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VITAMIN K-DEPENDENT CARBOXYLATION WITH RIBOSOMES FROM ESCHERICHIA COLI

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SUMMARY: A vitamin K-dependent carboxylation reaction has been demonstrated in vitro with crude ribosomal preparation from Escherichia coli. The vitamin K-dependent carboxylation was found to require NADH and an ATP generating system and was inhibited by warfarin. Irradiation (360 nm) of this facultative microorganism during growth has been shown to destroy the natural menaquinone, and thus is unable to synthesize a protein(s) containing γ-carboxyglutamic acid residues. Growth in the presence of light provides a natural substrate for studying the vitamin K-dependent carboxylation. The extent of vitamin K-dependent carboxylation was two- to three-fold in preparations obtained from irradiated cells as compared to those from non-irradiated cells.

INTRODUCTION: Vitamin K-dependent carboxylase is distributed widely in various tissues such as liver, kidney, placenta, lung, spleen, bone microsomes and chicken embryonic chorioallantoic membrane (1-6). Vitamin K-dependent carboxylation activity may parallel the occurrence of the vitamin K-dependent proteins containing γ-carboxyglutamic acid (Gla), which is synthesized as a post-translational vitamin K-dependent enzymatic carboxylation of specific glutamic acid residues in proteins. All Gla containing protein appear to be involved with calcium binding and metabolism. The biosynthesis of Gla and the vitamin K-dependent carboxylation reaction have been reviewed well by Olson and Suttie (7). Gla has been found also in ribosomes and cytoplasmic fractions of E. coli by Van Buskirk and Kirsh (8).

An enhancement of vitamin K-dependent carboxylation activity was observed in liver microsomes obtained from warfarin-treated or vitamin K-

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deficient rats (9). This stimulation of the carboxylation reaction presumably is due to either the increased endogenous protein substrate or the increased synthesis of enzyme(s) of the carboxylation system or both. In this communication we describe and compare a cell-free preparation which carries out a vitamin K-dependent carboxylation activity in ribosomes from E. coli cells grown with and without light (360 nm) treatment. The major advantage of the system obtained from the irradiated cells was the enhancement of vitamin K-dependent carboxylation reaction. The system obtained from bacterial cells grown in the presence of light may mimic the microsomal system obtained from warfarin-treated animals.

MATERIALS AND METHODS: Alumina, phosphocreatine, creatine phosphokinase, vitamin K_1 and o-phthalaldehyde were purchased from Sigma Chemical Co.; γ-carboxyglutamic acid and warfarin from Calbiochem; Dowex I (AG 1-X8, 200-400 mesh, chloride form) from Bio-Rad Laboratories; and [^{14}C]sodium bicarbonate from New England Nuclear Corp. E. coli type B was given to us by Dr. Margaret Lieb (USC, School of Medicine). All other chemicals were of reagent grade purity.

Preparation of ribosomes — Escherichia coli (type B) was grown in a minimal salts medium under limited aeration, overnight at 37°. The medium contained Bacto-Dextrose (Difco), 1.0 g; $(NH_4)_2SO_4$, 1.0 g; K_2HPO_4, 7.0 g; KH_2PO_4, 2.0 g; sodium citrate, 0.5 g; and $MgSO_4 \cdot 7H_2O$, 0.1 g in a total volume of 1.0 liter. Irradiated cells were grown under UV light at 360 nm using a series of GE lamps (type BLB black light). Ribosomes were prepared by a method similar to that of Staehelin and Maglott (10). The alumina-treated cell extract was centrifuged at 5,000 x g for 10 min to remove the alumina and cells. Then, the supernatant was centrifuged at 30,000 x g for 30 min following the further centrifugation of the supernatant at 140,000 x g for 60 min. Pellets (ribosomes) were washed twice with the extraction buffer by centrifugation and appeared to contain membrane fragments capable of carrying out a cyanide insensitive NADH oxidase activity.

Assay of vitamin K-dependent carboxylase — The incubation mixture was similar to that described by Suttie et al. (11). Each assay tube (13 x 100 mm) contained ribosomes (1.0 to 1.5 mg of protein), 50 µl of ATP-generating system (5 mM ATP, 50 mM phosphocreatine, 250 µg/ml creatine phosphokinase and 5 mM NADH), 0.4 ml of 25 mM imidazole, pH 7.2 (containing 0.25 M sucrose and 80 mM KCl), and 20 µl of [^{14}C]-$NaHCO_3$ (5 µCi, specific activity 54 mCi/mmol) in a total volume of 0.5 ml. The reaction was initiated by the addition of 10 µl of vitamin K_1 (10 mg/ml in ethanol). The samples were sealed with parafilm and incubated in a constantly shaking water bath at 25° for 15 min. The reaction was stopped by the addition of 4.0 ml of ice-cold 10% trichloroacetic acid. After the further addition of 0.5 ml of saline containing 10 mg/ml of bovine serum albumin, the protein precipitate was left for 30 min in ice bath. The precipitate was collected by centrifugation, dissolved in 1.0 ml of 0.2 M Na_2CO_3 and precipitated with 4.0 ml of trichloroacetic acid. The precipitation and centrifugation

TABLE I

Vitamin K-dependent carboxylation in ribosomes from E. coli cells grown without aeration and with irradiation.

Added	- Vitamin K_1	+ Vitamin K_1	Stimulation	Inhibition
	cpm/mg protein		fold	%
Cells grown without aeration				
Complete System	362	1,321	3.7	100
" + Warfarin (10 mM)	-	348	-	26
" - NADH	-	256	-	19
" - ATP	-	218	-	17
Cells grown with irradiation (360 nm)				
Complete System	245	1,807	7.4	100
" + Warfarin (10 mM)	-	280	-	15
" - NADH	-	156	-	9
" - ATP	-	127	-	7

The reaction mixture and the assay of carboxylation were similar to that described under "Materials and Methods" by using 1.0 mg of ribosomal preparation. The values given above represent the mean of three to five determinations.

process was repeated and the pellet was transferred to 10 ml of Bray's solution. The radioactivity was determined in a liquid scintillation spectrometer.

Detection of Gla — The endogenous protein (trichloroacetic acid precipitate) was hydrolyzed with alkali (2 N KOH) for 22 hr at 110°. After neutralizing the hydrolysate with $HClO_4$, the labeled Gla was detected in the eluted fractions of Dowex anion exchange chromatography and in the fluorescent detection as described by Gundberg et al. (12). Fluorescence was monitored with MPF-4 Perkins-Elmer spectrofluorometer. Excitation was carried out at 340 nm while the emission was set at 455 nm.

Protein estimation — Protein concentration was determined by a modification of the Biuret method (13) with bovine serum albumin as standard.

RESULTS AND DISCUSSION: Vitamin K-dependent carboxylation — The vitamin K-dependent incorporation of added $H^{14}CO_3^-$ was observed in ribosomal preparation from E. coli. As shown in Table I, irradiation of the cells during growth with light at 360 nm resulted in a stimulation of the vitamin K-dependent carboxylation. The extent of this carboxylation activity in ribosomes in the presence of vitamin K_1 was approximately 2.0 fold higher

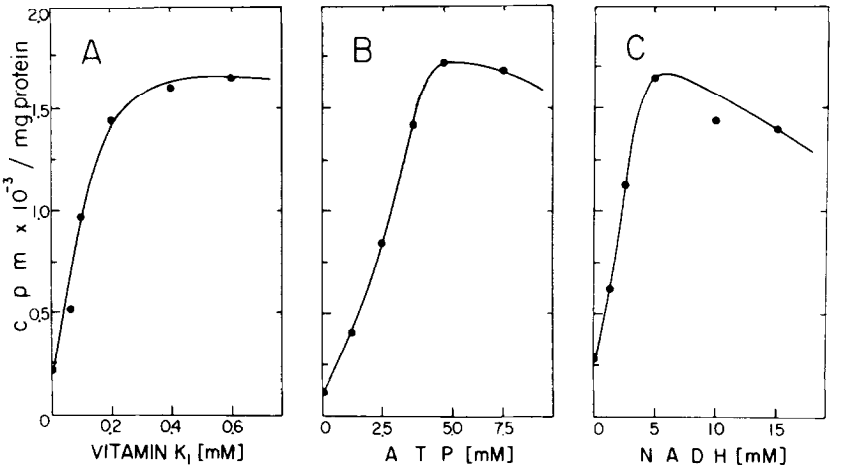
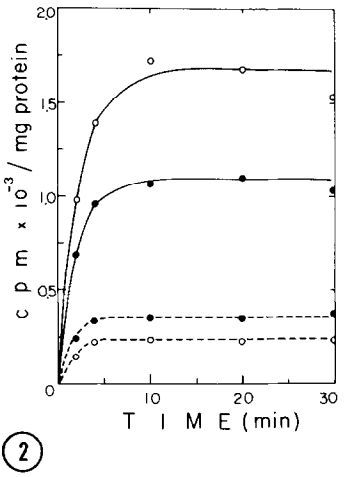


Fig. 1 The effect of various concentrations of vitamin K_1, ATP and NADH on the carboxylation reaction.
The reaction mixture and the assay of carboxylation were similar to that described under "Materials and Methods" by using 1.0 mg of ribosomal preparation from irradiated cells, except with indicated concentrations of vitamin K_1 (A); of ATP, (B); and of NADH, (C).

in irradiated cells as compared to non-irradiated cells. However, the carboxylation activity, in the absence of vitamin K_1 was lower in irradiated cells than in non-irradiated cells. Since UV irradiation has been shown previously to destroy the natural menaquinone (14,15), the results of vitamin K-dependent carboxylation suggested that the UV light grown cells, which lack the natural menaquinones, contain an endogenous substrate for the carboxylation reaction. The vitamin K-dependent carboxylation reaction in E. coli was found to require NADH and an ATP generating system and was inhibited by warfarin (Table I).

As shown in Fig. 1-A, an in vitro carboxylation of ribosomal preparation requires relatively high concentrations of vitamin K_1 (0.2 mM). The carboxylation reaction did not occur in the absence of NADH or ATP. The data shown in Fig. 1-B and C indicate that the optimum concentration for ATP or NADH appeared to be 5 mM, respectively. The addition of the supernatant obtained after removal of the ribosomes to the reaction mixture showed no significant effect on the carboxylation reaction. Although the ribosomal preparation had been washed twice, some contaminated membrane fragments were



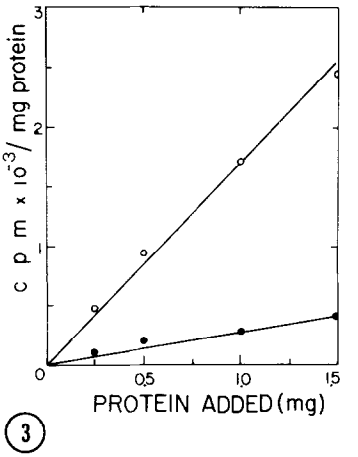


Fig. 2. The time course of vitamin K-dependent carboxylation.
The reaction mixture and assay of carboxylation were similar to that described under "Materials and Methods" by using 1.2 mg of ribosomal preparation. o —— o, irradiated + vitamin K_1; o . . . o, irradiated - vitamin K_1; ● —— ●, non-irradiated + vitamin K_1; ● . . . ●, non-irradiated - vitamin K_1.

Fig. 3. The level of carboxylation with various amounts of ribosomal protein from irradiated cells.
The reaction mixture and assay of carboxylation were similar to that described under "Materials and Methods" by using various amounts of protein as indicated above. o —— o, with vitamin K_1; ● —— ●, without vitamin K_1.

required for the oxidation of NADH or NADPH by a cyanide insensitive pathway. Time course of vitamin K-dependent carboxylation showed that the maximal carboxylation was reached at 10 min (Fig. 2).

The effect of warfarin on the carboxylation reaction — As shown in Table II, the vitamin K-dependent carboxylation reaction was inhibited by warfarin. Almost complete inhibition of the endogenous protein carboxylation occurred at the concentration of warfarin near 10 mM. Vitamin K analogues such as menadione and lapachol could not replace vitamin K_1 (data not shown), suggesting that there are structural requirements of naphthoquinones for the carboxylation activity.

The carboxylation reaction as a function of the ribosomal protein — The level of endogenous protein carboxylation was proportionate to the

TABLE II
The effect of warfarin on the carboxylation reaction.

Warfarin added	Carboxylation	% of inhibition
mM	cpm/mg protein	
0	1,820	100
3.0	1,450	80
10.0	280	15
20.0	210	12

The reaction mixture and the assay of carboxylation were similar to that described under "Materials and Methods" by using 1.2 mg of ribosomal proteins from irradiated cells. Warfarin was preincubated with ribosomes in the reaction mixture at the indicated concentrations for 10 min at 25°.

amount of protein added, up to 1.5 mg (Fig. 3). With concentrations over 1.5 mg, the level of carboxylation slowly reached a plateau with the preparations used in this study.

In order to confirm the formation of Gla as a product of carboxylation, the endogenous protein (trichloroacetic acid precipitate) was hydrolyzed with alkali, and the formed Gla was determined. As shown in Fig. 4, the labeled Gla was detected in the fractions (No. 15-17) eluted with low pH buffer (buffer C). On the other hand, the decarboxylated Gla sample, which was hydrolyzed with 6 N HCl, failed to show labeled Gla or fluorescence in the same fractions. A peak of fluorescence was found to match the elution peak of the alkali hydrolyzed sample as well as standard Gla.

In this study we have shown that the vitamin K-dependent carboxylation occurred in the endogenous protein from the ribosomal preparations of *E. coli*. Further, it has been shown that the level of carboxylation is enhanced in UV irradiated cells during growth. This system may be useful in studying the nature and mechanism of the vitamin K-dependent carboxylation reaction. The bacterial system may be useful in helping to understand the mechanisms of the vitamin K-dependent carboxylation reaction. The finding of the carboxylation reaction in bacteria is significant and

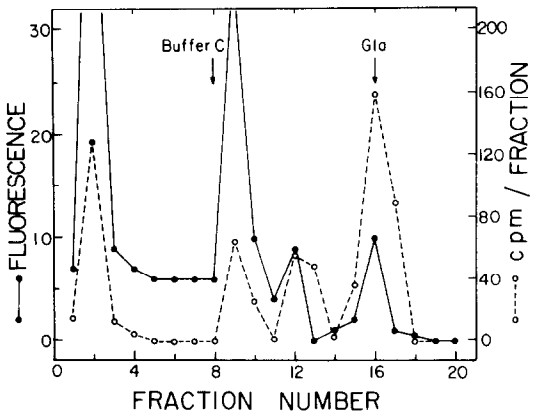


Fig. 4. Dowex I column chromatography and fluorescence change of o-phthaldehyde.

Dowex I-X8 column (6 x 0.7 cm) was previously equilibrated with an 0.01 M Hepes buffer at pH 10.0 (buffer A). After subjected the samples on column, 40 ml of 0.02 M Hepes buffer, pH 5.0 (buffer B) was passed through the column at a flow rate of 25 ml/hr. Following a 50 ml of 0.02 M Hepes containing 0.02 M $MgCl_2$ at pH 4.5 (buffer C) was passed through the column and collected in 5 ml fractions. A 0.5 ml aliquot of these fractions was transferred to 10 ml of Bray's solution, and the radioactivity was determined in a liquid scintillation spectrometer. Fluorescence was assayed in 3.0 ml of reaction mixture containing a 1.5 ml of column fractions and 1.5 ml of 6.0 mM o-phthalaldehyde in 0.4 M boric acid solution which titrated to pH 9.7 with KOH containing 0.2% of 2-mercaptoethanol. The fluorescence was determined by excitation at 340 nm and emission at 455 nm, and the fluorescence expressed by arbitrary units.

further indicates that nature tends to preserve reactions which are chemically and thermodynamically feasible. Although all of the proteins containing the Gla are involved in calcium binding or metabolism, the role of the Gla protein in E. coli is unknown and remains to be elucidated.

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