The Apparent Absence of Involvement of Biotin in the Vitamin K-Dependent Carboxylation of Glutamic Acid Residues of Proteins

By PAUL A. FRIEDMAN and MICHAEL A. SHIA

Longwood Area Hospital Clinical Pharmacology Unit and Department of Pharmacology,
Harvard Medical School, Boston, MA 02115, U.S.A.

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The mechanism of the vitamin K-dependent post-translational carboxylation of the γ -carbon atom of glutamic acid residues in proteins remains obscure. Experiments were performed *in vivo* and *in vitro* in an attempt to establish a role for biotin in the transfer of the carboxyl group. Weanling male rats were fed on a biotin-deficient diet until severe biotin deficiency was induced. Their degree of biotin deficiency was documented by assaying for liver acetyl-CoA carboxylase activity, which was about 15% of normal. However, one-stage and two-stage prothrombin times measured on the plasmas were normal. In addition, the liver microsomal fraction did not contain any more prothrombin precursor than did that of normal rat liver. Experiments were done *in vitro* in which vitamin K-dependent fixing of $^{14}\text{CO}_2$ was measured in the liver microsomal fraction from vitamin K-deficient male rats in the presence or absence of avidin. No evidence for an avidin-sensitive critical biotin-containing site was obtained. Thus neither series of experiments suggests a role for biotin; the data are compatible with carboxyl transfer occurring either through a carboxylated vitamin K intermediate, or via a yet to be identified intermediate, or perhaps via CO_2 itself.

The addition of single carboxyl groups to the γ -carbon atoms of the first ten glutamic acid residues in the polypeptide chain of prothrombin is the vitamin K-dependent step in the synthesis of the pro-coagulant (Stenflo *et al.*, 1974; Nelsesteun *et al.*, 1974). These modifications occur after translation of this portion of the prothrombin molecule. In addition to the likely presence of these γ -carboxyglutamate residues in the other vitamin K-dependent coagulation factors (VII, IX and X), γ -carboxyglutamate residues have been identified in a bovine plasma protein of unknown function, designated protein C (Stenflo, 1976) in proteins solubilized from bone (Hauschka *et al.*, 1975; Price *et al.*, 1976) and in protein from kidney (Hauschka *et al.*, 1976). That the synthesis of protein C and the bone protein is vitamin K-dependent is suggested by the diminution of γ -carboxyglutamate content in these proteins isolated from animals fed on coumarins, which are vitamin K antagonists. When the kidney microsomal fraction from rats fed on a vitamin K-deficient diet is incubated in the presence of $\text{NaH}^{14}\text{CO}_3$ and vitamin K, the vitamin K-dependent formation of labelled γ -carboxyglutamate can be demonstrated in the microsomal protein after incubation (Hauschka *et al.*, 1976).

The mechanism by which the vitamin leads to this carboxylation remains unknown. Although vitamin K may transfer the carboxyl group directly via a carboxylated vitamin K intermediate, it could also function in some ancillary manner with another compound directly involved in carboxyl transfer.

Among well-characterized enzymic carboxylation reactions, those in which biotin transfers the carboxyl group are well known. One study, published before the discovery that the vitamin K-dependent post-translational modification was a carboxylation, reported that young rats fed on a biotin-deficient diet developed modest prolongation of one-stage prothrombin times, whereas rats fed on a normal diet or the deficient diet supplemented with biotin maintained normal prothrombin times (Pitrelli & Marsili, 1973). However, the deficient diet was also quite low in vitamin K, and it was found that if excess of vitamin K (as menadione) was added to the diet, the prothrombin times were not prolonged. The data suggested that biotin deficiency caused an inefficient utilization of vitamin K. Yet, although signs of biotin deficiency, such as failure to gain weight, hair loss and unsteady gait, were apparent in these animals, no biochemical documentation of the degree of biotin deficiency was reported. With the knowledge that a vitamin K-dependent modification of a variety of proteins is a carboxylation, it seemed important to re-examine the possibility that biotin might have a role in the reaction. In this study we have looked for evidence *in vivo* and *in vitro* of biotin involvement in the carboxylation.

Experimental

Materials

Normal rat diet was from Purina (St. Louis, MO, U.S.A.) and biotin-deficient diet from ICN Pharma-

ceuticals (Cleveland, OH, U.S.A.). Acetyl-CoA, biotin, avidin, dithiothreitol and *Echis carinatus* (viper) venom were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); $\text{NaH}^{14}\text{CO}_3$ (specific radioactivity 50mCi/mmol) was from New England Nuclear (Billerica, MA, U.S.A.). Thromboplastin was from Ortho Diagnostics (Raritan, NJ, U.S.A.).

Feeding of animals

Weanling male CD rats (60g), from Charles River Farm (Wilmington, MA, U.S.A.), were placed in two groups in wire-bottomed cages and fed on either regular or biotin-deficient diet for 50 days. Since an earlier study (Petrelli & Marsili, 1973) had established that rats fed on the biotin-deficient diet supplemented with biotin showed none of the signs of biotin-deficiency nor any prolongation of prothrombin time, it was decided not to include this third group in the present study.

Treatment of plasma and preparation of a liver microsomal fraction

After the 50-day feeding period the rats were deprived of food for 18 h and plasma and a liver microsomal fraction obtained from each group. For this, the rats were lightly anaesthetized with diethyl ether, a laparotomy was performed, and 1.8 ml of blood was withdrawn from the aortic bifurcation by using a 22-gauge needle and a plastic syringe containing 0.2 ml of 3.8% (w/v) trisodium citrate. The one-stage prothrombin time described by Quick (1940) and the two-stage prothrombin time of Ware & Seegers (1948) were determined on the fresh plasmas obtained from this sample of blood. The rats were then killed by cutting the aorta and exsanguinated; the livers were quickly excised and placed in ice-cold 0.25 M-sucrose. Liver was washed with cold 0.025 M-imidazole/HCl, pH 7.2, containing 0.25 M-sucrose (buffered sucrose) and then homogenized in 2 vol. of buffered sucrose by two or three passes with a Potter-Elvehjem homogenizer rotating at 2000 rev./min. The homogenate was centrifuged at 12500g for 10 min and the resulting supernatant was then diluted with 5 vol. of buffered sucrose and centrifuged at 100000g for 60 min to obtain the microsomal pellet. The pellet was resuspended into buffered sucrose with a loose fitting Dounce homogenizer to 20% of the volume of the undiluted 12500g supernatant. The microsomal fraction was assayed for the presence of prothrombin precursor by using *E. carinatus* venom as described by Suttie (1973).

Acetyl-CoA carboxylase purification

Portions of the livers from each group were frozen and later assayed for acetyl-CoA carboxylase

activity after purification as far as the first $(\text{NH}_4)_2\text{SO}_4$ step by using a slight modification of the procedure of Inoue & Lowenstein (1972). In their original procedure, the frozen livers used were obtained from rats whose acetyl-CoA carboxylase activity had been induced by depriving the animals of food for 2 days and then feeding them on a diet high in fructose for the subsequent 5 days before death. This dietary manipulation was not used in our experiments for obvious reasons. Whereas Inoue & Lowenstein (1972) homogenized their liver in a Waring blender at top speed for 10 s, we used several passes on a Potter-Elvehjem homogenizer rotating at 2000 rev./min. The remainder of the partial purification was unchanged from the published method (Inoue & Lowenstein, 1972), and the $(\text{NH}_4)_2\text{SO}_4$ fractions were assayed for acetyl-CoA carboxylase activity immediately after the dialysis step.

Acetyl-CoA carboxylase assay

The assay for acetyl-CoA carboxylase was also modified slightly from that reported by Inoue & Lowenstein (1972). A sample of the enzyme preparation was first incubated for 30 min at 37°C in 0.11 ml of 80 mM-Tris/HCl buffer, pH 7.5, containing 23 mM- MgCl_2 , 27 mM-potassium citrate and 1.54 mM- β -mercaptoethanol, in plastic tubes (12 mm $\times$ 75 mm). These tubes were cooled on ice; the volume was then increased to 0.165 ml with ATP (5 mM), acetyl-CoA (0.2 mM when present) or an equivalent volume of water, and $\text{NaH}^{14}\text{CO}_3$ (10 mM, specific radioactivity 0.5 $\mu\text{Ci}/\mu\text{mol}$). After a second incubation (5 min at 37°C) the reaction was terminated with an equal volume of 10% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation, and 0.3 ml of the clear supernatant was placed in a scintillation vial and dried at 50°C under a stream of N_2 . To the residue was added 10 ml of Instagel (Packard Instrument Co., Downers Grove, IL, U.S.A.), and the sample was counted for radioactivity in a Packard liquid-scintillation spectrometer with an 85% efficiency for ^{14}C .

Assay for vitamin K-dependent carboxylation

When vitamin K-dependent carboxylation of protein was to be assayed, 200 g male CD rats were fed on vitamin K-deficient diet for 10 days; their liver microsomal fraction was obtained as described above and resuspended with a loose-fitting Dounce homogenizer into buffered sucrose to 60% of the volume of the undiluted 12500g supernatant. The carboxylation assay was performed as follows (Friedman & Shia, 1976). Incubations were at 37°C for 30 min in capped glass test tubes (10 mm $\times$ 75 mm). Reaction volume was 0.5 ml and contained: 0.3 ml of microsomal fraction; 25 mM-imidazole/HCl,

pH 7.2; 250 mM-sucrose; 50 mM-KCl; 2.5 mM-magnesium acetate; 0.02 ml of ethanol or 0.25 μ g of menaquinone-3 added in 0.02 ml of ethanol. The last addition was $\text{NaH}^{14}\text{CO}_3$ (5×10^6 c.p.m.; specific radioactivity 50 mCi/mmol). Reactions were stopped by addition of 0.025 ml of 10% (v/v) Triton X-100 and 0.035 ml of 4.4 M-acetic acid. The tubes were placed in a desiccator containing a KOH trap and kept at room temperature (22°C) for 30 min under reduced pressure, after which 0.4 ml of 1 M- NaHCO_3 was added to each tube. After 20 min 0.8 ml samples were placed in scintillation vials containing 2 ml of 0.3 M- Na_2CO_3 , and the protein was precipitated with 15 ml of ice-cold 10% trichloroacetic acid. After 30 min at 5°C the vials were centrifuged at low speed in a cold swinging-bucket centrifuge, the trichloroacetic acid was discarded, NCS solubilizer (1 ml) was placed on top of the pellets, and, after solubilization of the protein, 10 ml of Instagel was added, and the vials counted for radioactivity.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Results

Effect of biotin deficiency on plasma coagulation parameters

After 50 days on the biotin-deficient diet, that group of rats showed the usual signs of biotin deficiency: loss of hair, dermatitis, spectacled eyes, unsteady gait, increased irritation and failure to gain weight at the normal rate (Table 1). When the plasmas were assayed, there was no difference in the one-stage or two-stage prothrombin times between the rats on normal diet and the group on biotin-deficient diet. The biotin-deficient diet used here contained an excess of menadione. Thus our results with the plasma-clotting assays are compatible with the conclusion drawn by Petrelli & Marsili (1973) that biotin deprivation indirectly affects coagulation by preventing efficient use of menadione in the diet.

Assay for microsomal prothrombin precursor

Vitamin K-deprived rats accumulate a prothrombin precursor in the liver microsomal fraction that is characterized by little physiological activity, but which can be converted readily into thrombin with *E. carinatus* venom (Suttie, 1973). The possibility existed that at the degree of biotin deficiency induced here the prothrombin time might be normal and yet the content of precursor in the microsomal fraction might be higher than in the liver microsomal fraction from rats fed on a normal diet. Table 2 shows that

Table 1. Comparison of the prothrombin times of rats fed on a normal diet with rats fed on a biotin-deficient diet

The one-stage prothrombin times were done in 0.3 ml volumes, containing 0.1 ml of fresh plasma, 0.1 ml of thromboplastin and 0.1 ml of 0.02 M- CaCl_2 , by using a fibrometer to detect clotting. The two-stage prothrombin times were obtained on 10-fold dilutions of plasma in 0.145 M- NaCl .

Diet	Weight of animal at end of experiment (g)	Prothrombin times (s)	
		One-stage	Two-stage
Normal	380	14.6	11.0
	325	13.8	10.6
	315	15.1	12.0
	290	15.4	11.2
Average	328	14.7	11.2
Biotin-deficient	180	13.9	11
	190	15.1	11.8
	150	15.7	11.4
	200	14.6	11.6
Average	180	14.8	11.5

Table 2. Potential thrombin activity in the microsomal fractions from rats fed on normal diet, biotin-deficient diet, or vitamin K-deficient diet

Portions (2 g) of liver from each of four rats fed on the diet indicated were pooled, and the three pools were individually homogenized in 2 vol. of buffered sucrose. The microsomal suspensions were obtained as described in the Experimental section. Each suspension was adjusted to 0.33% Triton X-100 with a 10% stock solution of the detergent and then gently agitated at room temperature for 10 min. To 0.21 ml of suspension was added 0.04 ml of a 1 mg/ml solution of *E. carinatus* venom. After incubation for 10 min at 37°C, a 0.1 ml sample was assayed for thrombin activity (Suttie, 1973). The thrombin units reported were calculated from a thrombin standard curve obtained by using National Institutes of Health (lot B-3) thrombin. The usual log-log relationship of thrombin concentration and clotting times was used.

Diet	Time on diet (days)	Clotting times (s)	Thrombin (unit/assay)
Normal	50	45.5	0.039
Biotin-deficient	50	44.5	0.041
Vitamin K-deficient	10	12.5	0.500

this is not the case. Included for comparison is a clotting time of 12.5 s obtained from a microsomal suspension prepared in identical manner from the livers of rats fed on a vitamin K-deficient diet for 10 days.

Assay for liver acetyl-CoA carboxylase activity

Table 3 compares the activity of acetyl-CoA carboxylase in the rats fed on the two diets. As shown, the

activity per unit volume of the enzyme preparation from the livers of rats fed on the biotin-deficient diet is only 15% of the activity of the preparation from normal liver and the specific activity is only 13% of that of the normal preparation. Although we have not measured directly the biotin content of the livers, the marked decrease in activity of this biotin-containing enzyme is presumptive evidence that the livers from the rats fed on the biotin-deficient diet contained substantially less biotin than did livers from the normal rats. It should be noted that the acetyl-CoA carboxylase activity found in our preparation is low compared with values found in similar preparations from livers of rats fed on diets designed to induce the carboxylase, such as the high-fructose diet described by Inoue & Lowenstein (1972).

Effect of avidin on vitamin K-dependent carboxylation

Although we could not show hypoprothrombinemia by inducing a biotin deficiency *in vivo*, it was possible that an even more marked deficiency might

be required to obtain such an effect. With this in mind, experiments *in vitro* were designed to look for a role for biotin in the vitamin K-dependent carboxylation of liver microsomal protein by attempting to inhibit the carboxylation with avidin, the egg-white protein with a remarkably high affinity for free or enzyme-bound biotin (Green, 1963).

As shown in Table 4, vitamin K-dependent carboxylation in the microsomal fraction treated with 0.33% Triton X-100 was not affected by avidin, which was added to and incubated with the microsomal fraction before initiation of the carboxylation reaction. Carboxylation in an intact microsomal fraction consistently was decreased by 10–12% in experiments in which avidin was included. Since this degree of inhibition is not relieved by the use of avidin preincubated with excess of free biotin, the slight inhibition seen with avidin is not the result of the interaction of avidin with a critical biotin site in the intact microsomal fraction. That this is indeed the case is shown by experiments in which partially purified acetyl-CoA carboxylase was added to identical incubations and its activity assayed for in the absence of vitamin K. Avidin completely abolished activity, whereas avidin previously exposed to excess of free biotin had no inhibitory effect.

Discussion

Thus we find no evidence for a role for biotin in the vitamin K-dependent carboxylation reaction. Several limitations to a study of this nature exist, however. First, it is possible that at no degree of biotin deficiency induced either during the feeding experiments or *in vitro* with avidin did we reach a point at which a biotin-dependent enzymic step became the rate-limiting step in the series of reactions involved in carboxylation. Secondly, it is possible that the avidin never reached the critical biotin-containing site in the experiments *in vitro*.

Table 3. *Acetyl-CoA carboxylase activity in livers from rats fed on normal or biotin-deficient diets*

Samples (0.05 or 0.1 ml) of the enzyme preparation were assayed as described in the Experimental section. All incubations were run in duplicate. Blanks were incubations without acetyl-CoA. Results are expressed both as $^{14}\text{CO}_2$ fixed/ml of the enzyme preparation and as $^{14}\text{CO}_2$ fixed/mg of protein in the preparations. The protein concentrations were 5.75 and 7.13 mg/ml for the normal and biotin-deprived preparations respectively.

Source of liver for enzyme preparation	$^{14}\text{CO}_2$ fixed (c.p.m./ml)	$^{14}\text{CO}_2$ fixed (c.p.m./mg of protein)
Normal rats	65200	11300
Biotin-deprived rats	10600	1490

Table 4. *Effect of avidin on vitamin K-dependent carboxylation of rat liver microsomal protein*

Preparation of microsomal fractions and assay for vitamin K-dependent carboxylation was done as described in the Experimental section. The vitamin used was menaquinone-3 at a concentration of 0.5 $\mu\text{g}/\text{ml}$. In (b) 10% Triton X-100 was added to the microsomal suspension to give a concentration of 0.33%; this microsomal fraction was kept at room temperature for 10 min and then placed in ice until use. When avidin was used, 0.5 unit was added to the microsomal suspension, which was kept in ice for 20 min before initiation of carboxylation with menaquinone-3 and $\text{NaH}^{14}\text{CO}_3$. If avidin was preincubated with biotin, 20 μg of biotin was added for each unit of avidin. After 5 min in ice, a volume containing 0.5 unit of avidin was added to the microsomal fraction. In (a) and (b) the radioactivity found in the absence of added menaquinone-3 was the same in the experiments in which either avidin or avidin preincubated with biotin was included. Results are the average of duplicate incubations, which differed by less than 10%.

Microsomal fraction	$^{14}\text{CO}_2$ fixed (c.p.m.)			
	–Vitamin K	+Vitamin K	+Vitamin K+avidin	+Vitamin K+avidin preincubated with biotin
(a) Intact	70	435	390	380
(b) Detergent-treated	110	576	577	556

Without elucidation of all steps involved in carboxylation, it is not possible to eliminate the possibility of biotin involvement. What can be said is that all data fail to reveal such a role and remain compatible with the hypothesis that vitamin K is a cofactor which directly transfers the carboxyl group to glutamic acid residues.

A mechanism in which vitamin K transfers a carboxyl group would be novel, and thus the structure of the putative carboxynaphthoquinone intermediate unknown. In an alternative mechanism the vitamin could function in some manner to facilitate breaking the C-H bond of the γ -carbon atom, which in glutamate is α to a free carboxyl group, and as such quite stable. In the latter instance, the manner in which the carboxyl group is added is even less apparent, but could involve a yet to be identified carboxylated intermediate or perhaps just CO₂ itself.

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