No Strict Coupling of Vitamin K₁ (2-Methyl-3-phytyl-1,4-naphthoquinone)-Dependent Carboxylation and Vitamin K₁ Epoxidation in Detergent-Solubilized Microsomal Fractions from Rat Liver

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NAD(P)H dehydrogenase ('DT-diaphorase', EC 1.6.99.2) and vitamin K epoxidase were removed by affinity chromatography from detergent-solubilized microsomal fractions. Thereby the microsomal fractions normally carrying out vitamin K₁-dependent carboxylation of the microsomal precursor proteins of the prothrombin complex were inactivated. Purified NAD(P)H dehydrogenase added to this system restored carboxylation in the presence of vitamin K₁ (2-methyl-3-phytyl-1,4-naphthoquinone) plus NADH. Vitamin K₁ hydroquinone (2-methyl-3-phytyl-1,4-naphthoquinol) had no effect, in contrast with its effect in the intact system, where it can substitute for vitamin K₁ plus NADH. The ability of NAD(P)H dehydrogenase to restore carboxylation in a system without vitamin K epoxidase activity shows that there is no obligatory coupling of the vitamin K₁-dependent carboxylation with vitamin K₁ epoxidation. These results suggest that the form of vitamin K₁ that is active in the carboxylation reaction can be produced independently in two reactions: by NAD(P)H dehydrogenase in the reduction of the quinone and by vitamin K epoxidase in the epoxidation of the hydroquinone.

Vitamin K₁ (2-methyl-3-phytyl-1,4-naphthoquinone) is involved in a postribosomal modification of liver microsomal protein precursors to form the active clotting factors that make up the prothrombin complex (Factors II, VII, IX and X) (Suttie & Jackson, 1977; Prydz, 1977). The modification involves carboxylation of specific glutamic acid residues, which are transformed into γ -carboxyglutamic acid residues (Stenflo *et al.*, 1974; Nelses-tuen *et al.*, 1974; Magnusson *et al.*, 1974; Stenflo & Suttie, 1977) responsible for the binding of Ca²⁺ which is necessary for optimal activation of the coagulation system (Suttie & Jackson, 1977).

The isolation of subcellular systems carrying out the carboxylation reaction showed that the enzyme system involved was located in the liver microsomal fractions (Shah & Suttie, 1974; Mack *et al.*, 1976; Vermeer *et al.*, 1976). The system has been quite well characterized (Sadowski *et al.*, 1976; Esmon & Suttie, 1976; Esmon *et al.*, 1975b; Friedman & Shia, 1976). The enzyme(s) involved in carboxylation is bound to the rough-endoplasmic-reticulum membranes (Helgeland, 1977; Wallin & Prydz, 1978), and has not yet been purified.

At present it is generally believed that the reduced form of vitamin K₁ (vitamin H₁ hydroquinone) is the active component which mediates carboxylation (Sadowski *et al.*, 1976; Friedman & Shia, 1976),

since the hydroquinone can replace the enzymic reduction of vitamin K₁ quinone, a reaction carried out either by NAD(P)H dehydrogenase (EC 1.6.99.2) in the presence of NAD(P)H (Wallin *et al.*, 1978) or possibly by vitamin K reductase in the presence of dithiothreitol (Whitlon *et al.*, 1978). The discovery by Sadowski *et al.* (1977) that the hydroquinone and not the quinone is the substrate for vitamin K epoxidase also supports this view. The formation of vitamin K₁ 2,3-epoxide in microsomal preparations by vitamin K epoxidase (Matschiner *et al.*, 1970; Willingham & Matschiner, 1974) has been suggested to be directly coupled to the vitamin K-dependent carboxylation reaction, since the requirements of these reactions are very similar (Willingham & Matschiner, 1974), as are the effects of the inhibitors tetrachloro-4-pyridinol and 2-chloro-3-phytyl-1,4-naphthoquinone (Willingham *et al.*, 1976).

Wallin *et al.* (1978) found that NAD(P)H dehydrogenase was retained when detergent-solubilized microsomal fractions were passed through a column of Sepharose 4B to which vitamin K₃ (menadione) had been chemically coupled. In the present paper I report that vitamin K epoxidase is also retained. This has enabled an investigation of the relationship between epoxidation and vitamin K₁-dependent carboxylation to be made. The results reported in this paper suggest that the epoxidation of

the vitamin is not an absolute requirement of the vitamin K-dependent carboxylation of microsomal precursor protein.

Materials and Methods

Animals

Male Wistar-Möll rats (200–300 g body wt.) were from Möllgaard, Ejby, Denmark. Vitamin K-deficiency was obtained by feeding rats on a vitamin K-deficient diet (General Biochemicals, Chagrin Falls, OH, U.S.A.) for 8–9 days while they were kept in coprophagy-preventing cages. Their deficiency state was monitored with prothrombin determinations as described by Gladhaug & Prydz (1970).

Preparation of solubilized microsomal fractions

Livers were washed in ice-cold 25 mM-imidazole buffer (pH 7.2) containing 250 mM-sucrose and 2 mM-benzamidine (buffer A) and homogenized in 2 vol. of the same buffer in an ice-cooled Potter-Elvehjem homogenizer. The subsequent steps were carried out at 4°C. The homogenate was centrifuged at 10 000g for 10 min and the postmitochondrial supernatant obtained was recentrifuged at 100 000g for 60 min in a Beckman L2-65B ultracentrifuge. The microsomal pellet was rinsed with 2 × 1 ml of buffer A and resuspended by a loose-fitting Dounce homogenizer in buffer A containing 80 mM-KCl and 2% Triton X-100 to a final volume equal to that of the post-mitochondrial supernatant. Undissolved material was removed by centrifugation at 100 000g for 60 min. The supernatant was used in the experiments and referred to as solubilized microsomal fractions.

Chromatography of solubilized microsomal fractions

Sepharese-menadione was synthesized as previously described (Wallin *et al.*, 1978). Two columns of the same bed volume (14 ml) were packed with either Sepharese-menadione or Sepharese 4B (Pharmacia, Uppsala, Sweden) respectively and equilibrated in 50 mM-Tris/HCl (pH 7.4) containing 0.2% Triton X-100. The same volume of solubilized microsomal fractions was applied to both columns and subsequently eluted with the equilibration buffer. The eluate was assayed for vitamin K-dependent carboxylation, vitamin K epoxidase activity, warfarin-sensitive NAD(P)H dehydrogenase and the content of prothrombin precursor. These were related to protein concentration as measured by the absorbance at 280 nm.

Vitamin K₁ and inhibitors

The unlabelled vitamin K₁ quinone added to the incubation mixtures was a commercial solution (Aquamephyton) obtained from Merck, Sharp and Dohme, Hoddesdon, Herts., U.K. The hydroquinone

form of the vitamin (vitamin K₁H₂) and the epoxide (vitamin K₁ 2,3-epoxide) were prepared from vitamin K₁ (Sigma Chemical Co., St. Louis, MO, U.S.A.) by reduction and oxidation respectively as described by Wallin *et al.* (1978) and Fieser *et al.* (1941). Vitamin K₁H₂ was stored under nitrogen at -70°C and dissolved in ethanol (1 mg/ml) immediately before use. 5,6,7,8-³H-labelled vitamin K₁ (specific radioactivity 22.5 μCi/mg) was kindly given by Dr. J. W. Suttie, Department of Biochemistry, University of Wisconsin, Madison, WI, U.S.A. The hydroquinone form of the labelled form was prepared as described for the unlabelled form. Water-soluble vitamin K₃ (menadione sodium bisulphite) was from Nyegaard, Oslo, Norway. 2,3,5,6-Tetrachloro-4-pyridinol monohydrate was kindly provided by Dr. F. Marshall, Dow Chemicals, Midland, MI, U.S.A.

Assay systems

Vitamin K-dependent incorporation of H¹⁴CO₃⁻ into microsomal protein was measured as previously described (Wallin *et al.*, 1978). NAD(P)H dehydrogenase was purified from rat liver cytosol as previously described (Wallin *et al.*, 1978). Determination of vitamin K epoxidase activity was based on procedures described by Friedman & Smith (1977) and Sadowski *et al.* (1977) with some modifications. The test sample (1.6 ml) was diluted in buffer A to a final volume of 2.0 ml. Other additions specified in the text were dissolved in buffer A. Then 2.2 nmol of ³H-labelled vitamin K₁ hydroquinone (dissolved in ethanol, 0.2 mg/ml) was added and reactions were carried out in open tubes at 37°C for 20 min with constant shaking. Reactions were stopped by the addition of 5 ml of propan-2-ol/hexane (3:2, v/v). The mixture was shaken vigorously and centrifuged at 2000 rev./min for 5 min. The upper hexane phase was collected, dried under a stream of N₂ and reconstituted in 100 μl of hexane containing unlabelled vitamin K₁ and vitamin K₁ 2,3-epoxide (1 mg/ml each). Portions (10 μl) of each extract were applied to plastic sheets (5 cm × 20 cm) coated with silica gel G containing fluorescence indicator (Polygram Sil G/UV₂₅₄; Macherey Nagel und Co., Düren, Germany) and previously impregnated with 10% (v/v) paraffin in diethyl ether. Analysis by partition t.l.c. was carried out as described by Matschiner *et al.* (1970) in acetone/water (23:2, v/v) as the mobile phase. In this system the quinone and the hydroquinone moved with the same R_F value. The spots of epoxide and vitamin were detected under u.v. light, cut out and placed in separate counting vials, mixed with 10 ml of scintillation fluid {toluene containing 5 g of PPO (2,5-diphenyloxazole) and 0.05 g of dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene]/litre; Koch-Light, Colnbrook, Bucks., U.K.} and the radioactivity was determined in a Packard Tri-Carb liquid-scintillation counter.

The biologically inactive prothrombin precursor that builds up in the liver microsomal fractions of vitamin K-deficient rats (Willingham & Matschiner, 1974; Esmon *et al.*, 1975a) was assayed as thrombin activity liberated after incubation with *Echis carinatus* venom (Sigma) as described by Nelsestuen & Suttie (1972).

Protein determinations

Protein in incubation mixtures was measured by the method of Schaffner & Weissmann (1973), with bovine serum albumin (Sigma) as standard.

Results and Discussion

Chromatography of solubilized microsomal fractions on Sepharose 4B and Sepharose-menadione is shown in Fig. 1. Protein eluted from the Sepharose 4B column (Fig. 1b) carried out carboxylation in response to the addition of vitamin K₁ hydroquinone or vitamin K₁ quinone plus NADH respectively, as did the intact solubilized microsomal system (Table 1). After passage through the Sepharose-menadione column (Fig. 1a) no vitamin K-dependent carboxyl-

ation was observed unless NAD(P)H dehydrogenase was added to the eluted fractions, in accordance with previous results (Wallin *et al.*, 1978). However, the important new observation was the inability of vitamin K₁ hydroquinone to mediate carboxylation in solubilized microsomal fractions subjected to chromatography on Sepharose-menadione (Fig. 1a).

The warfarin-sensitive microsomal NAD(P)H dehydrogenase was bound to Sepharose-menadione and eluted in buffer containing dioxan (Fig. 1a) (Wallin *et al.*, 1978). In eluates from Sepharose 4B this enzyme was co-eluted with the other activities (Fig. 1b). The contents of prothrombin precursor in fractions containing unretarded protein from the two columns were similar (Figs. 1a and 1b), suggesting that vitamin K₁ under the conditions used for chromatography is not bound to the precursor protein while performing its action.

To carry out a more detailed study of the effect of such chromatography on vitamin K-dependent carboxylation, fractions from the two columns were pooled as shown by the bar in Fig. 1 and samples tested for H¹⁴CO₃⁻ fixation in response to the addition of vitamin K₁ hydroquinone, vitamin K₁

Fig. 1. Chromatography of solubilized microsomal fractions on Sepharose-menadione (a) and Sepharose 4B (b). A 10ml portion of solubilized microsomal fractions derived from vitamin K-deficient rat liver was applied to each column (1.7cm × 6cm) equilibrated in 50mM-Tris/HCl (pH7.4) containing 0.2% Triton X-100 and subsequently eluted in the equilibration buffer. NAD(P)H dehydrogenase bound to Sepharose-menadione was eluted in the equilibration buffer containing 50% (v/v) dioxan as indicated by the arrow (a). ●, A_{280} ; ▲, thrombin activity after *Echis carinatus* venom activation; ■, vitamin K-dependent carboxylation (¹⁴C incorporation) of protein in unretarded fractions in response to vitamin K₁ quinone plus NADH. Purified NAD(P)H dehydrogenase (1.5 μkat) was added to each fraction eluted from Sepharose-menadione (a) before incubation. □, Vitamin K-dependent carboxylation (¹⁴C incorporation) in response to vitamin K₁ hydroquinone; ○, warfarin-sensitive NAD(P)H dehydrogenase activity.

Table 1. *Vitamin K-dependent carboxylation in solubilized microsomal fractions exposed to chromatography*

Solubilized microsomal fractions from vitamin K-deficient rats were prepared as described in the Materials and Methods section and chromatographed on a column of either Sepharose-menadione or Sepharose 4B. Both columns had been equilibrated in 0.05 M-Tris/HCl (pH7.4) containing 0.2% Triton X-100. Fractions of unretarded protein were pooled (25 ml) as shown by the bars in Fig. 1 and samples tested for vitamin K-dependent carboxylation. Incubation mixtures (1 ml) contained 0.5 ml of pooled eluate or solubilized microsomal fractions, $\text{NaH}^{14}\text{CO}_3$ (5 $\mu\text{Ci/ml}$) and the additions indicated. All values are the averages of duplicate incubations differing by less than 10%. For each incubation the incorporation in the absence of vitamin K_1 or vitamin K_1 hydroquinone (background) has been subtracted. Backgrounds were: pooled eluate 350–370 c.p.m.; solubilized microsomal fractions, 650 c.p.m.

Incubations	Protein (mg/ml)	Additions				Vitamin K-dependent ¹⁴ C incorporation (c.p.m.)
		Vitamin K ₁ (μg)	Vitamin K ₁ H ₂ (μg)	NADH (mM)	NAD(P)H dehydrogenase (μkat)	
Triton X-100 solubilized micro- somal fractions	8.4	50		2.5		1908
	8.4		10			1941
Pooled eluate from Sepharose-menadione	4.4	50		2.5	1.5	480
	4.4	50		2.5	0.5	334
	4.4	50		2.5		20
	4.4		10		0.5	11
	4.4		10			18
Pooled eluate from Sepharose 4B	4.6	50		2.5		957
	4.6		10			1006

plus NADH and purified NAD(P)H dehydrogenase. As expected, pooled eluate from Sepharose-menadione exhibited no significant incorporation of $H^{14}CO_3^-$ in response to vitamin K₁ plus NADH unless purified NAD(P)H dehydrogenase was added (Table 1). The values 20, 11 and 18 were not significantly above the background.

An increase in incorporation was observed when more NAD(P)H dehydrogenase was added, supporting the hypothesis that this enzyme functions as the quinone reductase responsible for driving the vitamin K-dependent carboxylation reaction by NADH in a detergent-solubilized system (Wallin *et al.*, 1978). Thus in the model of the vitamin K cycle proposed by Whitton *et al.* (1978) this enzyme fits in very well as the second vitamin K₁ quinone reductase [NAD(P)H supported] which is active in intact microsomal fractions.

In agreement with results obtained by Friedman & Shia (1976) and Sadowski *et al.* (1976) vitamin K₁ hydroquinone was found to substitute for vitamin K₁ plus NADH in the intact system (Table 1). However, in pooled eluates from the Sepharose-menadione column, vitamin K₁ hydroquinone had no effect even in the presence of purified NAD(P)H dehydrogenase (Table 1). On the other hand, pooled eluates from Sepharose 4B exhibited vitamin K-dependent carboxylation in response to vitamin K₁ hydroquinone or vitamin K₁ plus NADH, and the incorporation of radioactivity per mg of protein was increased (2–3-fold) compared with the incorporation obtained in

pooled eluates from Sepharose-menadione in the presence of added purified NAD(P)H dehydrogenase vitamin K₁ and NADH (Table 1).

A possible explanation for the lack of effect of vitamin K₁ hydroquinone on carboxylation in the eluate from Sepharose-menadione might be the removal of one or more factors from the solubilized, chromatographed microsomal fractions in addition to the removal of NAD(P)H dehydrogenase. Since vitamin K₁ hydroquinone is the substrate for vitamin K epoxidase (Sadowski *et al.*, 1977), I looked for epoxidase activity in solubilized microsomal fractions and in eluates from chromatography of such microsomal fractions on columns of Sepharose 4B and Sepharose-menadione.

Vitamin K-deficient rats had a 3–4-fold higher epoxidase activity than normal rats, and the activity was inhibited by 2,3,5,6-tetrachloro-4-pyridinol, as reported by Willingham *et al.* (1976) (Table 2). Menadione sodium bisulphite did not inhibit the activity.

Eluates from Sepharose 4B showed good epoxidase activity (Table 2), whereas those from Sepharose-menadione did not contain measurable amounts of epoxidase activity (Table 2). Thus the epoxidase activity must have been bound by the affinity column and consequently removed from the eluate. Menadione sodium bisulphite (10mM) eluted warfarin-sensitive microsomal NAD(P)H dehydrogenase from the column, but the eluate contained no epoxidase activity.

Wallin *et al.* (1978) reported that vitamin K₁

Table 2. *Vitamin K epoxidase activity in solubilized microsomal fractions*

Solubilized microsomal fractions were prepared as described in the Materials and Methods section. The Triton X-100 extract from vitamin K-deficient rats was passed through a column of Sepharose-menadione or Sepharose 4B, equilibrated in 0.05M-Tris/HCl (pH 7.4) containing 0.2% Triton X-100. Fractions of unretarded protein were pooled as indicated by the bar in Fig. 1 and tested for vitamin K epoxidase activity. Incubations contained 1.6ml of pooled eluate, 0.4ml of 25mM-imidazole buffer (pH 7.2) containing 250mM-sucrose and 2mM-benzamidine, and 2.2nmol of 3H -labelled vitamin K₁ hydroquinone. Other additions are indicated. The results are the average of duplicate incubations differing by less than 10%.

Incubations	Protein (mg/ml)	Additions		Epoxide formed (pmol/mg of protein)
		Menadione sodium bisulphite (mM)	2,3,5,6- Tetrachloro- 4-pyridinol (μ g/ml)	
Normal rat liver	13.5	5		7.3
	13.5	5		7.4
	13.5		50	0.5
Vitamin K-deficient rat liver	13.5			28.6
Pooled eluate from Sepharose-menadione	7.1			0
Pooled eluate from Sepharose 4B	7.4			17.3

Fig. 2. Model for the action of vitamin K₁ in solubilized microsomal fractions

Abbreviations: vit K₁, vitamin K₁ quinone; vit K₁H₂, vitamin K₁ hydroquinone; vit K₁O, vitamin K₁ 2,3-epoxide; vit K₁•, vitamin K₁ semiquinone.

hydroquinone partially restored the carboxylase activity of the unretarded eluate of solubilized microsomal fractions obtained from a column of Sepharose-menadione. Reinvestigation showed that trace amounts of epoxidase activity were present in the eluate and probably responsible for the effect. To avoid even this minute release of epoxidase, the chromatography conditions were changed by substituting imidazole buffer (pH 7.2) for Tris buffer (pH 7.4) and omitting sucrose and KCl.

The results presented in this paper do not support the hypothesis of an obligatory coupling of the reactions of vitamin K₁ epoxidation and carboxylation (Willingham & Matschiner, 1974). In a comparative study of these two reactions Sadowski *et al.* (1977) also failed to demonstrate a complete parallelism, although the requirements for the two reactions were similar under a variety of experimental conditions.

In conclusion, I have shown that the enzymes involved in the reduction and epoxidation of vitamin K₁ can be specifically removed from a solubilized system carrying out vitamin K-dependent carboxylation. Thus the relationship between the carboxylation reaction and these two activities can easily be studied. The ability of purified NAD(P)H dehydrogenase (Wallin *et al.*, 1978) to restore carboxylation in such a system clearly demonstrates that: (1) the microsomal system loses no components essential for carboxylation other than NAD(P)H dehydrogenase and vitamin K epoxidase, and (2) no strict coupling of the reactions of epoxidation and carboxylation takes place. However, the increased incorporation of H¹⁴CO₃⁻ in the presence of the epoxidase (Table 1) indicates some role for this activity in carboxylation. The simultaneous inhibition of the reactions of vitamin K₁ epoxidation and carboxylation by 2,3,5,6-

tetrachloro-4-pyridinol and 2-chloro-3-phytyl-1,4-naphthoquinone (Willingham *et al.*, 1976; Sadowski *et al.*, 1976) does not constitute evidence for coupling, since the inhibitors may act on each reaction separately.

Girardot *et al.* (1976) have shown that the spin-trapping agent 5,5-dimethyl-1-pyrroline *N*-oxide inhibits carboxylation in a system containing vitamin K₁ plus NADH as well as when vitamin K₁ hydroquinone is present. They suggested that vitamin K₁ exerts its action through the semiquinone radical. A possible interpretation of these observations is that a semiquinone intermediate of the vitamin is formed both in the quinone-to-hydroquinone and in the epoxidation reactions, as suggested in Fig. 2. A common intermediate produced in both reactions would explain the observations of Girardot *et al.* (1976) as well as the present data and allows the prediction that addition of purified vitamin K epoxidase and vitamin K₁ hydroquinone should restore activity to solubilized microsomal fractions inactivated by Sepharose-menadione chromatography. The semiquinone may be the direct hydrogen-abstracting agent which allows the reaction of HCO₃⁻ with the resulting γ-carbon radical of the glutamic acid residue.

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