Vitamin K Dependent Carboxylase: Subcellular Location of the Carboxylase and Enzymes Involved in Vitamin K Metabolism in Rat Liver†

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ABSTRACT: Vitamin K dependent carboxylation of an exogenous peptide substrate and endogenous protein substrates, vitamin K epoxidation, and reduction of vitamin K epoxide were measured in subcellular fractions from rat liver. The rough microsomal fraction was highly enriched in all four activities; lower levels were found in smooth microsomes. Mitochondria, nuclei, and cytosol had negligible activities. The addition of 0.2% Triton X-100 to intact microsomes resulted in a 10–20-fold stimulation in carboxylation of a peptide substrate. This marked latency suggests that the active site of the carboxylase may be accessible only from the lumen of the microsomal membrane. A lumen-facing orientation of the carboxylase was also supported by its inaccessibility to trypsin in intact microsomes contrasted with marked inhibition by trypsin in detergent-permeabilized microsomes. Vitamin K epoxidase and epoxide reductase activities were also inhibited by trypsin much more effectively in permeabilized than in intact microsomes, although some degree of exposure at the cytosolic surface was also indicated. These data suggest that carboxylation is an early event in prothrombin synthesis occurring primarily on the lumen side of the rough endoplasmic reticulum membrane. The location of the vitamin K epoxidation–reduction cycle enzymes is consistent with their possible role in the carboxylation reaction.

Vitamin K functions in the posttranslational carboxylation of specific glutamyl residues in microsomal protein precursors to form biologically active proteins containing γ-carboxyglutamic acid. These vitamin K dependent proteins include prothrombin and coagulation factors VII, IX, and X, as well as more recently discovered proteins of unknown function in plasma and other tissues (Suttie & Jackson, 1977; Stenflo, 1978; Suttie, 1978). It has been demonstrated (Esmon et al., 1975b) that rat liver microsomes will support a vitamin K dependent carboxylation reaction. This unique carboxylase, which requires the reduced (quinol) form of vitamin K, O_2, and CO_2, has now been studied in a number of laboratories, and its properties have recently been reviewed (Suttie, 1979).

Preparations of rat liver microsomes will also support three reactions of vitamin K metabolism that are relevant to the carboxylation reaction. These are oxidation of the vitamin to its 2,3-epoxide, reduction of the 2,3-epoxide to vitamin K, and reduction of the quinone to the hydroquinone. Willingham & Matschiner (1974) have proposed that the epoxidation reaction might be an obligatory part of the carboxylase system, and the data which relate to this relationship between epoxidation and carboxylation have recently been reviewed (Suttie et al., 1978). The vitamin K epoxide reductase (Zimmerman & Matschiner, 1974) of rat liver microsomes appears (Whitlon et al., 1978; Bell, 1978) to be the enzyme responsible for the inhibition of vitamin K action by the coumarin anticoagulants. The physiologically important reductant for this enzyme is not known, but its in vitro action is driven by dithiothreitol. The vitamin K epoxidase and epoxide reductase, together with a dithiothreitol–vitamin K quinone reductase which is also sensitive to warfarin, comprise a vitamin K "redox cycle" which appears to be significant in understanding the action of vitamin K and its inhibition by coumarin anticoagulants. NADH can also supply reducing equivalents to drive in vitro carboxylation, and Wallin et al. (1978) have demonstrated that a microsomal form of the largely cytosolic DT-diaphorase can catalyze an NADH + vitamin K quinone driven carboxylation in a detergent-solubilized in vitro system.

Vitamin K is rather widely distributed (Bell & Matschiner, 1969; Nyquist et al., 1971; Knauer et al., 1976) among membrane organelles and concentrated in microsomes and mitochondria. A number of variables, including warfarin treatment, have been found to be without effect on this distribution (Thierry & Suttie, 1971). The distribution of vitamin K epoxide parallels that of the vitamin. In the present study, the subcellular distribution of vitamin K dependent carboxylation and of vitamin K epoxidation and epoxide reduction has been determined in rough and smooth microsomes, mitochondria, nuclei, and cytosol. In addition, the transverse orientation of these enzymes in the microsomal membrane was investigated. A knowledge of the location of these enzymes is crucial to a complete understanding of this posttranslational event in prothrombin synthesis and is important in evaluating the relationships between epoxidation and carboxylation and between the epoxidation–reduction cycle and warfarin inhibition of vitamin K action.

Experimental Procedure

Subcellular Fractions. Vitamin K deficiency was produced in adult male Holtzman strain rats as previously described (Suttie et al., 1976). Livers from rats killed by decapitation were washed in ice-cold homogenization medium, and subcellular fractions were prepared concurrently from the pooled livers of four rats. Rough and smooth microsomes were prepared by rate sedimentation through a sucrose step gradient as described by Dallner (1974). The clear middle third of the upper layer provided the cytosol fraction. The smooth microsome layer from three to six tubes was diluted to 17 mL with homogenization medium, and the smooth microsomes were pelleted at 165000*g* for 45 min to remove cytosol protein. Mitochondria were prepared by the method of Sottocasa et al. (1967) from the homogenate obtained after four strokes of a Potter–Elvehjem homogenizer operated at 400–800 rpm. Mitochondria were washed by hand resuspension with a cold Teflon pestle. Method III of Spelsberg et al. (1974) was used to purify nuclei. Washing in Spelsberg's solution C, which

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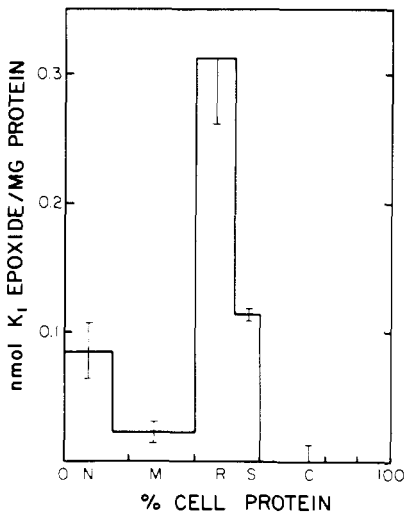

FIGURE 1: Glucose-6-phosphatase distribution among subcellular fractions. Subcellular fractions were prepared from fasted rats, and glucose-6-phosphatase was assayed as described under Experimental Procedure. The values shown are the mean ± standard error of between 5 and 10 preparations of each fraction. See legend to Figure 3 for details.

removes the outer nuclear membrane, was omitted, and the centrifugation through 1.7 M sucrose performed at 25000*g* was repeated unless the initial nuclear pellet was white. All solutions for preparation of subcellular fractions were modified from the literature description by the inclusion of dithiothreitol (0.001 M). Procedures were carried out at 0–6 °C unless otherwise stated.

Glucose-6-phosphatase was assayed as described by Morre (1971), and protein was assayed as described by Lowry et al. (1951), with crystalline bovine serum albumin as standard. RNA was assayed as suggested by Fleck & Munro (1962) using an $E_{1cm}^{1\%}$ of 213 at 260 nm for rat liver RNA. The yields (milligrams of protein per gram of liver ± SEM) of the subcellular fractions, estimated from the number of experiments indicated in parentheses, were as follows: rough microsomes, 12.8 ± 0.6 (26); smooth microsomes, 4.8 ± 0.2 (24); mitochondria, 26 ± 2 (7); nuclei, 1.75 ± 0.08 (9); cytosol, 63 ± 2 (8); homogenate, 212 ± 7 (6). Separation of rough and smooth microsomes was satisfactory as indicated by weight ratios of RNA/protein of 0.22 ± 0.02 for rough and 0.06 ± 0.01 for smooth microsomes. Gram (1974) has determined by electron microscopy that the rough microsomal fractions made by this method contain 25–30% smooth vesicles.

The distribution of glucose-6-phosphatase, a microsomal marker enzyme, was measured to determine the contamination of nonmicrosomal fractions with microsomes (Figure 1). The glucose-6-phosphatase activity seen in the nuclear fraction (15% that of rough microsomes) indicates that it contained ~10% microsomal protein, since activity endogenous to the nuclear membrane can account for a specific activity of only ~5% that of microsomes (Kasper, 1974). Very little contamination of mitochondria or cytosol with microsomes was detected.

Incubation Conditions. Mitochondria and rough and smooth microsomes were resuspended in 0.25 M sucrose, 0.025 M imidazole, and 0.001 M dithiothreitol, pH 7.2, made 0.5 M in KCl (buffer A) to a concentration of ~25 mg of protein per mL by using eight strokes of a loose-fitting pestle in a Dounce homogenizer. The cytosol fraction was also Dounce treated. An equal volume of 0.4% (w/v) Triton X-100 in buffer A was added and the Dounce treatment gently repeated. Portions (0.4 mL) of the resulting suspension were dispensed to the incubation tubes. Nuclei were similarly resuspended to 31 mg of protein per mL in KCl-free buffer A, and 0.16 mL was dispensed to the incubation tubes. To each tube containing nuclei was added 0.24 mL of 0.33% (w/v) Triton X-100 in buffer A with 0.83 M KCl with gentle vortex mixing. Incubations for the assay of vitamin K dependent carboxylation were composed of 0.4 mL each of Triton X-100 treated subcellular fraction containing 5 mg of protein, 0.1 mL of a mixture containing 0.0025 M peptide substrate (Phe-Leu-Glu-Glu-Leu), 100 μCi/mL $NaH^{14}CO_3$ (60 mCi/mmol, Amersham/Searle), 0.2% Triton X-100 in buffer A, and 0.01 mL of ethanol containing 50 μg of vitamin K_1 hydroquinone. Controls received ethanol only. The tubes were sealed with plastic film and incubated at 27 °C for 30 min with slow circular mixing.

Epoxidation of vitamin K was measured by addition of 5.1 μg of [^{3}H]vitamin K_1 hydroquinone (~0.05–0.1 μCi) in 0.010 mL of ethanol to 0.5-mL incubation mixtures prepared as for carboxylation with the addition of sodium warfarin (0.1 mg/mL) to block reduction of the epoxide formed. Peptide substrate and [^{14}C]bicarbonate were omitted. Reduction of vitamin K_1 epoxide to vitamin K was measured in incubations similar to those for epoxidation except that the warfarin was omitted, [^{3}H]vitamin K_1 2,3-epoxide (5.1 μg, ~0.05–0.1 μCi) was added in 0.01 mL of ethanol, and the reactions were incubated under a nitrogen atmosphere. Exclusion of oxygen is necessary to prevent reepoxidation of vitamin formed during the incubation. Incubations for 30 min at 27 °C were conducted as previously described (Whitlon et al., 1978).

Carboxylase, Vitamin K Epoxidase, and Vitamin K Epoxide Reductase Assays. Vitamin K dependent incorporation of $H^{14}CO_3^-$ into endogenous microsomal precursors (protein carboxylation) was measured as trichloroacetic acid (Cl_3AcOH) precipitable radioactivity. A 0.02- or 0.05-mL portion of each incubation mixture was added to 0.2 mL of 0.9% NaCl containing 2 mg of bovine serum albumin, and protein was precipitated with 3 mL of ice-cold 10% Cl_3AcOH and collected by centrifugation. The pellet was dissolved in 0.8 mL of Na_2CO_3 (0.2 M), precipitated with Cl_3AcOH twice, and then dissolved in 0.7 mL of NCS solubilizer and rinsed into a shell vial with two 1.9-mL portions of Econofluor. Radioactivity was determined in a liquid scintillation spectrometer. Incorporation of $H^{14}CO_3^-$ into the peptide substrate was measured as Cl_3AcOH-soluble, nonvolatile radioactivity after the addition of 1.00 mL of Cl_3AcOH to 0.2-mL portions of incubation mixtures essentially as described previously (Suttie et al., 1976). Vitamin K and vitamin K epoxide were measured as described by Whitlon et al. (1978). Water (0.5 mL) was added to each assay tube during extraction of the vitamin and its metabolites with 2.0 mL of 2-propanol–hexane. The vitamin K_1, vitamin K_1 hydroquinone, and vitamin K_1 2,3-epoxide were prepared as previously described (Whitlon et al., 1978). Ethanol solutions of vitamin K hydroquinone were stored under nitrogen in sealed vials at −20 °C.

Trypsin Treatment of Microsomes. Total microsomes (Sadowski et al., 1977) from two to four rat livers were resuspended (eight strokes with the loose, type A pestle in a Dounce homogenizer) to 1 mL/g of liver. Incubations contained 2.5 mL of microsomes, 0.75 mg of trypsin, and/or 10 mg of Triton X-100 in 5.0 mL of buffer A. After 15 h on ice, 0.05 mL of soybean trypsin inhibitor (50 mg/mL) was added with mixing; 30 min later, 0.10 mL of 10% Triton X-100 (or buffer A for those tubes already containing Triton) was added with gentle vortex mixing. Portions of 0.4 mL were assayed in duplicate or triplicate as described elsewhere.

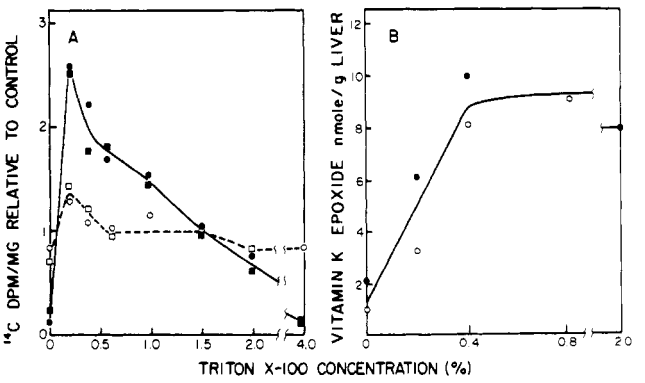

FIGURE 2: Effect of detergent concentration on vitamin K dependent CO_2 incorporation into peptide substrates and endogenous proteins and on vitamin K epoxidation. Microsomes from vitamin K deficient rats were resuspended and assayed as described under Experimental Procedure. Various concentrations of detergent were mixed with portions of microsomes in a Dounce homogenizer prior to assay. (A) Peptide (● and ■) and protein (○ and □) carboxylation. Values from two experiments are presented as the ratio of observed carboxylation to that seen under standard conditions for the peptide carboxylase assay described by Suttie et al. (1979), 1.5% Triton X-100 at 10 mg of protein per mL. Circles represent values from experiment 1 (control = 2.7×10^3 dpm/mg of protein); squares represent values from experiment 2 (control = 1.9×10^3 dpm/mg of protein). (B) Vitamin K epoxidation. Values from two experiments (○ or ●) are presented as nanomoles of vitamin K epoxide formed by microsomes derived from 1 g of liver.

Chemicals. Trypsin (Type I from bovine pancreas) and soybean trypsin inhibitor were from Sigma (St. Louis, MO). Phe-Leu-Glu-Glu-Leu was synthesized by Vega-Fox Biochemicals (Tucson, AZ). Triton X-100 (B grade) and dithiothreitol were from Calbiochem (San Diego, CA). Aquasol and Econofluor were supplied by New England Nuclear (Boston, MA), and NCS solubilizer was supplied by Amersham/Searle (Arlington Heights, IL). Unless otherwise stated, all chemicals were reagent grade or of the highest purity available from local suppliers.

Results

Preliminary experiments established detergent concentrations which gave optimum carboxylation and also allowed the assay of the other enzymes. Maximum carboxylation of both peptide substrate and endogenous protein occurred at 0.2% Triton X-100 when the microsomal protein concentration was 10 mg of protein per mL (Figure 2A). Further increases in detergent concentration progressively inhibited peptide carboxylation and slightly inhibited carboxylation of endogenous protein. Epoxidation of vitamin K was also stimulated by addition of detergent (Figure 2B).

When microsomes were diluted from 15 to 5 mg of protein per mL at a constant 1:1 weight ratio of Triton X-100 to microsomal protein, a three- to fourfold increase in specific activity of peptide substrate carboxylation was observed, but protein carboxylation was relatively unaffected. The specific activity of the vitamin K epoxidase also increased about three- to fourfold on dilution from 10 to 5 mg of protein per mL in 0.2% Triton X-100. The assay conditions were therefore standardized at 10 mg of protein per mL and 0.2% Triton X-100. The vitamin K epoxide reductase, although inhibited by high concentrations of Triton (Whitlon et al., 1978), was also active under these circumstances. Dithiothreitol, which may protect sulfhydryl groups essential to the carboxylase (Mack et al., 1976; Friedman & Shia, 1976), can also supply reducing equivalents to the vitamin K and vitamin K epoxide reductase (Zimmerman & Matschiner, 1974; Whitlon et al., 1978) and was included in the media for subcellular fractionation and in the final assay mixtures. Vitamin K was supplied as the hydroquinone in order to ensure that the various vitamin K reductase activities in the microsomal preparation were not limiting for carboxylation or epoxidation.

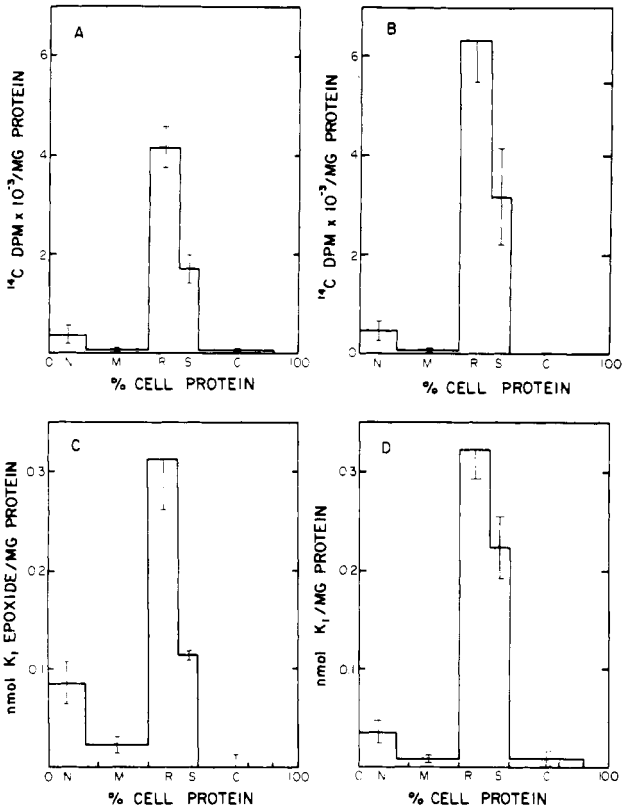

FIGURE 3: Subcellular distribution of vitamin K dependent CO_2 incorporation into endogenous microsomal proteins (A) and peptide substrates, (B) vitamin K_1 epoxidation (C), and vitamin K_1 epoxide reduction (D) in vitamin K deficient rats. Subcellular fractions were prepared from three to four rat livers and assayed as described under Experimental Procedure. The specific activity of the enzyme is indicated on the ordinate. The abscissa indicates what percentage of the total protein each organelle contributes in the intact cell (Steck, 1972). On the assumption that the isolated fraction contains a representative sample of the organelles found in vivo, the area of each bar represents the total activity located in the organelle. The abbreviations are N = nuclei, M = mitochondria, R = rough microsomes (rough endoplasmic reticulum), S = smooth microsomes, and C = cytosol. The values represent the mean ± standard error for three to seven experiments, each experiment involving duplicate or triplicate determinations. Protein carboxylation in mitochondria was measured in two experiments only.

Location of the Vitamin K Dependent Carboxylase. Vitamin K dependent carboxylation of endogenous protein was highly enriched in rough microsomes derived from vitamin K deficient rats, present at ~40% of this specific activity in smooth microsomes and at considerably lower specific activity in the nuclear fraction (Figure 3A). Mitochondria and cytosol were essentially devoid of activity. The distribution of endogenous protein carboxylation reflects the location of both the carboxylase enzyme system and its protein substrates, since both must be present for carboxylation to occur. In contrast, use of the peptide substrate Phe-Leu-Glu-Glu-Leu provided an assay in which the substrate concentration was controlled. The vitamin K dependent carboxylation of this peptide (Figure 3B) was about twofold concentrated in rough relative to smooth microsomes, present in low amounts in the nuclear fraction, and not detected in mitochondria or cytosol.

The rats used in the experiments described above were vitamin K deficient in order to ensure a uniformly high level of the prothrombin precursors and other endogenous substrates.

Table I: Vitamin K Dependent Carboxylation of a Peptide Substrate and Endogenous Proteins in Rough and Smooth Microsomes from Control and Vitamin K Deficient Rats[a]

	vitamin K dependent carboxylation (dpm × 10^{-3}/mg of protein)			
	peptide substrate	R/S	endogenous protein	R/S
control				
rough (R)	3.0		1.3	
		2.0		1.8
smooth (S)	1.5		0.7	
vitamin K deficient				
rough (R)	7.4		5.1	
		2.5		2.3
smooth (S)	3.0		2.3	

[a] Liver from control and vitamin K deficient rats (three in each group) was processed as described under Experimental Procedure. Values are means of duplicates differing by less than 15% for peptide and 8% for protein carboxylation.

The rough microsomes of vitamin K sufficient rats also carboxylated more peptide (2.0-fold) and endogenous protein (1.8-fold) than the smooth fraction (Table I). As previously observed (Shah & Suttie, 1978), vitamin K deficiency was accompanied by a two- to threefold increase in both protein and peptide carboxylase activities. This increase in protein and peptide carboxylation was found to occur preferentially in the rough membrane fraction, enhancing the distribution seen in vitamin K sufficient rats (Table I).

The concentration of peptide substrate used in these experiments was below its apparent K_m of $\sim 4 \times 10^{-3}$ M (Suttie et al., 1979), and the time course of peptide carboxylation falls off somewhat from linearity at the end of the 30-min incubation. Protein carboxylation occurs rapidly and is essentially complete by the end of the incubation period (Suttie et al., 1979). The possible effect of these limitations on the results obtained using this assay system was investigated. In several experiments, early rates of CO_2 fixation were measured; in others, the effect of higher peptide concentrations on the carboxylation was tested. The preferential distribution of peptide carboxylase into rough microsomes was about three- to fourfold when based on early rates, compared to about two- to threefold based on the 30-min incubation (Figure 4A). The relative carboxylation of the endogenous microsomal precursors in the two fractions (Figure 4B) was not significantly altered by varying the periods of incubation. If the apparent distribution of carboxylation were due to different peptide substrate affinities in the rough and smooth membrane fractions, then the ratio of CO_2 incorporation (rough/smooth) would be expected to decrease markedly as the concentration of peptide was increased in the region of the apparent K_m values. That this is not the case (parts C and D of Figure 4) also supports the conclusion that the vitamin K dependent carboxylase activity is enriched in rough microsomes.

Location of Vitamin K Metabolizing Enzymes. Two enzymes which metabolize vitamin K have been proposed to be intimately involved in the carboxylation reaction. The subcellular distributions of these enzymes, the vitamin K epoxidase and the vitamin K epoxide reductase, were studied under conditions similar to those used for the study of carboxylation. These experiments (Figure 3C) revealed an enrichment of vitamin K epoxidation in the rough membrane fraction. Smooth membranes had substantial activity, but no epoxidation was detected in the cytosol. The small amount of epoxidation measured in the mitochondrial fraction could be due to contamination with microsomes, but substantial epoxidation was apparently present in the nuclear fraction. The highest vitamin K epoxide reductase specific activity was again found in rough microsomes (Figure 3D). Smooth microsomes were slightly less active, mitochondria and cytosol contained very low activity, and the nuclear fraction had low activity comparable to the relative amount of epoxidation found there.

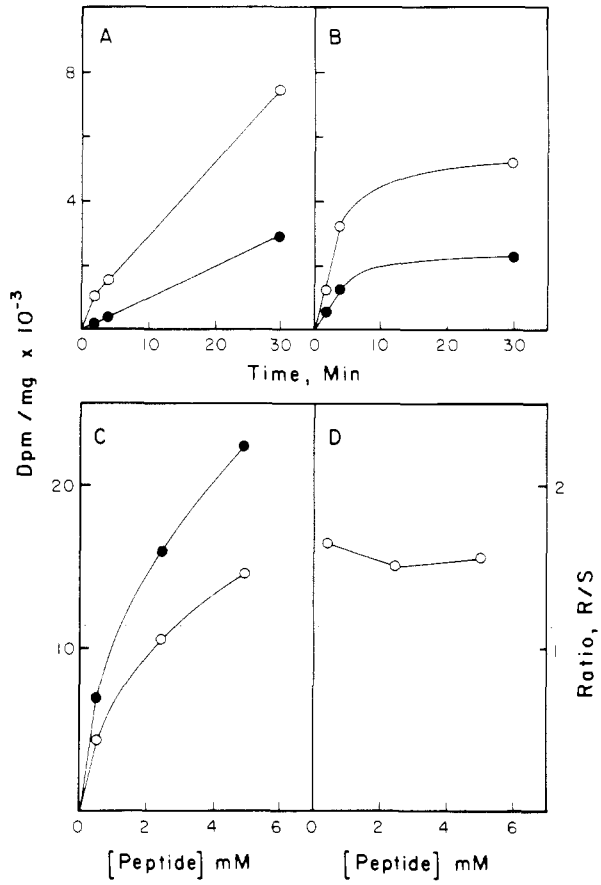

FIGURE 4: Assay of peptide substrate and endogenous protein carboxylation in rough and smooth microsomes. Early rates of peptide substrate (A) and endogenous protein (B) carboxylation in rough (○) and smooth (●) microsomes. Parallel incubations of assay mixtures containing rough or smooth microsomes from the pooled livers of four vitamin K deficient rats were terminated at the indicated times by chilling in ice slush and immediate sampling into cold Cl_3AcOH. (C) Dependence of peptide substrate carboxylation in rough (○) and smooth (●) microsomes on the concentration of peptide substrate. The peptide (Phe-Leu-Glu-Glu-Leu, NH_4^+ salt) was dissolved in buffer containing Triton X-100 and the pH adjusted to 7.2 with NaOH. Peptide (0.30 mL in 0.35% Triton buffer) was mixed with 0.20 mL of microsomes, and the reaction mixtures were completed by the addition of 0.02 mL of 500 μCi/mL $H^{14}CO_3^-$ and 0.01 mL of vitamin K hydroquinone in ethanol. (D) Ratios (rough/smooth) of the specific activity of peptide carboxylation at increasing peptide concentration from panel C.

Transverse Orientation of the Carboxylase and the Vitamin K Metabolizing Enzymes in the Microsomal Membrane. Impermeant probes which affect the activity of membrane enzymes provide an indirect approach to their orientation in the membrane. Trypsin does not penetrate microsomes (Ito & Sato, 1969; DePierre & Ernster, 1977), and the sensitivity of vitamin K dependent enzymes to trypsin digestion in intact and detergent-permeabilized microsomes was tested. Vitamin K dependent carboxylation of the peptide substrate was not significantly affected by either detergent or trypsin alone but was strongly inhibited by the simultaneous addition of both (Figure 5). The same pattern was observed for carboxylation of endogenous protein with the exception that a small but reproducible inhibition of protein carboxylation occurred with

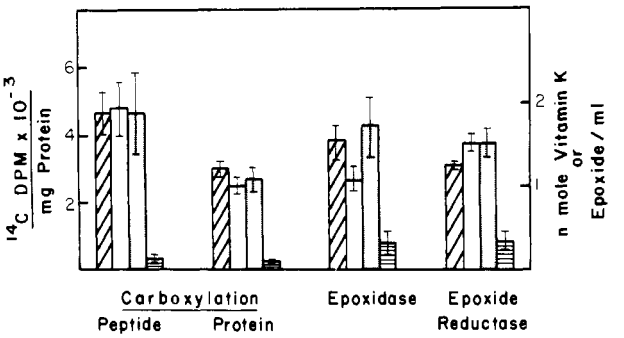

FIGURE 5: Effect of trypsin treatment, with or without Triton X-100, on carboxylation of peptide substrate or endogenous protein, vitamin K epoxidation, and epoxide reduction in microsomes from vitamin K deficient rats. Microsomes were prepared, treated, and assayed as described under Experimental Procedure. From left to right, the bars indicate the following treatment: control (diagonal hatching); trypsin (open); Triton X-100 (shaded); trypsin and Triton X-100 (horizontal hatching). About 25% (2.0 of 8.5 mg/mL) of the protein of intact, salt-washed microsomes was released by the trypsin digestion. For each enzyme, means ± standard errors for three to five experiments are represented.

trypsin alone. Digestion of intact microsomes with a sevenfold higher concentration of trypsin followed by pelleting and resuspension in fresh buffer still did not inhibit peptide carboxylase activity, although most of the cytochrome b_5, an outside-facing enzyme, was removed by this procedure (data not shown).

Vitamin K epoxidase activity responded somewhat differently to trypsin than did the carboxylase (Figure 5). Trypsin alone consistently inhibited epoxidase by ~30%; detergent alone had no significant effect, but the combination inhibited ~85% of epoxidase activity. Harsher trypsin digestion, followed by separation of released proteins from the microsomes, did not increase the inhibition over that seen with the milder treatment. This suggests that the partial inhibition of epoxidase seen in the presence of the normal trypsin concentration was not due to incomplete proteolysis of some component of the system. Epoxide reductase activity was consistently stimulated nearly 20% by trypsin alone (Figure 5). The presence of detergent alone during the 15-h incubation either stimulated (three experiments) or had little effect (two experiments), and the combined trypsin and detergent treatment markedly inhibited the activity.

The interpretation of the latency and trypsin inhibition data in terms of permeability of microsomes rests on the assumption that the membrane is intact until detergent was added. This assumption was tested by using the method of Arion et al. (1976). The low K_m mannose-6-phosphatase of microsomes is known to face the lumen, and mannose-6-phosphatase activity in microsomes treated as in the trypsin digestion experiments was 2.0 nmol min^{-1} (mg of protein)$^{-1}$ in the presence of 0.2% Triton X-100. Control microsomes (soybean trypsin inhibitor only) expressed only 5% of the phosphatase activity of detergent-treated vesicles, and trypsin treatment increased this to ~15%. These data verify that most of the microsomes did remain impermeable to small molecules under the conditions of the experiment.

Discussion

These data indicate that vitamin K dependent carboxylation, vitamin K epoxidation, and reduction of vitamin K epoxide are located primarily in the rough endoplasmic reticulum of hepatocytes. Carboxylation of endogenous microsomal substrate of the enzyme is enriched at least 2.5-fold in protein of rough relative to smooth microsomes of vitamin K deficient rats. The apparent enrichment becomes about threefold after correction for the presence of 25–30% smooth vesicles in the rough membrane fraction (Gram, 1974). On the basis of the estimates (Steck, 1972) that rough endoplasmic reticulum contains ~12% and smooth endoplasmic reticulum ~8% of liver cell protein, 85% of the total carboxylation of protein would reside in the rough endoplasmic reticulum. The small amount of carboxylation found in the nuclear fraction can be explained by endoplasmic reticulum contamination, and no significant carboxylation was detected in other fractions.

Helgeland (1977) and Wallin & Prydz (1979) have also reported a study of the carboxylation of endogenous protein by microsomal subfractions. The results expressed on the basis of protein carboxylation recovered per gram of liver or liver protein showed that most of the recovered carboxylation occurred in rough microsomes. The availability of a synthetic peptide substrate for the vitamin K dependent carboxylase (Suttie et al., 1976, 1979) allowed an assay of this activity independent of the endogenous substrate. The results obtained using this assay strongly support the localization of the carboxylase enzyme in rough endoplasmic reticulum. On the basis of early linear rates of carboxylation, rough membranes had about 3 to 4 times more activity than smooth microsomes. When corrected for the estimated contamination of rough with smooth vesicles, the ratio of specific activities in the two fractions was ~4–6, corresponding to the presence of ~90% of the total carboxylase activity in the rough endoplasmic reticulum. These data indicate that the carboxylation of prothrombin occurs at a relatively early stage in the transport of this protein through the cell. Disulfide bond formation, the cleavage of a presumed "signal peptide", and addition of core oligosaccharide are also thought to be early events in the synthesis of secretory glycoprotein, but the addition of sialic acid and galactose moieties very probably follows carboxylation.

Epoxidation of vitamin K correlates well with the carboxylation reaction under a wide variety of conditions, and the evidence to support an involvement of epoxidation in the carboxylation event has been reviewed (Suttie et al., 1978). The current experiments demonstrate that the epoxidation of vitamin K supplied as hydroquinone occurs primarily in membranes derived from rough rather than smooth endoplasmic reticulum. The similar distribution of this activity and the carboxylase (Figure 3) is consistent with the hypothesis that they may be closely related events. The enrichment of epoxidase in rough microsomes contrasts with the distribution of a number of mixed-function oxygenase activities catalyzed by cytochrome P-450 and related cytochromes which are enriched in smooth microsomes (Gram, 1974). Two other oxidases involved in posttranslational modification, prolyl and lysyl hydroxylase, are also located in the rough endoplasmic reticulum (Kao & Lee, 1978). Although absent in cytosol and mitochondria, the specific activity of the epoxidase in the nuclear fraction is sufficiently greater than that of the microsomal marker enzyme to suggest that this enzyme might be endogenous to the nucleus (Figures 1 and 3). However, the specific activity of the microsomal epoxidase did increase during dilution, and this might lead to an overestimation of the small amount of microsomal material contaminating the nuclear fraction. A comparison of the general subcellular distribution of epoxide formed in vivo (Nyquist et al., 1971) with the restricted distribution of the epoxidase reported here suggests that equilibration of epoxide among various organelles must occur in vivo. The warfarin-sensitive, vitamin K epoxide reductase activity was found to be less markedly enriched in

the rough (as opposed to smooth) microsomes than the other three activities. No significant reductase activity was detected in mitochondria, cytosol, or nuclei. The location of this activity is consistent with the hypothesis that the epoxidation–reduction cycle is in some way involved in maintaining the carboxylation reaction.

The most direct and reliable technique to determine the membrane orientation of endoplasmic reticulum enzymes is visualization of an immunospecific label by electron microscopy (Olson & Prockop, 1974). Specific antibodies to these enzymes are not available, and indirect techniques were used to study the orientation of the carboxylase and vitamin K metabolizing enzymes. Trypsin digestion inhibited the carboxylation of endogenous protein in detergent-disrupted but not intact microsomes, suggesting that most of the carboxylase is exposed at the cisternal face of the vesicles. Helgeland (1977) has reported a 40% inhibition of carboxylation of endogenous protein by either trypsin or deoxycholate alone, contrasted with 94% inhibition by the two together. This inhibition was interpreted as "a removal from the cytoplasmic side of vitamin K dependent carboxylase activity not connected with the formation of prothrombin". The assay employed used vitamin K and DTT or NADH, and, although Helgeland's explanation is not excluded, trypsin could have been inhibiting the microsomal vitamin K reductase activity. Vitamin K hydroquinone was used in the present study to avoid this uncertainty. With this reservation, Helgeland's data are essentially in agreement with the data on carboxylation of endogenous substrate reported here. Trypsin digestion drastically inhibited carboxylation of a peptide substrate in microsomes permeabilized by detergent but had no effect in sealed microsomes, again supporting the hypothesis that the carboxylase faces the lumen of the endoplasmic reticulum. This charged peptide should be excluded by the microsomal membrane (Nilsson et al., 1973), and the observation that detergent addition stimulated peptide carboxylation 10–20-fold (Figure 2) again strongly suggests that the active site is accessible only from the microsomal lumen. The demonstrated latency of mannose-6-phosphatase in these preparations is a stringent criterion for intact microsomal membranes, and the similar detergent stimulation of mannose-6-phosphatase and the peptide carboxylase is consistent with the view that disruption of the permeability barrier between substrate and enzyme was the primary effect of detergent treatment.

Vitamin K epoxidase also appears to be associated with microsomes as an intrinsic enzyme. Trypsin caused a reproducible and apparently limited inhibition of the epoxidase in intact microsomes, but detergent allowed much more extensive inhibition, consistent with a contribution of proteins on both sides of the membrane to epoxidase activity. The vitamin K epoxide reductase in intact vesicles was stimulated by trypsin treatment, demonstrating that reductase activity could be affected from the cytoplasmic side. However, trypsin digestion inhibited strongly when detergent was included, suggesting that a second effect of trypsin on reductase required exposing the lumen of the microsomes. These observations are consistent with an interaction of some component of the epoxide reductase at both sides of the membrane.

The evidence discussed above suggests a model in which the carboxylase active site is exposed on the cisternal side of the endoplasmic reticulum. The majority of both vitamin K epoxidase and the vitamin K epoxide reductase also appears to be on the cisternal side, but the data are consistent with some exposure of each on the cytoplasmic side as well. Exposure of the epoxide reductase to the cytoplasm is also supported by the recent observations (Siegfried, 1978) that it can be stimulated by a cytosolic protein. The evidence does not exclude exposure of a portion of the carboxylase at the cytoplasmic surface also, but any protein exposed there must be either resistant to trypsin or dispensable for catalysis. Whether these enzyme activities correspond in each case to one or to more than one enzyme catalyzing the same reaction also remains an open question. Kreibich et al. (1974) have found that most microsomal proteins studied were exposed to the outside and that no major proteins which were exposed only at the inner membrane face or completely buried could be detected. The dozen enzymes whose transverse orientation has been reviewed by DePierre & Ernster (1977) also appear to be assymetrically localized. The prothrombin precursors (Esmon et al., 1975a; Grant & Suttie, 1976) appear to be cisternal proteins. The more acidic (p*I* 5.8) precursor appears to be largely free in the lumen and the p*I* 7.2 precursor mostly associated with the membrane. The orientation of the carboxylase is probably related to its function in the posttranslational modification of these or related proteins secreted through the lumen of the endoplasmic reticulum to the plasma. The similar orientation of the vitamin K epoxidase is consistent with the view that it may be closely involved with the carboxylation reaction.

Vitamin K and its metabolites are sufficiently hydrophobic that their concentration by membranous organelles in vivo is not surprising. Their orientation in the membrane and the rates at which they may diffuse across it are less well understood. Of the other cosubstrates for the carboxylase, both oxygen and CO_2 would be expected to diffuse relatively rapidly through the membrane. Dithiothreitol, used as a reductant in in vitro studies, probably penetrates microsomes freely (Nilsson et al., 1973). NAD(P)H and vitamin K quinone drive carboxylation in intact microsomes in vitro. If, as seems likely, NAD(P)H does not penetrate the membrane, it must bind either to part of the carboxylase enzyme system or to a vitamin K quinone reductase at the outside of the microsome. A direct involvement of vitamin K hydroquinone in carboxylation would imply that it acts at the cisternal side. Electrons from NAD(P)H must then cross the membrane, either as vitamin K hydroquinone itself or via electron transport proteins which reduce the quinone at the cisternal side. It is also possible that vitamin K hydroquinone drives the carboxylation only indirectly by passing its reducing equivalents on to the carboxylase. This raises the possibility that a transmembrane electron transport chain might intervene between the hydroquinone at the cytosolic face and the carboxylase at the cisternal face. The present results require that, if present, such a chain must survive trypsin digestion of intact microsomes.

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Characterization of the Ribulosebisphosphate Carboxylase–Carbon Dioxide–Divalent Cation–Carboxypentitol Bisphosphate Complex†

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ABSTRACT: Ribulosebisphosphate carboxylase forms a stable quaternary complex with CO_2, divalent cation, and carboxypentitol bisphosphate. Incorporation of nonexchangeable CO_2 into the complex requires the presence of a divalent cation. Mg^{2+}, Mn^{2+}, or Co^{2+} supports stoichiometric binding of CO_2 activator. When the quaternary complex is formed in the presence of saturating CO_2, stoichiometric amounts of cation are bound in a nonexchangeable fashion. Incorporation of Mn^{2+} into an enzyme–CO_2–Mn^{2+}–carboxypentitol bisphosphate complex permitted investigation of cation environment by electron spin resonance (ESR) techniques. Measurements at 9 and 35 GHz suggest rhombic distortion of the coordination sphere of bound Mn^{2+}. A complex inner sphere liganding of the cation bound in the quaternary complex would account for both the ESR spectra and the marked stability of the complex with respect to cation exchange.

The primary reaction in photosynthetic carbon fixation is catalyzed by ribulosebisphosphate carboxylase, an enzyme which requires a divalent metal cation (M^{2+}) for M^{2+} (Weissbach et al., 1956; Siegel et al., 1972). Kinetic (Lorimer et al., 1976) and physical (Miziorko & Mildvan, 1974) evidence suggests that there is an ordered addition of CO_2 and M^{2+} to the purified enzyme. Upon formation of this ternary complex, the enzyme becomes catalytically competent and the dissociation constant of the cation approaches the activator constant determined kinetically. Observation of an activation process prompted speculation that the enzyme binds an activator CO_2 molecule that is distinct from the CO_2 which is ultimately fixed in the carboxylation reaction. Kinetic turnover experiments (Lorimer, 1979) and the demonstration of simultaneous stoichiometric binding of CO_2 and the transition-state analogue carboxypentitol bisphosphate (CPBP)[1] to

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