Assembly of the Warfarin-sensitive Vitamin K 2,3-Epoxide Reductase Enzyme Complex in the Endoplasmic Reticulum Membrane*  

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The reduced form of vitamin K₁ (vitamin K₁H₂) functions as a cofactor for the vitamin K-dependent carboxylase (γ-carboxylase), an enzyme which resides in the endoplasmic reticulum (ER) membrane and participates in post-translational γ-carboxylation of secretory proteins (1, 2). The carboxylase converts a limited number of glutamic acid residues in the targeted proteins to γ-carboxyglutamic acid residues (1, 2). The vitamin K-dependent proteins include the coagulation factors prothrombin, factors VII, IX, and X, and proteins S and C, which are produced by the liver (2), and several proteins synthesized in extrahepatic tissues, some of which have been identified. They are osteocalcin (3), matrix γ-carboxyglutamic acid protein (3), protein S (4), and Gas6 (5). γ-Carboxylation of one Glu residue is coupled stoichiometrically to formation of 1 molecule of the vitamin K metabolite, vitamin K₁ 2,3-epoxide. The epoxide is reduced by liver enzyme(s) back to the hydroquinone cofactor form of the vitamin, vitamin K₁H₂, and this cyclic conversion establishes a redox cycle for vitamin K in liver, known as the vitamin K cycle. Two unrelated vitamin K-reducing enzymes have been identified as part of the cycle (6). The flavoprotein DT-diaphorase (EC 1.6.99.2) reduces the quinone form of vitamin K₁ but not the epoxide (7). On the other hand, the warfarin-sensitive enzyme vitamin K epoxide reductase (VKOR) reduces the epoxide as well as the quinone and thus is essential for operation of the cycle (8).

Because of the importance of warfarin as an anticoagulant in prophylactic medicine (9) and as a poison in rodent pest control (10), numerous attempts have been made to understand the molecular mechanism underlying warfarin-sensitive vitamin K 2,3-epoxide reduction. These studies have demonstrated repeatedly that thiol groups are involved in the enzyme reaction (11) and in recognition of warfarin as an enzyme inhibitor (12, 13). Nevertheless, the molecular components that constitute the enzyme have not been identified.

In a search for protein components that would be involved in this reaction we designed an in vitro γ-carboxylation test system where the warfarin-sensitive VKOR enzyme produces the cofactor for the γ-carboxylase. Dissection of this system by chromatographic techniques has identified a member(s) of the gluthathione S-transferase gene family as one component of the VKOR enzyme complex in the endoplasmic reticulum membrane. The affinity-purified glutathione S-transferase(s) was sensitive to warfarin but lost its warfarin sensitivity and glutathione transferase activity upon association with lipids in the presence of Mn²⁺ or Ca²⁺. In the γ-carboxylation test system, loss of warfarin-sensitive glutathione S-transferase activity coincided with formation of the VKOR enzyme complex. It is proposed that formation of VKOR in the endoplasmic reticulum membrane resembles formation of the lipoxygenase enzyme complex where the glutathione S-transferase-related FLAP protein binds cytosolic lipoxigenase to form a membrane enzyme complex.

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The abbreviations used are: vitamin K₁H₂, reduced form of vitamin K₁; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylycerine; CDNB, 1-chloro-2,4-dinitrobenzene; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio-1-propanesulfonate; DTT, dithiothreitol; GST, glutathione S-transferase; mGST, microsomal glutathione S-transferase; VKOR, warfarin-sensitive vitamin K 2,3-epoxide reductase; mEH, microsomal epoxide hydrolase; ER, endoplasmic reticulum; IAM, immobilized artificial membrane.

Animals—Normal male Sprague-Dawley rats weighing 250–300 g were purchased from Zivic Miller Laboratories, Inc., Zelienople, PA. Vitamin K-dependent Enzyme Assays—γ-Carboxylation activity was measured as incorporation of 14CO₂ into the carboxylase substrate peptide Phe-Leu-Glu-Glu-Leu using chemically reduced vitamin K₁H₂ to trigger the reaction (14). The reduced vitamin was prepared by dithionite reduction as described previously (15). To prevent vitamin K epoxide reduction by the vitamin K cycle, 10 μM warfarin was added to the test system. Warfarin-sensitive VKOR activity was measured as described elsewhere (8) by estimating the percent conversion of vitamin K₁ 2,3-epoxide to vitamin K₁. The vitamin and the epoxide were separated by high performance liquid chromatography on a reversed phase C18 column in methanol and quantified against external standards. Vitamin K₁ 2,3-epoxide was prepared by H₂O₂ oxidation (16) and added to the test system in ethanol to give a final concentration of 8 μM.
VKOR-supported γ-carboxylase activity was measured as described above for γ-carboxylase activity, except that warfarin was not added to the test system and the reaction was triggered with 8 μM vitamin K₁, 2,3-epoxide and 5 mM DTT. The reaction was carried out at 26 °C. These concentrations of epoxide and DTT were shown previously to be saturating for the enzyme reaction (6). All three assays were linear for 30 min at 26 °C.

Other Enzyme Assays—GST activity was measured as described by Andersson et al. (17) using CDNB (1 mM) as a substrate in the presence of 1 mM GSH. Warfarin-sensitive GST activity was determined after addition of 5 mM warfarin to the assay mixture. Microsomal epoxide hydrolase (mEH) activity was measured as the rate of conversion of styrene 1,2-epoxide to the corresponding diol (18).

Preparation of Microsomes, Detergent Extraction, and Reconstitution of the γ-Carboxylation System—Rat liver microsomes were prepared as described previously (6) and stored frozen at −85 °C. Prior to use, microsomes were resuspended in ice-cold 0.025 M imidazole-HCl buffer, pH 7.6, containing 0.5% CHAPS (buffer A) and 2 mM diisopropyl fluorophosphate with six strokes in a tight fitting Dounce homogenizer to give a protein concentration of 7 mg/ml.

After resuspension, the microsomal homogenate was left on ice for 15 min and then centrifuged at 100,000 × g for 45 min. The supernatant was collected, and the remaining pellet surface washed with buffer A. The pellets remaining after extraction were resuspended in: 1) buffer A (control); 2) the buffer A extract, or 3) purified proteins from the extract dissolved in buffer A, respectively. In each set of experiments the final protein concentration of the pellet was identical in all samples. Before measuring vitamin K-dependent enzyme activities, 10 mM MnCl₂ was added to each test sample. Warfarin was added at a concentration of 10 μM.

Purification of Warfarin-sensitive GSTs—The microsomal extract in buffer A was gel-filtrated on a Bio-Gel P-100 column equilibrated in the same buffer. The retained fraction (fraction B-P(100)) was loaded onto a cellulose DE52 anion exchanger (Whatman) equilibrated in buffer A. The unretained fraction (fraction A, see Fig. 3) was collected and loaded onto an agarose-GSH affinity column equilibrated in buffer A. All chromatographic steps were carried out at 4 °C.

Table-Embedded Artificial Membrane (IAM). Chromatography—Pre-packed columns of silica beads covered with immobilized phosphatidylcholine (PC) (REGIS Technologies, Inc., Morton Grove, IL) were equilibrated in buffer A without detergent. GSTs purified by agarose-GSH affinity chromatography were first stirred for 1 h with SM2 beads (Bio-Rad) to remove excess detergent. The beads were removed by filtration, and the clear solution was loaded onto the IAM column. The unretained protein fraction was collected, and the column was washed with several bed volumes of buffer A without detergent before the fractions of retained GSTs were eluted from the column in 250 mM imidazole, 0.5 mM KCl, 0.75% CHAPS, pH 7.85.

Additional Methods—Liposomes were prepared by sonication of asolectin as described previously (19). SDS-PAGE in 10 and 15% gels was carried out according to Laemmli (20) and stained with Coomassie Blue as described previously (21). For Western blotting, proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and immunocomplexes were detected with alkaline phosphatase-conjugated secondary antibodies as described previously (14, 22). N-terminal sequence analysis was performed by the Protein Analysis Core Laboratory at The Bowman Gray School of Medicine. Protein sequence comparisons were carried out using the Genetics Computer Group Sequence Analysis Software Package Version 8.0.

Materials—A rabbit polyclonal antiserum against microsomal glutathione S-transferase (mgGST) was kindly provided by Dr. Ralf Morgenstern, Division of Toxicology, Karolinska Institutet, Stockholm, Sweden. A rabbit polyclonal antiserum raised against recombinant human eGSTAI was a gift from Dr. Alan Townsend, The Bowman Gray School of Medicine. Vitamin K₁, GSH, agarose-GSH, and Sepharose-iminodiacetic acid were from Sigma. Asolectin from soybeans was from Fluka, Buchs, Switzerland. [3H]Styrene 1,2-epoxide (specific activity, 314 × 10⁶ cpm/mmol) was a gift from Dr. Thomas Guenther, Department of Pharmacology, University of Illinois, Chicago.

RESULTS

Reconstitution of the γ-Carboxylation System by Metal-induced Lipid Aggregation—To identify individual proteins that contribute to the activity of the multicomponent vitamin K-dependent γ-carboxylation system (11), it was necessary to develop a reconstitution assay system. A prerequisite for such a system to work was the finding of experimental conditions where the γ-carboxylase and the VKOR components of the system were both active and could be separated and assembled again for reconstitution of the system. Consistent with earlier reported work on VKOR (23), we found that a variety of experimental conditions which retained γ-carboxylase activity destroyed VKOR activity. Most of the commercially available detergents inactivates VKOR. However, when rat liver microsomes were resuspended in an isotonic buffer (buffer A) containing the zwitter ionic detergent CHAPS, γ-carboxylase and VKOR were both active. Furthermore, when increasing concentrations of MnCl₂ was added to microsomes resuspended in buffer A, a 14-fold increase in VKOR supported γ-carboxylase activity was observed (Fig. 1, triangles). MnCl₂ addition also stimulated VKOR (squares), and γ-carboxylase (circles) activities 6- and 12-fold, respectively (see Fig. 1). Calcium was found to be equally effective as Mn²⁺ but Mg²⁺ was only half as effective (data not shown). Aggregation of negatively charged lipids with divalent cations will occur in isotonic buffers (24) and provided a mechanism for reconstitution of the γ-carboxylation system. The cation attracts negative phospholipids present in different detergent micelles resulting in a merger of these lipid-protein detergent micelles to form lipid aggregates (24) where proteins are inserted as in a lipid bilayer (25). Based on the data shown in Fig. 1, 10 mM MnCl₂ was used to trigger reconstitution of the γ-carboxylation system enzyme activities. In this system no nonenzymic reduction of vitamin K₁ 2,3-epoxide took place at pH 7.6 and there was no hydroxy-vitamin K₁ formation (26). Optimal VKOR supported γ-carboxylase activity was also measured at this pH.

Having established a reconstitution test system, the next objective was to use the system to identify essential components of the γ-carboxylation system. Separation of components belonging to the system was achieved by extracting microsomes with buffer A. Reassembly of the extract and the extracted
**FIG. 2. Detergent extraction of VKOR from the ER membrane.** Microsomes were resuspended in buffer A containing various concentrations of CHAPS and insoluble and solubilized material (phases) separated by centrifugation. Panel A, enzyme activities were measured in the microsomal particles remaining after extraction. Symbols for enzyme activities are the same as used in Fig. 1. Panel B, distribution of VKOR activity between soluble (black bars) and insoluble phases (white bars). Panel C, distribution of γ-carboxylase activity between soluble (black bars) and insoluble phases (white bars).

Microsomes could then be tested in the reconstitution system. Fig. 2A shows the effect of buffer A extraction on vitamin K-dependent enzyme activities remaining in the extracted microsomes when various concentrations of CHAPS were present in the buffer. γ-Carboxylase activity (circles) increased 11-fold by extracting microsomes with CHAPS above its critical micelle concentration of 0.5%. High concentrations of CHAPS (1.5%) resulted in loss of γ-carboxylase activity, suggesting that the γ-carboxylase was either inactivated or extracted by these detergent concentrations. On the other hand, detergent extraction resulted in a decrease in VKOR, and VKOR supported γ-carboxylase activities by 50% and 80%, respectively (1.0% CHAPS, see Fig. 2A). This suggested that a significant portion of the VKOR enzyme pool in microsomes had been removed from the microsomal vesicles or inactivated by the detergent extraction. Therefore the distribution of VKOR and γ-carboxylase activities in the detergent extract and the remaining pellets at each detergent concentration was examined. As shown in Fig. 2B, 50% of VKOR in microsomes was brought into solution by detergent extraction. A kinetic analysis of VKOR in the extract and pellet showed that the enzymes had the same $K_m$ values (2.5 μM). Both enzymes were also equally sensitive to inhibition by warfarin and the thiol blocker N-ethylmaleimide, which suggests that the same VKOR enzyme is present in the extract and the remaining membrane fragments (pellet). In contrast, the γ-carboxylase was not solubilized by concentrations of CHAPS in buffer A ranging from 0 to 1.5% (Fig. 2C). As summarized in Table I, combining extracted microsomes with the soluble fraction in the presence of Mn$^{2+}$ to induce lipid aggregation restored all vitamin K-dependent enzyme activities to control levels found in microsomes resuspended in buffer A.

**Fractionation of Components of the γ-Carboxylation System**—To understand the 11-fold increase in γ-carboxylase activity measured in detergent-extracted microsomes (Fig. 1), the possibility that inhibitory components of the γ-carboxylase were present in microsomes was investigated. To determine if these components were metals or other low molecular weight compounds present in microsomes, the buffer A extract was subjected to gel-filtration on a column of Bio-Gel P-100 in buffer A. The void volume fraction A(P-100) and the retained fraction B(P-100) were recombined, respectively, with the buffer A-extracted microsomes for determination of vitamin K-dependent enzyme activities. When the extracted microsomes were combined with fraction A(P-100), the γ-carboxylase activity was the same as extracted microsomes resuspended in buffer A (see Table I). Combination of extracted microsomes with fraction B(P-100) showed that the inhibitory components were present in the retained fraction (see Table I). When metals were removed from fraction B(P-100) by passing the fraction over a metal chelating column of Sepharose-iminodiacetic acid prior to combining it with extracted microsomes, a significant recovery of γ-carboxylase activity was observed (67%). Thus, endogenous metals accounted for some of the γ-carboxylase inhibitory component in microsomes. Surprisingly, the main protein containing fraction A(P-100) from the Bio-Gel P-100 column did not restore VKOR and VKOR supported γ-carboxylase activities (Table I), while the retained fraction B(P-100) did (Table I). The component(s) in fraction B(P-100) which stimulated VKOR and VKOR-supported γ-carboxylase activities was inactivated by boiling and proteinase K treatment, and could be concentrated on a YM-10 filter. These qualitative tests indicate that the active component(s) is a protein.

**Purification of the Active Protein Component(s) in Fraction B(P-100)**—To further purify the protein which reconstituted VKOR activity, fraction B(P-100) was applied to a cellulose DE52 anion exchanger equilibrated in buffer A. As shown in Fig. 3, the protein was not retained by the ion exchanger. SDS-PAGE in a 10% gel of proteins in the unretained fraction from the column (fraction A, Fig. 3) is shown in lane A on the inset in Fig. 3. Coomassie Blue-stained protein bands with apparent molecular masses 74, 66, 32, and 29 kDa were seen.

To further purify the protein of interest, we subjected each Coomassie-stained band to N-terminal protein sequencing to gain information about the proteins which could be helpful in deciding the next purification step. The most useful information which resulted from these analyses was the finding of a sequence (PGSDLHLYQDKGREK) in the 32-kDa band which, in 14 overlaps, were 57.1% identical to the YC2 subunit of the α-class of GST enzymes (27). These enzymes are found nor-

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**TABLE I**

| Fractions | γ-Carboxylase activity | VKOR activity |
|-----------|------------------------|---------------|
|           | $dpm/ml \times 10^{-2}$ | nmol/ml |
| Resuspended microsomes | 1,013 ± 50 | 982 ± 40 | 4.80 ± 0.20 |
| Extracted microsomes | 9,112 ± 360 | 372 ± 16 | 2.26 ± 0.09 |
| + buffer A | 1,115 ± 44 | 987 ± 37 | 4.75 ± 0.18 |
| Extracted microsomes | 9,240 ± 382 | 385 ± 21 | 3.20 ± 0.12 |
| + fraction A (P-100) | 1,230 ± 52 | 1,005 ± 42 | 4.81 ± 0.19 |
| Extracted microsomes | 1,230 ± 52 | 1,005 ± 42 | 4.81 ± 0.19 |
| + fraction B (P-100) | 1,230 ± 52 | 1,005 ± 42 | 4.81 ± 0.19 |
ically in cytosol as dimeric proteins of variable subunit composition (27). The indication of a GST enzyme in the void (fraction A) from the cellulose DE52 column determined the next purification step. The void fraction was applied to an agarose-GSH affinity column. To avoid thiol reactions between proteins and the GSH-affinity ligand, an affinity resin was used where GSH was attached to the matrix via its thiol group. The affinity column retained all enzymes expressing GST activity and the GSTs could be eluted with 100 mM GSH in buffer A (Fig. 4A, triangles). The ability of the enzymes in the retained fraction to restore VKOR supported γ-carboxylase activity is also shown in Fig. 4A (squares). As expected, the VKOR supported γ-carboxylase activity was inhibited by warfarin, but surprisingly, the GST activity in the retained fraction was also found to be inhibited by the drug (see the bar graph in Fig. 4A). An \( I_{50} \) value of 0.7 mM warfarin was estimated for warfarin inhibition of the purified GST protein(s) in a test system where CDNB and GSH are present at 1 mM. As shown in Fig. 4B, the affinity-purified GST enzyme(s) in the retained fraction contained subunits with apparent molecular masses of 32 and 29 kDa.

Since the 32- and 29-kDa proteins were purified from fraction B(P-100), which was the protein fraction retained by the Bio-Gel P-100 column, the unretained fraction A from the Bio-Gel P-100 column was also tested for GST activity. Actually more than 90% of the total GST activity was recovered in the void fraction A(P-100), but this activity was not inhibited by warfarin (data not shown). Thus, the Bio-Gel P-100 column had separated the warfarin-sensitive GST enzymes from the overall pool of the GSTs present in the buffer A extract of microsomes. Based on its ability to reconstitute VKOR-supported γ-carboxylase activity, we estimated that the GST enzyme(s) was purified 6,213-fold from the detergent extract (Table II). The greatest single purification step was the Bio-Gel P-100 column (2,091-fold). For unknown reasons the Bio-Gel resin retained the warfarin-sensitive GST enzyme(s) more than the majority of the other proteins present in the buffer A extract.

**Association of Cytosolic GSTs and VKOR with the ER Membrane**—The presence of a dimeric GST enzyme(s) in the buffer A extract of microsomes suggests that these GSTs may have resulted from cytotoxic contamination of the microsomal preparations. This conclusion is supported by the data in Fig. 5, which shows that 70% of the GST activity in microsomes was found in the supernatant after resuspending the microsomes in buffer A without detergent (circle on the y axis at zero detergent concentration). In contrast, release of VKOR from the ER membrane required detergent (squares). The detergent concentrations used were also shown to release the two ER integral membrane proteins mGST and mEH, which are both anchored to the membrane by one transmembrane domain (28, 29). This shows that some of the VKOR protein components are firmly attached to the ER membrane. As shown in Fig. 5A, the VKOR activity profile (squares) in the detergent extracts follows the activity profile of mEH (triangles). This is of interest, because previous data, obtained from experiments with monospecific antibodies against mEH (19), have shown that mEH participates in vitamin K 2,3-epoxide reduction as a component of the VKOR enzyme complex (19).

The question as to what extent cytotoxic GSTs are tightly associated with microsomes was addressed by examining the GSTs in microsomes after extraction with buffer A, with and
without 0.5% CHAPS. The cytosolic enzyme DT-diaphorase (30) was used as a marker enzyme for cytosolic contamination. Since DT-diaphorase has been regarded as a protein that sticks to the ER membrane and was indeed, in the past, thought of as a microsomal protein (31), it is an appropriate marker for cytosolic contamination. DT-diaphorase activity could not be measured in microsomes extracted with buffer A. GST activities measured in 1) microsomes resuspended in buffer A, 2) microsomes extracted with buffer A without detergent and resuspended in buffer A, and 3) buffer A-extracted microsomes resuspended in buffer A were 1.21 ± 0.06, 0.49 ± 0.02, and 0.30 ± 0.01 μmol/min/ml, respectively. Inhibition of each of these activities with 5 mM warfarin was 23, 40, and 41%. These data show that a significant portion of the GST enzymes in microsomes are tightly associated with the ER membrane and that the warfarin-sensitive GST enzymes are the most difficult to remove from the membrane. To determine if members of the α-class of GST enzymes could be found in detergent-extracted microsomes we subjected buffer A-extracted microsomes to SDS-PAGE and Western blotting with α-GST1 polyclonal antibodies that recognize all known members of the GST α-class. Fig. 6, lane A, shows a Coomassie Blue stained preparation of buffer A-extracted microsomes to SDS-PAGE and Western blotting with α-GST1 polyclonal antibodies that recognize all known members of the GST α-class. The blot identified 32 (Yc) and 29 (Ya) kDa subunits (27) of α-class GSTs. Two unknown lower molecular weight proteins were also recognized by the antibodies. This experiment confirmed the tight membrane association of certain dimeric GSTs with the ER membrane. To explore the possibility that these GSTs interacted with the lipid components of the membrane, we first isolated the membrane associated dimeric GSTs by agarose-GSH affinity chromatography and then explored their binding to a lipid surface by IAM chromatography where PC was the immobilized ligand. This experiment confirmed the tight membrane association of certain dimeric GSTs with the ER membrane. To explore the possibility that these GSTs interacted with the lipid components of the membrane, we first isolated the membrane associated dimeric GSTs by agarose-GSH affinity chromatography and then explored their binding to a lipid surface by IAM chromatography where PC was the immobilized ligand. Fig. 7, lane C, shows Coomassie Blue staining of the affinity-purified dimeric GSTs. Lane D shows GSTs retained by the IAM column. A protein band of intermediate size between the Ya and Yc subunits of α-GSTs was also seen among the affinity-purified and the IAM-bound proteins. This suggests that members of classes of GSTs other than the α-class (27) are also firmly attached to the ER membrane. Forty percent of the membrane-associated affinity-purified dimeric GSTs actually bound to the column, indicating that only a subpopulation of the isolated GSTs had high affinity binding to PC on the IAM column.

Strong association of some dimeric GSTs with the ER membrane that cannot be explained by cytosolic contamination has also been observed by other investigators (32, 33). Our findings led to the hypothesis that these GSTs interact with the ER membrane to create catalysts intended for specific metabolic activities including vitamin K 2,3-epoxide reduction.

Reconstitution of VKOR Results in Loss of Warfarin-sensitive GST Activity—The possibility that the property of the GST enzyme(s) that are involved in formation of the VKOR enzyme complex changes on association with the ER membrane to create the VKOR enzyme complex was investigated. For these experiments, microsomes that had been extracted with buffer A without detergent were used, because cytosolic GSTs that were not strongly associated with the ER membrane were removed by this extraction step (Fig. 5), and all VKOR activity remained attached to the ER membrane. The extracted microsomes were resuspended in buffer A. Total GST, warfarin-sensitive GST, and VKOR activities were determined before and after reconstitution of the VKOR enzyme complex by Mn²⁺-induced lipid aggregation. The data are summarized in Table III. Addition of 10 mM Mn²⁺ to the resuspended microsomes resulted in a 40% decrease in GST activity from 79 to 47 nmol/min/mg, which was
the same activity loss measured when 5 mM warfarin was added without Mn$^{2+}$. This loss of GST activity by Mn$^{2+}$ addition was recovered when EDTA was added to the Mn$^{2+}$-containing sample (see Table III). The effect of Mn$^{2+}$ addition to the test system had the opposite effect on VKOR activity than it had on GST activity. VKOR activity increased 4.4-fold (see Table III) on addition of Mn$^{2+}$, and EDTA addition returned this activity to the activity measured without Mn$^{2+}$. Several control experiments were carried out. These experiments showed that Mn$^{2+}$ had no effect on: 1) warfarin-sensitive GST activity expressed by the affinity-purified GSTs, 2) this activity when the enzymes were added to extracted microsomes previously aggregated with Mn$^{2+}$, and 3) the activity of another ER membrane-associated enzyme, cytochrome P-450 reductase. Thus, the changes in VKOR and warfarin-sensitive GST activities observed in the reconstitution system appears to reflect changes in enzyme functions and not changes in enzyme behavior due to changes in the lipid detergent environment induced by Mn$^{2+}$.

A Subpopulation of the Membrane-Associated GSTs Is Inhibited by Lipid and Warfarin—The experiment with washed microsomal membranes suggested that aggregated endogenous lipids inactivated the warfarin-sensitive GST enzymes (Table III). To test this hypothesis, we investigated the effect of lipids, lipid aggregation with Mn$^{2+}$, and warfarin on the activity of affinity-purified membrane associated dimeric GSTs. As shown in Fig. 7, panel A, liposomes prepared from crude soy bean lipids inhibited GST activity. Aggregation of these lipids with Mn$^{2+}$ enhanced the lipid inhibition. The warfarin sensitivity of the affinity-purified GSTs is shown in Fig. 7, panel B. Maximal inhibition was achieved when >5 mM warfarin was present in the test system. Approximately the same inhibition of GST activity was measured with aggregated lipids (see Fig. 7, panel A). These data indicate that lipids inactivate the warfarin-sensitive GSTs. Fig. 8 provides evidence for this hypothesis. As shown, lipid addition to samples containing 5 mM warfarin did not further enhance inhibition of the remaining activity but completely neutralized the part of the activity that was sensitive to warfarin.

Vitamin K1 2,3-Epoxide and Vitamin K1 Quinone Are Putative Substrates for GST Enzymes in Microsomes—CDNB, which is a substrate for most GST enzymes (27), was found to inhibit VKOR in microsomes re suspended in buffer A (Fig. 5B). This finding strengthened our hypothesis that GST is indeed one part of the reductase enzyme complex. As shown in Fig. 9A, 0.1–1.0 mM GSH also inhibited VKOR-supported γ-carboxylase activity triggered with vitamin K1 2,3-epoxide and DTT (squares), but these GSH concentrations had no effect on γ-carboxylase activity in the test system triggered with chemically reduced vitamin K1H2 (triangles). Glutathione inactivation of VKOR-supported γ-carboxylase activity may indicate formation of vitamin K-GSH adducts, which are not accepted as cofactors for the γ-carboxylase.

**DISCUSSION**

Since its discovery in 1970 by Bell and Matchiner (34), purification of the VKOR enzyme of the vitamin K cycle has presented a challenge to researchers in the vitamin K field. Although the consensus has been that VKOR is an enzyme complex which easily is disrupted by chromatographic manipulations, firm evidence for this hypothesis has not been presented. This paper presents the first data to show the validity of this hypothesis. The key to this finding was: 1) the fact that 50% of the active VKOR enzyme could be brought in solution by extraction of microsomes with an isotonic buffer containing the detergent CHAPS, and 2) the disruption of the VKOR enzyme complex when the extract was gel filtrated on a Bio-Gel P-100 column in the same CHAPS-containing buffer. Evidence that a warfarin-sensitive member of the GST superfamily of proteins is one of the components of the enzyme complex is also presented.

Partial extraction of VKOR, but not the γ-carboxylase, from the microsomal vesicles defined a reconstitution test system where components of VKOR present in the detergent extract could be tested for their ability to restore VKOR and VKOR-supported γ-carboxylase activity in the microsomal particles remaining after detergent extraction. Reconstitution of the γ-carboxylation system was accomplished by lipid aggregation induced by addition of MnCl2 to the test system. We have shown previously (19) by phospholipase A2 treatment of a partially purified preparation of the VKOR enzyme complex that phospholipids are essential for the activity of the complex. Thus, the lipid bilayer in the ER membrane appears to have a

**Table III**

Reconstitution of VKOR activity results in loss of warfarin-sensitive GST activity

Microsomes extracted with buffer A without detergent were used for the experiments. The extracted microsomes were resuspended in buffer A. GST and VKOR activities were measured as described under “Experimental Procedures.” Additions to each test system were as described under “Experimental Procedures.” Each number is the average of three parallel incubations ± S.D.

| Fractions          | GST activity | VKOR activity |
|--------------------|--------------|---------------|
|                    | −Mn$^{2+}$   | +Mn$^{2+}$     |
|                    | ( + Mn$^{2+}$  + EDTA) | ( + Mn$^{2+}$  + EDTA) |
| Washed microsomes, normal rats | 79 ± 3 | 47 ± 2 | 78 ± 3 |
| − Warfarin         | 48 ± 1 | 46 ± 2 | 47 ± 2 |
| + Warfarin         | 209 ± 6 | 878 ± 18 | 205 ± 7 |
|                    | 12.5 ± 0.6 | 70 ± 4 | 14 ± 0.5 |
dual function for the γ-carboxylation system by 1) providing lipid as an essential component(s) for the enzymic mechanism and 2) providing an environment for necessary assembly and correct orientation of the protein components of the system.

The finding of a warfarin-sensitive GST enzyme(s) as one of the components needed for reconstitution of VKOR has made a link between vitamin K metabolism and the GST superfamily of proteins. Four membrane proteins of the GST family have been identified. They are: 1) mGSTI (35), the 14-kDa protein investigated in this study by antibodies; 2) leukotriene C₄ synthase (36), a 16.6 kDa protein which conjugates leukotriene A₄ with GSH to form leukotriene C₄; 3) 5-lipoxygenase-activating protein (FLAP) (37), an 18-kDa protein localized to the nuclear envelope membrane, which in the presence of Ca²⁺ forms a membrane bound complex with cytosolic 5-lipoxygenase (38); and 4) a recently discovered 16.6-kDa mGSTII enzyme with unknown function(s) (39). The four known GST membrane-spanning proteins have molecular masses between 14 and 18 kDa (39), which are significantly below the apparent molecular masses for the warfarin-sensitive GSTs shown in this work to reconstitute VKOR activity. The data support the hypothesis that a different and dimeric GST enzyme(s) “docks” with lipid and protein components of the ER membrane to form the VKOR enzyme complex. A subpopulation of dimeric GSTs is shown in this work and by others (32, 33) to be firmly attached to the ER membrane and we have shown that a member(s) of this subpopulation will reconstitute VKOR activity. Purified cytosolic GSTs will not substitute for this enzyme (data not shown). The mechanism for attachment of this subpopulation of dimeric GSTs to the ER membrane remains to be established.

The analogy between the model for formation of the lipoxygenase enzyme complex and our proposed model for formation of the VKOR enzyme complex is striking. The warfarin-sensitive dimeric GST may combine with a second protein in the ER membrane in a similar way FLAP forms a complex with 5-lipoxygenase (38). We propose that one function of the warfarin-sensitive dimeric GST is directed toward formation of the VKOR enzyme complex. Our hypothetical model of VKOR and its link to the γ-carboxylase in the ER membrane is shown in Fig. 10. VKOR is a membrane associated enzyme complex formed between the warfarin-sensitive membrane associated GST and mEH which is attached to the ER membrane by one transmembrane spanning domain (19). VKOR harbors two binding sites to support reduction of vitamin K₂,3-epoxide (Kᵢₒ). One binding site, which would be involved in GSH binding when the warfarin-sensitive GST is not a part of the complex, has become inaccessible to GSH and now functions as the warfarin-sensitive thiol redox center known to exist in VKOR (13). A second vitamin K₂,3-epoxide binding site is located on the complex. The existence of this site is consistent with our earlier finding that mEH harbors a binding site for the epoxide (19). We propose that DTT, because of its easy penetration into lipid environments (40), can reduce the red/ox
center which will initiate a flow of electrons to vitamin K₁ 2,3-epoxide (K > O) for its reduction to vitamin K₁H₂. Although lipoamide (41) and the thioredoxin system (42) have been suggested as possible reducing agents for VKOR, the physiological reduc tant is still not known with certainty. Since reduced vita min K₁H₂ would be easily oxidized in the ER (43), the γ-carboxylase must be in close proximity to VKOR for efficient cofactor transfer. There is no covalent link between the γ-carboxylase and VKOR (19).

Finally our data show that vitamin K₁ 2,3-epoxide and vit amin K₁ quinone are putative substrates for GSTs present in microsomes. Based on the variety of compounds that are known to be substrates for GSTs (27), our conclusion seems plausible and raises questions about the physiological roles of GSTs in vitamin K metabolism. In support of this hypothesis is the finding that vitamin K₃ has been shown to be a substrate for GSTs in hepatocytes (44). The exact nature of GSTs participation in vitamin K metabolism and identification of the warfarin-sensitive GST that becomes a part of the VKOR enzyme complex is currently being studied in our laboratory.

REFERENCES
1. Suttie, J. W. (1985) Annu. Rev. Biochem. 54, 459–477
2. Furie, B., and Furie, C. (1988) Cell 53, 505–518
3. Hauschka, P. V., Lian, J. B., Cole, D. E., and Gundberg, C. M. (1989) Physiol. Rev. 69, 990–1047
4. Maillard, C., Bertrand, M., Serve, C. M., Dechavanne, M., and Dalmas, P. (1992) Endocrinology 130, 1599–1604
5. Stitt, T. N., Conn, G., Gore, M., Lai, C., Bruno, J., Radziejewski, C., Mattsson, K., Fischer, J., Gies, D. R., and Jones, P. F. (1995) EMBO J. 14, 2827–2837
6. Wallin, R., and Martin, L. F. Biochem. J. 241, 389–396
7. Wallin, R., Gebhardt, O., and Prydz, H. (1978) Biochem. J. 169, 95–101
8. Wallin, R., and Martin, L. F. (1985) J. Clin. Invest. 76, 1879–1884
9. Suttie, J. W. (1987) in Vitamin K Research (Suttie, J. W., ed.) pp. 381–397, Elsevier, New York
10. Suttie, J. W. (1978) in Handbook of Lipid Research (Deluca, H. F., ed), pp. 211–277, Plenum Press, New York
11. Suttie, J. W. (1985) Annu. Rev. Biochem. 54, 459–477
12. Fasco, M. J., Principe, L. M., Walsh, W. A., and Friedman, P. A. (1983) Biochemistry 22, 2246–2252
13. Lee, J. J., and Fasco, M. (1984) Biochemistry 23, 2246–2252
14. Wallin, R., and Martin, L. F. (1988) J. Biol. Chem. 263, 9994–10001
15. Sadowski, J. A., Émond, C. T., and Suttie, J. W. (1976) J. Biol. Chem. 251, 2770–2776
16. Tishler, M., Fiers, L. F., and Wendler, N. L. (1940) J. Am. Chem. Soc. 62, 2966–2971
17. Andersen, C., Piemonte, F., Mosialou, E., Weinander, R., Sun, T.-H., Lundqvist, G., Adang, A. E. P., and Morgenstern, R. (1995) Biochim. Biophys. Acta 1247, 277–283
18. Greulich, T. M., Benley, P., and Oesch, F. Methods Enzymol. 77, 344–351
19. Wallin, R., and Guenther, T. M. (1997) Methods Enzymol. 282, 395–403
20. Laemml, U. K. (1970) Nature 227, 680–685
21. Wallin, R., Culp, E., Coleman, D. B., and Goodman, S. R. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 4095–406
22. Stanton, C., Taylor, R., and Wallin, R. (1991) Biochem. J. 277, 59–65
23. Hildebrandt, F., Preusch, P. C., Patterson, J. L., and Sutjie, J. W. (1984) Arch. Biochem. Biophys. 226, 480–492
24. Verkjei, A. J. (1984) Biochim. Biophys. Acta 779, 43–63
25. Lenaz, G. (1977) in The Molecular Biology of Membranes (Fleisher, S., Hatefi, Y., MacLennan, D. H., Tragolof, A., eds), pp. 137–162, Plenum Press, New York
26. Fasco, M. J., Preusch, P. C., Hildebrandt, E., and Sutjie, J. W. (1983) J. Biol. Chem. 258, 4372–4380
27. Hayes, J. D., and Pulford, D. J. (1995) Crit. Rev. Biochem. Mol. Biol. 30, 446–460
28. Friedberg, T., Lollmann, B., Becker, R., Holler, and Oesch, F. (1994) Biochem. J. 303, 967–972
29. Anderson, C., Weinander, R., Lundqvist, G., DePierre, J. W., and Morgenstern, R. (1994) Biochim. Biophys. Acta 1240, 298–304
30. Ernster, L. (1967) Methods Enzymol. 10, 309–361
31. Lind, C., and Hjärberg, B. (1981) Arch. Biochem. Biophys. 207, 217–224
32. Morgenstern, R., Guthenberg, C., Mannervik, B., and DePierre, J. W. (1983) FEBS Lett. 160, 264–280
33. Friedberg, T., Bentley, P., Stasiecki, D., Glatt, H. R., Raphael, D., and Oesch, F. (1979) J. Biol. Chem. 254, 12028–12033
34. Bell, R. G., and Matchiner, J. T. (1970) Arch. Biochem. Biophys. 141, 473–476
35. Morgenstern, R., DePierre, J. W., and Jornvall, H. (1985) J. Biol. Chem. 260, 13976–13983
36. Samuelson, B. (1983) Science 220, 568–575
37. Dixon, R. A., Diehl, R. E., Opas, E., Rand, E., Vickers, P. J., Evans, J. F., Gilliard, J. W., and Miller, D. K. (1990) Nature 343, 252–254
38. Abramovitz, M., Wong, E., Cox, M. E., Richardson, C. D., Li, C., and Vickers, P. J. (1993) Eur. J. Biochem. 215, 105–111
39. Jakobsson, P.-J., Mancini, J. A., and Ford-Hutchinson, A. W. (1996) J. Biol. Chem. 271, 2203–2210
40. Breakman, I., Helenius, J., and Helenius, A. (1992) EMBO J. 11, 1717–1722
41. Thøjesen, H. H. W., Jannsen, Y. P. G., and Vervoort, L. M (1994) Biochem. J. 297, 277–280
42. Soutie, A. M., Groenen-van Dooren, M. M. C. L., Holmgren, A., Lundstrom, J., and Vermeer, C. (1992) Biochem. J. 281, 255–259
43. Freedman, R. B. (1989) Cell 57, 1069–1072
44. Ross, D., Thor, H., Orrenius, S., and Moldeus, P. (1985) Chem. Biol. Interact. 55, 177–184