Biochem. J. (1989) 260, 879–884 (Printed in Great Britain)

Vitamin K_1 reduction in human liver

Location of the coumarin-drug-insensitive enzyme

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The antidotal effect of vitamin K in overcoming poisoning by coumarin anticoagulant drugs is mediated by a vitamin K-reducing enzyme of the endoplasmic reticulum [Wallin & Martin (1987) Biochem. J. **241**, 389–396]. With microsomes obtained from human liver biopsies, we have investigated the localization and the transverse orientation of this enzyme in the endoplasmic reticulum and compared its orientation to that of the other enzymes of the vitamin K-dependent carboxylation system. All enzymes were protected by the microsomal membrane and thus appear to have a luminal orientation in the endoplasmic reticulum, consistent with their role in the vitamin K-dependent modification of secretory glycoproteins. Separation of rough and smooth microsomes showed that vitamin K-dependent carboxylase activity was 6-fold higher in rough than in smooth microsomes. Vitamin K_1 reduction by the coumarin-drug-sensitive (pathway I) and -insensitive (pathway II) enzymes of the vitamin K-dependent carboxylation system was the same in rough and smooth microsomes. The data suggest a close association between the pathway I and II enzymes in the endoplasmic reticulum. These pathways may be partial reactions of a multienzyme complex which carries out the various activities associated with the vitamin K-dependent carboxylation system.

INTRODUCTION

Vitamin K is essential for the synthesis of the functional coagulation factors II, VII, IX, X, protein C and proteins S by the liver [1]. These factors are secretory glycoproteins which are modified postribosomally by the vitamin K-dependent carboxylase in a γ-carboxylation reaction, and this reaction requires reduced vitamin KH_2 as cofactor [2,3].

The postribosomal modification reaction results in conversion of specific glutamic acid residues (Glu) to γ-carboxyglutamic acid residues (Gla), which gives Ca^{2+}-binding properties to the proteins [1]. In the liver, vitamin K is reduced to the active cofactor by enzymes belonging to two independent reduction pathways. These are: (1) pathway I, a pathway catalysed by vitamin K epoxide reductase, an enzyme which is highly sensitive to inhibition by coumarin anticoagulant drugs; and (2) pathway II, a pathway catalysed by pyridine-nucleotide-dependent enzymes that are less sensitive to inhibition by these drugs [4]. Warfarin, one of the coumarin drugs, has been shown to inhibit pathway I irreversibly [3,5]. Pathway II is responsible for the antidotal effect of vitamin K in overcoming warfarin inhibition of pathway I [3,6], and the pathway serves an important salvage function in that it can be used to prevent fatal bleedings caused by overdosage with coumarin anticoagulant drugs [7].

In rat liver, at least two dehydrogenases are responsible for the activity of pathway II [3,8]. Studies *in vitro* with rat liver microsomes have shown that DT-diaphorase (EC 1.6.99.2) contributes about 50% to the total activity of pathway II [9]. The remaining activity is accounted for by an NADH-specific dehydrogenase(s) which is insensitive to warfarin and also to other coumarin anticoagulant drugs [3,5].

In human liver, the specific activity of DT-diaphorase is $> 1\%$ of the activity measured in rat liver [5,10]. Therefore, in human liver, the coumarin-insensitive NADH-specific dehydrogenase appears to be the physiologically important enzyme mediating the antidotal effect of vitamin K in overcoming poisoning by these drugs. The enzyme has resisted purification attempts and the identity of the enzyme has not yet been established [9]. It has been shown, however, that the enzyme is a membrane protein of the endoplasmic reticulum and thus has a similar attachment to this membrane system as the other enzymes of the vitamin K-dependent carboxylation system [3,5]. In order to learn more about this enzyme and its relationship to the other enzymes of the vitamin K-dependent carboxylation system, we have investigated the distribution and transmembrane orientation of these enzymes in rough and smooth microsomes obtained from human liver biopsies. Our data suggest a close association between the coumarin-insensitive enzyme and the other enzymes of the system. The data propose the existence of an enzyme complex which can carry out the various reactions currently associated with the vitamin K-dependent carboxylation system.

MATERIALS AND METHODS

Human liver

Human liver biopsies were obtained from morbidly obese patients undergoing gastric bypass surgery. The

Abbreviations used: Chaps, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate; DTT, dithiothreitol; Vitamin K_1H_2, fully reduced vitamin K_1.

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study was approved by the Clinical Investigation Committee of the Milton S. Hershey Medical Center, Pennsylvania State University. It followed the guidelines of the National Institutes of Health. Subjects participated only after signing a form of consent.

Preparation of microsomes

Liver biopsies were rinsed in ice-cold saline and homogenized in 80 mM-KCl/200 mM-sucrose/20 mM-Tris/HCl/5 mM-benzamidine, pH 7.8, as described [5]. When the biopsy was used for preparation of microsomes designated for treatment with proteolytic enzymes, benzamidine was omitted from the homogenization buffer.

The homogenate was centrifuged twice at 10000 *g* for 10 min. The postribosomal supernatant after the last 10000 *g* spin was centrifuged for 60 min at 100000 *g*. The microsomal pellet after the 100000 *g* spin was resuspended in 250 mM-sucrose/25 mM-imidazole/5 mM-benzamidine, pH 7.2 (SI-buffer) and pelleted a second time by centrifugation at 100000 *g* for 60 min. Again, if microsomes were designated for treatment with proteolytic enzymes, benzamidine was omitted from the SI-buffer. Microsomes were stored in liquid N_2.

Treatment of microsomes with proteolytic enzymes and detergent

Microsomes (5–8 mg of protein/ml) were resuspended in the SI-buffer and incubated for 18 h at 4 °C in the presence of 150 μg of either trypsin or chymotrypsin. This treatment of microsomes with trypsin or chymotrypsin was also carried out in the presence of 0.5% Chaps. Controls were identical incubations without the proteolytic enzyme. Enzymic proteolysis was stopped by addition of 0.5 mg of soybean trypsin inhibitor/ml to the incubations. Soybean trypsin inhibitor was also added to the controls. The treated microsomes were either prepared directly for vitamin K-dependent carboxylase activity measurements or centrifuged at 100000 *g* for 60 min. The supernatants from centrifuged incubations were collected for cytochrome *c* reductase activity measurements. The pellets were resuspended in the SI-buffer and the suspension was centrifuged for 60 min at 100000 *g*. The supernatants were discarded and the pellets were resuspended in the SI-buffer and centrifuged one more time at 100000 *g* for 60 min. The final pellets were resuspended in SI-buffer containing 0.5% Chaps and prepared for vitamin K-dependent carboxylase measurements.

Preparation of rough and smooth microsomes

Rough and smooth microsomes were prepared by using a sucrose/CsCl gradient as described by Dallner [11]. Microsomes were suspended in 250 mM-sucrose/5 mM-benzamidine/15 mM-CsCl, and 7.0 ml of the suspension was layered above 5 ml of 1.3 M-sucrose/5 mM-benzamidine/15 mM-CsCl. Centrifugation was carried out at 4 °C in a SW 41 rotor at 39000 rev./min ($r_{av.}$ = 11.27 cm) for 3 h. The pellet (rough microsomes) and the particles sedimenting in the interface (smooth microsomes) [11] were collected in different tubes, resuspended in the SI-buffer and pelleted again by centrifugation at 100000 *g* for 60 min. The pellets were resuspended in the SI-buffer containing 0.5% Chaps and prepared for vitamin K-dependent carboxylase activity measurements.

Vitamin K-dependent carboxylase and vitamin K epoxide reductase activity measurements

All samples prepared for vitamin K-dependent carboxylase activity measurements contained 5 mM-dithiothreitol (DTT) and 1 mM-$MnCl_2$ [5]. Activity was measured as $^{14}CO_2$ incorporation into the synthetic pentapeptide Phe-Leu-Glu-Glu-Leu as described by Esmon & Suttie [12]. Incubations contained a 2 mM concentration of the pentapeptide. Incubations with chemically-reduced vitamin K_1H_2 contained 100 μg of the reduced vitamin/ml. Carboxylase activity supported by pathways I and II were measured as follows. For measurement of pathway I-supported activity, incubations contained 100 μg of vitamin K_1 quinone/ml; for pathway II-supported activity, incubations contained 100 μg of vitamin K_1 quinone/ml, 2 mM-NADH and 50 μM-warfarin for neutralization of pathway I.

Vitamin K expoxide reductase activity was measured as conversion of vitamin K_1 2,3-epoxide to vitamin K_1 quinone using an h.p.l.c. system for separation of the two components. The assay with human microsomes was carried out as described [5].

Other assays

Cytochrome *P*-450 was measured as described by Omura & Sato [13] using the CO-difference spectrum of dithionite-reduced microsomes. Cytochrome *c* reductase activity was measured at room temperature in 0.05 M-potassium phosphate, pH 7.5, containing Tween-20 (0.8 mg/ml), 75 mM-cytochrome *c* and 0.1 mM-NADPH. Enzyme activity was measured as the reduction of cytochrome *c* monitored at 550 nm. An extinction coefficient of 18.5 $mM^{-1} \cdot cm^{-1}$ was used for calculation of enzyme activity. Phospholipids were measured as inorganic phosphate as described by Barlett [14]. A factor of 25 was used for estimation of phospholipids in the samples. RNA was estimated based on the dual-wavelength method described by Monro & Fleck [15] with the modifications described by Ashford & Pain [16]. RNA was calculated from the equation: RNA (μg/ml) = $(32.9 \times A_{260} - 6.11 \times A_{232})$. Protein was measured according to Shaffner & Weissmann [17].

Materials

Vitamin K_1 used for synthesis of reduced vitamin K_1H_2 was purchased from Sigma (St. Louis, MO, U.S.A.). The vitamin was reduced with dithionite as described by Sadowski *et al.* [18]. Vitamin K_1 quinone used in the incubations was the commercial preparation Aquamephyton obtained from Merck Sharp and Dohme. The pentapeptide Phe-Leu-Glu-Glu-Leu was from Vega Fox Biochemicals (Tucson, AZ, U.S.A.). $NaH^{14}CO_3$ (60 mCi/mmol) was from the Amersham Corporation (Arlington Heights, IL, U.S.A.). Trypsin (type I), chymotrypsin (type II) and soybean trypsin–chymotrypsin inhibitor were from Sigma. All other chemicals were of reagent grade or better.

RESULTS

Human microsomes, when suspended in buffer without the detergent Chaps, exhibited no carboxylase activity when the activity was measured as $^{14}CO_2$-carboxylation of the charged pentapeptide substrate. However, significant activity was measured when 0.5% Chaps was

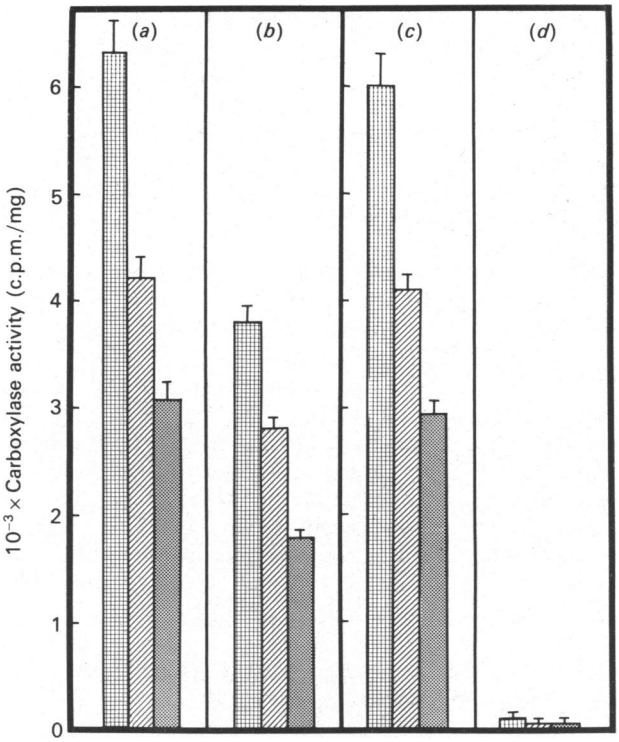

Fig. 1. Vitamin K-dependent carboxylase activity in trypsin- and detergent-treated microsomes

Carboxylase activity was measured in microsomes (*a*), in microsomes treated with trypsin (*b*), in microsomes treated with 0.5% Chaps (*c*) and in microsomes treated with 0.5% Chaps and trypsin (*d*). Carboxylase activity was supported by chemically-reduced vitamin K_1H_2(▦), vitamin K_1 + DTT (pathway I) (▨) and vitamin K_1 + NADH (pathway II) (■) respectively. Results are shown as the average of three parallel incubations + S.D. ($n = 3$). Incubations in the absence of vitamin K_1 served as controls.

present in the test system (see Table 3). This suggested that the carboxylase in human liver has, as has been shown in rat liver [19,20], a luminal orientation in the endoplasmic reticulum, thus making the enzyme inaccessible to the charged pentapeptide substrate. To confirm this, microsomes were incubated with trypsin in the presence and absence of Chaps. When incubated with trypsin in the absence of Chaps, trypsin did not destroy vitamin K-dependent carboxylase activity. However, as shown in Fig. 1(*b*), trypsin treatment resulted in a 35% lower carboxylase activity (triggered with chemically-reduced vitamin K_1H_2) than that measured in untreated microsomes (Fig. 1*a*). The activity when supported by pathways I and II was 15% and 40% lower respectively than the controls (Fig. 1*b*; controls, Fig. 1*a*). Since the activity of the vitamin K-dependent carboxylase enzyme was reduced by 35% in trypsin-treated microsomes, a lower activity supported by the pathways was expected to be measured in these microsomes. On the other hand, all carboxylase activity was lost when microsomes were incubated with trypsin in the presence of Chaps (Fig. 1*d*). Fig. 1(*c*) shows the activities that were measured in a control incubation with only Chaps present. This experiment demonstrated that the human vitamin K-dependent carboxylase, when accessible to trypsin, as in the detergent-treated microsomes, is proteolytically destroyed by trypsin. The experiment presents evidence for a luminal orientation of the human vitamin K-dependent carboxylase in the endoplasmic reticulum.

The susceptiblity of the pathway I and II enzymes to trypsin could not be decided by the experiment described above. For this, specific test systems for the individual enzymes were required. We measured the activity of the pathway I enzyme, the vitamin K epoxide reductase [5]. When microsomes were incubated for 20 min in the presence of 5 mM-DTT and 10 μM-vitamin K 2,3-epoxide [5], human vitamin K epoxide reductase reduced 141 pmol of the epoxide per mg of microsomal protein. In microsomes treated with trypsin, this activity was reduced by 15%, but the activity was destroyed when microsomes were incubated with trypsin and 0.5% Chaps. The experiment demonstrated that the human pathway I enzyme was also protected by the microsomal membrane and has, like the human carboxylase, a luminal orientation in the endoplasmic reticulum. No specific test system was available for the unidentified pathway II enzyme. We could only determine its activity by measuring its ability to provide the carboxylase with reduced vitamin K_1H_2-cofactor. To decide whether or not this enzyme has a cytoplasmic orientation in the endoplasmic reticulum, we digested human microsomes with trypsin or chymotrypsin and tested the potential release of the pathway II enzyme from the microsomal membrane. Digested microsomes were washed extensively before they were used for carboxylase activity measurements (see legend to Fig. 2). As shown in Fig. 2(*b*), washed trypsin-treated microsomes had lost 31% of their carboxylase activity when compared with just-washed microsomes (Fig. 2*a*). However, the data clearly demonstrate that trypsin did not remove the pathway I and II enzymes (see Fig. 2*b*). When trypsin was exchanged for chymotrypsin in these experiments, no significant differences in the results were found (results not shown). Thus neither trypsin nor chymotrypsin was able to release the pathway II enzyme from the outside microsomal membrane, suggesting a luminal orientation also for the coumarin-drug-insensitive enzyme.

To verify whether trypsin indeed had activity towards human microsomal membrane proteins, the ability of trypsin to release cytochrome *c* reductase from the microsomal membrane was investigated. When untreated microsomes were suspended in buffer and subjected to centrifugation, the pellet and the supernatant showed 136.1 nmol/min per mg and 10.2 nmol/min per mg of cytochrome *c* reductase activity respectively (Table 1). After trypsin treatment of microsomes, the activity in the pellet was reduced to 0.4 nmol/min per mg, but the activity in the supernatant had increased to 328.0 nmol/min per mg (Table 1). Also shown in Table 1 is the finding that trypsin did not destroy cytochrome *c* reductase activity in either the absence or presence of the detergent Chaps. The experiment demonstrated that trypsin indeed released cytochrome *c* from the cytosolic side of the microsomal membrane, suggesting that a similar activity would also be exerted towards other susceptible membrane proteins.

Separation of human microsomes into rough and smooth particles was achieved with sucrose/CsCl density centrifugation, and separation was verified using two criteria, the RNA/phospholipid ratio and the distribution of cytochrome *P*-450. The RNA/phospholipid ratio in rough microsomes was 1.5-fold higher than this

ratio in smooth microsomes. However, we measured the same RNA/phospholipid ratio in rough microsomes as we measured in unfractionated microsomes (Table 2). The cytochrome *P*-450 distribution appeared to be a better criterion. We measured 0.59 nmol of cytochrome *P*-450 per mg of protein in human microsomes (Table 2), a value which is in close agreement with what has been reported by others [21,22]. The concentration of cytochrome *P*-450 in rough microsomes was < 0.06 nmol per mg of protein. On the other hand, smooth microsomes had 0.26 nmol of cytochrome *P*-450 per mg of protein. Although this number was smaller than for unfractionated microsomes, the measured distribution of cytochrome *P*-450 in rough and smooth microsomes agrees with what is known about the distribution of this cytochrome in subcellular particles isolated from animal livers [23].

Table 3 shows carboxylase activity measured in rough and smooth microsomes and also in unfractionated microsomes. The activity was triggered with chemically-reduced vitamin K_1H_2, vitamin K_1 + DTT, and vitamin K_1 + NADH in order to measure the activity of the carboxylase enzyme, as well as the activity of this enzyme when supported by pathway I or pathway II respectively. When triggered with chemically-reduced vitamin K_1H_2, rough microsomes exhibited the highest specific carboxylase activity, and this activity was 6-fold higher than the activity measured in smooth microsomes. When we

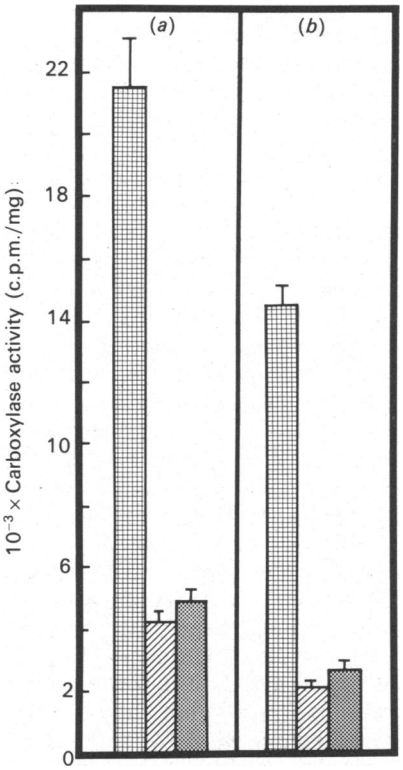

Fig. 2. Vitamin K-dependent carboxylase activity in trypsin-treated and washed microsomes

Microsomes were washed in SI-buffer by ultracentrifugation as described in the Materials and methods section. Carboxylase activity measured in washed microsomes is shown in (*a*). Panel (*b*) shows carboxylase activity in trypsin-treated microsomes after extensive washing in SI-buffer (see the Materials and methods section). Carboxylase activity was measured as described in the legend to Fig. 1. Results are shown as the average activity measured in three parallel incubations + S.D. ($n = 3$).

Table 1. Cytochrome *c* reductase activity of liver microsomes treated with trypsin and detergent

Human liver microsomes were treated with detergent and trypsin as described in the Materials and methods section. Untreated and trypsin-treated microsomes were centrifuged at 100000 *g* for 60 min to obtain a fraction of soluble protein (supernatant) and a fraction of insoluble protein (pellet). Specific cytochrome *c* reductase activity (nmol/min per mg) was measured as described in the Materials and methods section.

Samples	Cytochrome *c* reductase activity (nmol/min per mg)
Microsomes:	123.8
+ Trypsin	168.5
+ Chaps	130.8
+ Trypsin + Chaps	154.6
Microsomes after centrifugation:	
Pellet	136.1
Supernatant	10.2
Trypsin-treated microsomes after centrifugation:	
Pellet	0.4
Supernatant	328.0

Table 2. Determination of markers for rough and smooth microsomes

Concentrations of protein, phospholipid (PL), RNA and cytochrome *P*-450 were determined according to standard procedures (see the Materials and methods section). Numbers are the average of four parallel measurements which differed by less than 10%.

Fraction	Protein (mg/ml)	PL (mg/mg*)	RNA (μg/mg*)	RNA/PL	Cytochrome *P*-450 (nmol/mg*)
Microsomes	2.1	1.4	95	0.07	0.59
Rough microsomes	0.35	3.1	214	0.07	< 0.06
Smooth microsomes	1.2	2.1	88	0.04	0.26

* mg of microsomal protein.

Table 3. Vitamin K-dependent carboxylase activity in rough and smooth microsomes

Carboxylase activity was measured as described in the Materials and methods section when the activity was supported by chemically-reduced vitamin K_1H_2 (vit. K_1H_2), vitamin K_1 + DTT (pathway I) or vitamin K_1 + NADH (pathway II). Numbers are the means of three parallel incubations differing by less than 10%. Incubations in the absence of vitamin K_1 served as controls.

Fraction	Carboxylase activity (c.p.m./mg*)			Ratio		
	Vit. K_1H_2	Vit. K_1 + DTT	Vit. K_1 + NADH	Vit. K_1H_2 / Vit. K_1 + DTT	Vit. K_1H_2 / Vit. K_1 + NADH	Vit. K_1 + DTT / Vit. K_1 + NADH
Microsomes	6650	4380	3330	1.5	2.0	1.3
Rough microsomes	17960	6810	4010	2.6	4.5	1.7
Smooth microsomes	2830	2500	1560	1.1	1.8	1.6

* mg of microsomal protein

calculated the ratio between carboxylase activity triggered with chemically-reduced vitamin K_1H_2 and either vitamin K_1 + DTT or vitamin K_1 + NADH, these ratios were different and also signifcantly higher for rough microsomes (2.6 and 4.5) than for smooth microsomes (1.1 and 1.8) (Table 3). However, when we caculated the ratio between pathway I- and pathway II-supported carboxylase activity in rough and smooth microsomes, these ratios were 1.7 and 1.6 respectively, and the difference was within the experimental error (Table 3). Thus, the pathway I and pathway II enzymes appear to be equally distributed in rough and smooth microsomes isolated from human liver, suggesting a close association between these enzymes in the endoplasmic reticulum.

DISCUSSION

Our data on the subcellular location and the transmembrane orientation of the vitamin K-dependent carboxylase in human liver cells are consistent with data from rat experiments [19,20,24]. The human carboxylase is clearly enriched in the rough endoplasmic reticulum of the liver cells and the enzyme faces the luminal side of this membrane system. This location of the carboxylase is consistent with its role in the early processing of secretory glycoproteins [25]. Significant carboxylase activity was also measured in smooth microsomes, but the specific activity of the enzyme was only 16% of the specific activity measured in rough microsomes. The activity measured in smooth microsomes is unlikely to result from contamination with rough particles in our preparation of smooth microsomes. Dallner's method [11] for separation of rough and smooth microsomes by CsCl gradient centrifugation has been shown to yield < 1% contamination of rough particles in the smooth microsomal fraction [26]. Separation was based on two criteria, the RNA/phospholipid ratio and the distribution of cytochrome *P*-450. The concentration of cytochrome *P*-450 in rough microsomes was below the limit for estimation by our spectrophotometer. On the other hand, smooth microsomes had 0.26 nmol of the cytochrome per mg of protein, which was however a lower specific concentration than was measured in unfractionated microsomes. Since the cytochrome *P*-450 content of rough microsomes was extremely low, one would expect to have measured an increase in the specific concentration of cytochrome *P*-450 in smooth microsomes. However, a lower concentration in smooth microsomes was measured in several independent experiments. A possible explanation for this discrepancy may be loss of cytochrome *P*-450 when human microsomes were subjected to fractionation according to Dallner's method. On the other hand, our data demonstrate a significant difference in the concentration of cytochrome *P*-450 in rough and smooth human microsomes. Therefore, it appears that cytochrome *P*-450 is an excellent marker also for human smooth microsomes, as it is for smooth microsomes isolated from animal sources.

Our data do not allow any conclusions to be drawn about the distribution of the pathway II enzyme in the endoplasmic reticulum. In our γ-carboxylation test system only the apparent activity of the pathway II enzyme could be measured. The activity was dependent upon the presence of the vitamin K-dependent carboxylase. However, our data showed that carboxylase activity could be supported by pathways I and II in rough as well as smooth microsomes, and, most importantly, we measured the same ratio between these activities in rough and smooth microsomes, indicating an equal distribution of the pathway I and II enzymes in the endoplasmic reticulum. In unfractionated microsomes pathway I appeared to be more active than pathway II (see vitamin K_1 + DTT/vitamin K_1 + NADH ratio in Table 3). A lower activity of pathway I in rough and smooth microsomes could result from some loss of vitamin K epoxide reductase activity after CsCl gradient centrifugation of crude microsomes.

Carlisle & Suttie [19] have shown that the enzyme activity profiles of the vitamin K-dependent carboxylase, the vitamin K epoxide reductase (the pathway I enzyme) and the vitamin K epoxidase in rough and smooth microsomes from rat liver are quite similar. Their data suggest that the enzymes involved in vitamin K function and vitamin K metabolism appear together in the endoplasmic reticulum. Since it has been established that this enzyme system is similar in rat and human liver [3], it is reasonable to assume that there is also a close association in human liver between the enzymes belonging to this system. An additional similarity between

the rat and the human system is evident from the finding of a luminal orientation also for the vitamin K epoxide reductase.

Our data do not provide conclusive evidence, but strongly suggest that the pathway II enzyme also faces the luminal side of the endoplasmic reticulum. Since the pathway I and II enzymes had the same distribution in rough and smooth microsomes, we propose that the pathway II enzyme is part of the vitamin K-dependent enzyme system and appears together with the other enzymes of the system. These enzymes may be parts of an ordered multienzyme complex.

Previous attempts to purify the NADH-specific pathway II enzyme on dehydrogenase-selective affinity resins have failed [9]. We have, however, provided evidence that the pathway II enzyme is not identical to cytochrome *P*-450 reductase or cytochrome *b* reductase, two enzymes which have been considered as candidates for vitamin K_1 reduction in liver [9]. This evidence is strengthened by the demonstration in this paper that trypsin did not release the pathway II enzyme from the microsomal vesicles. However, trypsin treatment of microsomes resulted in a 35% loss of carboxylase activity. A similar loss of carboxylase activity in trypsin-treated rat microsomes has been reported by Helgeland [20], but was not observed by Carlisle & Suttie [19]. Since we could not measure any γ-carboxylation of the synthetic pentapeptide when intact microsomes were used for the assay, it is unlikely that the 35% loss of carboxylase activity represents a pool of the carboxylase facing the cytosolic side of the endoplasmic reticulum. The trypsin effect may be triggered by loss of membrane proteins which affect carboxylase activity.

In conclusion, it appears from our data that the pathway I and II enzymes are closely associated enzymes in the endoplasmic reticulum. Thus, the tentative enzyme complex for vitamin K function and vitamin K metabolism appears to have an associated enzyme (the pathway II enzyme) which can, at high liver concentrations of vitamin K_1, override inhibition of γ-carboxylation by coumarin anticoagulant drugs.

This work was supported by NIH Grant HL32070 and a grant from the American Heart Association, Pennsylvania, Harrisburg affiliate. We want to thank Mary Ann Maxfield for typing this manuscript.

Received 17 October 1988/6 March 1989; accepted 6 March 1989

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