Vitamin K-dependent Carboxylation and Vitamin K Metabolism in Liver

Effects of Warfarin

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Abstract

The systems involved in vitamin K-dependent carboxylation and vitamin K metabolism have been extensively studied in rat liver. To determine how clinically applicable this information is, similar in vitro studies were completed using human liver. One major difference exists in the pathways that provide reduced vitamin K₃ cofactor for the carboxylation reaction. The coumarin-sensitive DT-diaphorase (EC.1.6.99.2) in human liver appears to play a relatively minor role in the dehydrogenase pathway. However, similar to rat liver, the human liver contains a warfarin-insensitive enzyme in this dehydrogenase pathway. The data suggest that this enzyme is responsible for the antibiotic effect of vitamin K₃ in cases of coumarin intoxication. Human vitamin K epoxide reductase, which constitutes the other pathway for vitamin K₃ reduction, has kinetic and enzymological characteristics that are very similar to the rat enzyme. This enzyme exhibited similar activity in rat and human microsomes. Initial velocities for vitamin K₃ epoxide reduction in rat and human microsomes were 20 and 32 pmol/mg·min, respectively. The human enzyme is highly sensitive to warfarin inhibition. The mechanism for this inhibition appears to be similar to what has been proposed for the rat enzyme. Also, a vitamin K₃-dependent carboxylation system is described that allows both pathways to support the carboxylation reaction with reduced vitamin K₃ cofactor. The effect of warfarin on this in vitro system is consistent with the current model for the mechanism of action of coumarin anticoagulant drugs in the rat.

Introduction

In the liver, reduced vitamin K is a necessary cofactor for an enzyme that converts precursor forms of the vitamin K-dependent blood clotting factors to active coagulation zymogens (1, 2). This is achieved by carboxylation of specific glutamic acid residues in the precursor proteins (1, 2). Concomitant with this reaction, vitamin K is converted to vitamin K 2,3-epoxide (1, 2). In vitro, the epoxide can be reduced back to the active cofactor form of the vitamin by dithiol reagents in a reaction catalyzed by vitamin K epoxide reductase (1, 2). The endogenous liver reductant for this enzyme, however, is not known. Vitamin K epoxide reductase thus establishes a redox cycle for vitamin K in the liver (1, 2).

Vitamin K is absorbed by the intestine and received by the liver as the quinone form (3). It has been shown in vitro that vitamin K quinone can also be reduced to the hydroquinone by liver microsomes in a dithiothreitol (DTT)¹-supported reaction (4). It has been proposed that the same enzyme is responsible for reduction of the quinone form as well as the epoxide form of the vitamin (5), but no firm evidence has been presented to support this hypothesis.

Vitamin K epoxide reductase is highly sensitive to inhibition by coumarin anticoagulant drugs and is believed to be the target for their anticoagulant effect (1, 2). We have shown in rat liver that, in addition to the reductase, there are pyridine nucleotide-dependent dehydrogenases that also can reduce vitamin K quinone (6). These enzymes are far less sensitive to coumarin drugs and constitute an important pathway for vitamin K reduction in cases of coumarin intoxication (7).

In order to understand the mechanism of anticoagulation by coumarins, the system described above has been extensively studied in various vitro systems from rat liver (1, 2). Since these drugs are used so frequently in patients, we decided to study the system in human liver. Our goal was to determine how closely the enzyme system in human liver corresponded to the model established for the action of vitamin K and coumarins in rat liver.

Methods

Preparation of microsomes and cytosol. Liver biopsies were obtained from morbidly obese patients undergoing gastric bypass surgery. The study was approved by the Clinical Investigation Committee of the Milton S. Hershey Medical Center, The Pennsylvania State University. It followed the ethical guidelines of the National Institutes of Health. Subjects participated only after signing a form of consent.

The biopsies were rinsed in ice-cold saline, blotted to remove excess saline, weighed, minced, and suspended in 2 vol (vol/wt) of the homogenization buffer: 50 mM KCl, 200 mM sucrose and 20 mM Tris·HCl, pH 7.8 (8). After addition of 1 mM of the insecticide 2,6-dichlororinyl dimethyl phosphor (VAPONA), the suspension was dispersed by using a polytron for 10 s at a low setting. The slurry was then homogenized in a Potter-Elvehjem homogenizer with a tight-fitting pestle. The homogenate was centrifuged twice at 10,000 g for 10 min. The postmitochondrial supernatant was centrifuged 60 min at 100,000 g. The microsomal pellets were surface washed with S1 buffer (25 mM sucrose and 25 mM imidazole, pH 7.2 [SI]) and stored frozen in liquid N₂. The supernatant (liver cytosol) was stored frozen at −20°C. Rat liver microsomes and cytosol were prepared as described by Suttie et al. (9).

Assays. Vitamin K epoxide reductase activity was measured in mi-

1. Abbreviations used in this paper: CHAPS, 3-[[(chlomidopropyl)dimethylammonio]-1-propanesulfonate; DCPIP, 2,6-dichlorophenolin-
dophenol; DTT, dithiothreitol; FAD, flavin adenine dinucleotide; HPLC, high performance liquid chromatography; SI, 25 mM sucrose, 25 mM imidazole, pH 7.2; VAPONA, 2,6-dichlororinyl dimethyl phosphate; vitamin K₃, epoxide, vitamin K₁, 2,3-epoxide; vitamin K₃H₂, fully reduced vitamin K₃.
crosomes resuspended in SI buffer. Vitamin K<sub>2</sub>, 2,3-epoxide (vitamin K<sub>1</sub> epoxide) in 10 μl 95% ethanol was added to 0.5 ml of the microsomal suspension. After preincubation for 1 min, 20 μl DTT in SI buffer was added and the suspension incubated in open tubes under the various conditions described in the text. When DTT was added to the test system before vitamin K epoxide, preincubation was also carried out for 1 min. Reactions were stopped by the addition of 1 ml isopropanol/hexane (3: 2:vol/wt) and mixed into the test system using a vortex mixer. The mixture was subjected to a brief centrifugation. 300 μl of the upper hexane phase was removed and evaporated to dryness at room temperature. The residue was dissolved in 100 μl of isopropanol and 20 μl was analyzed by high performance liquid chromatography (HPLC) using a Rainin gradient system equipped with a Gilson 704 HPLC system manager. Separation was achieved on a Rainin Micro-Hichrom (300 x 4.6 mm, 10 micron) reversed-phase column, eluted in 100% methanol. Absorbance of eluting materials was measured at 254 nm with an ISCO UM-5 detector equipped with an ISCO HPLC 10-μl flow cell. Retention times for vitamin K<sub>1</sub> and vitamin K<sub>2</sub> epoxide were 5.8 and 3.6 min, respectively, at a flow rate of 2 ml/min. Quantitation was based on the integrated absorption peaks when compared with peaks from standards of vitamin K<sub>1</sub> and vitamin K<sub>2</sub> epoxide. Reduced vitamin K<sub>1</sub> was not measured in our system. Enzymatically reduced vitamin K<sub>1</sub> was reoxidized to the quinone before being analyzed on HPLC. Reoxidation was achieved by evaporation of the hexane layers in open tubes over night at room temperature in the dark. Reoxidation of chemically reduced vitamin K<sub>1</sub> served as control. Reduced vitamin K<sub>1</sub> (100 μM) was added to control incubations. These were extracted immediately with hexane, and these extracts were allowed to evaporate overnight with the test samples. No nonenzymatic reduction of vitamin K<sub>1</sub> epoxide occurred in the hexane phases. Control samples from zero time incubations contained no vitamin K<sub>1</sub>.

The concentrations of vitamin K<sub>1</sub> and its epoxide were determined spectrophotometrically in 95% ethanol using extinction coefficients of 30,000 M<sup>-1</sup>·cm<sup>-1</sup> at 266 nm for the epoxide, and 18,900 M<sup>-1</sup>·cm<sup>-1</sup> at 248 nm for vitamin K<sub>1</sub> (10). DT-diaphorase activity was measured at room temperature as described by Dallner (11) with 2,6-dichlorophenol-indophenol (DCPIP) as the electron acceptor. The reaction was followed at 600 nm. An extinction coefficient of 21,000 M<sup>-1</sup>·cm<sup>-1</sup> for DCPIP was used to calculate enzyme activity. Cytochrome c reductase activity was measured at room temperature in 0.05 M potassium phosphate, pH 7.5, containing 0.8 mg/ml Tween-20, 75 μM cytochrome c, and 0.1 M NADPH. The reaction was followed at 550 nm. An extinction coefficient of 18,500 M<sup>-1</sup>·cm<sup>-1</sup> for cytochrome c was used to calculate enzyme activity (12).

Vitamin K-dependent carboxylase activity was measured in microsomes resuspended in 25 mM imidazole, pH 7.2, containing 0.5% 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate (CHAPS). The assay, which measures [<sup>14</sup>C]CO<sub>2</sub>-incorporation into the synthetic peptide Phe-Leu-Glu-Glu-Leu, was described as Esmon and Suttle (13). All incubations contained 2 mM of the pentapeptide and 5 mM DTT. When added to the test system, fully reduced vitamin K<sub>1</sub> (vitamin K<sub>1</sub>H<sub>2</sub>) and vitamin K<sub>1</sub> (Aquamephyton) were present in 100 μg/ml concentration. Incubations with NADH contained 2 mM of the pyridine nucleotide. Test samples were incubated 30 min at 25°C.

**Chromatography.** Human cytosol (1 ml) was gel filtrated on a column of Sephadex G-25 fine (1.7 x 15 cm) in SI buffer. **Chemicals.** Vitamin K<sub>1</sub> was purchased from Sigma Chemical Co., St. Louis, MO. The vitamin was reduced to vitamin K<sub>1</sub> hydroquinone with dithionite as described by Sadowski et al. (14). Aquamephyton (vitamin K<sub>1</sub>; 10 mg/ml) was obtained from Merck Sharp and Dohme, West Point, PA. Vitamin K<sub>1</sub>, 2,3-epoxide was prepared from vitamin K<sub>1</sub> (Sigma Chemical Co.) as described by Tishler et al. (15). Warfarin, CHAPS, glutathione, and lipico acid (reduced form) were from Sigma Chemical Co. The pentapeptide Phe-Leu-Glu-Glu-Leu was from Vega Fox Biochemicals Div. (Tucson, AZ). NaH<sub>14</sub>CO<sub>3</sub> (60 mC/mmol) was from Amersham Corp., Arlington Heights, IL. Sephadex G-25 was from Pharmacia Fine Chemicals (Uppsala, Sweden). The insecticide VAPONA was a gift from the Shell Oil Co. (Houston, TX). Protein was measured with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

**Results**

Preparation of rat liver microsomes for studies on vitamin K metabolism is normally carried out in 250 mM sucrose (1). When we applied the same procedure to the human liver, centrifugation at 100,000 g for 60 min did not result in a solid pellet of microsomal particles. However, lowering the sucrose concentrations to 200 mM resulted in a pellet that could easily be separated from cytosol. To verify that we had isolated human microsomes, we measured the activity of cytochrome c reductase, which is a marker enzyme for microsomes. The specific activity of this enzyme in our preparations from human liver was found to be 72 nmol reduced cytochrome c formed per minute per milligram of microsomal protein. In rat liver microsomes, the specific activity was 114 nmol/min·mg.

**The dehydrogenase pathway.** We have shown previously in rat liver that DT-diaphorase (EC.1.6.99.2) is a vitamin K<sub>1</sub>-reducing enzyme that can provide reduced vitamin K<sub>1</sub> cofactor for the carboxylation reaction (16, 17). This enzyme, which is present in rat liver microsomes and cytosol (18), is inhibited by coumarin anticoagulant drugs (19). We compared the activity of DT-diaphorase in cytosol and microsomes from human and rat liver. When diaphorase activity was measured in human cytosol, significant activity was measured without addition of NADH to the test system (Table I). This background activity was not found in rat liver cytosol (Table I). Gelfiltration of human cytosol on Sephadex G-25 resulted in a void volume fraction, where the background activity was reduced by 75% (Table I). The void volume fraction was therefore used to measure diaphorase activity in human cytosol. It could be shown that the retention volume from the Sephadex column contained a reductant that, when added to the diaphorase test system, reduced DCPIP nonenzymatically. Apparently this material was responsible for the high background activity.

Diaphorase activity was significantly higher in rat liver than in human liver (Table II). Warfarin (3 mM) inhibited this activity 96 and 11% in rat and human liver cytosol, respectively (Table II). The activities in rat and human microsomes were inhibited 37% and <3% (Table II). The ability to reduce the dye DCPIP, and thus express diaphorase activity, is shared by many liver dehydrogenases. However, DT-diaphorase (EC.1.6.99.2) is sensitive to coumarin drugs, and thus the activity of this enzyme

**Table I. DT-Diaphorase Activity in Human and Rat Liver Cytosol**

<table>
<thead>
<tr>
<th></th>
<th>Cytosol</th>
<th>Gelfiltrated cytosol</th>
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<tbody>
<tr>
<td>Liver</td>
<td>−NADH(\hat{h})</td>
<td>+NADH</td>
</tr>
<tr>
<td></td>
<td>nmol/mg·min</td>
<td>nmol/mg·min</td>
</tr>
<tr>
<td>Human</td>
<td>4.17</td>
<td>18.3</td>
</tr>
<tr>
<td>Rat</td>
<td>0</td>
<td>208.8</td>
</tr>
</tbody>
</table>

* DT-diaphorase activity was measured as described in Methods with DCDIP as electron acceptor.  
† DT-diaphorase activity was measured in human cytosol gelfiltrated on Sephadex G-25 as described in Methods.  
§ DT-diaphorase activity was measured without addition of NADH to the test system.
Table II. Warfarin Inhibition of DT-Diaphorase Activity in Cytosol and Microsomes from Human and Rat Liver

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Rat</th>
<th>Human</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No addition</td>
<td>Warfarin (3 mM)</td>
</tr>
<tr>
<td>Cytosol</td>
<td>208.4</td>
<td>8.3</td>
</tr>
<tr>
<td>Microsomes§</td>
<td>69.3</td>
<td>43.6</td>
</tr>
</tbody>
</table>

* DT-diaphorase activity was measured as described in Methods with DCPIP as electron acceptor.
‡ DT-diaphorase activity was measured in human cytosol gelfiltrated on Sephadex G-25 as described in Methods.
§ DT-diaphorase activity was measured in microsomes solubilized in 0.25 M sucrose, 0.5 M KCl, 1.5% Triton X-100, and 0.025 M imidazole, pH 7.2.

...can be measured as the warfarin-sensitive part of total diaphorase activity (21). Our data showed that DT-diaphorase in rat liver cytosol accounted for 96% of the measured diaphorase activity, whereas the enzyme in human liver cytosol accounted for <3% of the activity. Edwards et al. (20) have reported that a partial purification of human DT-diaphorase required addition of flavin adenine dinucleotide (FAD) to express enzyme activity. We added various concentrations of FAD to cytosol from human liver but could not measure any enhanced DT-diaphorase activity. Thus, the low DT-diaphorase activity measured in human liver cytosol was due to inactivation of the enzyme by loss of FAD cofactor. DT-diaphorase isolated from livers of various species are rather stable enzymes. Thus, it is also unlikely that our treatment of the liver biopsies was responsible for the low enzyme activity. We also measured DT-diaphorase activity in cytosol from several individual biopsies with the same results. The significantly lower activity of this enzyme in human liver suggested that the dehydrogenase pathway for vitamin K\textsubscript{1} reduction is different in human and rat liver.

*The vitamin K epoxide reductase pathway.* Before this report, no data was available on vitamin K epoxide reductase in human liver. Thus, in order to compare this pathway for vitamin K\textsubscript{1} reduction in rat and human liver it was necessary to characterize the human enzyme. Human vitamin K epoxide reductase was assayed in open tubes. When we compared the activity obtained under a N\textsubscript{2} atmosphere with that obtained in open tubes, no difference was found. Similar results have been reported by Fascio et al. (21) in rats maintained on a normal diet. Thus, formation of vitamin K epoxide due to carboxylation of endogenous precursor proteins did not effect our test system (21).

Reduction of vitamin K\textsubscript{1} epoxide by the enzyme was dependent on temperature as shown in Fig. 1. The enzyme was rapidly inactivated at temperatures above 40°C and exhibited a temperature optimum of 33°C. The rat enzyme has a temperature optimum of 25°C (22).

The activity of the human enzyme was studied in the pH range (6.5–8.5). The enzyme showed little variation in activity from pH 7.0–8.5, and no distinct pH optimum was observed. However, the enzyme rapidly lost activity at pH values < 6.5.

Some kinetic studies were also carried out with the human enzyme. As shown in Fig. 2, reduction of vitamin K\textsubscript{1} epoxide was linear for the first 10 min of incubation. With 10 \textmu M vitamin K\textsubscript{1} epoxide and 5 mM DTT present in the test system, the initial rate was calculated to be 32 pmol vitamin K\textsubscript{1} formed per minute per milligram of microsomal protein. Under the same conditions, initial rate for the rat enzyme was found to be 20 pmol/mg·min. Also, we measured initial rates of vitamin K\textsubscript{1} formation at varied concentrations of vitamin K\textsubscript{1} epoxide. With saturating concentrations of DTT (5 mM) present as reductant, an apparent \(K^m\) value for vitamin K\textsubscript{1} epoxide of 8 \textmu M was calculated for the human enzyme. Higher concentrations of DTT (up to 50 mM) had no effect on the activity. When enzyme activity was measured vs. enzyme concentration, the relationship was not linear (Fig. 3). The specific activity of the enzyme increased upon dilution of the microsomal suspension, suggesting that an inhibitor was present in human microsomes.

![Figure 1](image1.png)

**Figure 1.** Dependence of human vitamin K epoxide reductase activity on incubation temperature. Human microsomes were incubated at various temperatures for 30 min in the presence of 10 \textmu M vitamin K\textsubscript{1} epoxide and 5 mM DTT. Activity was measured as described in Methods. Activity (micromolar vitamin K\textsubscript{1} formed) is presented as the average of duplicate or triplicate incubations differing <5%.

![Figure 2](image2.png)

**Figure 2.** Dependence of human vitamin K epoxide reductase activity on incubation time. Human microsomes were incubated for various times at 33°C in the presence of 10 \textmu M vitamin K\textsubscript{1} epoxide and 5 mM DTT. Activity was measured as described in Methods and in the legend to Fig. 1.
The potential of various thiol compounds to serve as reductants for rat liver vitamin K epoxide reductase have been investigated (23). We repeated experiments with two diithiols (DTT and lipoic acid) and two monothiols (mercaptoethanol and glutathione) to determine their effect on human vitamin K epoxide reductase. As shown in Table III, lipoic acid was a better reductant for the human enzyme than mercaptoethanol and glutathione. DTT, however, was superior to all other thiol compounds tested. Thus, similar to what has been observed in rat liver, diithiols appear to be better reductants for the human enzyme than monothiols.

Low concentrations of warfarin inhibited the human enzyme (Fig. 4). When microsomes were preincubated with 10 µM vitamin K₁ epoxide, 10 µM warfarin caused a spontaneous 90% inhibition which lasted for up to 30 min of incubation (Fig. 4). However, preincubation with DTT antagonized warfarin inhibition. As shown in Fig. 4, addition of 5 mM DTT before warfarin resulted in only 65% inhibition. In this case, the inhibition by warfarin was time dependent, and maximum inhibition was obtained at ~10 min. After that there was a slow recovery of activity. With 15 mM of DTT, warfarin inhibition was 25% less at 30 min than at 7.5 min of incubation (Fig. 4). This was in clear contrast to microsomes preincubated with vitamin K epoxide.

The carboxylation system. Normally, Triton X-100 has been used to solubilize microsomes in order to study vitamin K-dependent carboxylation using the synthetic pentapeptide as substrate (1). The pentapeptide does not reach the luminal side of the microsomal vesicle where the carboxylase is located unless detergents are present (1). However, Triton X-100 inactivates the vitamin K epoxide reductase (1).

We used the Zwitter ionic detergent CHAPS to prepare our in vitro system. This resulted in a carboxylation system that was active with the pentapeptide and also retained good vitamin K epoxide reductase activity. Reductase activity was found to be 70% of the activity measured in microsomes suspended in SI buffer. As shown in Table IV, in the CHAPS system, both DTT and NADH were able to provide reduction equivalents for the enzymes catalyzing reduction of vitamin K₁. Thus, the microsomal carboxylation system in CHAPS expressed vitamin K₁ reduction by both pathways known to participate in vitamin K₁ reduction in rat liver (1). The system in human liver is, therefore, similar to the system in rat liver. Fig. 5 shows the effect of warfarin on carboxylation in the CHAPS system. The small effect of warfarin on carboxylation supported by reduced vitamin K₁ showed that the drug had little effect on human carboxylase. On the other hand, 10 µM warfarin neutralized the DTT-supported activity. This is consistent with its known effect on vitamin K epoxide reductase. When warfarin was added to incubations containing [NADH + DTT + vitamin K₁], only 46% of the

**Table III. Ability of Various Thiol Compounds to Support Human Vitamin K Epoxide Reductase Activity**

<table>
<thead>
<tr>
<th>Thiol compound</th>
<th>Enzyme activity* (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>100</td>
</tr>
<tr>
<td>Lipoic acid (reduced)‡</td>
<td>66</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>42</td>
</tr>
<tr>
<td>Glutathione</td>
<td>18</td>
</tr>
</tbody>
</table>

* Vitamin K epoxide reductase activity was measured in microsomes incubated at 33°C for 30 min with 10 µM vitamin K₁ epoxide present in the test system. Activities were measured relative to the activity obtained with DTT.
‡ All thiol compounds were present in 5 mM concentration.
§ Lipoic acid was added to the test system dissolved in isopropanol.

**Table IV. Carboxylase Activity in Human Microsomes**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Carboxylase activity* cpm/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin K₁H₂</td>
<td>4,560</td>
</tr>
<tr>
<td>Vitamin K₁</td>
<td>2,320</td>
</tr>
<tr>
<td>Vitamin K₁ + NADH</td>
<td>3,260</td>
</tr>
</tbody>
</table>

* Carboxylase activity was measured as described in Methods as carboxylation of the peptide Phe-Leu-Glu-Glu-Leu. All incubations contained 5 mM DTT. Final concentrations of vitamin K₁ and vitamin K₁H₂ were 100 µg/ml. NADH was present in 2 mM concentration. 
‡ Activity is given as cpm/mg of microsomal protein. The numbers are the average of duplicate incubations differing <5%. Incubations in the absence of the vitamin served as controls.
activity was inhibited (Fig. 5). The remaining NADH-supported carboxylase activity was unaffected by high concentrations of warfarin (1 mM) (Fig. 5).

From studies of in vitro systems from rat liver it is known that 1 mM warfarin neutralizes DT-diaphorase and results in a significant inhibitory effect on [vitamin K\(_i\) + NADH]-supported carboxylation (6, 24). Thus, the apparent lack of warfarin inhibition of this activity in human liver suggests that DT-diaphorase has no significant role in the dehydrogenase pathway for vitamin K reduction in human liver.

**Discussion**

These data suggest that the systems involved in vitamin K-dependent biosynthesis of blood clotting factors are quite similar in human and rat liver. However, some differences were observed. The most striking difference was the pyridine nucleotide pathway for vitamin K reduction. In contrast to rat liver, the human liver exhibited very low warfarin-sensitive DT-diaphorase activity. Based on the specific activities in rat and human liver cytosol, it could be estimated that per milligram of protein this enzyme activity in human cytosol was only 0.8% of the activity measured in rat cytosol. Edwards et al. (20) have studied DT-diaphorase in water extracts of various human tissues. Their data are consistent with the relatively low concentration of the enzyme we have measured in human liver. Thus, it appears that the warfarin-sensitive DT-diaphorase in human liver plays only a minor role in the dehydrogenase pathway for vitamin K\(_i\) reduction. In support of this assumption is our data on the effect of warfarin on [NADH + vitamin K\(_i\)]-supported carboxylase activity. High concentrations of warfarin had no significant effect on this activity. In addition, these data present evidence for the existence of a warfarin-insensitive dehydrogenase in human liver that can reduce vitamin K\(_i\) and provide reduced vitamin K\(_i\) cofactor for the carboxylation reaction. Previously, we have presented evidence for a similar warfarin-insensitive dehydrogenase in rat liver (6). Because the vitamin K epoxide reductase pathway in rat liver has been shown to be essentially irreversibly blocked by warfarin (25), it appears that this enzyme is responsible for the antidote effect of vitamin K\(_i\) in cases of coumarin intoxication (26). A more detailed study of the dehydrogenase pathway in human liver is currently being conducted in our laboratory.

Human liver vitamin K epoxide reductase exhibited characteristics that were very similar to the rat liver enzyme. A \(K_m\) value for K\(_e\) epoxide for the rat enzyme has been reported to be 6 \(\mu\)M (22). For the human enzyme we calculated a \(K_m\) value of 8 \(\mu\)M. Because of similar \(K_m\) values and similar specific microsomal activities (20 and 32 pmol/mg·min), this suggests that the rat and the human enzyme have about the same activity in vivo.

The human enzyme was also inhibited by warfarin in a manner similar to what has been reported for the rat enzyme (23). Warfarin inhibition, however, was antagonized by preincubating microsomes with DTT. The results from these experiments are in accordance with the rat model proposed by Fasco et al. (21) for the mechanism of action of coumarin drugs on vitamin K epoxide reductase. This suggests that the human enzyme, as well as the rat enzyme, is dependent upon essential thiol groups which, when oxidized, are blocked by coumarin drugs.

The requirements for thiol reductants were basically the same for the human enzyme as has been reported for the rat enzyme (23). However, mercaptoethanol resulted in a significantly higher activity than has been reported for the rat enzyme (23). The reason for the high activity measured with this monothiol is presently unclear.

The in vitro carboxylation system described in this paper appears to have all the essential activities that are known to be involved in liver vitamin K metabolism and vitamin K-dependent carboxylation. Importantly, the synthetic pentapeptide is accepted as substrate for the carboxylase in this system, which also retains good vitamin K epoxide reductase activity. This in vitro carboxylation system provided evidence for the existence of two vitamin K\(_i\) reducing pathways in human liver. The vitamin K epoxide reductase pathway was neutralized by low concentrations of warfarin. However, it was also clear that high concentrations of vitamin K\(_i\) could provide the carboxylase with cofactor when the epoxide reductase pathway was blocked by warfarin. These results demonstrated the potential of the dehydrogenase pathway in human liver to serve as a salvage pathway in cases of coumarin intoxication.

In conclusion, the systems involved in vitamin K-dependent carboxylation and vitamin K-metabolism in human and rat liver appear to be similar. Thus, the rat model is applicable to the human liver. However, the dehydrogenase, which appears to be the physiologically important enzyme in the salvage pathway, is not yet identified.

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