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VITAMIN K EPOXIDE AND QUINONE REDUCTASE ACTIVITIES

EVIDENCE FOR REDUCTION BY A COMMON ENZYME

SHARYN L. GARDILL* and J. W. SUTTIE†
Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, WI 53706, U.S.A.

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Abstract—Vitamin K hydroquinone formation in rat liver can be catalyzed by a thiol-dependent quinone reductase activity which shares several characteristics with the vitamin K 2,3-epoxide reductase activity. The possibility that a single enzyme catalyzes both reductions was investigated. Values of V_{max}/K_m for several different vitamin K analogs were determined and found to be similar for both reductase activities. Several different coumarins were also shown to achieve 50% inhibition at similar concentrations for both enzyme activities. The chloro analog of menaquinone-2 was shown to inhibit both reductases, and the presence of either the quinone or epoxide form of the vitamin protected both activities from inactivation. Thioredoxin was shown to function as a reductant for both reductase activities, although the maximum enzyme activity achieved by this reductant was only half that achieved with dithiothreitol as a reductant. Cofractionation of the two reductase activities on a variety of column matrices was also observed. These data strongly support the hypothesis that one microsomal enzyme is capable of catalyzing both reduction of vitamin K 2,3-epoxide to the quinone, and the quinone to vitamin K hydroquinone.

The reduced form of vitamin K (vitamin KH_2) is a co-substrate for an enzyme catalyzing the post-translational modification of specific glutamyl residues to γ-carboxyglutamyl residues in a limited number of proteins. These proteins include the plasma clotting factors II, VII, IX, and X, plasma proteins C and S, two well-characterized bone proteins, and uncharacterized proteins in other tissues [1]. Enzymatic formation of γ-carboxyglutamic acid is associated with the formation of vitamin K 2,3-epoxide (vitamin KO) [2], and the epoxide is converted subsequently to the vitamin K quinone (vitamin K) through the action of a microsomal vitamin KO reductase [3]. Formation of the naphthohydroquinone form of the vitamin (vitamin KH_2) in rat liver is catalyzed by DT-diaphorase and/or other NADH-linked microsomal quinone reductases [4, 5] and by a reduced dithiol-dependent enzyme which shares several characteristics with the vitamin KO reductase.

The reduction of both vitamin K and vitamin KO is inhibited by 4-hydroxycoumarins and lapachol [6, 7], and both reductions demonstrate a reduced sensitivity to warfarin (3-α-phenyl-β-acetylethyl-4-hydroxycoumarin) [6] in anticoagulant-resistant rat strains [8]. It has been demonstrated that warfarin interacts with a thiol group on the enzyme and that the presence of vitamin KO protects against this inactivation [9]. Both reductase activities are inhibited by sulfhydryl modifying reagents such as *N*-ethylmaleimide, and either vitamin KO or vitamin K will protect both enzymatic activities from this inactivation [10]. Vitamin K, vitamin KO, or warfarin has also been shown to protect a 14–17 kD warfarin-sensitive protein from modification by [^{3}H]*N*-ethylmaleimide [11]. Vitamin K can act as an effective competitive inhibitor of vitamin KO reductase activity in whole rat liver microsomes [12], and an incomplete product of the vitamin KO reductase, 2(3)-hydroxyvitamin K, is formed as a product of this enzyme in livers from warfarin-resistant rats [13]. These similarities and kinetic analysis of the reactions [14] imply that warfarin, dithiothreitol (DTT), vitamin K, and vitamin KO could all interact at a common site.

We have now investigated the possibility that both quinone and epoxide reductions are catalyzed by the same enzyme through a further definition of the similarities or differences between the two reductase activities. Several vitamin K analogs have been used to compare the structural requirements of the two reductase activities, and the sensitivity of the two activities to inhibition by a number of 4-hydroxycoumarins and by 2-Cl-3-geranyl-1,4-naphthoquinone (Cl-MK-2) has been determined. Thioredoxin has been shown to be capable of acting as a reductant for the vitamin KO reductase [15, 16], and its ability to act as a reductant for the vitamin K reductase activity has been studied. Co-fractionation of the two reductase activities by a number of procedures has been demonstrated.

MATERIALS AND METHODS

Materials. HPLC procedures utilized Waters (Bedford, MA) models 6000A and M45 pumps, a 440 absorbance detector, a UK-6 injector, and a

* Present address: Department of Physiological Chemistry, University of Wisconsin-Madison, Madison, WI 53706.
† To whom correspondence should be addressed.

Table 1. Retention times and solvent systems for vitamin K metabolites

	Retention time (min)		
	Quinone*	Epoxide*	Naphthohydroquinone†
Phylloquinone	8.0	5.3	14.8
Menaquinone-2 (MK-2)	15.2	10.6	14.1
2-Ethyl-MK-2	15.8	11.2	14.5
5-Methyl-MK-2	15.3	11.5	13.9
6-Methyl-MK-2	10.1	7.03	14.4
7-Methyl-MK-2	10.0	7.45	15.2
8-Methyl-MK-2	15.2	11.4	
2-Methyl-3-undecyl-1,4-naphthoquinone (undecyl-K)	17.2	11.5	16.3

* Flow rate of 2.0 mL/min and 97:3 (methanol:H_2O) for phylloquinone, 1.5 mL/min and 90:10 (methanol:H_2O) for 5 and 7-Me-MK-2, 1.4 mL/min and 80:20 (methanol:H_2O) for all other forms of vitamin.

† Flow rate of 2.0 mL/min and 80:12 (methanol:H_2O) for phylloquinone, 1.0 mL/min and 80:20 (methanol:H_2O) for all other forms of the vitamin.

710C WISP-automatic injector. Gradient elutions were achieved with a Waters model 660 system controller. Fluorometric detection was achieved using a Perkin–Elmer model 650-10LC fluorescence spectrophotometer set at 430 nm excitation and 340 nm absorption and equipment with a flow cell. Separation of vitamin K metabolites was achieved on a Waters C18 μBondapak 10 μm analytical column with the solvent systems, flow rates, and retention times shown in Table 1. Vitamin extinction coefficients (in hexane) utilized to quantitate standards were: 30,880 $M^{-1}cm^{-1}$ at 225 nm for vitamin KO [17]; 18,900 $M^{-1}cm^{-1}$ at 248 for vitamin K [18]; 34,600 $M^{-1}cm^{-1}$ for hydroxy viamin K (vitamin KHOH) [19]. The extinction coefficient for vitamin KH_2 was taken to be 0.898 that of vitamin K at 254 nm in 95% methanol:5% water [20]. All manipulations of vitamin K derivatives were conducted under yellow light or in a darkened room. Fast protein liquid chromatography (FPLC) of proteins utilized an all Pharmacia (Piscataway, NJ) system consisting of an ACT 100 injector, P-500 pumps, a single path UV monitor, and a GP-250 gradient programmer. A Pharmacia high pressure HR 5/10 column was used with this system.

Phylloquinone, TAPS [*N*-tris(hydroxymethyl)-methyl-3-amino-propane sulfonic acid] buffer, CHAPS (3-[(3-cholamidopropyl)dimethyl-ammonio] 1-propane sulfonate), Lubrol PX, polyethylene glycol (PEG), glutathione, glutathione reductase, NADH, NADPH, cyanogen bromide, DTNB [5,5'-dithiobis(2-nitrobenzoic acid)], and Sepharose 4B were all purchased from the Sigma Chemical Co. (St. Louis, MO). DTT was from Boehringer Mannheim (Indianapolis, IN), and sodium cholate from Kodak (Rochester, NY); 0.22 micron filters were from Millipore (Bedford, MA), and HPLC grade solvents from American Burdick & Jackson (Muskegon, MI). The chromatographic matrices, phenyl Sepharose, and lentil lectin-Sepharose 4B were all purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Thioredoxin was obtained from Calbiochem (San Diago, CA). Whatman DE-52, microgranular, preswollen anion exchanger was purchased from American Scientific Products (McGraw Park, IL), and Emulgen 91 was obtained from KAO Atlas (Tokyo, Japan). Warfarin was obtained from the Wisconsin Alumni Research Foundation (Madison, WI), and other 4-hydroxycoumarins were a gift from Dr. Colin Schroeder (D&S Associates, Madison, WI). These compounds were purified by HPLC on a 10 μm C18 μBondapak column. All other chemicals were of analytical reagent grade.

Preparation of vitamin K derivatives. Analogs of menaquinone-2 (MK-2) [21] and Cl-MK-2 were synthesized by Dr. Alex Cheung. The 2,3-epoxides of vitamin K analogs were prepared by alkaline hydrogen peroxide oxidation according to the method of Tishler *et al.* [17] and purified by preparative HPLC on a Waters C18 μBondapak 10 μm semipreparative column. Vitamin KHOH was synthesized as previously described [13], and vitamin KH_2 was prepared by dithionite reduction [22].

Preparation of microsomes. Male 250 g rats were obtained from the Holtzman Co. (Madison, WI) or the Sprague–Dawley Co. (Madison, WI) and fed a vitamin K-deficient diet [23] in coprophagy-preventing cages [24] for 7 days, fasted overnight, and killed by decapitation. The livers were excised, rinsed, and weighed. Homogenates (50%, w/v) were prepared in 0.25 M sucrose/0.025 M imidazole buffer (pH 7.2) using a polytron homogenizer (Brinkmann Industries, Des Plaines, IL). The homogenate was centrifuged at 10,000 *g* for 15 min using a Beckman JA-21 rotor and centrifuge, and the postmitochondrial supernatant fraction was centrifuged at 105,000 *g* for 1 hr using a Ti-65 ultracentrifuge rotor and a Sorval model OTD75B ultracentrifuge. The microsomal pellets were surface washed with sucrose/imidazole buffer and stored in liquid nitrogen.

Assays. Vitamin K quinone and epoxide reductase activities were assayed in a total volume of 0.5 to 1 mL of 0.25 M sucrose/0.010 M TAPS/0.15 M KCl/0.6% cholate and 1 mM DTT (pH 8.8) containing 10 μM vitamin in 1% Emulgen 911. The reaction was incubated at 25° for 5 min during which time product

formation was linear. Epoxide reductase reactions were carried out in air and terminated with 2 vol. of isopropanol: hexane (3:2, v/v), the hexane layer was removed, dried under nitrogen, and dissolved in methanol, and quinone formed was quantitated by HPLC. Quinone reductase assays were run under nitrogen in sealed vials and quenched with 1 vol. of ice-cold isopropanol. After low speed centrifugation, vitamin KH_2 in a fraction of the isopropanol phase was quantitated by HPLC analysis. Initial rate data were obtained from time courses of product formation, and V_{max}/K_m values were determined from double-reciprocal plots of initial rate data and fitted to the hyperbola $v = VA/(K + A)$ using Cleland's program HYPERO [25]. Protein concentrations were determined by the method of Lowry *et al.* [26] or spectrophotometrically at either 254 nm or 280 nm.

Thioredoxin studies. Epoxide and quinone reductase activities were partially purified from microsomes by PEG precipitation and dye-ligand chromatography. Enzyme activities were assayed as previously described with 150 μM thioredoxin replacing 1 mM DTT. The reaction mixture also contained 1.8 units of thioredoxin reductase and 10 mM NADPH. Thioredoxin reductase was purified (and quantitated) according to the method of Luthman and Holmgren [27].

Anion exchange chromatography. Rat liver microsomes solubilized in 0.010 M TAPS/0.250 M sucrose/1 mM DTT (pH 8.3) containing 1% sodium cholate were passed through a Millipore 0.22 micron filter. The filtrate (containing 50–80 mg of protein) was applied to a 1 cm × 12.7 cm gravity packed Whatman DE-52 anion exchange column previously equilibrated in 0.01 M TAPS/0.25 M sucrose/1 mM DTT (pH 8.3) containing 0.2% Lubrol PX (buffer A). FPLC chromatography was carried out at a flow rate of 1 mL/min utilizing a complex salt gradient made by using a solvent programmer. The gradient consisted of 50% buffer B (buffer A containing 1 M NaCl) incorporated into buffer A linearly for 9 min, maintenance of this concentration for 9 min, and a linear gradient of from 50 to 100% buffer B achieved over an additional 42-min time period. The entire preparative procedure was carried out at room temperature.

Thioredoxin affinity chromatography. A thioredoxin affinity column was prepared according to the method of Cuatrecasas and coworkers [28]. Rat liver microsomes were solubilized in 0.01 M TAPS/0.25 M sucrose/0.15 M KCl (pH 8.8) containing 1% sodium cholate and applied to a 1 cm × 3.5 cm thioredoxin affinity column which was equilibrated previously in 0.01 M TAPS/0.25 M sucrose/0.15 M KCl (pH 8.8). Chromatography was carried out at a flow rate of 0.5 mL/min. Elution of activity was achieved with a 5-mL application of 20 mM DTT. The entire procedure was carried out at 4°.

Lectin chromatography. Rat liver microsomes were resuspended in 0.01 M TAPS/0.15 M KCl and 20% glycerol (pH 8.8) and re-pelleted by centrifugation at 105,000 *g* for 1 hr. The washed microsomes (30 mg protein) were solubilized in 0.01 M TAPS/0.5 M KCl/20% glycerol/1 mM DTT (pH 8.8) containing 1% sodium cholate and applied to a 1 cm × 4 cm lentil lectin-Sepharose 4B column previously equilibrated with solubilizing buffer. Chromatography was carried out at a rate of 0.5 mL/min at 4°. Elution of activity was achieved using 0.01 M TAPS/0.2 M KCl/1% sodium cholate/1 mM DTT and 0.5 M sucrose (pH 8.8).

Hydrophobic chromatography. Rat liver microsomes were solubilized in 0.01 M TAPS/0.25 M sucrose/0.8 M NaCl/1 mM DTT (pH 8.3) containing 1% sodium cholate and passed through a 0.22 micron filter. The filtrate (containing 30–50 mg protein) was applied to an FPLC apparatus equipped with a phenyl-Sepharose column. The 1 cm × 12.7 cm column was equilibrated previously with 0.01 M TAPS/0.25 M sucrose/0.8 M NaCl/1 mM DTT and 0.2% Lubrol PX. Chromatography was carried out at a flow rate of 1 mL/min. Elution was achieved with a step gradient of 0–5% DMSO (dimethyl sulfoxide) in equilibration buffer. The procedure was carried out at room temperature. The eluant was concentrated using an Amicon apparatus equipped with a PX 10 filter.

Dye-ligand chromatography. Rat liver microsomes were solubilized in 0.01 M TAPS/0.25 M sucrose/0.15 KCl (pH 8.8) containing 1% sodium cholate. PEG was added to a final concentration of 5% (w/v). After centrifugation at 105,000 *g* for 30 min, the supernatant fraction was discarded, and the pellet (containing approximately 15 mg protein) was resolubilized in solubilization buffer and applied to an Affigel blue column (1 cm × 4 cm). The column was equilibrated and developed in 0.01 M TAPS/0.25 M sucrose/0.15 M KCl (pH 8.8). The procedure was carried out at room temperature at a flow rate of 0.5 mL/min.

RESULTS

Substrate specificity for reductases. The activities of several vitamin K analogs as substrates for the vitamin K 2,3-epoxide reductase were determined, and the kinetic constants are presented in Table 2. Substitution of MK-2 with a methyl group at the 6- or 7-position had little effect on substrate activity, but the 5- or 8-methyl derivatives were markedly less active. These two analogs also had K_m values that were at least an order of magnitude higher than those of the other epoxides assayed. The 2-ethyl-MK-2 and 3-undecyl vitamin K analog had kinetic constants in the same range as the phylloquinone and MK-2 epoxides. Similar data for the quinone reductase activity exhibited by the same analogs are also presented in Table 2. Both K_m and V_{max} values for the reduction of the quinone analogs to the hydronaphthoquinone form are in the same range as those determined for epoxide reduction. As was the case for epoxide reduction, the 5-methyl-MK-2 analog demonstrated a relatively low activity and a greatly increased K_m when compared to the other analogs. To compare the similarity between the vitamin K epoxide reductase and the quinone reductase activities, V_{max}/K_m values for both activities are plotted in Fig. 1. The catalytic efficiency for the various vitamin K analogs assayed was similar for the two enzyme activities. Although the V_{max}/K_m values for the various forms of the vitamin covered a wide range, there

Table 2. Kinetic constants for various vitamin K analogs as substrates for the epoxide reductase and quinone reductase activities of rat liver microsomes

Compound	Epoxide reductase		Quinone reductase	
	K_m (μM)	V_{max} (nmol/min)	K_m (μM)	V_{max} (nmol/min)
Phylloquinone	9.5 ± 1.1	3.3 ± 0.2	15.3 ± 3.5	7.3 ± 0.8
Undecyl-K	25.9 ± 0.8	2.1 ± 0.2	11.3 ± 0.9	0.9 ± 0.1
MK-2	11.2 ± 2.3	4.3 ± 1.1	14.5 ± 4.3	5.6 ± 1.9
2-Ethyl-MK-2	6.3 ± 0.05	2.1 ± 0.3	11.7 ± 3.4	3.9 ± 0.3
5-Methyl-MK-2	186.3 ± 11.5	0.46 ± 0.03	136 ± 23.1	0.73 ± 0.2
6-Methyl-MK-2	8.7 ± 0.9	3.0 ± 1.2	15.8 ± 2.1	6.2 ± 0.7
7-Methyl-MK-2	11.3 ± 1.2	5.1 ± 0.3	14.1 ± 0.9	4.5 ± 1.2
8-Methyl-MK-2	209 ± 15.3	0.70 ± 0.09		

Values are means ± SE for three or four measurements/compound.

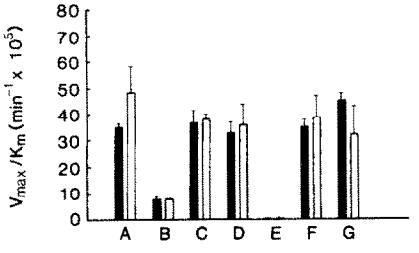

Fig. 1. Vitamin K analogs as substrates for microsomal vitamin KO and vitamin K reductase activities. Assays were conducted, initial rates of product formation were determined, and V_{max}/K_m values were derived as described in Materials and Methods. Key: (A) phylloquinone, (B) undecyl K, (C) menaquinone-2, (D) 2-ethyl menaquinone-2, (E) 5-methyl menaquinone-2, (F) 6-methyl menaquinone-2, and (G) 7-methyl menaquinone-2. Values are means ± SE for triplicate assays. Closed bars: vitamin KO reductase activity. Open bars: vitamin K reductase activity.

was remarkable similarity between the responses of the two enzyme activities.

Inhibition of reductase activities by 4-hydroxycoumarins. The concentrations of a number of 4-hydroxycoumarins required to reduce the activity of both the epoxide and quinone reductase by 50% (I_{50}) were also investigated (Table 3). As can be seen, 3-(α-ethyl-benzyl)-4-hydroxycoumarin was a more effective inhibitor for both reductase activities than warfarin. However, 3,3′-butylidene-bis-4-hydroxycoumarin-4,4′-diacetate demonstrated a 5-fold increase, and 3-phenyl-4-hydroxycoumarin and 6-hydroxywarfarin demonstrated a nearly 10-fold increase in concentration needed to cause a 50% reduction in both the epoxide and quinone reductase activities. An additional compound, 7,8-dihydroxycoumarin, was also tested as a potential inhibitor of both reductase activities. The activity of neither the epoxide reductase nor the quinone reductase could be reduced by more than 10% at millimolar coumarin concentrations of this potential inhibitor.

Reductase inhibition by Cl-MK-2. Inhibition of the epoxide and quinone reductase activities by a different class of inhibitors (Cl-MK-2) was also investigated, and the I_{50} was found to be between 0.3 and 0.4 μM for both activities. This inhibition (Fig. 2) was dependent upon prereduction of the enzyme, and incubation of dithiothreitol-reduced microsomes with either vitamin K quinone or vitamin K epoxide prior to Cl-MK-2 addition diminished the extent of inhibition of both activities by 80–90% (Fig. 3).

Thioredoxin as a reductant for both reductase activities. The extent of the NADPH-dependent vitamin K reductase activity in crude microsomes was reduced by partial purification of this activity by PEG precipitation and dye-ligand chromatography. This protocol removed approximately 75% of the NADPH-dependent hydroquinone formation (data not shown). It was observed that a reaction mixture containing partially purified enzyme, thioredoxin,

Table 3. Effects of various 4-hydroxycoumarins on the epoxide reductase and quinone reductase activities of rat liver microsomes

Compound	Epoxide reductase I_{50} (μM)	Quinone reductase I_{50} (μM)
Warfarin	2.6 ± 0.4	2.3 ± 0.8
3-(α-Ethyl-benzyl)-4-OH-coumarin	1.0 ± 0.5	1.4 ± 0.1
3-Phenyl-4-OH-coumarin	23.7 ± 3.3	29.7 ± 6.8
6-OH-warfarin	20.0 ± 2.7	18.4 ± 0
3,3′-Butylidene-bis-4-OH-coumarin-4,4′-diacetate	12.0 ± 1.7	13.8 ± 1.1

Values are means ± SE for three or four measurements/compound.

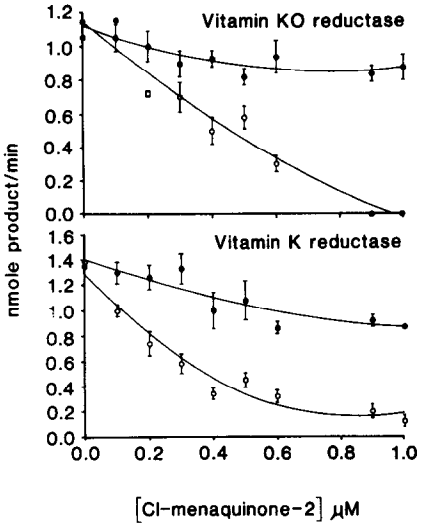

Fig. 2. Effect of the concentration of the 2-chloro analog of menaquinone-2 on the rates of vitamin KO and vitamin K reduction. Assays were performed as described in Materials and Methods, but DTT was omitted from the reaction mixture. The following were added at 1-min intervals: 0.1 mM DTT or an equal volume of buffer to prereduce microsomes; Cl-MK-2 or an equal volume of ethanol (final concentration less than 5%); 5 mM DTT to destroy excess Cl-MK-2 and to serve as a cofactor for the uninhibited enzyme; 20 μM vitamin K 2,3-epoxide. Each point is the average of triplicate incubations ± SE. Key: (—○—) microsomes pretreated with DTT, and (—●—) no pretreatment.

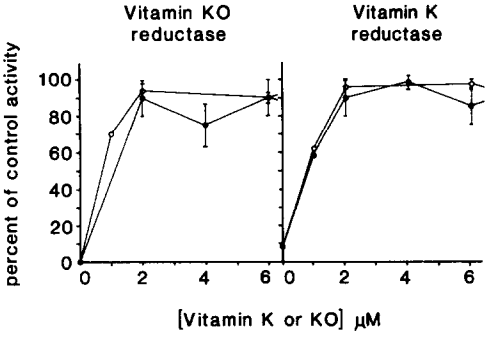

Fig. 3. Effects of vitamin K or vitamin K 2,3-epoxide on Cl-MK-2 inhibition of vitamin K 2,3-epoxide and vitamin K quinone reduction in reduced rat liver microsomes. Assays were conducted as described in Materials and Methods, but DTT was omitted from the reaction mixture. The following were added at 1-min intervals: 0.1 mM DTT to prereduce the microsomes; 0–6 μM vitamin K (—●—) or 0–6 μM vitamin KO (—○—); 2 μM Cl-MK-2; 5 mM DTT to destroy excess Cl-MK-2 and to serve as a cofactor for the ininhibited enzyme; 20 μM vitamin KO (KO reductase activity) or 20 μM vitamin K (K reductase activity). Data are expressed as a percentage of the vitamin K formation or vitamin KH_2 formation in the absence of Cl-MK-2. The control rate of vitamin KO reduction was 1.3 nmol/min, and the control rate of vitamin K reduction was 1.1 nmol/min. Each point is the average of duplicate incubations and the range of values.

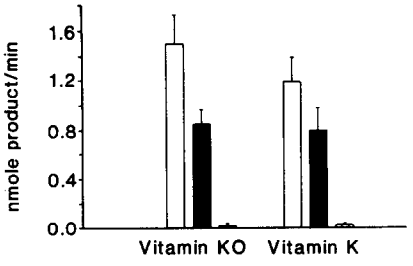

Fig. 4. Utilization of thioredoxin, DTT, or glutathione as a reductant for vitamin KO and vitamin K reduction. Assays were conducted as described in Materials and Methods. Either 1 mM DTT (open bars); 150 μM thioredoxin, 0.8 units thioredoxin reductase, 0.01 mM NADPH (closed bars); or 8 mM glutathione, 20 units glutathione reductase, 10 mM NADPH (hatched bars) were incubated for 5 min prior to the addition of the partially purified reductase activities and substrate. The results are the average of three separate experiments ± SE.

thioredoxin reductase, and NADPH would serve as a reductant for both the vitamin KO and vitamin K (Fig. 4). The maximum rate of reduction of both metabolites was reached at a thioredoxin concentration of approximately 150 μM. This rate of reduction was about 50% that obtained with 1 mM DTT. Glutathione in the presence of glutathione reductase and NADPH was not able to function as a reductant for either of the reductase activities.

Cofractionation of reductase activities. Solubilized microsomes were subjected to fractionation by anion exchange chromatography, hydrophobic chromatography, or affinity to immobilized thioredoxin, lentil lectin, or Affigel blue by procedures described in Materials and Methods. The enzyme activity that could be recovered in a peak fraction and the extent of purification achieved by these various column chromatographic procedures varied considerably. However, the enrichment of enzyme activity was essentially the same for vitamin KO reduction and vitamin K reduction in all cases (Table 4).

DISCUSSION

Vitamin KO formed from vitamin KH_2 by the liver microsomal γ-glutamyl carboxylase reaction is recycled via two reductase activities that share many of the same characteristics; they are both microsomal, thiol dependent, inhibited by 4-hydroxycoumarins, and demonstrate a reduced susceptibility to warfarin in warfarin-resistant rat strains. These observations and studies of the properties of the two enzyme activities have suggested that the vitamin K, 2,3-epoxide and quinone reductase activities are carried out by the same enzyme. The results presented in this report further substantiate and extend this hypothesis.

The data in Fig. 1 demonstrate that V_{max}/K_m values for six different vitamin K analogs of varying substrate efficiency were similar for both reductase activities. This value, the apparent first-order rate constant for the enzymatic reaction at very low substrate concentrations, reflects the ability of an

Table 4. Cofractionation of reductase activities

Matrix	Fold increase in specific activity	
	Vitamin KO reductase	Vitamin K reductase
DE-52 (anion exchange)	78	73
Phenyl-Sepharose (hydrophobic)	40	43
Thioredoxin-Sepharose (thiol affinity)	42	38
Lentil lectin (glyco affinity)	1.4	2.1
Affigel blue (dye ligand)	10	10

Chromatography of solubilized crude microsomes on each of these column supports was carried out as described in Materials and Methods, and the specific activity of both activities in the fraction that was most enriched for the vitamin KO reductase activity was compared to that of the crude microsomes.

enzyme and its substrate to form a complex, and the consistency of this value for both reductase activities provides additional evidence that one enzyme is catalyzing both reactions. Although *cis*- or *trans*-phylloquinone have been shown previously to be equally competent as a substrate for the vitamin KO reductase activity [29], the enzyme has been shown [30] to demonstrate some selectivity for 2,3-substituted naphthoquinones. The current study (see Table 2) indicates that there is little selectivity for a methyl substituent at the 6- or 7-position of the vitamin but that the addition of a methyl group at the 5- or 8-position leads to a substantial decrease in activity. These results are similar to those observed when these analogs were assayed as substrates for the vitamin K-dependent carboxylase [21]. However, in contrast to the results reported here, the 5- and 8-methyl analogs had very low K_m values as substrates for the carboxylase. Although the 2-ethyl MK-2 analog was a good substrate for both the vitamin KO and vitamin K reductase and quinone reductase activities, it was a very poor substrate for the carboxylase.

Inhibition of the thiol-dependent vitamin K reductase(s) is thought to be the pharmacologically important site of 4-hydroxycoumarin action. Table 3 demonstrates that a variety of different 4-hydroxycoumarins achieve 50% inhibition at the same concentration for both enzyme activities, lending additional support to a model where both reductase activities are carried out by the same enzyme. However, the possibility that the physiologically relevant enzymes of the vitamin K cycle exist as individual parts of a multienzyme complex and that 4-hydroxycoumarins bind to a common site on a subunit separate from that of the enzyme(s) catalytic site(s) has not been ruled out.

Mechanistic studies have implied sulfhydryl group involvement in both vitamin KO and vitamin K reduction [10, 19, 31] and chloro analogs of vitamin K can form thiol adducts with several low molecular weight thiol compounds [32]. As seen in Fig. 2, Cl-MK-2 inhibited the thiol-dependent vitamin K reductases. This result as well as the observation that either substrate can protect either reductase activity from Cl-MK-2 inhibition (Fig. 3) lends additional support to a single active site catalyzing both reductase reactions. However, the possibility of a common disulfide transferring reducing equivalents to separate catalytic sites to reduce the appropriate vitamin substrate still exists. Because inhibition is dependent upon prereduction of the microsomes, it would appear that, at least in this system, Cl-MK-2 functions as a sulfhydryl modifying reagent.

Mammalian thioredoxin, although not studied as extensively as the bacterial form, has been implicated in a number of metabolic systems [33]. Recently, it has been suggested [15, 16] that thioredoxin is the physiological reductant for the metabolism of vitamin KO. Figure 4 demonstrates that in the presence of thioredoxin reductase and NADPH it can function as a reductant for both the partially purified vitamin K epoxide and quinone reductase activities. This observation lends additional support to both reductase reactions being catalyzed by a single enzyme. Thioredoxin is only one-half as effective as DTT as a reductant for both vitamin KO and vitamin K reductase activities, and whether or not thioredoxin is the endogenous reductant to the vitamin K cycle remains to be proven.

Vitamin K 2,3-epoxide reductase is an integral membrane protein that has not been purified, but several procedures have been reported for the solubilization and partial purification of this activity [20, 31, 34, 35]. Several fractionation procedures were used in this study, and the results in Table 4 demonstrate that both vitamin KO and vitamin K reductase activities cofractionate on a variety of column supports. This cofractionation along with the other results in this report clearly support the hypothesis that both the vitamin K 2,3-epoxide reductase and the vitamin K quinone reductase activities are carried out by the same enzyme. Final proof of this hypothesis, however, will depend on obtaining a single purified enzyme that is capable of catalyzing both reactions.

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