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Vitamin K-dependent carboxylation and vitamin K epoxidation

Evidence that the warfarin-sensitive microsomal NAD(P)H dehydrogenase reduces vitamin K_1 in these reactions

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Passage of a Triton X-100-solubilized microsomal fraction ('microsomes') through a Sepharose–menadione affinity column resulted in loss of the ability to carry out vitamin K-dependent carboxylation and to convert vitamin K_1 into vitamin K_1 2,3-epoxide. The addition of purified cytosolic NAD(P)H dehydrogenase (EC 1.6.99.2) or the equivalent activity isolated from microsomes to the unretarded fraction from the affinity column restored epoxidation and carboxylation in the presence of reduced nicotinamide nucleotides. Other NAD(P)H-linked microsomal activities, cytochrome *P*-450 reductase and cytochrome b_5 reductase, were unable to reduce vitamin K_1. These data provide evidence that the only known enzyme that can reduce vitamin K_1 in the Triton X-100-solubilized microsomal systems *in vitro* that are used to study these reactions is the warfarin-sensitive NAD(P)H dehydrogenase.

Vitamin K is required for the synthesis of four plasma-clotting factors (prothrombin and Factors VII, IX and X) as well as other proteins of unknown function. The molecular role of the vitamin is as a cofactor for a microsomal enzyme that carries out the carboxylation of specific glutamate residues in precursor proteins to convert them into γ-carboxyglutamate residues (Suttie & Jackson, 1977; Olson & Suttie, 1978; Suttie, 1978). This modification introduces Ca^{2+}-binding properties in these proteins that are required for their physiological function. Various microsomal systems that carry out the vitamin K-dependent carboxylation reaction *in vitro* have been developed, and their properties have been reviewed (Suttie, 1980*a*,*b*). The enzyme requires O_2, CO_2 and reduced vitamin K. Vitamin K can be added to these systems *in vitro* as the chemically reduced hydroquinone, or the quinone form of the vitamin may be reduced by enzymes present in the microsomal preparations. In the Triton X-100-solubilized microsomal systems that are widely used to study this reaction, this enzymic reduction requires reduced nicotinamide nucleotides.

Wallin *et al.* (1978) demonstrated that when the warfarin-sensitive NAD(P)H dehydrogenase activity in Triton X-100-solubilized microsomal fractions ('microsomes') was removed by affinity chromatography, vitamin K + NADH would no longer support the carboxylation reaction. However, addition of purified cytosolic NAD(P)H dehydrogenase (EC 1.6.99.2) to the inactive system would restore carboxylation in the presence of vitamin K + NADH. These data demonstrated that this purified enzyme (also termed DT-diaphorase) could reduce vitamin K and replace the function of the removed microsomal dehydrogenase(s). Microsomes contain two other dehydrogenases that require NADPH (cytochrome *P*-450 reductase) or NADH (cytochrome b_5 reductase) for activity (Gillette *et al.*, 1972). As NADPH, as well as NADH, will support vitamin K-dependent microsomal carboxylation, it was of interest to establish if these two enzymes could contribute to the enzymic reduction of vitamin K_1. This report presents evidence that the warfarin-sensitive NAD(P)H dehydrogenase(s) in Triton X-100-solubilized microsomes is the only NAD(P)H-linked dehydrogenase activity present that can reduce vitamin K_1. Furthermore, it is shown that NAD(P)H dehydrogenase restores vitamin K_1 epoxidation as well as carboxylation in a system where this activity had been removed by affinity chromatography.

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Materials and methods

Male Holtzman-strain rats (250–300 g) were kept in coprophagy-preventing cages (Metta *et al.*, 1961) and fed a vitamin K-deficient diet (Mameesh & Johnson, 1959) for 8 days. The animals were killed by decapitation and liver microsomal pellets were prepared as described by Suttie *et al.* (1976). The pellets were solubilized in SIK-Triton buffer (250 mM-sucrose; 25 mM-imidazole; 0.5 M-KCl; 1.5% Triton X-100; pH 7.2). Undissolved material was removed by centrifugation at 100000 *g* for 60 min. Microsomes solubilized in SIK-Triton buffer were desalted on Sephadex G-25 in buffer A (25 mM-imidazole; 0.2% Triton X-100; pH 7.2) and subsequently applied to a Sepharose–menadione column (Wallin *et al.*, 1978) pre-equilibrated in buffer A. The column was washed with 2 bed vol. of buffer A. Protein retained by the affinity resin was eluted either with 25% dioxan in buffer A or by successive elution with 1 M-NaCl in buffer A followed by 25% dioxan in buffer. A. The first procedure resulted in recovery of an unretarded protein fraction (fraction A; Fig. 1*a*) followed by elution of retained protein in a single protein fraction (fraction B; Fig. 1*a*). The second procedure resulted in elution of two protein fractions in addition to fraction A (fractions C and D; Fig. 1*b*). Fractions were pooled as indicated in Fig. 1 and subsequently dialysed overnight against SIK-Triton buffer containing 1 mM-dithiothreitol.

Vitamin K-dependent carboxylase activity was measured at 17°C as $^{14}CO_2$ incorporation into the synthetic peptide substrate Phe-Leu-Glu-Glu-Leu (Vega Biochemicals, Tucson, AZ, U.S.A.) (Suttie *et al.*, 1979) or into endogenous microsomal protein (Esmon & Suttie, 1976). Incubations contained 0.4 ml of the dialysed fractions in a total volume of 0.57 ml. Vitamin K_1 (50 μg) was added in 10 μl of ethanol. Other additions are specified in the text. Vitamin K epoxidase activity was assayed at 27°C as described by Wallin & Suttie (1980). ^{3}H-labelled vitamin K_1 (2.2 nmol) was added to each incubation in 5 μl of ethanol. NAD(P)H dehydrogenase activity was measured at room temperature as described by Dallner (1964) with 2,6-dichlorophenol-indophenol as hydrogen acceptor. A molar-absorption coefficient for dichlorophenol-indophenol of 21 $mM^{-1} \cdot cm^{-1}$ at 600 nm was used to calculate enzyme activity. NADPH–cytochrome *c* reductase and NADH–ferricyanide reductase activities were measured at room temperature as described by Comai & Gaylor (1973). Absorption coefficients at 19.6 $mM^{-1} \cdot cm^{-1}$ (550 nm) and 1.02 $mM^{-1} \cdot cm^{-1}$ (420 nm) were used to calculate enzymic reduction of cytochrome *c* and ferricyanide respectively. Cytosolic NAD(P)H dehydrogenase was purified to electrophoretic homogeneity as described by Wallin (1979).

Vitamin K_1, NADH and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Vitamin K_1 was purified (Matschiner *et al.*, 1967) by silicic acid chromatography before use. ^{3}H-labelled vitamin K_1 was synthesized from tetrasodium 2-methyl-1,4-[5,6,7,8-^{3}H]naphthoquinol diphosphate (80 Ci/mmol; Amersham/Searle) to a specific radioactivity of 5.3 Ci/mmol as described by Matschiner (1970) and was a gift from Dr. C. Siegfried. $NaH^{14}CO_3$ (sp. radioactivity 60 Ci/mol) was from Amersham (Arlington Heights, IL, U.S.A.). Sodium warfarin was from WARF Institute Inc., Madison, WI, U.S.A. All other chemicals were reagent grade or better.

Results

Chromatography of Triton X-100-solubilized microsomal proteins on a Sepharose–menadione affinity column resulted in the elution profiles shown in Fig. 1. In agreement with previous results (Wallin

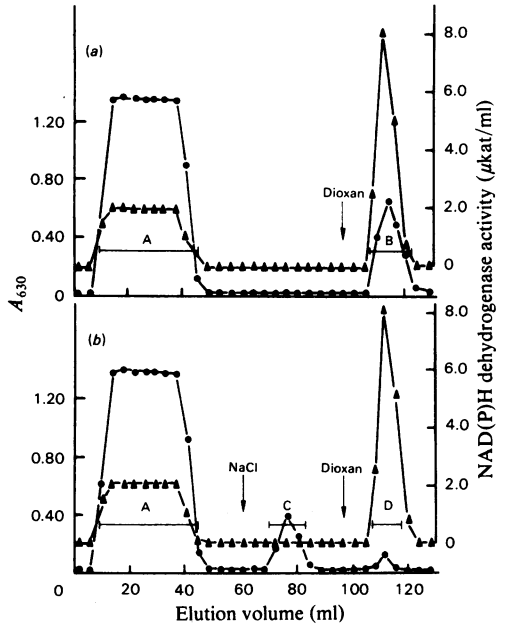

Fig. 1. *Chromatography of Triton X-100-solubilized microsomes on Sepharose–menadione*

Microsomes were solubilized in SIK-Triton buffer, desalted on Sephadex G-25 in buffer A and 20 ml was applied to a column (1.5 cm × 8 cm) of Sepharose–menadione pre-equilibrated in buffer A. The affinity column was eluted as described in the Materials and methods section. The arrows indicate the start of elution of retained protein with either 25% dioxan (*a*) or 1 M-NaCl followed by 25% dioxan (*b*) in buffer A. Protein (●) was measured by A_{630}, and NAD(P)H dehydrogenase activity (▲) was measured by 2,6-dichlorophenol-indophenol reduction of 50 μl portions from each tube. Protein fractions were pooled as indicated by the bars.

et al., 1978), an appreciable part of the NAD(P)H dehydrogenase activity was not retained by the affinity column (fraction A: Fig. 1). When the column was eluted in a single step with dioxan, the NAD(P)H dehydrogenase that was bound by the column resin co-eluted with the protein fraction (fraction B; Fig. 1*a*). However, NAD(P)H dehydrogenase retained by the column could be separated from the majority of other retained proteins by stepwise elution with NaCl to remove most of the protein (fraction C; Fig. 1*b*) followed by dioxan to remove the dehydrogenase activity (fraction D; Fig. 1*b*).

About 60% of the NAD(P)H dehydrogenase activity in solubilized microsomes was inhibited by warfarin additions (Table 1). However, warfarin had no inhibitory effect on the dehydrogenase activity that passed through the Sepharose–menadione column (fraction A: Fig. 1). Thus the affinity column removed all warfarin-sensitive NAD(P)H dehydrogenase activity in solubilized microsomes. All of the NAD(P)H dehydrogenase activity retained by the affinity resin (fraction D; Fig. 1*b*) was inhibited by 1 mg of warfarin/ml. Fraction A also exhibited good cytochrome *c* reductase and ferricyanide reductase activities; and, as shown in Table 1, these two activities were not inhibited by warfarin. The cytochrome *c* reductase and ferricyanide reductase assay systems express the activities of microsomal cytochrome *P*-450 reductase and cytochrome b_5 reductase respectively (Strobel & Dignam, 1978; Mihara & Sato, 1978). The specific activity of cytochrome *c* reductase in fraction A was not different from that in solubilized microsomes, and the specific activity of ferricyanide reductase was about 14% lower than in solubilized microsomes. Although a poor electron acceptor, ferricyanide has been shown by Ernster *et al.* (1962) to accept electrons from NAD(P)H dehydrogenase. Purified NAD(P)H dehydrogenase exhibited 10% activity with ferricyanide as an electron acceptor as it did in the standard dichlorophenol-indophenol test system, indicating that there is some overlap in these two assays. The lower activity of ferricyanide reductase in fraction A is, therefore, probably due to removal of NAD(P)H dehydrogenase by the affinity resin.

Removal of the warfarin-sensitive NAD(P)H dehydrogenase activity from solubilized microsomes also resulted in complete loss of [vitamin K + NAD(P)H]-dependent carboxylase and epoxidase activity (Table 2). Since cytochrome *P*-450

Table 1. *Dehydrogenase activities in Triton X-100-solubilized microsomes before and after chromatography on Sepharose–menadione*

NAD(P)H dehydrogenase, cytochrome *c* reductase and ferricyanide reductase activities were measured in SIK-Triton-solubilized microsomes and fraction A from Sepharose–menadione (Fig. 1) as described in the Materials and methods section. Warfarin inhibition (+Warf) was measured in the presence of 1 mg of sodium warfarin/ml. Activities are given as specific activities in the respective fractions.

	Enzyme activity (μkat/mg)					
	NAD(P)H dehydrogenase		Cytochrome *c* reductase		Ferricyanide reductase	
Fractions	Control	+Warf	Control	+Warf	Control	+Warf
SIK-Triton-solubilized microsomes	1.05	0.42	1.13	1.12	14.2	11.4
Fraction A from Sepharose–menadione	0.50	0.49	1.16	1.16	12.5	12.4

Table 2. *Nicotinamide nucleotide-dependent carboxylation and epoxidation in Triton X-100-solubilized microsomes before and after chromatography on Sepharose–menadione*

Vitamin K-dependent carboxylation of an added peptide substrate and of endogenous microsomal proteins were measured in the presence of 2.5 mM-NADH or -NADPH in incubations containing 50 μg of vitamin K_1 and expressed as d.p.m./mg of microsomal protein. Vitamin K epoxidase activity was measured as pmol of ^{3}H-labelled vitamin K_1 (2.2 nmol) converted into epoxide in the presence of 2.5 mM-NADH or NADPH. Results are the average of duplicate incubations differing by less than 2%.

	SIK-Triton-solubilized microsomes			Fraction A from Sepharose–menadione		
	Carboxylation (d.p.m./mg)			Carboxylation (d.p.m./mg)		
Additions	Of peptide	Of protein	Epoxidation (pmol/mg)	Of peptide	Of protein	Epoxidation (pmol/mg)
NADH	6562	3430	48	0	0	0
NADPH	6184	2960	43	0	0	0

Table 3. *Nicotinamide nucleotide-dependent carboxylation and epoxidation in Triton X-100-solubilized microsomes after chromatography on Sepharose–menadione*

Enzymic activities were measured in fraction A from Sepharose–menadione (Fig. 1) under the same conditions as described in the legend to Table 2. Other additions to the incubations were: pooled fraction C (Fig. 1*b*) (18 μg of protein), pooled fraction D (Fig. 1*b*) [1.25 μkat of NAD(P)H dehydrogenase activity], purified cytosolic NAD(P)H dehydrogenase (2.5 μkat). The numbers are the means of duplicate incubations differing by less than 2%.

Additions to fraction A	Carboxylation (d.p.m./mg) Peptide	Carboxylation (d.p.m./mg) Protein	Epoxidation (pmol/mg)
Fraction C + NADH	0	0	0
+ NADPH	0	0	0
Fraction D + NADH	4210	2050	25
+ NADPH	4030	1972	21
NAD(P)H dehydrogenase + NADH	7340	3863	55
+ NADPH	6895	3680	50

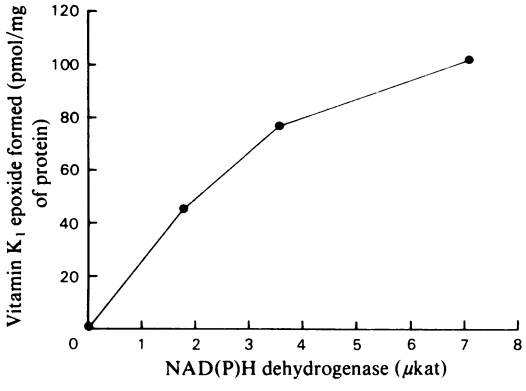

Fig. 2. *Effect of NAD(P)H dehydrogenase on vitamin K epoxidase activity*

Purified cytosolic NAD(P)H dehydrogenase was added to portions of fraction A from the Sepharose–menadione column (Fig. 1) before addition of ^{3}H-labelled vitamin K_1 (5 nmol) and NADH (2.5 mM). Epoxidation was measured, as described in the text, as pmol of vitamin K_1 converted into vitamin K_1 epoxide. Each point is the average of two independent incubations differing by less than 2%.

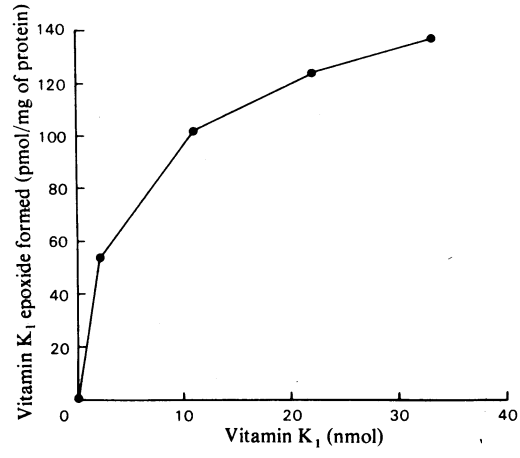

Fig. 3. *Dependence of NAD(P)H dehydrogenase-restored vitamin K_1 epoxidase activity on vitamin K_1 concentration*

Purified cytosolic NAD(P)H dehydrogenase (2.5 μkat) was added to portions of fraction A from the Sepharose–menadione column (Fig. 1) and epoxidation was determined in the presence of various concentrations of ^{3}H-labelled vitamin K_1 and 2.5 mM-NADH. Each point is the average of two incubations differing by less than 2%.

reductase and cytochrome b_5 reductase are present and active in fraction A, it is clear that these enzymes cannot reduce vitamin K_1. However, as previously reported, microsomal NAD(P)H dehydrogenase (fraction D: Fig. 1*b*) and/or purified cytosolic NAD(P)H dehydrogenase could restore carboxylation in fraction A (Table 3).

The data in Table 3 also demonstrate the ability of NAD(P)H dehydrogenase to restore vitamin K epoxidation activity to fraction A. Increasing the amount of purified NAD(P)H dehydrogenase added to portions of fraction A resulted in an increased conversion of vitamin K_1 into its epoxide (Fig. 2). At a fixed concentration of added dehydrogenase and NADH, the epoxidase activity was dependent on vitamin K concentration as shown in Fig. 3. Purified NAD(P)H dehydrogenase was shown to have no epoxidase activity in the absence of fraction A.

Discussion

Although menadione (vitamin K_3) has long been recognized as a good electron acceptor for

NAD(P)H dehydrogenase (DT-diaphorase), there has been a controversy regarding the ability of this enzyme to reduce the 3-phytyl derivative of menadione, vitamin K_1 (Weber *et al.*, 1958; Ernster *et al.*, 1962; Koli *et al.*, 1969). Martius *et al.* (1975) have, however, reported the successful reduction of vitamin K_1 by cytosolic DT-diaphorase when the vitamin was incorporated into liposomes, and their data support the view that a biological function of this enzyme is to reduce membrane-bound vitamin K_1. This NAD(P)H dehydrogenase is strongly inhibited by warfarin, which is a competitive inhibitor of the enzyme with respect to NADH (Ernster *et al.*, 1962). Although largely cytoplasmic, this enzyme is also found associated with microsomes, and Raftell & Blomberg (1980) have reported that antibodies raised against purified rat liver cytosolic NAD(P)H dehydrogenase cross-reacted with the enzyme present in microsomes. Their observation supports these data, which demonstrate that purified cytosolic enzyme can replace the microsomal enzyme in two systems *in vitro*.

Reduction of vitamin K_1 is an obligatory event before its participation in the carboxylation and epoxidation reactions. Cytosolic NAD(P)H dehydrogenase and microsomal NAD(P)H dehydrogenase restored carboxylation and epoxidation in microsomal preparations that have had the warfarin-sensitive dehydrogenases removed by affinity chromatography. The data strongly suggest that the warfarin-sensitive NAD(P)H dehydrogenase activity in microsomes is the only enzyme activity that can reduce vitamin K_1 in the systems studied *in vitro*. The data also strongly suggest that the microsomal NAD(P)H dehydrogenase activity commonly called DT-diaphorase participates in the vitamin K-dependent carboxylation reaction by providing reduced vitamin K. It is possible that the affinity column has also removed another vitamin K-specific warfarin-sensitive reductase, and that the enzymes that were added back merely substitute for this activity. At the present time, there is no way to assess the possible existence of such an activity. The presence of cytochrome *P*-450 reductase and cytochrome b_5 reductase in the inactive fraction A from the affinity column exclude these dehydrogenases as possible candidates for vitamin K_1-reducing enzymes in microsomes. The vitamin K epoxide reductase activity of liver microsomes, which may be very important in the physiological metabolism of the vitamin, is not active in these preparations (Whitlon *et al.*, 1978). If vitamin K reduction is the role of the microsomal diaphorase, no physiological function has yet been established for the cytosolic enzyme (Raftell & Blomberg, 1980). It is possible that this enzyme maintains a steady-state concentration of reduced vitamin in the cell and that cytoplasmically reduced vitamin K may diffuse across the membrane to the carboxylase and epoxidase, which appear to be located (Carlisle & Suttie, 1980) on the luminal surface.

The observation that both the carboxylation and epoxidation activities were restored to fraction A (Fig. 1) by addition of purified NAD(P)H dehydrogenase shows that no essential component that participates in either of these reactions is removed by the Sepharose–menadione column. This similarity in behaviour of these two reactions is consistent with the current view (Suttie *et al.*, 1978, 1980; Friedman & Smith, 1979; Carlisle & Suttie, 1980) that they are carried out by the same enzyme complex.

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