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NAD(P)H Dehydrogenase and its Role in the Vitamin K (2-Methyl-3-phytyl-1,4-naphthaquinone)-Dependent Carboxylation Reaction

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A simple three-step method was established for the purification of NAD(P)H dehydrogenase (quinone) ('DT-diaphorase', EC 1.6.99.2) from rat liver by affinity chromatography with a recovery of above 50%. The final enzyme preparation was purified about 750-fold and was electrophoretically homogeneous. Gel filtration showed that the enzyme had a mol.wt. of about 55000, and one molecule of FAD was found per 55000 mol.wt. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis gave a mol.wt. of about 27000. Two *N*-terminal amino acids, asparagine/aspartic acid and glutamine/glutamic acid, were found in about equal yield, suggesting the presence of two non-identical polypeptide chains in the enzyme. NAD(P)H dehydrogenase was selectively removed by this affinity-chromatographic method from a microsomal carboxylation system. The system, which was solubilized by detergent and is dependent on vitamin K (2-methyl-3-phytyl-1,4-naphthaquinone or analogues with other side chains), lost its activity on the removal of the enzyme. The activity can be completely restored to the system by adding purified cytoplasmic NAD(P)H dehydrogenase or by using the quinol form of vitamin K_1 (2-methyl-3-phytyl-1,4-naphthaquinol).

It is now established that the vitamin K (2-methyl-3-phytyl-1,4-naphthaquinone)-dependent step in the biosynthesis of the blood-coagulation Factors II, VII, IX and X is a post-translational carboxylation of a number of glutamic acid residues (Stenflo *et al.*, 1974; Magnusson *et al.*, 1974; for reviews see Suttie & Jackson, 1977; Prydz, 1977).

A subcellular system carrying out this carboxylation has been described by Suttie & Shah (1974), who used a post-mitochondrial fraction from rat liver. The system has been further developed (Esmon *et al.*, 1975; Mack *et al.*, 1976), but, although several studies have been reported (Chung *et al.*, 1975; Jones *et al.*, 1976; Bell & Stark, 1976; Vermeer *et al.*, 1976; Friedman & Shia, 1976; Girardot *et al.*, 1976; Sadowski *et al.*, 1976; Suttie *et al.*, 1976), neither the components of the system nor the molecular reaction mechanisms have been fully characterized.

NAD(P)H dehydrogenase ('DT-diaphorase', EC 1.6.99.2) has long been thought to be a key enzyme in the action of vitamin K because menadione is a good substrate and the enzyme is highly sensitive to the coumarin and indanedione vitamin K antagonists (Martius & Strufe, 1954; Märki & Martius, 1960, 1961; Ernster & Navazio, 1958; Ernster *et al.*, 1960). We report here a simple and efficient method for the purification of NAD(P)H dehydrogenase, based on affinity chromatography. By this method the enzyme has been selectively removed from a detergent-solubilized microsomal vitamin K-dependent carboxylating system. The effect on the system has been studied.

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Materials and Methods

Animals

Male Wistar–Møll rats (Møllgaard, Ejby, Denmark) (200–300 g body wt.) were fed on a vitamin K-deficient diet (General Biochemicals, Chagrin Falls, OH, U.S.A.) for 8–9 days and kept in coprophagy-preventing cages. Their deficiency state was monitored with prothrombin determinations.

Chemicals

Chemicals were obtained as follows. Sephadex G-25, G-100 and Sepharose 4B from Pharmacia, Uppsala, Sweden; *N*-ε-t-butoxycarbonyl-L-lysine (Boc-lysine), *N*-acetylhomocysteine thiolactone, menadione, β-NADH, benzamidine, avidin (14.1 units/ml) (1 unit will bind 1 μg of D-biotin) and dithiothreitol from Sigma Chemical Co., St. Louis, MO, U.S.A.; Triton X-100 from BDH, Poole, Dorset, U.K.; 2,6-dichlorophenol-indophenol sodium salt from Koch–Light, Colnbrook, Bucks., U.K.;

phylloquinone (Aquamephyton) from Merck, Sharpe and Dohme, Hoddesdon, Herts., U.K.; menadione sodium bisulphite and sodium warfarin from Nyegaard, Oslo, Norway; trifluoroacetic acid and dichloromethane from Merck, Darmstadt, Germany; and Dilusolve from Packard Instruments, Zürich, Switzerland.

2,3,5,6-Tetrachloro-4-pyridinol monohydrate was kindly provided by Dr. F. Marshall, Dow Chemicals, Midland, MI, U.S.A.

$NaH^{14}CO_3$ (60.2mCi/μmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

All other chemicals were reagent grade or better.

Preparation of Sepharose–menadione for affinity chromatography

The method of Johnston & Eisen (1974) was used with some modifications. Usually 270mg of Boc-lysine was coupled to 50g of Sepharose 4B as described by March *et al.* (1974). The product was washed with 3vol. of water, 2vol. of 100% ethanol and 2vol. of 40% trifluoroacetic acid in dichloromethane. The suspension was shaken in 40% trifluoroacetic acid in dichloromethane for 60min at room temperature (21°C) to remove the Boc group exposing the ε-amino group of lysine. After washing in 3vol. of 100% ethanol and 10vol. of water, the slurry was suspended in 100ml of cold 0.2M-Na_2CO_3, pH10.7, containing 500mg of *N*-acetylhomocysteine thiolactone. The homocysteinyl groups were attached to the free ε-amino groups by shaking the suspension for 3h at 4°C, and the thiolated particles washed with 3vol. of water and 5vol. of 0.1M-sodium acetate buffer, pH5.0. Menadione was then bound by shaking the thiolated Sepharose in the dark for 20h at room temperature in 100ml of 0.1M-sodium acetate, pH5.0, containing 60mg of menadione. The final product had a yellow colour and was protected from light. Unbound menadione was removed by repeated washings on a filter with 50% (v/v) dioxan followed by large volumes of water.

Preparation of subcellular fractions

Vitamin K-deficient rats deprived of food overnight were anaesthetized with diethyl ether. Blood was drawn from the vena cava into 0.1vol. of 2.85% sodium citrate, and the liver was removed immediately. Only livers from rats with a prothrombin activity below 15% of normal values were used. The livers were washed in ice-cold 25mM-imidazole buffer (pH7.2) containing 0.25M-sucrose and 2mM-benzamidine (buffer A) and homogenized in 2vol. of the same buffer in a Potter–Elvehjem homogenizer. The subsequent procedure was carried out at 4°C. The homogenate was centrifuged at 10000*g* for 10min in the SS-34 rotor of the Sorvall RC2-B centrifuge. The supernatant was further centrifuged at 100000*g* for 60min in a Beckman L2-65B ultracentrifuge with a Ti 65 rotor. The supernatant from this centrifugation was used as the starting material for the purification of NAD(P)H dehydrogenase.

The microsomal pellet was resuspended by a loose-fitting Dounce homogenizer in buffer A [containing 2% (w/v) Triton X-100 and 80mM-KCl] to a final volume equal to that of the 10000*g* supernatant. Undissolved material was removed by centrifugation at 100000*g* for 60min and the supernatant used as the solubilized microsomal fraction.

System for incorporation of $H^{14}CO_3^-$ into vitamin K-dependent proteins

The incubation system was based on those described by Esmon & Suttie (1976) and Girardot *et al.* (1976). The Triton X-100-solubilized microsomal pellet was mixed at 0°C with an equal volume of 80mM-KCl in 25mM-imidazole buffer (pH7.2), containing NADH and $NaH^{14}CO_3$ to give final concentrations in the incubation mixture of 2mM and 5μCi/ml respectively. Vitamin K_1 (Aquamephyton; 50μg/ml) was added and the mixture incubated for 15min at 37°C. To measure ^{14}C incorporation the reaction was stopped by adding 1ml of ice-cold 10% (w/v) trichloroacetic acid to the incubation mixture. The precipitate after 30min at 4°C was dissolved in 2% Na_2CO_3 and reprecipitated in 10% trichloroacetic acid as described by Girardot *et al.* (1976). The final precipitate from 1ml of incubation mixture was dissolved in 1ml of 2% Na_2CO_3, transferred to 10ml of Dilusolve and counted for radioactivity in a Packard Tri-Carb liquid-scintillation counter after standing protected from light at 4°C until stable count rates were reached. The ^{14}C incorporation in the absence of added vitamin K was always tested and has been subtracted from all values given.

Purification of NAD(P)H dehydrogenase

Triton X-100 was added to the 100000*g* supernatant to a final concentration of 0.2% and the mixture was dialysed against 50mM-Tris/HCl, pH7.4, containing 0.1M-NaCl and 0.2% Triton X-100. When the supernatant had been stored frozen, undissolved material was removed by centrifugation at 100000*g* for 60min. The clear supernatant (50–70ml) was applied to a column of Sepharose 4B–vitamin K_3 (bed vol. 40ml) equilibrated to the same Tris/HCl/NaCl/Triton X-100 buffer. The column was washed with 4 bed volumes of the equilibration buffer followed by 5 bed volumes of the same buffer without Triton X-100. For elution of the bound protein 50% dioxan in 50mM-Tris/HCl (pH7.4) was used. The fractions containing NAD(P)H dehydrogenase activity were pooled and dioxan was removed by gel filtration through Sephadex G-25 equilibrated and eluted with 2.5mM-potassium phosphate, pH7.0.

The void-volume fractions containing the enzyme were pooled and applied to a hydroxyapatite column (bed volume 9ml) equilibrated with 2.5mM-potassium phosphate, pH7.0. A linear gradient (2.5–200mM) of potassium phosphate, pH7.0, was used for elution. In some experiments 50mM-potassium phosphate was used instead of 2.5mM. The activity and most of the protein were eluted in a single peak at a buffer concentration of about 90mM.

Affinity chromatography of the Triton X-100-solubilized microsomal pellet

This was carried out in two different ways. Method A was used to investigate whether microsomal fractions contain a warfarin-sensitive NAD(P)H dehydrogenase. Method B was used to obtain specific removal of NAD(P)H dehydrogenase from the solubilized microsomal fractions when the non-adherent material was intended for studies of its vitamin K-dependent carboxylation capacity.

In method A a column of Sepharose 4B–vitamin K_3 gel (bed volume 20ml) was equilibrated with 50mM-Tris/HCl, pH7.4, containing 0.2% Triton X-100 and 0.1M-NaCl. The supernatant from the solubilized microsomal fraction (5–10ml) was applied to the column, followed by 5 bed volumes of the same buffer. Dioxan (50%) in 50mM-Tris/HCl, pH7.4, was used for elution.

In method B an identical column was equilibrated with 25mM-imidazole buffer, pH7.2, containing 0.25M-sucrose, 80mM-KCl and 0.1% Triton X-100. The supernatant from the solubilized microsomal fraction (5–10ml) was applied to the column, followed by 5 bed volumes of the same buffer. The unretarded material (fraction I) was collected. The bound material (fraction II) was eluted with 25% (w/v) dioxan and 1% Triton X-100 in 25mM-imidazole buffer, pH7.2.

Other methods

Vitamin K_1 dissolved in diethyl ether (10mg/ml) was reduced by shaking with $NaBH_4$ (aq. 5%, w/v, solution), the ether layer was taken off and dried under N_2 and the final residue dissolved in 10ml of ethanol.

NAD(P)H dehydrogenase was assayed as described by Dallner (1963), with 2,6-dichlorophenol-indophenol as hydrogen acceptor at room temperature. A molar extinction coefficient for dichlorophenol-indophenol of 21000 litre·mol^{-1}·cm^{-1} at 600nm was used to calculate enzyme activity.

Prothrombin was determined as described by Gladhaug & Prydz (1970). The activity in 1ml of normal citrated rat plasma was taken as 100 units.

Hydroxyapatite was prepared by the method of Bernardi (1971). *N*-Terminal analysis was carried out by the dansyl method as described by Weiner *et al.* (1972).

Barium sulphate adsorption was carried out as described by Hjort (1957) on incubation mixtures diluted with 1vol. of veronal-buffered saline (Hjort, 1957) containing 20mM-sodium oxalate.

Analytical polyacrylamide-gel electrophoresis was performed at pH9.4 as described by Davis (1964). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out by the method of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue R 250 as described by Bjørklid *et al.* (1973). Gel filtration was carried out on Sephadex G-100 equilibrated and eluted with 50mM-Tris/HCl, pH7.4, containing 0.1M-NaCl. For molecular-weight estimations, ovalbumin, bovine serum albumin (Sigma) and the heavy and light chains of immunoglobulin G (Kabi, Stockholm, Sweden) were used as standards.

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. FAD was determined as described by Faeder & Siegel (1973).

Results

Purification of NAD(P)H dehydrogenase

Supernatants from four rat livers were combined for each purification experiment. A typical elution diagram for the Sepharose–vitamin K column (Fig. 1) shows the unretarded bulk protein peak (fraction I) and the peak eluted with 50% (v/v) dioxan (fraction II). In both peaks there was NADH oxidase activity when 2,6-dichlorophenol-indophenol was the hydrogen acceptor, but the activity in fraction I was less than 2% of the fraction-II activity, and the former was not inhibited by 0.6μM-warfarin, whereas the fraction-II activity was completely inhibited.

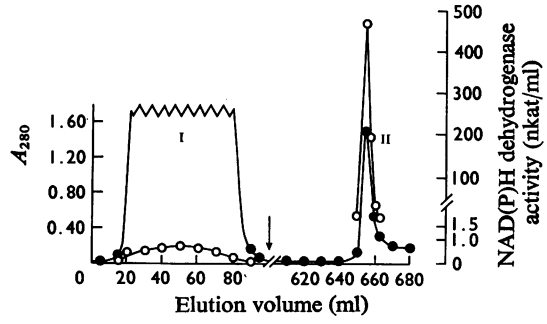

Fig. 1. *Affinity chromatography of* 100000*g supernatant from rat liver on Sepharose 4B–vitamin* K_3 *column*
The column (gel bed vol. 40ml) was equilibrated and washed with 50mM-Tris/HCl, pH7.4, containing 0.1M-NaCl and 0.2% Triton X-100. The arrow indicates the start of elution with 50% dioxan in 50mM-Tris/HCl, pH7.4. ●, A_{280}; ○, NAD(P)H dehydrogenase activity.

Table 1. *Purification of NAD(P)H dehydrogenase from rat liver 100000g supernatant*

Purification step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity at 20°C (nkat/ml)	Total activity (nkat)	Specific activity (nkat/mg)	Purification (fold)	Recovery (%)
100000*g* supernatant	65	27	1755	44.5	2892.5	1.6	1	100
Eluate from Sepharose–vitamin K and Sephadex G-25	33	0.048	1.58	53.2	1755.6	1111	694	67
Eluate from hydroxyapatite	32	0.042	1.34	51.7	1654.3	1235	772	57

NAD(P)H dehydrogenase was eluted as a sharp peak (fraction II). The most active fractions were yellow, and both NADH and NADPH were oxidized by the enzyme. Dioxan did not inactivate the enzyme and was easily removed by filtration through Sephadex G-25. Menadione bisulphite in 50ml of Tris/HCl, pH7.4, also eluted the enzyme completely. It was, however, difficult to remove menadione from the enzyme and dioxan was therefore preferred.

On analytical disc gel electrophoresis of fraction II, two bands were seen. The pooled active fractions from the affinity column were therefore submitted to hydroxyapatite chromatography, whereby the faint low-mobility protein band was removed.

The results from a typical purification are given in Table 1. Affinity chromatography with Sepharose–vitamin K gels in the presence of Triton X-100 is the essential step in the procedure. The enzyme stuck to the gel even in the presence of 2% Triton X-100. The detergent decreased non-specific hydrophobic binding and markedly improved the purification in this step.

When the Sepharose–vitamin K gel with purified enzyme bound was incubated at room temperature in the presence of NADH, the gel became colourless, showing that menadione was reduced to the quinol form on the column. The affinity column in this reduced form did not bind the enzyme under the conditions described, and it was possible to elute the enzyme with NADH.

The final enzyme preparation gave a single band on analytical polyacrylamide disc gel electrophoresis. A single band was also seen on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, corresponding to a mol.wt. of about 27000, whereas gel filtration in the absence of detergent gave a mol.wt. of about 55000. Dansylation gave two different *N*-terminal residues (asparagine/aspartic acid and glutamine/glutamic acid) in about equal yield. The enzyme contained 1 molecule of FAD per 55000 mol.wt. Warfarin (0.3mM) inhibited the reduction of 2,6-dichlorophenol-indophenol by the purified enzyme more than 90%.

Carboxylating system in vitro

The activity of the solubilized microsomal system from vitamin K-deficient rats was usually monitored by the vitamin K-dependent incorporation of ^{14}C from $NaH^{14}CO_3$ into trichloroacetic acid-precipitable material. To check the specificity of the system, the effects of avidin, tetrachloropyridinol, warfarin and dithiothreitol were tested (Table 2). Avidin had essentially no effect on the system, whereas tetrachloropyridinol completely blocked the incorporation. Warfarin was moderately inhibitory at the high doses used. Dithiothreitol had only a very limited effect in the absence of NADH and none in its presence.

We also measured the factor-II activity and the ^{14}C incorporation in proteins eluted after $BaSO_4$ adsorption to ensure that the system gave increased factor-II activity and increased specific and total ^{14}C activity of these proteins (results not shown). Varying the final concentration of Triton X-100 from 0.1 to 2% did not affect the system.

Table 2. *Effect of avidin, tetrachloropyridinol and dithiothreitol on vitamin K-dependent carboxylation in vitro*

The standard system is described in the Materials and Methods section. The subtracted background incorporation was unaffected by the inhibitors and slightly (20–25%) decreased by dithiothreitol. All values are the mean of two separate incubations differing less than 10%.

Additions/omissions	^{14}C incorporation (c.p.m.)	Inhibition (%)
Standard system (background 752 c.p.m.)	1722	0
+Avidin (50μg/ml)	1560	9.4
+Avidin (100μg/ml)	1570	8.8
+Tetrachloropyridinol (50μg/ml)	0	100
Standard system (background 843c.p.m.)	2105	0
+Warfarin (0.6mg/ml)	1130	47
+Warfarin (1.5mg/ml)	700	66
Standard system (background 578c.p.m.)	1056	0
+Dithiothreitol (5mM)	1094	0
−NADH+dithiothreitol (5mM)	392	74

Affinity chromatography of solubilized microsomal fractions

Repeated washing of the microsomal pellet by Dounce homogenization and re-centrifugation markedly decreased its NAD(P)H dehydrogenase activity, but there was still 4–7% of the total activity of the 10000*g* supernatant left. This activity was only slightly (less than 20%) or not at all inhibited by 0.3mM-warfarin, which inhibited the cytosol enzyme about 95%.

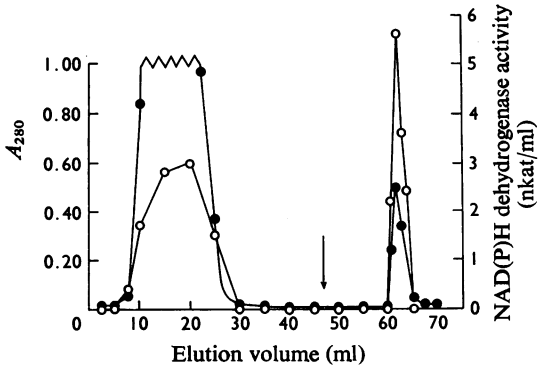

Fig. 2. *Affinity chromatography of supernatant from solubilized microsomal fraction on Sepharose 4B–vitamin K_3 column*

The column (gel bed vol. 20ml) was equilibrated and eluted as described in method A. The arrow indicates the start of elution with dioxan. ●, A_{280}; ○, NAD(P)H dehydrogenase activity.

Solubilized microsomal fractions were therefore chromatographed on the Sepharose–vitamin K column as described in method A. In preparations where the residual microsomal NAD(P)H dehydrogenase activity was sensitive to warfarin, two peaks of enzyme activity were found (Fig. 2). Only peak II (Fig. 2) was sensitive to warfarin. The amount of material in peak II decreased when the microsomal fractions were washed as described above before addition of Triton X-100, and peak II was completely absent when the enzyme activity of the washed microsomal preparation was totally warfarin-resistant.

To effect the specific and complete removal of the warfarin-sensitive NAD(P)H dehydrogenase and thus allow a study of its role in the vitamin K-dependent carboxylation, supernatants of solubilized microsomal preparations were submitted to chromatography on Sepharose–vitamin K columns as described in method B. The protein fraction passing unretarded through the column was pooled and used as the basis for a carboxylating system, and the effect of adding back isolated protein fractions or purified NAD(P)H dehydrogenase was studied (Table 3). The pooled unretarded fractions (fraction I) showed essentially no vitamin K-dependent incorporation of ^{14}C (i.e. <3%), whereas similar fractions from ordinary gel filtration through unsubstituted Sepharose showed 70% of control incorporation. When either fraction II (the pool of fractions containing the warfarin-sensitive NAD(P)H dehydrogenase) or cytosolic NAD(P)H dehydrogenase purified to electrophoretic homogeneity (Table 1) was added back (0.2ml to 0.8ml of fraction I) a marked vitamin

Table 3. *Effect of NAD(P)H dehydrogenase on vitamin K-dependent carboxylation*

Basic system consists of 0.8ml of pooled unretarded fraction (fraction I) from Sepharose–vitamin K chromatography of the supernatant of solubilized microsomal fractions (method B). Vitamin K_1 (50μg/ml), NADH (2mM) and $NaH^{14}CO_3$ (5μCi/ml) were added. Buffer I contains 1% Triton X-100 and 25% dioxan in 25mM-imidazole buffer, pH7.2. Buffer II is 10mM-potassium phosphate, pH7.0. Fraction II contained 3.6nkat/ml (Expts. II and III) and 17.3nkat/ml (Expt. IV) of NAD(P)H dehydrogenase activity. All values are averages of duplicate determinations. The incorporation in the absence of vitamin K_1 (background) has been subtracted.

	Additions (0.2ml) for basic system				Vitamin K-dependent ^{14}C incorporation	Background radioactivity
Expt.	Buffer I	Buffer II	Fraction II	NAD(P)H dehydrogenase*	(c.p.m.)	(c.p.m.)
I	+				0	762
			+		2800	764
II	+				32	432
			+		586	416
III	+				54	383
			+		1192	391
IV	+				18	430
			+		548	425
		+			15	416
				+	671	428
V		+			251	247
				+	1495	251

* Protein content 22 μg/ml (Expt. IV) and 32 μg/ml (Expt. V).

K-dependent incorporation took place. In a separate experiment, the addition of vitamin K_1 reduced by borohydride was about as effective (94%) as the addition of the fraction containing NAD(P)H dehydrogenase in restoring the vitamin K-dependent carboxylation.

Discussion

The preparations of NAD(P)H dehydrogenase obtained by the present purification method are electrophoretically homogeneous and give only two *N*-terminal amino acids (glutamine/glutamic acid and asparagine/aspartic acid) in about equal yield, which suggests the presence of two non-identical subunits in the enzyme. Our results thus confirm the previous finding of Rase *et al.* (1976) that the enzyme contains two subunits of about 27000 mol.wt. The recovery of enzyme is better than and the purity as good as in previously described methods (Hosoda *et al.*, 1974; Rase *et al.*, 1976) and the method is simpler. The different final specific activities may be caused by the differences in rat strains and assay methods used.

Affinity chromatography on Sepharose 4B–vitamin K_3 columns in the presence of Triton X-100 is the essential step in the procedure; the enzyme was bound even at 2% detergent concentration. This was exploited to eliminate unspecific hydrophobic interactions, and increased the purification obtained in this step. The binding of NAD(P)H dehydrogenase to the column, the reduction of Sepharose-bound vitamin K by NADH catalysed by the bound enzyme and the release of enzyme when the substrate is reduced show that vitamin K_3 coupled by the procedure used here can participate in specific enzyme–substrate binding. It also shows that the unreduced form of the enzyme binds the second substrate in the absence of NADH and that the enzyme can interact in a specific way with NADH when bound to the electron acceptor. These observations suggest that the enzymic mechanism may not be of the Ping Pong type as suggested by Hosoda *et al.* (1974). The sequence of binding of the two substrates (NADH and vitamin K_3) appears to be random, and a specific ternary complex is apparently formed, suggesting a random sequential mechanism.

It is generally accepted that NAD(P)H dehydrogenase is a cytosol enzyme with a small activity (2–5%) found also in the microsomal fraction (Ernster, 1967). The removal of all warfarin-sensitive Sepharose–vitamin K-binding NAD(P)H dehydrogenase activity by careful Dounce homogenization of the microsomal pellet may suggest that the enzyme is only loosely associated with the outside of the microsomal vesicles or trapped inside these vesicles as they are formed during the initial homogenization of the liver. Strain differences may be important here, since the liver NAD(P)H dehydrogenase activity of the various Wistar-derived strains is much lower than that of Sprague–Dawley rats (Lind *et al.*, 1973). A higher enzyme activity might therefore at any time be associated with the microsomal fraction of Sprague–Dawley rat livers, owing to a higher rate of synthesis.

The report by Martius *et al.* (1975) and our findings finally settle the question of whether vitamin K_1 is a physiological substrate for NAD(P)H dehydrogenase, and our results indicate a definitive role for the enzyme in providing reduced vitamin K for the vitamin K-dependent carboxylation reaction. Used as described here, the affinity column removes only two components from rat liver cytosol or solubilized microsomal fractions. One of these is NAD(P)H dehydrogenase, the other is without diaphorase activity. The resulting inactivation of the microsomal carboxylating system, and its restoration when reduced vitamin K_1 is added, suggest that NAD(P)H dehydrogenase may be the only physiologically important NAD(P)H–vitamin K_1 oxidoreductase in rat liver.

The solubilized microsomal system used in our experiments corresponds closely to those described by others (Esmon & Suttie, 1976; Girardot *et al.*, 1976; Mack *et al.*, 1976) with regard to composition and action of inhibitors. Avidin had no significant inhibitory effect, hence biotin is not involved. Tetrachloropyridinol, a known antagonist of vitamin K_1 *in vivo* (Ren *et al.*, 1974), blocked the vitamin K-dependent carboxylation completely. However, some differences were noted. In our hands dithiothreitol had no significant stimulatory effect, and warfarin at relatively high concentrations was inhibitory. Dithiothreitol replaces the requirement for exogenous NADH in the microsomal system (Sadowski *et al.*, 1977). In the solubilized microsomal system dithiothreitol cannot replace NADH (Sadowski *et al.*, 1977) or dithiothreitol is active but can be replaced by reduced vitamin K_1 (Girardot *et al.*, 1976). The mode of action of dithiothreitol is therefore probably to provide reducing equivalents for the reduction of vitamin K_1 via NADH, and dithiothreitol is thus only indirectly involved in the carboxylation reaction. The lack of effect of dithiothreitol in our system is most probably due to the presence of an adequate amount of NADH relative to the other components of the system. Here again the lower activity of NAD(P)H dehydrogenase and the concentrations of other NADH dehydrogenases present in the liver of Wistar rats may be of importance.

If the inhibition by warfarin is due to its inhibitory effect on NAD(P)H dehydrogenase, one might expect the effect to be decreased in the presence of NADH, since warfarin is a competitive inhibitor of this enzyme with respect to NADH (Ernster *et al.*, 1962; Hollander & Ernster, 1975). The various results reported with warfarin (Bell & Stark, 1976; Sadowski

et al., 1977; the present paper) may result from the use of different NADH/enzyme ratios in the different systems. Other interpretations are possible since the target for warfarin inhibition of prothrombin synthesis is still not clearly defined.

Varying the detergent concentration used to solubilize the microsomal pellet in the range 0.1–2% did not significantly change the activity of the system. Similarly, Mack *et al.* (1976) found essentially no change with the same detergent in the range 0.2–1%.

Finally, our results demonstrate clearly that NAD(P)H dehydrogenase participates in the reactions leading to vitamin K-dependent carboxylation by providing reduced vitamin K for the reaction. The role of this reduced vitamin K is presently unknown.

R. W. is a Research Fellow of the Norwegian Council on Cardiovascular Diseases.

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