes

Pretreatment[a]	Per cent inactivation
untreated	3
dithiothreitol (0.2 mM)	28[b]
dithioerythritol (0.7 mM)	28

[a]Microsomes (1.2 mg) resuspended in a total volume of 0.5 ml of 50 mM Tris-HCl buffer, pH 7.4 containing 250 mM sucrose, were pretreated at 25°C with dithiothreitol (0.2 mM), dithioerythritol (0.7 mM), or buffer for 2 min prior to incubation with NEM (1.0 mM) for 5 min. DTT (final concentration 5 mM) and vitamin K_1 epoxide (final concentration 40 μM) were then added, and the enzyme was assayed as described in Materials and Methods.

[b]The per cent inactivation was nil when 10 μM vitamin K_1 epoxide was added to the pre-reduced prior to addition of NEM.

TABLE II

Effect of 1.0 mM NEM on vitamin K_1 epoxide reductase activity in solubilized beef liver microsomes

Pretreatment[a]	Detergent	Per cent inactivation
untreated	CHAPS (1%)	23
dithiothreitol	CHAPS (1%)	35
untreated	Lubrol PX (0.2%)	44
dithioerythritol	Lubrol PX (0.2%)	65
untreated[b]	Lubrol PX (0.2%)	49
dithioerythritol[b]	Lubrol PX (0.2%)	52
untreated	sodium cholate (1%)	50
dithioerythritol	sodium cholate 1%)	50

[a]Same protocol as in Table I, except that the enzyme source was the supernatant obtained after centrifugation of detergent-solubilized microsomes for 90 min at 105,000 × g. Each assay mixture contained the above concentration of detergent, which gives maximum enzyme activity.

[b]Iodoacetamide (1.0 mM) was used as the sulfhydryl reagent instead of NEM.

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absence of reducing agent there was little inactivation by 1.0 mM NEM, but with DTT pre-treatment 76% of the enzyme activity was destroyed by 1.0 mM NEM. This suggests that the cause of the disparity in results is species related. The results with the rat enzyme were used by Lee and Fasco[4] to support the hypothesis of Silverman[2] that an active site disulfide is reduced in the first step of the enzyme-catalyzed reduction of vitamin K_1 epoxide. As was suggested recently,[7] the beef enzyme, then, may proceed by a different mechanism, for example, in which the dithiol does not reduce an active site disulfide, but, rather, is the reducing agent involved directly with vitamin K_1 epoxide.

These two mechanistic possibilities can be differentiated kinetically. Dithiol reduction of an active site disulfide followed by enzyme dithiol-catalyzed reduction of vitamin K_1 epoxide, should proceed by a ping-pong mechanism, whereas direct dithiol reduction of vitamin K_1 epoxide would not. This was the approach taken by Hildebrandt *et al.*[5] and Preusch and Brummet[6] who showed that the rat liver enzyme obeyed ping-pong kinetics. The same kinetic analysis with the beef liver enzyme was carried out, i.e., the initial velocity patterns of 1/V versus 1/[vitamin K_1 epoxide] (Figure 2A) for several fixed concentrations of DTT and 1/V versus 1/[DTT] (Figure 2B) for several fixed concentrations of vitamin K_1 epoxide. Parallel lines were obtained. This result also was obtained when reduced thioredoxin was substituted for DTT (data not shown). These results suggest that a ping-pong mechanism also is involved in the beef liver enzyme reaction. This conclusion was further supported by a product inhibition study with oxidized DTT (*trans*-4,5-dihydroxy-1,2-dithiane), which was found to be competitive with vitamin K_1 epoxide (K_i = 25 mM) and non-competitive with DTT (K_i = 20 mM), also as expected for the ping-pong

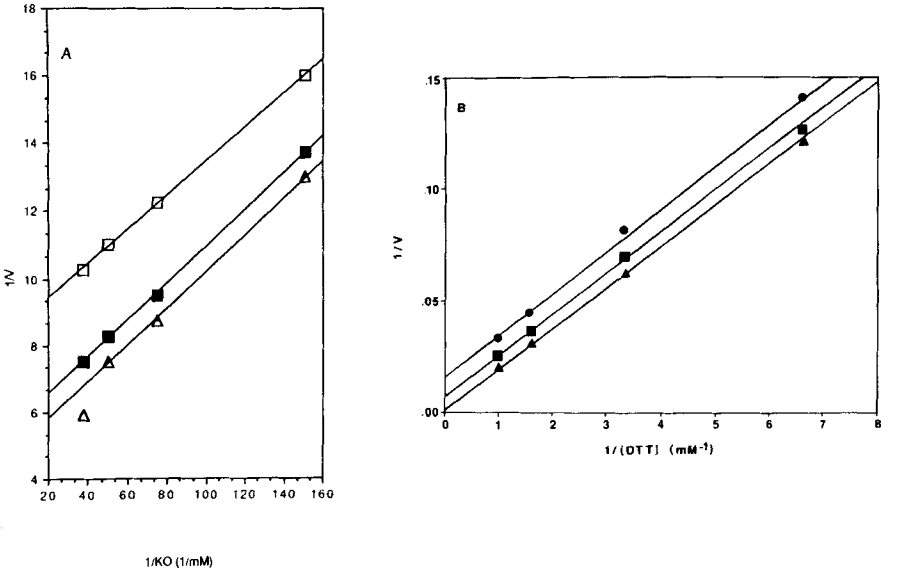

FIGURE 2 Kinetic analysis of beef liver microsomal vitamin K_1 epoxide reductase activity as a function of (A) vitamin K_1 epoxide concentration at fixed concentrations of DTT (□, 0.25 mM; ■, 0.5 mM; △, 1.0 mM) and (B) dithiothreitol (DTT) concentration at fixed concentrations of vitamin K_1 epoxide (●, 10 μM; ■, 15 μM; ▲, 20 μM). Assays were performed as described in Materials and Methods; 1.2 mg of microsomal enzyme was used for each experiment.

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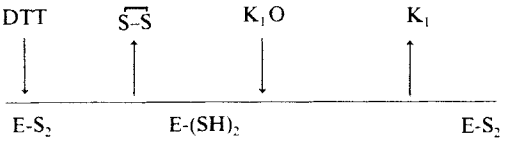

SCHEME 1 Kinetic diagram for the conversion of vitamin K_1 epoxide (K_1O) to vitamin K_1 (K_1) with oxidation of dithiothreitol (DTT) to oxidized DTT ($\overline{S\text{–}S}$) and reduction of oxidized enzyme (E-S_2) to reduced enzyme (E-$(SH)_2$)

mechanism shown in scheme 1. This result, then, contrasts the results described above for the NEM experiments with the beef and the rat liver enzyme, and suggests that the disulfide in the beef liver enzyme may be somewhat shielded so that reaction with NEM is slower than with the rat liver enzyme.

Although there is an apparent structural difference in the beef and rat liver microsomal enzymes, the ping-pong kinetics observed for both enzymes support the hypothesis of Silverman[2] that reduction of vitamin K_1 epoxide proceeds via initial thiol-catalyzed reduction of an active site disulfide bond, and that the thiol is not acting as the direct reductant.[7]

Acknowledgment

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